QUANTITATION OF MALE AND FEMALE DNA

IN MIXED BIOLOGICAL SAMPLES USING

QUANTITATIVE AMPLIFICATION OF

THE HUMAN AMELOGENIN LOCUS

By

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2006

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my research advisor, Dr. Robert Allen for his advice and guidance throughout my graduate experience and especially through the thesis process. I would also like to thank my additional committee members for their helpful recommendations in specific areas of my research, Dr. David Wallace for his help with statistical calculations, Dr. Gregory Sawyer for his guidance in optimizing conditions, Dr. Valerie Fuller at the Tulsa Police Department for her donation of samples and for her continued input and ideas, and Jane Pritchard for her advice on quality assurance issues. I am additionally grateful to the Oklahoma State University Center for Health Sciences and Dr. Robert Allen for the opportunity to conduct research in the Identity Testing Laboratory. I would like to extend my thanks to Phylis Emery, Jun Fu, Elizabeth Lienemann, and Adrea Tiller for their patience, friendship, and support throughout my research process and for making my time in Tulsa more enjoyable. In addition, I would also like to thank my family for their continued support and encouragement in everything I do.

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NOMENCLATURE

Alu	repetitive DNA elements found in primate DNA
Amelogenin	gene used in sex determination
ASCLD	American Society of Crime Laboratory Directors
bp	basepair
CCD	charge-coupled device
DNA	deoxyribonucleic acid
DTT	dithiothreitol
FBI	Federal Bureau of Investigation
hTERT	human telomerase reverse transcriptase gene
MGB	minor grove binding probes
NFSTC	National Forensic Science Technology Center
ng	nanogram
NIST	National Institute of Standards and Technology
OD	optical density
OSU	Oklahoma State University
PCR	polymerase chain reaction
pg	picogram
Q-TAT	quantitative template amplification technology
RB1	retinoblastoma gene

RFLP	restriction fragment length polymorphism
RFU	relative fluorescence unit
SDS	sodium dodecyl sulfate
SRY	sex-determining region Y
STR	short tandem repeat
THO1	STR marker within the tyrosine hydroxylase gene
TPD	Tulsa Police Department
TWGDAM	Technical Working Group on DNA Analysis Methods
U	units
UV	ultraviolet
VNTR	variable number of tandem repeats
Y-STR	Y-chromosomal short tandem repeats

I. INTRODUCTION

Deoxyribonucleic acid (DNA) isolated from biological fluids or tissues is commonly used to link a suspect to a crime. DNA is found in all nucleated cells of the body and is present in biological fluids such as blood, saliva, and semen. Within human cells, DNA is distributed among 22 pairs of autosomal chromosomes and one pair of sex determining chromosomes. Normal males receive an X chromosome from their mother and a Y chromosome from their father and therefore males carry the XY genotype. Normal females receive an X chromosome from their mother and the other X chromosome from their father and are genotypically XX (Butler, 2005) (Figure 1).

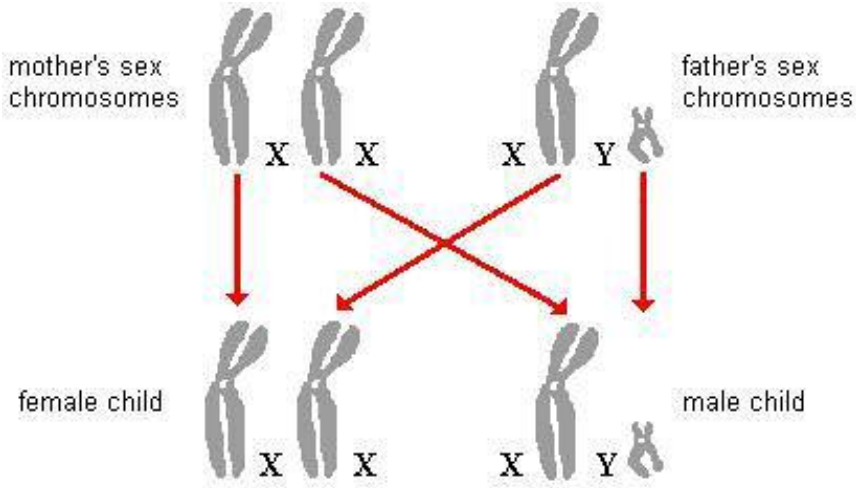

Figure 1: Sex differentiation (O'Neil, 2005).

Because only males contain a Y chromosome, it is possible to genetically distinguish the two sexes using chromosomal or molecular analysis. The Amelogenin gene is found on

both the X and Y chromosomes and encodes for a protein found in tooth enamel. The gene structure differs slightly on the two chromosomes (Nakahori et al., 1991) (Figure 2) and therefore molecular assays that reveal the structural differences of the Amelogenin gene can be used for sex determination.

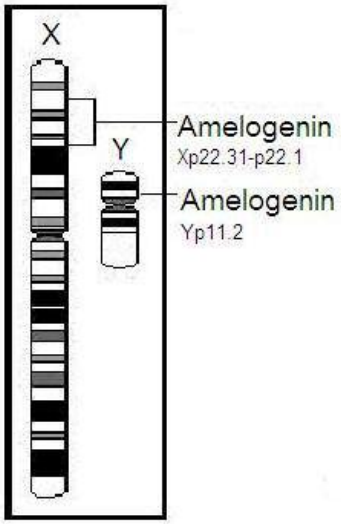

Figure 2: Location of Amelogenin gene on the X and Y chromosomes (Staveley, 2006).

Genetic markers are polymorphic traits that are encoded in the chromosome. Genetic markers differ between individuals and are therefore useful for human identification and forensic investigations. The variable forms of a genetic marker are known as alleles. Highly polymorphic DNA markers have been described in human DNA (Nakamura et al., 1987; Wong et al., 1987; Hammond et al., 1994) and usually exist in the heterozygous state among members of the population. Short tandem repeat (STR) markers are an example of DNA polymorphisms in human genomic DNA that consist of tandem repetitions of three to five basepair (bp) repeats within the chromosome (Hammond et al., 1994; Butler, 2005). Highly polymorphic STR markers are found in

noncoding regions of autosomal chromosomes and are useful for human identification purposes because they provide the ability to discriminate between samples.

When biological fluids are recovered (normally as stains) from a crime scene, DNA can be isolated from nucleated cells present in the fluid. Once DNA has been isolated, polymorphic STR markers within the genome can be detected and analyzed following polymerase chain reaction (PCR) amplification (Butler, 2005). Multiplex PCRs are designed to amplify several loci simultaneously, producing alleles from multiple STR loci used for identification in a sample PCR reaction (Edwards and Gibbs, 1994; Butler, 2005). Multiplex STR typing kits currently available commercially can co-amplify STR alleles from as many as 15 separate STR loci as well as the Amelogenin locus for sex determination (Applied Biosystems, 2006; Promega, 2006). The amplification of alleles using primers labeled with fluorescent dyes coupled with amplicon size differences, enables the STR loci to be resolved and identified using capillary electrophoresis with fluorescent detection. The combination of alleles present at each STR locus of interest is referred to as a DNA profile.

Each person has two alleles at each locus and therefore in a single source biological sample, a maximum of two alleles will be visualized for each STR locus analyzed. Unfortunately, stains recovered from a crime scene can contain DNA from multiple contributors. The DNA profile from a sample consisting of a mixture from two individuals can have up to four alleles present at each locus. As the number of individuals contributing to the mixture increases, the number of alleles potentially visualized at each locus will increase as well.

The interpretation of DNA profile data from evidentiary samples containing contributions from multiple donors can be a challenging task for the DNA scientist (Ladd et al., 2001). Among evidentiary sample mixtures, mixed male and female samples occur frequently in sexual assault cases and homicide cases. Violent crimes make up 62.5% of all crimes committed, with forcible rape being about seven times more frequent than homicide (Catalano, 2004). In 90% of all sexual assaults, rape victims are women and suspects are male (Catalano, 2004). Thus, most sexual assault evidence recovered from crime scenes has a high probability of being a mixture of male and female DNA.

Quantitation of genomic DNA present in a sample is an essential part of the DNA typing process. Quantitation helps ensure that a DNA profile of high quality is produced and therefore readily interpretable. Moreover, when dealing with evidence from sexual assaults, knowing the relative proportion of male and female DNA present in an evidentiary sample will assist an analyst in selecting the proper analytical method for examining the evidence. For example, it is common to see low amounts of male DNA and high quantities of female DNA in sexual assault evidence. When low quantities of male DNA are present in a mixture containing high amounts of female DNA, Y chromosome analysis, rather than autosomal STR analysis, can be useful for revealing the identity of the perpetrator (Prinz et al., 2001). In mixtures consisting of relatively equal DNA contributions from each donor, quantitation is useful to predict autosomal STR and Y-STR profile results.

DNA mixtures often occur in forensic cases and have proven to be difficult to interpret. The common methods of evaluating the presence of a mixture are time consuming and tedious or nonspecific and incomplete, and the new real-time PCR

methods use specialized instrumentation, require additional training, and only provide a limited amount of information about mixture composition. Quantitative template amplification technology (Q-TAT) is a method designed to quantitate human DNA using the Amelogenin locus on the X and Y chromosomes. Q-TAT uses common DNA profiling equipment and procedures, and has been shown to be a reliable method for quantitating DNA in single source samples (Allen and Fuller, 2006). Because the products amplified from the Amelogenin locus on the X and Y chromosome differ in size, Q-TAT also has the potential to individually quantitate male and female contributions of DNA present in mixtures. The purpose of this study was to **develop a method for evaluating the relative proportions of male and female DNA recovered from forensic evidence consisting of mixtures**. The specific aims of this project were:

- To verify that Q-TAT can be used to quantitate single source male and female DNA samples with equal accuracy.
- To determine whether Q-TAT can accurately estimate the proportions of male and female DNA in biological mixtures of known proportions.
- To determine Q-TAT's ability to resolve the relative proportions of male and female DNA in non-probative sexual assault samples donated by the Tulsa Police Department Laboratory (TPD).

II. REVIEW OF LITERATURE

II.A. Need for Quantitation

Quantitation of human chromosomal DNA recovered from biological evidence is a necessary step for forensic DNA typing. Optimal amplification of polymorphic DNA markers of the short tandem repeat (STR) variety is dependent on a fairly narrow range of input DNA template (Kline et al., 2005), mandating an analyst know the concentration of human genomic DNA recovered from a biological sample. Not only is quantitation important for ensuring the quality of typing results, but standards enforced by agencies that accredit forensic DNA typing laboratories mandate the quantitation of human DNA recovered from a sample before it is subjected to further testing. It is important that only the minimum amount of DNA sample be used in order to preserve the maximum amount of evidence for re-testing and confirmation purposes if desired.

II.A.i. Regulations

Standards for forensic DNA testing labs set forth by the Federal Bureau of Investigation (FBI) require that quantitation of DNA be conducted to determine the amount of DNA recovered by extraction. Standard 9.4.2.1 (DNA Advisory Board, 2000) states that "laboratories must use quantitation standards which estimate the amount of human nuclear DNA recovered by extraction." Not only is quantitation of DNA samples mandated by the FBI, but it is also required for accreditation by a number of

agencies. The American Society of Crime Laboratory Directors/Laboratory Accreditation Board (ASCLD-LAB) and the National Forensic Technology Center (NFSTC) audit quality assurance of labs and mandate the quantitation of human DNA recovered from evidentiary items that will be subjected to forensic DNA analysis (Allen and Fuller, 2006).

A variety of sample types are recovered from crime scenes and submitted for DNA analysis. Depending on the type and amount of sample submitted, varying amounts of DNA will be recovered. Small sample sizes or high levels of degradation can lead to low recovery of genomic DNA suitable for STR typing. A method must therefore be in place to determine exactly how much DNA was recovered from an item in order to optimally produce an STR profile. Quantitation requirements are in place to 1) ensure the quality of DNA-STR profiles, especially when dealing with multiplexes that are dependent on the quantity of DNA template used, 2) ensure specificity for human DNA, and eliminate the inaccuracy presented by non-specific contaminants, and 3) allow for the preservation of as much template as possible, should repeat testing be requested (Allen and Fuller, 2006).

II.A.ii. Quality Results

One of the biggest concerns in forensic DNA labs is achieving accurate DNA genotyping results while conserving as much sample as possible. By quantitating the amount of human DNA in a sample, accurate results can be achieved initially without wasting excess sample material. Too much DNA added to an amplification reaction can lead to off-scale fluorescent signal and a variety of polymerase chain reaction (PCR)

artifacts, including: allelic or locus imbalance in the multiplex PCR amplification, incomplete adenylation of PCR products, and enhanced strand-slippage or "stutter" of various forms (Kline et al., 2005). Too little DNA can result in stochastic amplification artifacts that cause imbalance within and between loci and even allele dropout (Kline et al., 2005). By quantitating the amount of DNA recovered from a sample before initiating STR typing, many of these problems can be eliminated.

II.B. Quantitation Methods

Quantitation methods that have routinely been used in forensic DNA laboratories to quantitate the amount of DNA present in an extract continue to evolve. Newly developed methods have increased the sensitivity and specificity for human genomic DNA quantitation. The production of reliable estimates of DNA concentrations results in the production of DNA profiles of high quality.

II.B.i. Current and Historical Methods

II.B.i.a. Spectrophotometry

Ultraviolet (UV) spectrophotometry is a standard method often used for the quantitation of nucleic acids. A spectrophotometer produces an optical density (OD) reading for the sample at a particular wavelength of UV or visible light. Nucleic acids absorb UV light maximally at a wavelength of 260nm (Molecular Diagnostic Lab Manual, 2005). However, all nucleic acids absorb at this wavelength and, as a result, spectrophotometry is unable to distinguish the amount of DNA in a sample from any RNA that maybe present. Hence, the concentration of DNA calculated by UV

spectrophotometry is not a specific representation of the amount of DNA present in the sample.

The ratio of absorption of UV light at 260 nm and 280 nm can be used to evaluate the purity of a DNA sample (Maniatis et al., 1982). Proteins absorb UV light maximally at 230 nm and 280 nm (Manchester, 1996). Purity of a DNA sample can therefore be estimated by dividing the absorbance of a DNA sample at 260 nm by the absorbance at 280 nm. If this ratio is below 1.6, the sample is probably contaminated with proteinaceous material and an accurate quantitation of nucleic acids in the sample is unlikely to be produced. If the 260/280 ratio is greater then 2.5, an accurate DNA quantitation is unlikely due to interference from RNA contamination of the sample (Molecular Diagnostic Lab Manual, 2005). If the 260/280 ratio is close to 1.8, the DNA is reasonably pure and its concentration can be accurately determined from the absorbance value. The quantity of DNA in a sample is equal to the absorbance at 260 nm using the constant of 1.0 A260 unit equal to 50 μg/mL of double stranded DNA (Maniatis et al., 1982).

Although this quantitation method has been used in DNA laboratories for many years, it is not a specific method of DNA quantitation. Technologies used in forensic testing laboratories demand a more sensitive and specific method for quantitation. UV spectrophotometry is only able to provide a rough estimate of the amount of DNA present in a sample. This method is also not able to provide any information as to the state of degradation or as to whether the DNA present is human or non-human.

II.B.i.b. Yield Gels

Yield gels also have a long history in DNA typing laboratories. Yield gels have been used to determine both the quantity and quality of DNA extracted from evidentiary and reference samples. A yield gel is simply an agarose gel in which a collection of DNA samples of known concentration, such as lambda DNA, are co-electrophoresed with samples of unknown quantity. Following electrophoresis, an intercalating agent, such as ethidium bromide, is added to the gel to stain the DNA. When the gel is exposed to UV light an analyst can visually compare the relative fluorescence in genomic DNA bands to the intensity of lambda DNA in the reference standards (Virginia Department of Forensic Science, 2006). The intensity of the fluorescence observed in each lane is roughly proportional to the amount of DNA present in each gel track (Allen and Fuller, 2006). Therefore, the samples with known quantities of lambda DNA are used for comparison to estimate the approximate concentration of DNA in unknowns.

Because yield gels separate DNA by size, degradation that may be present in the sample can also be revealed to an analyst. While yield gels quantitate and evaluate the integrity of DNA in the sample, the method lacks specificity. Yield gels do not distinguish between human and non-human DNA. Evidence collected from crime scenes are commonly contaminated by environmental microorganisms such as bacteria, fungi, and yeast. DNA from these organisms can be co-extracted with any human DNA that may be present. Therefore, in heavily contaminated samples the quantity of human DNA recovered may be overestimated. Moreover, based on results from a DNA Quantitation Study conducted by the National Institute of Standards and Technology (NIST) in 2004,

yield gels are too insensitive to give reliable information, even on DNA samples of fairly high concentration (Kline et al., 2005).

II.B.i.c. Hybridization Methods

To overcome problems associated with non-specific quantitation methods like yield gels or spectrophotometry, dot and slot blot methods have been routinely used by forensic laboratories for the specific quantitation of human DNA (Butler, 2001). A commercially available kit for slot blot quantitation commonly used by crime labs is the QuantiBlot Human DNA Quantification Kit available from Applied Biosystems (Foster City, CA). The QuantiBlot kit was developed from original work reported by Walsh et al. (1992).

To perform slot-blot quantitation, DNA samples are immobilized on a nylon membrane and then hybridized to a biotinylated oligonucleotide probe complementary to a primate-specific, alpha satellite DNA sequence (D17Z1) (Walsh et al., 1992; Applied Biosystems, 2004). Following hybridization and washing of the membrane, it is soaked in a solution containing streptavidin conjugated horseradish peroxidase, which ultimately allows for colorimetric or chemiluminescent detection and quantitation of bound probe (Walsh et al., 1992). Similar to yield gels, the signal intensity of DNA in an unknown sample bound to the membrane can be compared against the signal intensity produced by a known standard, allowing concentration estimates to be made (Walsh et al., 1992). Walsh et al. (1992) automated slot blot methods somewhat by describing a computerized method for estimating concentrations in unknowns when using chemiluminescent

detection. Since chemiluminescent detection involves the use of X-ray film, films could be scanned and silver grain density patterns captured by computer.

QuantiBlots are able to detect primate-specific DNA in a range of 0.15 ng to 10 ng within a two to three hour time period (Applied Biosystems, 2004). While the sensitivity and specificity of QuantiBlot is better than with yield gels, QuantiBlots do not give an indication of the quality of DNA present in the sample and have been reported to underestimate the quantity of nuclear DNA in degraded samples and samples with high levels of microbial contamination (Timken et al., 2005). In addition, slot blots provide no predictive information on the existence of inhibitors that might interfere with the PCR, (Timken et al., 2005), do not utilize instrumentation and technology commonly used for genotyping, and are not amenable to extensive automation.

II.B.ii. New Methods for DNA Quantitation

II.B.ii.a. End Point PCR

The need to increase throughput in forensic DNA genotyping has led to growing interest in developing new DNA quantitation methods that are more efficient than the UV spectrophotometry, yield gel and slot blot hybridization methods currently used in many forensic DNA labs (Timken, et al., 2005). A new method for quantitation, recently developed in this laboratory involves quantitation of human genomic DNA through quantitative amplification of the Amelogenin locus (Allen and Fuller, 2006). This method known as quantitative template amplification technology (Q-TAT), estimates the quantity of human DNA present in an extract by comparing the relative fluorescence in X and Y amplicons amplified from samples of unknown concentration with counterparts

produced from DNA samples of known concentration. Thus, standard DNA of known quantity is serially diluted and amplified to produce a standard curve of X and Y amplicon fluorescence which can then be used to estimate the concentration of DNA in samples of unknown quantity.

Fluorescently labeled primers direct the amplification of the Amelogenin locus present on both the X and Y chromosomes. The X amplicon is six basepairs shorter (210 bp) than the amplicon produced from the Y chromosome (216 bp), and thus X and Y chromosome amplicons can be distinguished electrophoretically (Allen and Fuller, 2006). Quantitation of fluorescence in X and Y products is achieved using a charge-coupled device (CCD) camera that is part of a genetic analyzer.

The ABI 310 Gene Analyzer (Applied Biosystems, Foster City, CA) is especially suited to separate and quantitate fluorescent DNA molecules. Fluorescently labeled DNA fragments can be separated with high resolution (< 1 bp) by capillary electrophoresis. As electrophoresis occurs, the fluorescent dyes incorporated into the DNA amplicons are excited by a laser and their fluorescent emissions detected by a CCD camera. Fluorescence associated with PCR products is therefore captured as a function of electrophoretic migration. The migration of the PCR products can be compared to the migration of a collection of fluorescently labeled size standards which are mixed and co-electrophoresed with amplicons in each sample. The size standards allow for precise estimation of the size of products amplified from input template.

GeneScan software supplied with the ABI 310 Gene Analyzer calculates the amount of fluorescence, expressed in relative fluorescent units (RFU), for the peak height and area of each DNA fragment. This information, within limits, is proportional to the

amount of amplicon present in a sample, which, in turn, is proportional to the quantity of template DNA added initially to the PCR reaction. Thus in the Q-TAT method, the peak area from X and Y amplicons amplified from the serially diluted standard reference DNA are used to form a linear plot which can then be used to estimate samples of unknown concentration (Allen and Fuller, 2006).

Unlike other quantitation techniques, Q-TAT uses the same methodology as STR typing. This is advantageous because it precludes the need for new instrumentation, additional training of technicians, or added quality assurance requirements. Q-TAT is able to detect 20 to 500 pg of human DNA which is more sensitive than QuantiBlot but less sensitive than real-time PCR techniques described below (Allen and Fuller, 2006). In fact, the principle limitation of Q-TAT is that its dynamic range is only 20-30 fold.

When comparing Q-TAT to QuantiBlot, there is reasonable agreement in estimates of human DNA concentration, the greatest disagreement occurring when quantitating either very high or very low quantities of DNA (Allen and Fuller, 2006). When looking at Q-TAT in relation to real-time PCR as DNA quantitation methods, a reasonable agreement was also observed. However, the real-time PCR estimates were consistently higher (Allen and Fuller, 2006). Since Q-TAT amplifies the Amelogenin locus found on the X and Y chromosomes, it has the potential to quantitate male and female DNA found in mixed samples (Allen and Fuller, 2006).

Another end point PCR method used for DNA quantitation is an Alu-based assay developed by Sifis, et al. (2002). Alu sequences in genomic DNA are primate specific and make up 5 to 10% of the human genome (Sifis et al., 2002). Alu sequences are well conserved among primates, and PCR methods to detect them do not cross-react with non-

primate DNA. The PCR assay developed by Sifis et al. (2002) has been shown to have a dynamic range for human DNA quantitation of 100 pg to 2.5 ng (Sifis et al., 2002). The assay incorporates labeled primers designed to produce a 229 bp PCR product from within the 282 bp consensus Alu sequence (Sifis et al., 2002). The products are analyzed with an ABI Prism 377 Gene Analyzer coupled to GeneScan software used for sizing and fluorescent quantitation (Sifis et al., 2002).

Like Q-TAT, a standard curve is produced from samples of known concentration and used for estimating DNA concentration in unknowns. Because Q-TAT and the Alu-based assay use PCR amplification, any PCR inhibitors that maybe present in a sample, will interfere equally with both DNA quantitation and DNA typing (Sifis et al., 2002; Allen and Fuller, 2006). In addition, since amplicons from the Amelogenin and Alu assays are similar in size to typical STR alleles, both assays give an indication of the extent of any DNA degradation that may exist in a sample (Sifis et al., 2002; Allen and Fuller, 2006). The one advantage of Q-TAT over the Alu assay is the ability of Q-TAT to determine the sex of the sample donor, and possibly in mixed male and female samples, the relative proportion of the two DNAs present in the mixture.

II.B.ii.b. Real-Time PCR

Another new method for quantitation, with an increased level of sensitivity, is real-time, quantitative PCR. The introduction of real-time PCR methods made it possible to accurately quantitate human DNA during PCR amplification without the need for post-PCR analysis (Tringal et al., 2004). There are numerous published reports describing

real-time procedures as well as commercially available kits that target various DNA loci in genomic DNA.

Two such commercially available kits are the Quantifiler Human DNA Quantification kit, which targets the total human telomerase reverse transcriptase gene (hTERT), and the Quantifiler Y Human Male DNA Quantification kit, which targets the sex-determining region Y gene (SRY) on the Y chromosome (both available from Applied Biosystems, Foster City, CA). Both of these kits are described as producing reliable and reproducible results, thereby minimizing the need for repeat STR analysis (Applied Biosystems, 2005; Green et al., 2005).

The technology used by the Quantifiler kit is based on the 5' nuclease assay characteristic of Taq DNA polymerase (i.e. TaqMan assay (Livak, 1999)) (Applied Biosystems, Foster City, CA). The Quantifiler methodology claims to be able to quantitate human DNA in the range of 0.023 ng/μL to greater than 50 ng/μL (Green et al., 2005). These kits offer sensitive, reliable methods for quantitation with minimal labor, accelerated time-to-results, no sample transfer, and the potential for automation (Applied Biosystems, 2005).

Additional targets selected for real-time PCR amplification and analysis are Alu (Nicklas and Buel, 2003; Nicklas and Buel, 2005), Amelogenin (Alonso et al., 2003; Alonso et al., 2004; Alonso and Martin, 2005), THO1 (Tringal, et al., 2004; Timken et al., 2005), and the Retinoblastoma gene (RB1) (Andreasson et al., 2002). Some real-time assays have even been developed to analyze nuclear DNA and mitochondrial DNA simultaneously (Andreasson et al., 2002; Alonso et al., 2004; Von Wurmb-Shwark et al., 2004; Timken et al., 2005).

Targeting Alu sequences allows DNA quantitation using less template or more dilute samples because it is not a single copy gene like other targets, and is instead present in multiple copies in the genome (Sifis et al., 2002; Nicklas and Buel, 2005). The Alu-based assay described by Nicklas and Buel (2003) is reported to be sensitive, specific, fast, quantitative, and has a dynamic range of 1 pg to 16 ng. In a more recent publication (Nicklas and Buel, 2005), minor grove binding (MGB) Eclipse probes (Epoch Biosciences, Bothell, WA) have been used as molecular beacons to improve the assay's dynamic range from 0.5 pg/μL to 128 ng/μL. MGB probes contain a 5' minor grove binder, coupled to the 3' fluor and 5' quencher, that assists in binding the probe to the PCR product (Afonina et al., 2002).

The Amelogenin locus, like Alu sequences, has been the amplification target of both end point PCR and real-time PCR assays. An Amelogenin real-time PCR assay developed by Alonso et al. (2004, 2005) produces amplicons of 106 bp from the X chromosome and 112 bp from the Y chromosome. The amplicons produced by the real-time assay are designed to be shorter than the ones produced by the end point assay. However, the X and Y amplicons can both be separated and identified because of the six bp difference, allowing these assays not only to be used for quantitation but also for sex determination.

Like the real-time Alu assay, the Amelogenin assay uses MGB probes to target the six bp X deletion/Y insertion and help improve dynamic range. With this assay, allele drop out was observed with DNA input of less than 60 pg (Alonso et al., 2004). Quantitation data produced with slot blots and the Amelogenin real-time assays produced concordance of results 70% of the time for samples between 30 pg to 1 ng (Alonso et al.,

2004). Thirty percent of samples found to be devoid of DNA using slot blots were scored as positive for DNA using real-time PCR. When comparing the real-time assay with end point PCR assays, sex determination was concordant 100% of the time (Alonso et al., 2004).

Andreasson et al. (2002) described a real-time PCR method using the retinoblastoma gene (RB1) as the amplifiable target for nuclear DNA. Like other real-time PCR assays, this one has also been shown to be very sensitive. In most experiments, the target could be detected down to a single copy and when 0.1 DNA copy was analyzed, the assay had a success rate of 44% (Andreasson, et al., 2002).

Andreasson et al. (2002) chose to use a short target sequence in order to detect the total amount of DNA present in the sample, including degraded fragments. This method is highly sensitive and has a large dynamic range; however it is not as informative as other real-time methods since degraded DNA will produce results. In addition, the RB1 target has not been shown to be primate-specific and thus the assay will cross-react with DNA from other species (Timken et al., 2005).

Another target for real-time PCR quantitation is the THO1 STR locus (Tringal et al., 2004; Timken et al., 2005). The authors claim the THO1 locus is an ideal target because it is one of the loci included in commercial STR typing kits, such as Identifiler (Applied Biosystems, Foster City, CA) and PowerPlex 16 (Promega Corp., Madison, WI). Because the quantitation method is used to determine the amount of template for input into STR typing assays, using an STR target could directly predict the success of STR genotyping for the sample (Tringal et al., 2004; Timken et al., 2005).

The assay developed by Timken et al. (2005) adapts the method described by Andreasson et al. (2002) to target a 170 bp – 190 bp sequence that spans the THO1 repeat region. Validation studies have shown this assay to be sensitive and precise down to 10 - 15 genome copies, have a dynamic range spanning three orders of magnitude, and exhibit no cross-reactivity with non-human DNA templates. The assay developed by Tringali et al. (2004) has a dynamic range from 25 pg to greater then 100 ng, with a correlation coefficient of 0.983 and a PCR efficiency of 99.9%.

When looking at DNA samples ranging from undegraded to moderately degraded, the results with the THO1 assay were similar to slot blot and Quantifiler. However for highly degraded samples, this method produced more accurate results. Quantifiler tended to overestimate the amount of DNA present, while the slot blot method showed an underestimate (Timken et al., 2005). Therefore for degraded samples the THO1 target sequence leads to improved STR typing results compared to Quantifiler and the slot blot technique.

Quantifiler's 62 bp target sequence most likely resulted in an overestimate of DNA because it was able to amplify both degraded and intact DNA in the sample. The THO1 assay uses a longer target and therefore only quantifies the amplifiable DNA in a sample (Tringal et al., 2004; Timken et al., 2005). For the purpose of STR typing, it is best to quantify only the amount of DNA that can be amplified and used to produce and STR profile, not the total DNA in the sample.

II.C. Analysis of DNA Mixtures

DNA samples consisting of mixtures provide a special challenge to a DNA analyst and the current methods for dealing with mixed DNA profiles have a number of limitations. Current methods designed to simplify the analysis of mixtures include procedures applied during DNA extraction (ie pre-analysis strategies) and procedures applied during the data analysis phase of the STR typing process (Butler, 2005). If an evidentiary sample consists of a mixture of male and female DNA, the relative proportions of male and female contributions to the sample will only be apparent after the STR profile has been produced and the typing process has reached the data analysis stage (Butler, 2005).

If there is limited sample available, knowing that a female:male mixture exists and also knowing the relative contributions of DNA to the mixture would allow an analyst to make decisions on how best to proceed with DNA typing to make the best use of the DNA available from an evidentiary sample. There are several DNA typing approaches that can be taken to produce a DNA profile from a limited sample of sexual assault evidence. However, in order to choose among the options, an analyst must 1) know that a mixture exists and 2) know the total amount of DNA in the sample, and 3) be aware of the relative contributions of male and female DNA present in the mixture.

The analysis of mixtures in sexual assault evidence can be simplified somewhat by using a specialized DNA extraction method known as differential extraction (Gill et al., 1985; McElfresh et al., 1989; Butler, 2005). The differential extraction methods exploit differences in DNA packaging in sperm and epithelial cells that allow separation of male and female contributions of DNA to a forensic sample that then allows profiling

of the DNA extracts individually. Differential extraction however, does not provide completely predictable levels of separation of male and female DNA. Often, the female fraction is contaminated with varying amounts of male DNA and occasionally, there will be female DNA present in the male fraction.

Moreover, bloodstain evidence consisting of female:male mixtures is not suitable for differential extraction and DNA from such evidence is still most efficiently processed if the relative proportion of DNA from the two donors is known prior to amplifying STR loci. For this reason, it would be useful for an analyst to be able to quantitate the relative proportions of male and female DNA in suspected mixed samples prior to performing STR analysis. Quantitation methods available to provide such information would allow the DNA analyst to use an optimal technique to produce an informative profile from an evidentiary sample.

II.C.i. Current Methods

II.C.i.a. Differential Extraction

Differential extraction allows for the separation of male and female portions of the sample during the extraction process (Gill et al., 1985; McElfresh et al., 1989; Butler, 2005) when performing DNA extraction on samples consisting of epithelial cells (from the victim) and spermatozoa (from a sexual assailant). The variations in DNA packaging in these cell types make them differentially sensitive to detergent and protease activity. In this method, the sample is first digested with proteinase K and sodium dodecyl sulfate (SDS) to rupture the epithelial cells and allow the DNA originating from a female victim to be released into solution. The sperm cells are not solubilized under these conditions

and will remain intact and can be recovered by centrifugation. A more stringent lysis buffer including proteinase K, SDS, and dithiothreitol (DTT) is then added to disrupt disulfide crosslinks reinforcing the sperm capsid and digest the sperm cells (Gill et al., 1985).

Because this process is time consuming and labor intensive, another approach has been applied to the separation of epithelial and sperm cells. This separation method also exploits the physical properties of sperm versus epithelial cells. A microfluidic device allows the epithelial cells to settle to the bottom of an inlet reservoir and adhere to a glass substrate. Using low flow rates, the sperm cells can be separated from the epithelial cells in the mixture (Horsman et al., 2005). Once the cells are separated with the microfluidic device, they can be extracted by conventional laboratory procedures. Even though the differential extraction techniques often succeed in separating most of the male sperm cells from the female epithelial cells, mixed profiles still persist and are a common problem for sexual assault cases (Prinz et al., 2001).

II.C.i.b. Y-Chromosome Analysis

The biggest problem for PCR typing on rape evidence is the generation of mixed profiles due to insufficient separation of sperm and epithelial cells during differential extraction or possibly due to the presence of more than one semen donor (Prinz et al., 2001). Since 1998, typing of STR markers residing on the Y chromosome (Y-STR markers) has been a valuable addition to the forensic DNA panel when dealing with samples containing low amounts of male DNA sometimes seen in male/female mixtures present in sexual assault cases.

When looking at male/female DNA mixtures, Y-STRs are advantageous because the Y chromosome lacks a homologous chromosome in the female (Prinz et al., 2001). Therefore, any Y-STR alleles visualized can be attributable to an alleged assailant. Any chromosomal DNA in the sample from the female victim will be ignored by the assay. Y-STR testing also has the ability to provide profiles on highly degraded samples containing male DNA.

A validation study was conducted by Prinz et al. (2001) following suggestions from the Technical Working Group DNA Analysis Methods (TWGDAM) to evaluate the detection limits of Y-STRs in mixed samples. Various ratios of male:male and male:female mixtures of known composition were produced for analysis. For male:male ratios the total amount of DNA template used was 2 ng, and for male:female mixtures the male component was held constant at 400 pg and combined with increasing amounts of female DNA. For the male:female DNA mixtures it was possible to detect the male component even with the highest ratio of female DNA, 1 male:4000 female (Prinz et al., 201). The male:male mixture results depended on the allele peak positions because of a degree of stutter for some alleles. If stutter was not a problem, the minor component could be detected at a ratio of up to 1:50; this could decrease as low as 1:10 with stutter peaks (Prinz et al., 2001).

Although Y-STR typing provides an additional tool for the evaluation of male contributors in mixed samples, there are also limitations inherent with this DNA analysis approach. One limitation is the inability of Y-STR typing to discriminate among male relatives. All males descended from a common patrilineal line will share the same Y-STR genotype. Another limitation relates to the tight linkage exhibited among alleles at

the different loci on the Y chromosome mandating the use of haplotypes rather than genotypes for statistical analysis. Since there is no homologue for the Y chromosome to recombine with during meiosis, STR alleles on the Y chromosome must be combined into haplotype frequencies for statistical interpretation of Y-STR typing results.

Population substructure is not a significant concern when typing autosomal STR alleles; however, because of the stability exhibited by haplotypes in different ethnic groups, it is a significant concern for Y-STR results. Thus, the prevalence of certain Y-STR profiles in specific population groups and subgroups due to founder effects and substructure (Redd et al., 2006) can lead to erroneous estimates of haplotype frequencies for a suspect included as the perpetrator of a crime. In addition, the magnitude of an inclusion estimate from a Y-STR profile will typically not achieve the same level of certainty as a match produced from 13-15 autosomal STR loci. Therefore, Y-STR typing is not as powerful a tool for identification as a collection of autosomal STR markers.

II.C.ii. New Methods

Recent studies by the National Institute of Standards and Technology (NIST) indicated that the accuracy of DNA quantitation significantly impacts the quality of STR typing, particularly when examining mixtures (Kline et al., 2005). Real-time PCR and end point PCR that target the Amelogenin locus are highly specific and sensitive assays that can be used for quantitation and sexual identification. Male and female body fluid mixtures are common forensic samples of high interest for analysis of sexual assault. The possibility of having an accurate estimate of male and female contributions of DNA in a mixed sample before performing STR analysis could be of great help in deciding on

a strategy that will maximize the chance for success for the detection of the male DNA profile from a sample that may contain an excess of female DNA (Alonso et al., 2004).

Of the quantitation methods previously described in this paper, the assays targeting the Amelogenin locus (Alonso et al., 2004; Allen and Fuller, 2006) have the potential to identify and characterize male and female mixed samples. The Q-TAT end point PCR method and the Amelogenin real-time technique are the only two assays that have the ability to simultaneously evaluate X and Y amplicons (Alonso et al., 2004; Allen and Fuller, 2006). Both of these methods therefore hold promise to quantitate male and female proportions in mixed samples. To date however, neither method has yet been evaluated for this purpose.

Q-TAT has an advantage over the real-time method because it uses the same methodology as STR typing; it does not require new instrumentation, training, additional allocation of space, or added quality assurance requirements. The Q-TAT method accurately quantitates human chromosomal DNA so as to ensure the production of an STR profile of high quality (Allen and Fuller, 2006). Q-TAT will not produce overestimates or underestimates of the amount of amplifiable DNA because inhibitors or degradation affecting the Q-TAT assay will affect the STR analysis to the same extent.

In contrast, real-time PCR technology uses dedicated instrumentation that is not commonly found in forensic laboratories. In addition, real-time PCR procedures require analysts to be trained and proficiency tested in this novel technology, which also requires additional quality assurance measures (Allen and Fuller, 2006). Moreover, the only commercially available kit for real-time quantitation of Y chromosomal DNA detects only the Y chromosome (Applied Biosystems, 2005). Thus, one kit available must be

used to estimate total DNA and another must be used to detect Y DNA. Subtracting the Y chromosomal DNA from the total presumably leave the female portion.

The major advantages of real-time methods are increased sensitivity and greater dynamic range. With real-time PCR, the analyst is able to view the accumulation of product after each PCR cycle. Also, because no post-PCR analysis is needed, the PCR tubes are never opened and the potential for post-PCR contamination reduced.

There are advantages and disadvantages to both the end point and real-time PCR techniques. However the potential ability to distinguish relative proportions of male and female contributors in mixed forensic samples gives both of these methods an advantage over other quantitation methods.

III. METHODOLOGIES

III.A. Method Background

III.A.i. Samples

The overall goal of this study was to assess the ability of the Q-TAT assay to accurately quantitate relative proportions of male and female DNA in mixed samples. To that end, one approach was to use the Q-TAT assay to estimate male and female DNA concentrations in mixed samples of known proportion. As a first step in this experimental approach, a panel of well characterized, single source male and female samples were produced and used to prepare a collection of mixed samples of known proportions. Ten single source male and ten single source female samples had their DNA concentrations estimated using yield gels. Any sample showing signs of DNA degradation based on yield gel analysis was eliminated from the study and substituted with another sample that was intact. In addition to assessing integrity, yield gels were also used to roughly quantitate DNA in samples which were more precisely quantitated using the Q-TAT assay. Only samples for which estimates produced by yield gel and Q-TAT were in agreement were used for further studies.

All samples used in these experiments were previously extracted from either blood or buccal swabs. In most cases, an organic extraction method was used to isolate the DNA from the sample. However, because the samples used were anonymous, the specific source of the initial sample and the extraction method used to recover DNA is not known. Having produced the repository of well characterized male and female

samples, mixtures of known proportion were then made ranging from 1 part female:1 part male to 1 part female:10 parts male and 10 parts female:1 part male.

Samples used in this study also consisted of non-probative evidentiary samples obtained from the Tulsa Police Department Laboratory (TPD). All samples were non-probative, anonymous, and contained unknown quantities of male and female DNA. The evidentiary samples were generally derived from sexual assault cases and consisted of vaginal swabs or stains on clothing. The exact source of each sample was unknown to this laboratory. All DNA samples obtained from TPD were isolated by organic DNA extraction methods.

III.A.ii. Techniques

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

The single source samples, known mixtures, and unknown evidentiary samples provided by TPD were quantitated using the Q-TAT method using published methods (Allen and Fuller, 2006). The Amelogenin locus is the PCR target for the Q-TAT assay and is present on both the X and Y chromosomes. The X amplicon amplified using the assay is 210 bp whereas the Y amplicon is 216 bp in length (Allen and Fuller, 2006). Because of the six bp length difference in the X and Y amplicons produced using the Amelogenin primers, male and female contributions to mixed samples can be detected and possibly quantitated accurately.

X and Y amplicons were produced through PCR amplification of genomic DNA samples using fluorescently labeled primers targeting the Amelogenin locus (upstream primer - 5'-ACCTCATCCTGGGCACCCTGG–3', downstream primer – 5'-AGGCTTG

AGGCCAACCATCAG–3'). The amplification primers used for this project were synthesized by Invitrogen (Chicago, IL) and are identical in sequence to those included in the sex typing kit commercially available from Promega Corp. (Madison, WI). For most of these studies, the primers synthesized by Invitrogen had the 5' end of the downstream primer labeled with fluorescein (FAM). Based on previous studies using upstream and downstream fluorescein-labeled Amelogenin primers, no significant difference in the qualitative or quantitative characteristics of the amplicons was observed (Pogemiller, 2006).

For each PCR sample, 1 μL of template DNA was combined with 1 μM of forward and reverse Amelogenin primers (Invitrogen, Chicago, IL), 1X Gold ST*R buffer (Promega, Madison, WI), and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a 12.5 μL reaction. The additional volume was made up with UV treated ultrapure water. The primer mix, buffer solution, and Taq polymerase were combined to form a master mix that was aliquotted into tubes containing the template DNA.

In order to determine the quantity of DNA present using the Q-TAT method, a standard curve was prepared using dilutions of a DNA reference sample of known concentration. The standard curve was produced by amplifying the Amelogenin locus in five dilutions of the reference male DNA (31.25 pg, 62.5 pg, 125 pg, 250 pg, and 500 pg). The fluorescence contained within X and Y amplicons is proportional to the amount of amplicon produced during PCR, which in turn, is proportional to the input amount of DNA template added to the PCR reaction initially. A new standard curve was produced for each experimental run.

To prepare the template used to produce the standard curve, the reference sample (at 50 μg/μL) was diluted 1:100 with UV treated ultrapure water to produce a 500 pg/μL sample. Two-fold serial dilutions of this sample were then made in ultrapure water to produce the remaining template amounts. One microliter of each diluted DNA standard was then added to the PCR reaction mix. All PCR amplifications were performed on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using the following program (Table1):

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

Table 1: PCR conditions for Q-TAT (Allen and Fuller 2006).

III.A.ii.b. PCR Amplification of STR Loci

The AmpFlSTR Profiler Plus kit (Applied Biosystems, Foster City, CA) was used to produce DNA profiles consisting of STR alleles from nine autosomal loci and the Amelogenin locus. The autosomal loci amplified using this kit include D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820. The multiplex PCR kit consists of primers attached to various fluorescent dyes that direct the amplification of the nine loci plus Amelogenin. Thus, amplicon size and color are combined to distinguish alleles from the different loci. The collection of STR alleles and Amelogenin amplicons together constitute the DNA profile of the sample under analysis.

For each PCR reaction, 0.750 ng to 1 ng of template DNA was combined with 2.75 μL of AmpFlSTR Profiler Plus primer set, 5.25 μL of AmpFlSTR PCR reaction mix

1.25 U of AmpliTaq Gold in a 12.5 μL reaction. The additional volume was made up with UV treated ultrapure water. AmpFlSTR control DNA 9947A is a positive control sample included in the Profiler Plus kit. This sample, along with a negative control consisting of only UV treated ultrapure water and reaction mix, was amplified along with all samples to evaluate the reagents and test for contamination. All PCR amplifications were performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA) using the following program (Table 2):

1 cycle	28 cycles			1 cycle	
Initial Incubation	Melt	Anneal	Extend	Melt	Anneal
95°	94°	59°	72°	60°	10°
11 min	1 min	1 min	1 min	45 min	Hold

Table 2: PCR conditions for Profiler Plus (Applied Biosystems, Foster City, CA).

III.A.iii. Instruments

Following amplification, 1μL of each amplified sample or allelic ladder was added to 24.5 μL of formamide containing 0.5 μL of ROX 350 or ROX 500 internal size standard (Applied Biosystems, Foster City, CA). All of the samples were then placed in a sample rack on the autosampler of an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) for electrophoresis and amplicon analysis (Figure 3). The ABI 310 Genetic Analyzer uses capillary electrophoresis technology to separate the amplicons present in each sample by size. The sample is electro-injected into a polymer filled capillary that separates DNA fragments by size; smaller amplicons move through the

capillary faster and are detected earlier in the run than are larger amplicons. Each run requires 24 or 27 minutes at 60° for Q-TAT and Profiler Plus electrophoresis respectively (Figure 4).

A fluorescently labeled internal size standard, ROX 350 or ROX 500, is included with each sample. Because the size of DNA fragments in the standard is known, their migration during electrophoresis can be used by the GeneScan software (Applied Biosystems, Foster City, CA) in conjunction with a local homology size algorithm (Elder and Southern, 1983) to precisely estimate the size of the unknowns in the sample to within a single base pair of resolution. During amplification, the fluorescent label on one member of each primer pair is incorporated into each amplicon. As that fragment migrates past a window in the capillary, a laser beam excites the flour and the Genetic Analyzer is able to capture and interpret this fluorescent label. The amount of fluorescence detected by the Genetic Analyzer is directly proportional to the amount of amplified DNA present in the sample. Therefore, using GeneScan software, the relative fluorescent units (RFU) for each amplicon can be quantitated and provides a measure of how much PCR product is present.

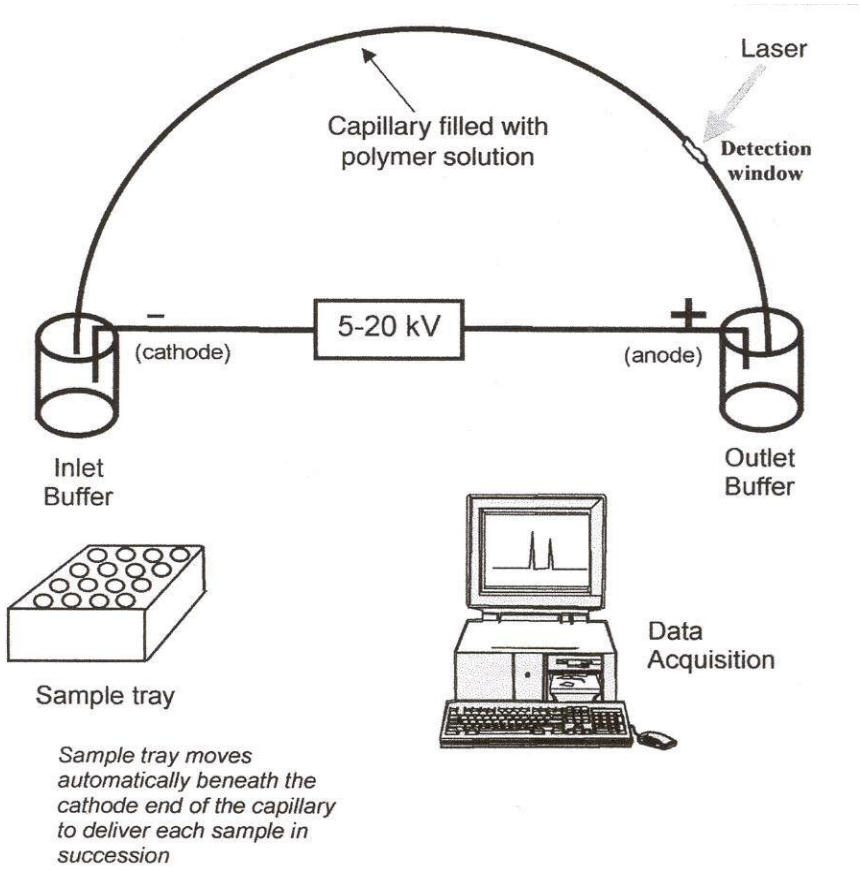

Figure 3: Capillary electrophoresis process (Butler, 2005).

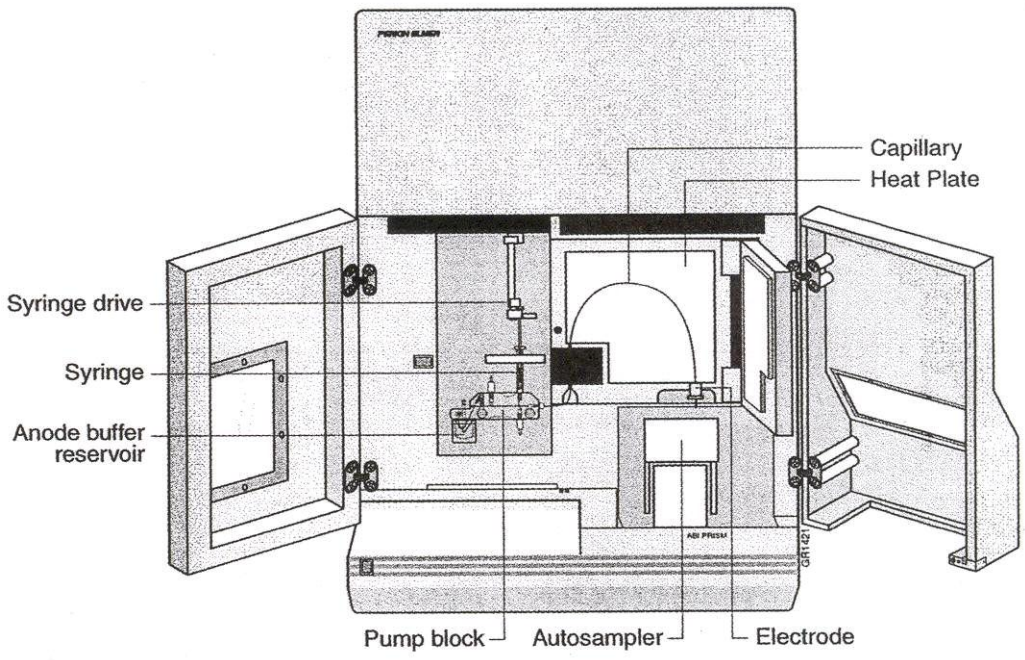

Figure 4: 310 Genetic Analyzer used for capillary electrophoresis (Budowle, 2000).

For the evaluation of single source samples with Amelogenin, the RFUs for the X peak area are proportional to the total amount of female DNA in a genomic DNA sample, whereas the sum of RFUs for the X and Y peak areas reflect the total amount of male genomic DNA in a sample. For mixed samples, the proportion of male and female DNA in the sample is therefore reflected by the X to Y ratio of RFUs.

For the Profiler Plus kit, Genotyper software (Applied Biosystems, Foster City, CA) is used to compare the alleles detected at each locus to the AmpFlSTR Profiler Plus allelic ladder (Applied Biosystems, Foster City, CA) consisting of a mixture of known alleles for each STR locus. The allelic ladder and the unknown samples are electrophoresed under identical conditions on the 310 Genetic Analyzer. The computer based comparison of the allelic ladder and the unknowns allows the software to match

unknowns with alleles in the allelic ladder thereby enabling the software to identify each allele expressed as the number of tandem repeats it contains (Applied Biosystems, 2005).

III.B. Analysis Methods

III.B.i. Part I – Evaluation of Single Source Samples

DNA extracted from samples containing either single source male or single source female DNA was quantitated using yield gel analysis, and, of these samples, ten male and ten female samples with no, or very limited degradation were selected for Q-TAT analysis. The DNA samples were diluted to 200 pg/μL based on the yield gel quantitations. The DNA samples and serially diluted reference standards were then amplified following the standard protocol for Q-TAT quantitation of genomic DNA in each sample (Allen and Fuller, 2006).

The X and Y amplicon peaks were identified and the RFUs contained within each peak were recorded in an Excel spreadsheet. To normalize different injections in a run, the RFUs within the ROX 200 bp peak was used. Since the ROX size standard is present in equal amounts in each sample within a given experimental run, the RFU in each size standard component is expected to be constant. Minor variations in electro-injection from one sample to the next, or CCD or laser fluctuations that could affect fluorescence will affect the ROX fluorescence along with fluorescence of X and Y amplicons. Therefore, normalizing all injections based upon a ROX labeled size standard should also normalize any variability in fluorescein emissions from the Amelogenin products (Allen and Fuller, 2006).

The normalized values for fluorescence in X and Y amplicons in each quantitation standard sample is divided by the known amount of input template DNA. The results are averaged for all standard curve points and a constant is produced. The constant is then multiplied by each normalized RFU value for X and Y amplicons in unknowns to estimate the quantity of DNA in that sample. Each single source sample was run at least two times and the quantities for each run were averaged. The average DNA quantities calculated using the Q-TAT assay were then compared to the initial quantity produced by yield gel analysis. The ratio between the X and Y peak areas was also evaluated to assess any possible imbalance present between the X and Y amplicons produced with this method.

Confirmation of the quantitation estimates for the single source male and female samples was performed with the production of DNA profiles using the Profiler Plus kit as the ultimate measure of the accuracy for the Q-TAT quantitation method. Seven hundred and fifty picograms represents an optimal amount of template DNA to amplify with the Profiler Plus kit (Applied Biosystems, 2005). This amount of DNA, based on the quantity estimate derived from the Q-TAT assay, was added to Profiler Plus amplifications and the resulting STR profiles were evaluated for quality to confirm that the quantity of genomic DNA estimated by Q-TAT was suitable for performing STR analysis with Profiler Plus. The DNA samples were amplified following the protocol for amplification using instructions provided with the Profiler Plus kit and the amplicons were separated by the 310 Genetic Analyzer as described above. Genotyper software was used to analyze the data.

It is known that some imbalance exists between the alleles produced with the commercially available Profiler Plus STR typing kit (Applied Biosystems, 2005). In order to ensure that the imbalance produced for the X and Y amplicons with Q-TAT was similar to that normally seen in STR profiles, the ratio of allelic peak areas was evaluated for the Amelogenin amplicons produced with Q-TAT and Profiler Plus, and also the autosomal alleles produced with the Profiler Plus STR kit. A ratio of the RFU peak areas for the alleles at each locus was computed. Because our proposed use of the X and Y peaks produced with Q-TAT was to be used to evaluate the amount of male and female DNA present in evidentiary samples that may represent mixtures, it is important that any imbalance between X and Y PCR is not significantly higher then the normal minor imbalance observed for alleles produced with commercial STR typing kits.

III.B.ii. Part II – Evaluation of Mixtures of Known Proportions

Five single source male and five single source female samples previously quantitated by Q-TAT were selected for use in preparing mixtures. Before mixing the male and female samples together, samples were diluted to 100 μg/mL based on their Q-TAT quantities to facilitate the mixing process. The first mixture analyzed contained equal parts of male and female DNA. Each of the five male DNA samples were combined in equal volume with each of the five female DNA samples, and each mixed sample was analyzed in duplicate. Amplification reactions were programmed with 200 pg of template and amplified using the Amelogenin PCR protocol as described above. Products were separated on the 310 Genetic Analyzer and analyzed using GeneScan

software. Total human chromosomal DNA (male and female) was calculated using the standard curve and Excel spreadsheets as previously described.

In order to evaluate the accuracy of the Q-TAT method for quantitating the relative proportions of male and female DNA in known mixtures, a ratio examining the RFUs contained within the X peak to the RFUs in the Y peak area was computed. Because males have one X and one Y, and females have two Xs, the molar X/Y ratio of a mixture containing 1 part female and 1 part male would be expected to be 3:1 (3X/1Y). Total fluorescence in X and Y amplicons was entered into an Excel spreadsheet. The X/Y molar ratio was determined by dividing the RFU value of the peak area for the X peak by the RFU value of the peak area for the Y peak.

This approach was repeated for mixtures containing male and female DNA in the following proportions: 3 parts female and 1 part male (7X/1Y), 1 part female and 3 parts male (5X/3Y), 5 parts female and 1 part male (11X/1Y), 1 part female and 5 parts male (7X/5Y), 10 parts female and 1 part male (21X/1Y), 1 part female and 10 parts male (12X/10Y). The X/Y molar ratio calculated by examining the RFUs for the X and Y peak areas was computed for each male and female mixed sample and compared against the expected X/Y molar ratio based on the known input ratio.

The Profiler Plus STR typing kit was also used to analyze female:male mixtures of varying proportions to evaluate the reliability of the Q-TAT procedure based upon the relative fluorescence of autosomal STR alleles produced from the DNA of the two donors. As stated above, the overall purpose of quantitating human DNA is to ensure that optimal STR DNA profiles are produced during PCR amplification. Optimal STR profiles produced from mixed samples are also the normal means by which mixtures are initially

identified (Butler, 2005). Furthermore, the relative proportion of each contribution of DNA to a mixed sample is usually calculated from the relative fluorescence in STR alleles for loci in which the two contributors share no alleles (ie 4 STR alleles are visible). Thus, STR typing of the known mixtures represented the "gold standard" with which to compare the Q-TAT estimates of male and female DNA.

For the Profiler Plus STR typing kit, 750 pg of input DNA was added to each PCR reaction based on Q-TAT quantitation results. The samples were amplified following the Profiler Plus amplification protocol and amplicons were separated and analyzed on the 310 Genetic Analyzer. Loci containing four alleles were the focus for the analysis. The fluorescence in alleles for loci containing four alleles were compared with the X and Y ratios produced with the Q-TAT assay to evaluate the reliability of the quantitation assay for mixed samples. For example, a locus revealing four alleles produced for a mixed genomic DNA template consisting of 5 female:1 male DNA should show two alleles containing about 5 fold more fluorescence than the other two alleles visible; consistent with the 5:1 mixture.

III.B.iii. Part III – Evaluation of Evidentiary Samples

Once the limitations of the Q-TAT method as applied to female:male mixtures were established, the technique was applied to unknown evidentiary samples. Samples obtained from the Tulsa Police Department (TPD) were amplified using the Q-TAT amplification protocol for the Amelogenin locus. Because the amount of DNA in the samples was unknown, 1 μL of template DNA from undiluted, 1:10 diluted, and 1:100 diluted samples were used for Q-TAT PCR reactions. The reactions were analyzed on

the 310 Genetic Analyzer and evaluated with GeneScan software. The total concentration of DNA in the sample was computed and relative male and female proportions were estimated from the X/Y ratio of the peak area RFUs. The template dilution providing the optimal conditions was used for all calculations.

After evaluation with Q-TAT, the total concentrations and relative proportions of male and female DNA present in the sample was compared with values computed by TPD using STR alleles in the profile as described above. TPD used QuantiBlot assays to determine the concentration of each DNA sample and Identifiler STR analysis to evaluate the relative proportions of male and female DNA in the sample. The amount of input template DNA used for Identifiler STR analysis at TPD was based on the QuantiBlot concentration results.

We also repeated the STR analysis using the Profiler Plus kit with the evidentiary samples from TPD to perform our own evaluation of the presumably mixed DNA samples. Based on Q-TAT quantitation results, 750 pg of DNA was added to each PCR and amplified using the Profiler Plus amplification protocol. The amplicons were separated by the 310 Genetic Analyzer and analyzed with Genotyper software. The allele peak sizes and areas were evaluated. The X/Y ratio was calculated for the Amelogenin amplicons produced with Q-TAT, Profiler Plus, and Identifiler. The X/Y ratio produced with Q-TAT was compared to the X/Y ratio produced with the commercially available kits. The input DNA template values for Profiler Plus were based on the Q-TAT quantitation method and input DNA template values for Identifiler were based on QuantiBlot results.

Because STR profiles are typically used for mixture evaluation, the major/minor peak areas for loci containing four alleles were used to produce a major/minor allele ratio. This ratio was compared to the major/minor ratio computed with Q-TAT from RFU values produced from the X and Y products of the Amelogenin locus. STR typing is presently the "gold standard" for the resolution of mixtures and therefore, it is important to ensure that Q-TAT is able to produce similar results.

IV. RESULTS

This study was designed to evaluate the ability of Q-TAT to detect male and female contributors in a mixed DNA sample and to estimate the relative proportions. Quantitation of single source samples was performed using yield gels and Q-TAT to produce a panel of well characterized single source male and female DNA samples for mixture preparation and to confirm the results of the study conducted by Allen and Fuller (2006). Fully characterized single source samples were combined in known proportions to challenge Q-TAT's ability as a quantitation method for the detection and resolution of mixtures. Knowledge obtained from analysis of single source samples and mixtures of known proportions was then applied to non-probative, forensic evidentiary samples.

IV.A. Analysis of Standard Curve

In order to determine the total quantity of human genomic DNA recovered from an evidentiary sample, fluorescence incorporated into Amelogenin products amplified from samples of unknown quantity were plotted against a standard curve of fluorescence incorporated into products amplified from analyzed known quantities of human DNA template. The standard curve is produced by plotting relative fluorescent units (RFUs) against the amount of input reference DNA. Serial two-fold dilutions from 32.5 pg to 500 pg were amplified and their products quantitated and used to create the standard curve. By plotting the RFUs in the total area in X and Y amplicons produced from

samples of unknown quantity against the standard curve, the amount of DNA present in unknowns can be estimated.

The greatest accuracy of the Q-TAT method occurs when unknown samples fall in the range of the standard curve, between 100 and 300 ng. Ideal peak area RFU values range from 10000 to 80000. Extremely high values cause the pixels of the CCD camera to become saturated and low values increase the chance of allele dropout (Pogemiller, 2005). In order to ensure the inclusion of only true Amelogenin peaks, the minimum peak height threshold for detection was set at 50 RFUs.

Shown in Figure 5 are examples of electropherograms containing size standards (in red) and X and Y amplicons (in blue) produced from the different dilutions of reference DNA. Input amounts of human DNA template are noted in the different panels in the figure, as are the locations of X and Y amplicons. The size range (in bp) of DNA fragments in each profile is shown across the top of the figure. In Figure 6, the information from Figure 5 is combined into a typical standard curve, plotting RFU in X and Y amplicons against the input amount of template DNA.

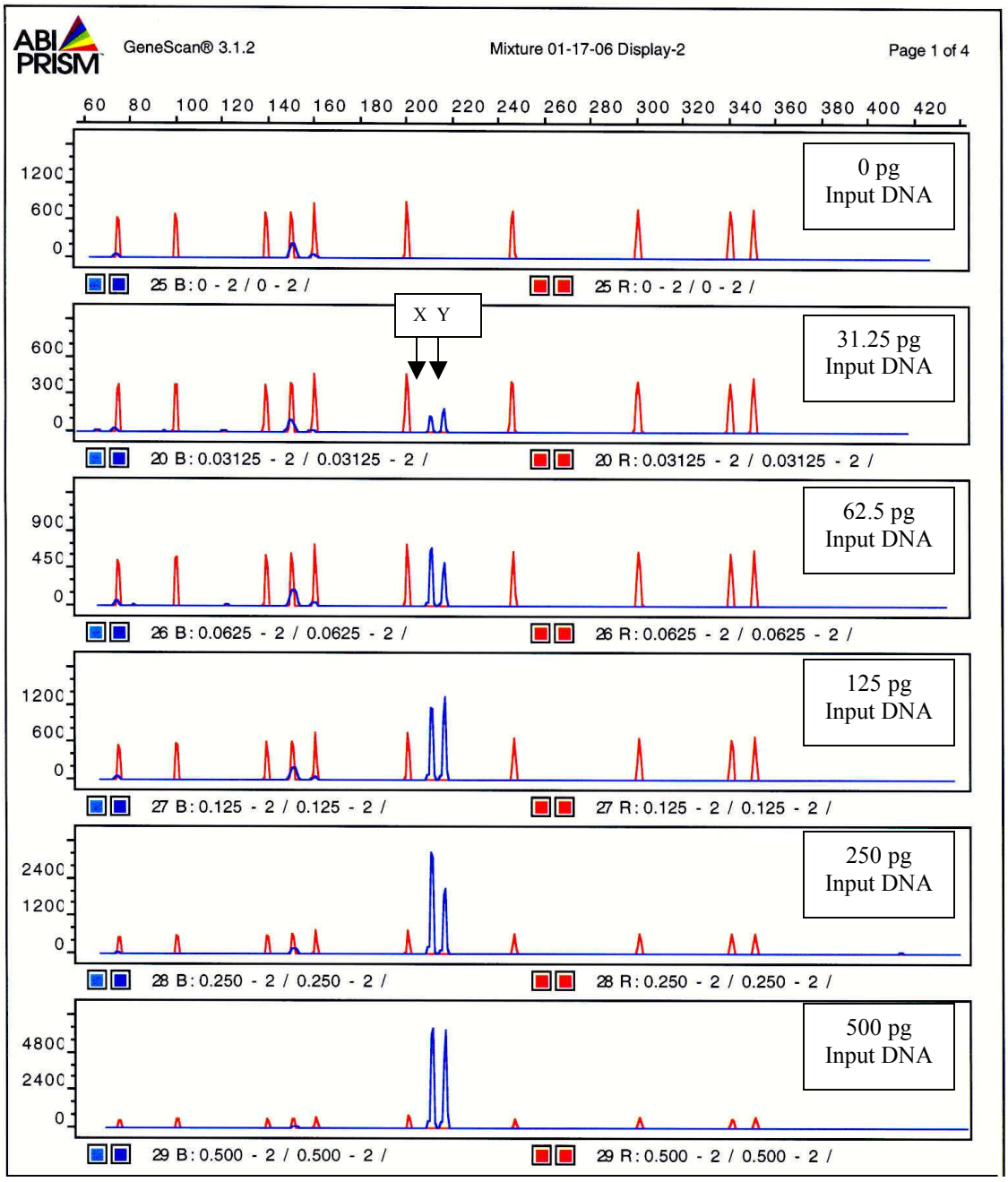

Figure 5: Electropherogram of X and Y Amelogenin peaks used to produce a standard curve. The red peaks represent the internal size standard, labeled with ROX fluor. The blue peaks present at 210 bp and 216 bp indicate the X and Y amplicons produced for the Amelogenin locus respectively. Input amounts of male template DNA are noted in the different panels. The size range (bp) of DNA fragments is shown across the top axis and the RFU value on the left axis. Notice the scale of the axis can change (automatically set by Genetic Analyzer) depending upon the highest fluorescence detected during a run.

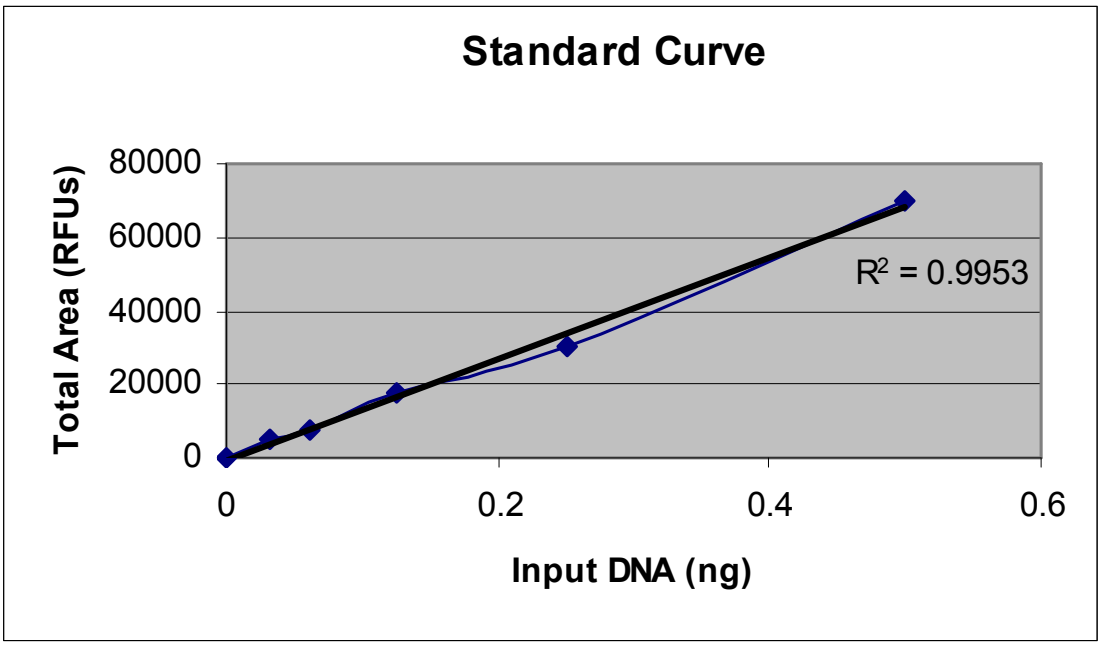

Figure 6: Example of a standard curve depicting the amount of input male reference DNA (ng) verses the total area of fluorescence incorporated into the Amelogenin X and Y amplicons (RFUs). Standard curve produced from two-fold serial dilutions of reference male DNA. Dilutions range from 32.5 pg to 500 pg.

IV.B. Analysis of Allele Imbalance

IV.B.i. Allele Imbalance at Varying DNA Concentrations

In order to effectively use Q-TAT to establish male and female DNA in mixed samples, the Amelogenin locus on the X and Y chromosomes must be amplified with reasonably equal efficiency so that the molar proportions of X and Y chromosomal DNA are estimated accurately. If amplification efficiency is not equal, the differences must be known and considered in the calculations. Therefore, a series of experiments were performed to assess the balance of X and Y Amelogenin products amplified from a collection of single source male DNA samples.

Q-TAT measures fluorescence in X and Y peaks and does not directly evaluate the male and female DNA proportions. Therefore X and Y amplicon peak imbalance will have an effect on proportions of male and female DNA estimated in a sample because male X DNA will be added to the female-X-DNA. To evaluate peak imbalance, an X/Y RFU ratio was computed by dividing the RFUs under the X peak by the RFU value for the Y peak. If the X and Y peaks are balanced, the X/Y ratio would be expected to be 1.0.

If the male X and Y peaks are essentially balanced, the RFU value for the Y peak can be subtracted from the X RFU value, leaving any remaining fluorescence in the X amplicon peak attributable to a female DNA contribution (XX) to a mixture. Similarly, the RFU in the Y peak can be doubled and plotted on the standard curve to estimate the DNA from the male contributor (XY). If the X/Y ratio is not balanced, the amount of imbalance must be taken into consideration when evaluating the male and female contributions in a mixture. Possible imbalance of X and Y peaks in male samples was assessed at varying concentrations of added template to determine if allele imbalance is affected by the amount of template amplified (Figure 7).

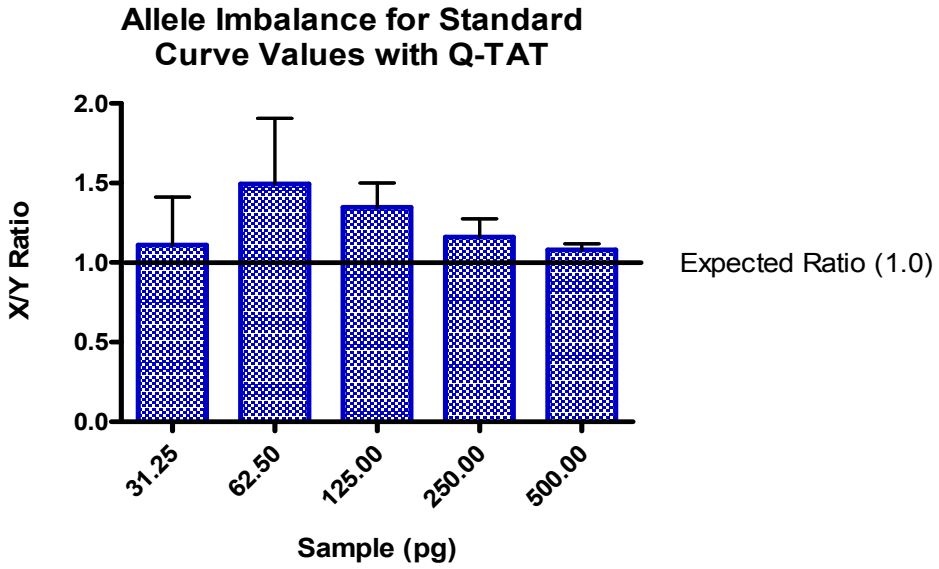

Figure 7: Allele imbalance observed for X and Y amplicons detected in replicate amplifications of the male DNA used to prepare the standard curve. Peak imbalance was evaluated by dividing the RFUs under the X peak by the RFU value for the Y peak to create an X/Y ratio.

Results in Figure 7 show that the X/Y ratios are similar for all concentrations of amplified male DNA. The greatest amount of variability for X/Y peak imbalance is seen at the lower concentrations of input DNA template (32.25 and 62.5 pg) as opposed to that observed for the higher concentrations of DNA (125, 250, and 500 pg). Therefore, DNA samples containing low amounts of male DNA will likely have increased error for mixture interpretation when compared to samples containing equal or high amounts of male DNA in the presence of female DNA.

IV.B.ii. Allele Imbalance with Q-TAT and STR Analysis

Small to moderate levels of allele imbalance are commonly observed at STR loci, especially when less than optimal amounts of genomic template DNA are amplified by PCR (Applied Biosystems, 2005; Figure 8). Therefore, comparable levels of imbalance

between Amelogenin X and Y amplicons are not unexpected. However, it was important to quantitatively evaluate the levels of imbalance between X and Y amplicons as compared to autosomal STR alleles. Experiments were performed with male single source samples to directly compare RFU ratios in X and Y amplicons produced using the Q-TAT assay versus autosomal STR alleles (Figure 9).

Levels of allele imbalance were assessed for single source male samples amplified using Q-TAT or the Profiler Plus STR typing kit (Applied Biosystems, Foster City, CA). The X/Y peak ratio was computed for the Q-TAT results and for the Amelogenin locus included in the Profiler Plus kit. Also, a ratio was calculated for all autosomal loci producing two alleles when analyzed with the Profiler Plus kit (Figure 8). The autosomal allele ratios for the two allele loci were averaged to produce a value for each of the five samples. The expected ratio for the Amelogenin locus and all autosomal loci was 1.0, indicating a balance between the two alleles. Knowing the typical level of allele imbalance observed for the Q-TAT assay enables an analyst to account for overestimates or underestimates of contributions in mixtures that may occur as a result.

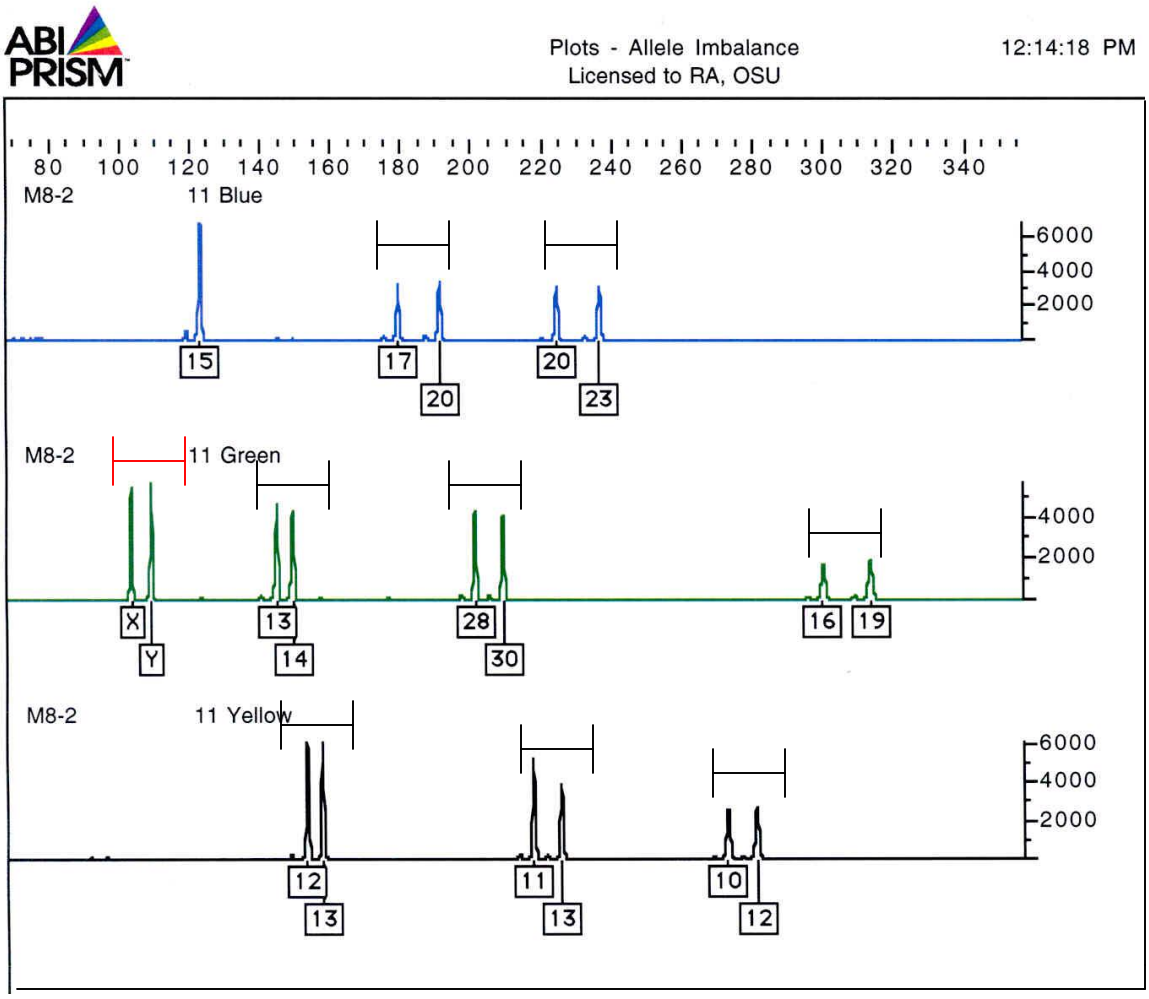

Figure 8: Example of Genotyper analysis for STR loci. For each locus showing two alleles, a ratio was calculated between the RFUs in each peak. The average ratio was computed for all loci containing two alleles at each concentration. The black brackets depict the two alleles for each heterozygous autosomal locus. The red bracket indicates the Amelogenin X and Y peaks also amplified in the Profiler Plus multiplex kit.

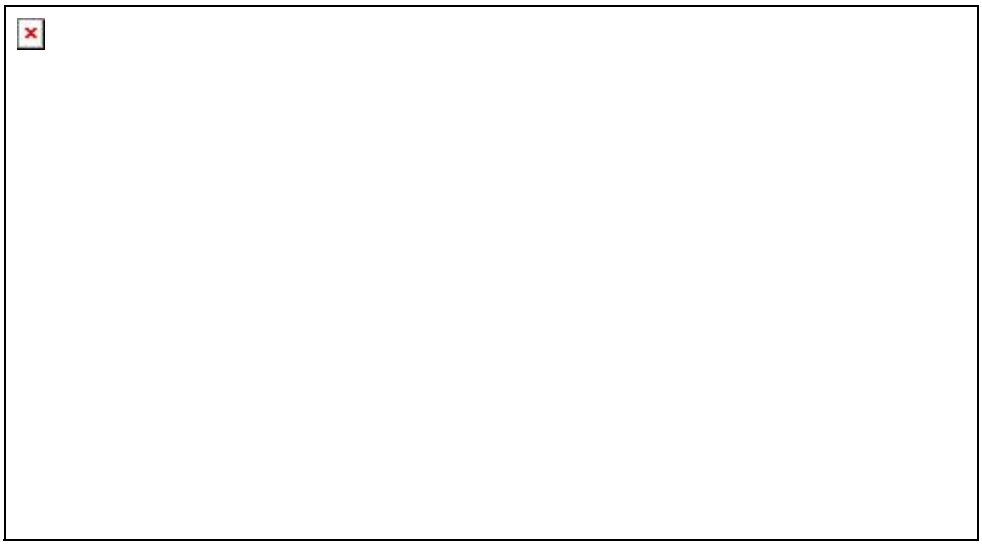

Figure 9: Comparison of allele imbalance observed for Amelogenin with Q-TAT and Profiler Plus and for autosomal alleles produced with the Profiler Plus kit. The horizontal line represents the expected value of 1.0 for the ratio. The analysis was conducted on the pure male samples. No significant differnce was observed between the methods used to evaluate the Amelogenin amplicons (p=0.3125). Results produced for the autosomal loci appeared to be similar to those produced for the Amelogenin locus.

Comparison of the X/Y ratios produced using Q-TAT with the X/Y ratio of the Amelogenin products produced with the Profiler Plus typing kit showed no significant difference between the two methods. A non-parametric, paired, Wilcoxon rank test produced a p-value of 0.3125 for comparison of the X/Y ratio produced with Q-TAT and X/Y ratio of Amelogenin amplicons co-amplified with STR loci by the Profiler Plus kit. Statistical analysis cannot be performed because of the differences in sample types. Because one ratio is produced for products from the Amelogenin locus whereas the autosomal ratio is a reflection of multiple loci, these values cannot be directly compared. However, after visually comparing the allele ratios produced by Q-TAT and the average ratio produced for the autosomal alleles, the results appear to be similar for the two methods. Therefore, the balance of X and Y amplicons amplified by Q-TAT appears to

be generally suitable for predicting the relative proportions of male and female DNA in mixed samples.

IV.B.iii. Optimization of Experimental Conditions

One possible source of imbalance in Amelogenin amplicons produced from X and Y templates are PCR conditions, particularly the temperature of the annealing step during cycling. The annealing temperature of 60^{o} used for this project was established by Promega Corp. (Madison, WI) for use with their sex typing kit. The sex typing kit was not utilized in this study; however, our primer sequences were identical. In order to ensure that the optimal PCR conditions were being used for this project, the standard curve was produced using varying annealing temperatures (57^{o}, 60^{o}, and 63^{o}).

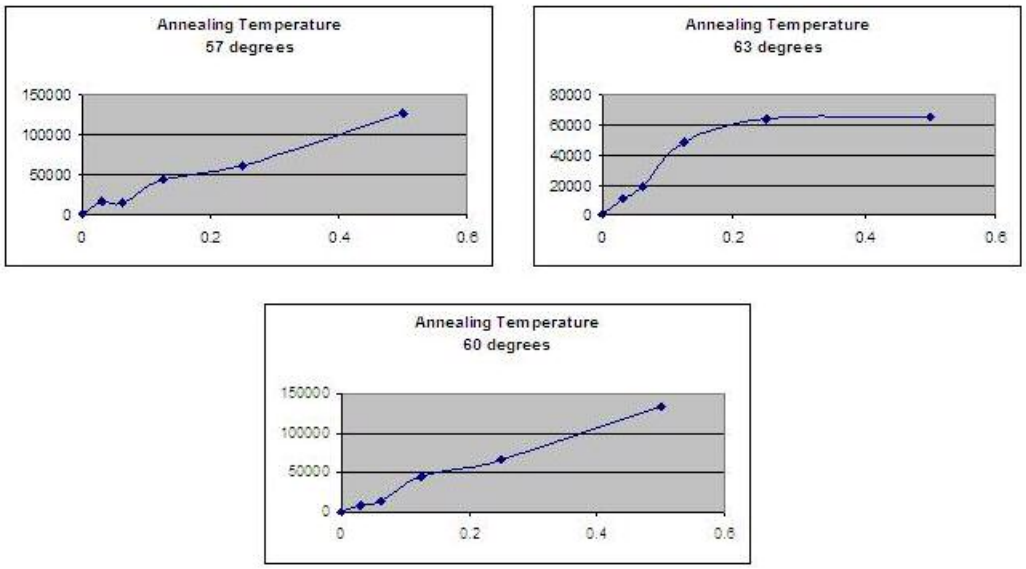

Figure 10: Optimization of annealing temperatures for amplification of the Amelogenin locus. Standard curve input values for male reference DNA were plotted against the total RFUs observed for the X and Y products for the Amelogenin locus.

Input DNA (ng)	Output DNA (ng)		
	57 degrees	60 degrees	63 degrees
0	0	0	0
31.25	51	29	36
62.5	44	49	66
125	139	164	174
250	191	242	232
500	401	495	235

Table 3: Optimization of annealing temperatures for amplification of the Amelogenin locus. Standard curve input values are listed along with calculated quantities produced at each annealing temperature.

Based on the shape of the standard curve plots (Figure 10) and estimates of input template amounts (Table 3), 60^{o} and 57^{o}, appear to be adequate annealing temperatures for the Q-TAT assay. An annealing temperature of 63^{o} was unable to produce optimal annealing temperature for the amplification of the Amelogenin locus. As a result of this experiment and literature findings (Promega Corp., 2006), no improvement in X and Y amplicon balance was apparent from altering the annealing temperature, and 60^{o} was chosen as the annealing temperature for use with the Q-TAT assay in this study.

IV.C. Part I – Evaluation of Single Source Samples

IV.C. i. Quantitation of Single Source Samples with Yield Gel and Q-TAT

Yield gel analysis is a standard method of quantitating the amount of total DNA in a sample. Yield gels can evaluate the level of degradation in a sample, but are not specific for human DNA. This portion of the study was designed to confirm the results of Allen and Fuller (2006) by evaluating Q-TAT's ability to estimate the total amount of

DNA in a sample. Yield gel analysis was performed to evaluate the quality and quantity of ten single source male and ten single source female DNA samples. Samples with adequate quality were chosen and their respective concentration estimates determined by yield gel. The same samples were then quantitated by Q-TAT to confirm or adjust yield gel estimates. Samples with degradation were eliminated from the study and replaced with intact DNA samples. The yield gel and Q-TAT results were compared to ensure that the standard and new quantitation methods were in agreement (Figure 11, 12).

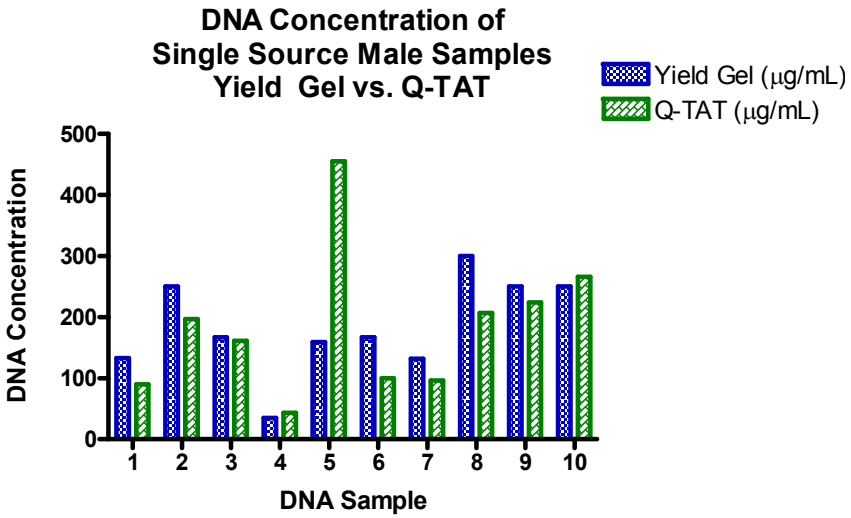

Figure 11: Quantitation of single source DNA samples using yield gel and Q-TAT. Ten single source male samples were analyzed by yield gel and Q-TAT (in duplicate). Using a paired t-test, no significant difference was found between the two quantitation methods (p=0.9910).

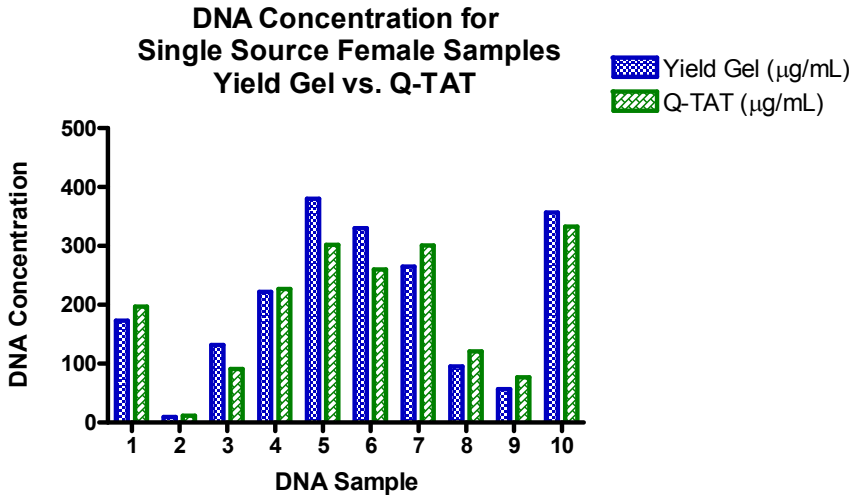

Figure 12: Quantitation of single source DNA samples using yield gel and Q-TAT. Ten single source female samples were analyzed by yield gel and Q-TAT (in duplicate). Using a paired t-test, no significant difference was found between the two quantitation methods (p=0.4630).

Based on a paired t-test analysis, there is no significant difference between Q-TAT and yield gel quantitation methods for the ten single source male samples (p=0.9910) and the ten single source female samples (p=0.4630) analyzed. Additionally, there is no apparent trend between the two quantitation methods, and there does not appear to be a consistent overestimate or underestimate with either method evaluated. Therefore, Q-TAT is able to produce quantitation values similar to those produced by the standard yield gel method.

IV.D. Part II – Evaluation of Mixtures of Known Proportions

IV.D.i. Quantitation of DNA Mixtures with Q-TAT

Mixtures of known proportions were produced to evaluate Q-TAT's ability to resolve biological samples consisting of mixed male and female DNA. Five single

source male samples and five single source female samples whose quantities produced with yield gel and Q-TAT analysis were in the best agreement were used to produce the collection of DNA mixtures. To evaluate the ability of Q-TAT to resolve mixtures with minor male contributors in the presence of excess female DNA, proportions of 1 female:1 male (3X/1Y), 3 female:1 male (7X/1Y), 5 female:1 male (11X/1Y), and 10 female:1 male (21X/1Y) were produced and evaluated (Figure 13, 15). For completeness, samples containing minor female contributions in the presence of excess male DNA in the following proportions were also produced and analyzed: 1 female:1 male (3X/1Y), 1 female:3 male (5X/3Y), 1 female:5 male (7X/5Y), and 1female:10male (12X/10Y) (figure 14, 16). The RFUs produced in the X peak were divided by the RFUs produced in the Y peak to produce an X/Y ratio for each sample. This ratio was compared against the expected X/Y ratio based upon the known proportion of male and female DNA in the amplified mixture.

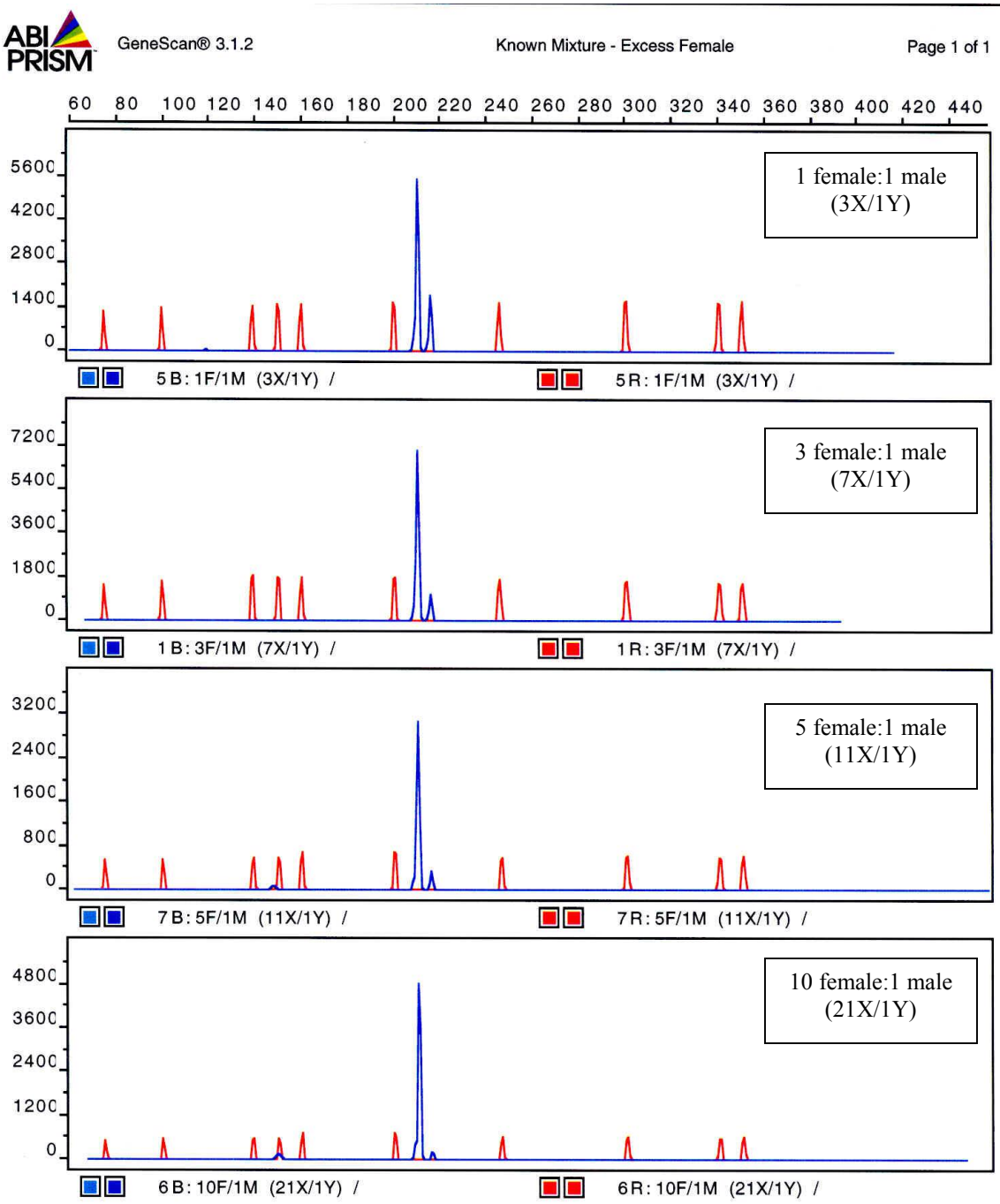

Figure 13: Sample electropherogram of Amelogenin peaks produced with Q-TAT. Results are for samples consisting of mixtures produced with known proportions of excess female DNA in the presence of male DNA. The red peaks represent the internal size standard, labeled with ROX fluor. The blue peaks present at 210 bp and 216 bp indicate the X and Y amplicons produced for the Amelogenin locus respectively. Known proportions of input DNA are noted in the different panels. The size range (bp) of DNA fragments is shown across the top axis and the RFU value on the left axis.

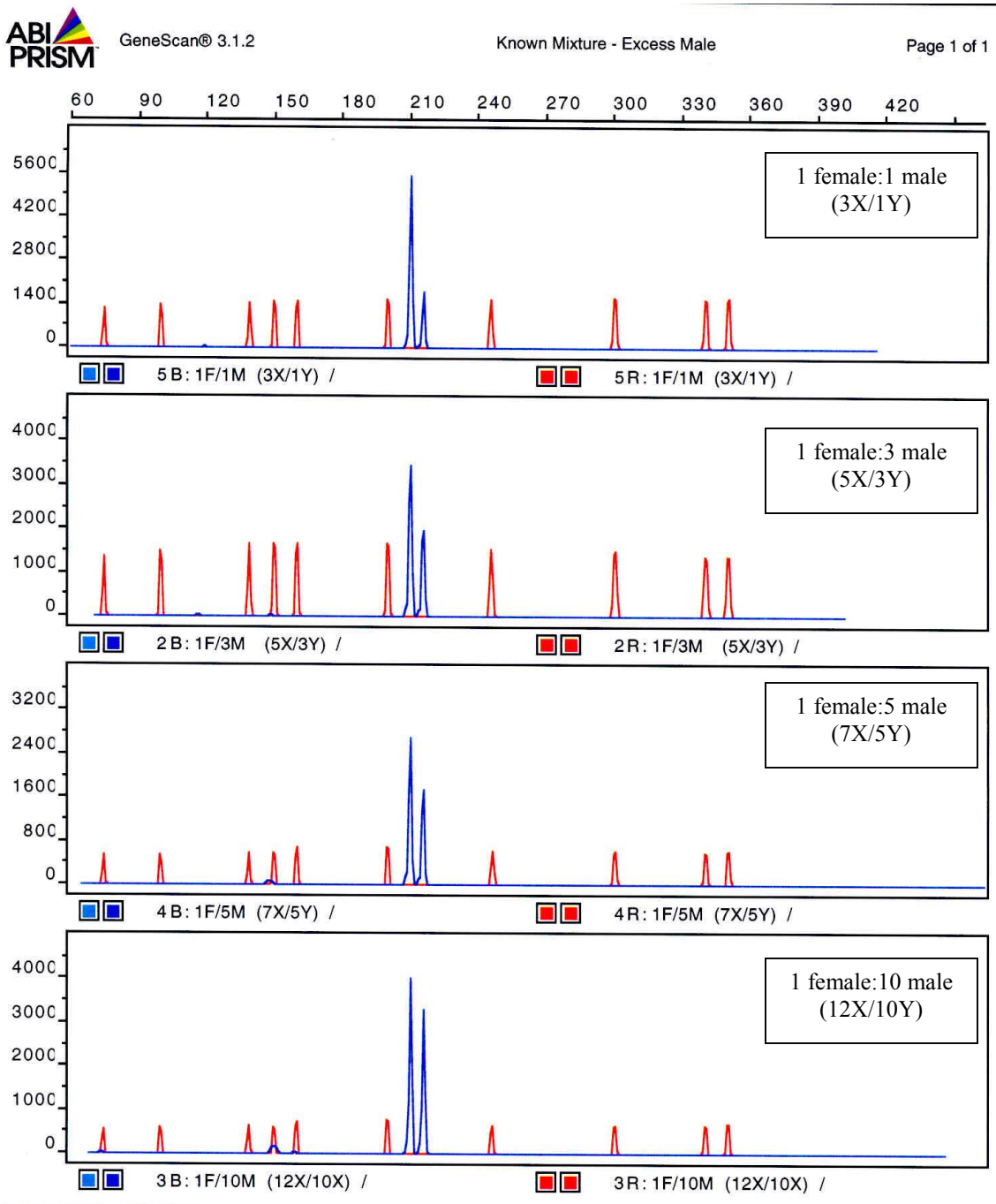

Figure 14: Sample electropherogram of Amelogenin peaks produced with Q-TAT. Results are for samples consisting of mixtures produced with known proportions of excess male DNA in the presence of female DNA. The red peaks represent the internal size standard, labeled with ROX fluor. The blue peaks present at 210 bp and 216 bp indicate the X and Y amplicons produced for the Amelogenin locus respectively. Known proportions of input DNA are noted in the different panels. The size range (bp) of DNA fragments is shown across the top axis and the RFU value on the left axis.

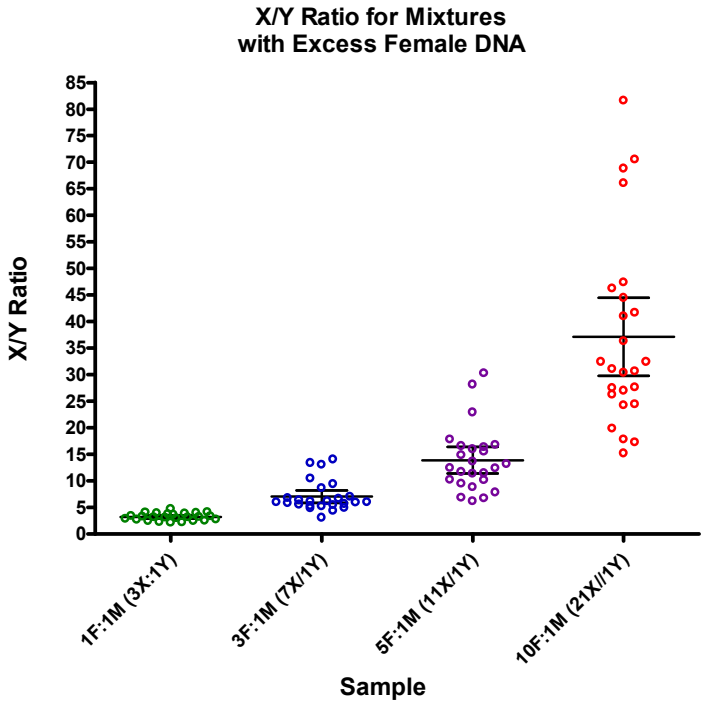

Figure 15: X/Y ratio of male and female mixtures with increasing amounts of female DNA. The solid bar across the middle represents the median for each group of samples at a female:male proportion and the additional bars depict the 95% confidence interval for each set of mixtures. Twenty-five different mixtures were run in duplicate for each of the known mixture proportions.

X/Y ratios produced for the different mixtures are summarized in Figures 15 and 16 and in Table 4. Figure 15 shows the results for samples in which the male was the minor contributor. No significant difference was observed between the samples composed of 1 female:1 male (3X/1Y) because the expected mean of 3.0 fell within the 95% confidence interval of 2.907 to 3.472 observed for the data. There is also no significant difference between the samples composed of 3 female:1 male (7X/1Y) because the expected mean of 7.0 fell within the 95% confidence interval of 5.849 to 8.317. There is a significant difference from expectation for samples composed of 5 female:1 male (11X/1Y) because the expected mean of 11.0 fell just below the 95%

confidence interval of 11.37 to 16.40. There also is a significant difference between samples composed of 10 female:1 male (21X/1Y) because the expected mean of 21.0 fell below the 95% confidence interval of 29.74 to 44.49. However, even though the expected ratios for the 10 female:1 male and 5 female:1 male mixtures fell outside the 95% confidence interval, Q-TAT still has the ability to identify DNA samples with minor male contributors. This information aids an analyst in deciding whether autosomal STR analysis or Y-STR analysis will provide the most useful information about the male DNA profile.

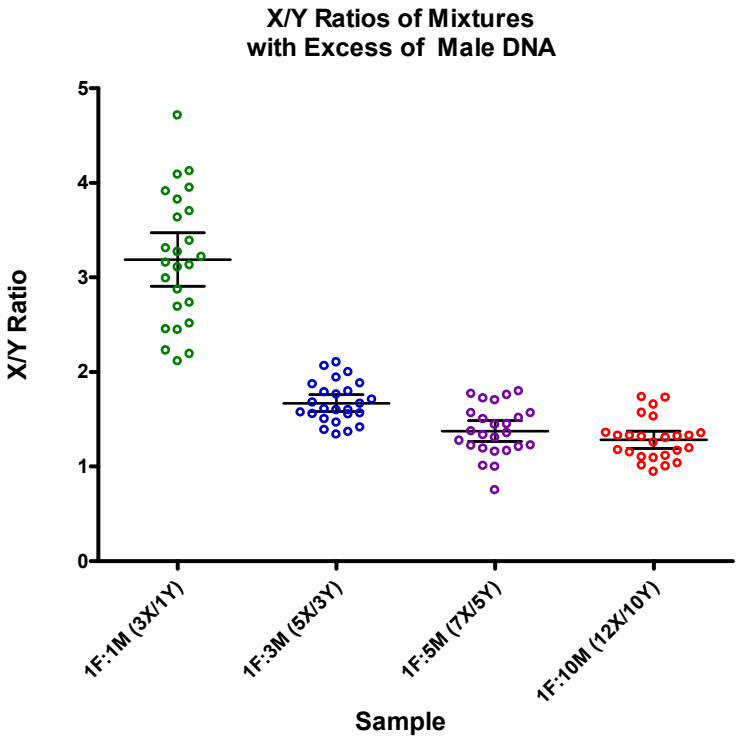

Figure 16: X/Y ratio of male and female mixtures with increasing amounts of male DNA. The solid bar across the middle represents the median for each group of samples at a female:male proportion and the additional bars depict the 95% confidence interval for each set of mixtures. Twenty-five different mixtures were run in duplicate for each of the known mixture proportions.

A comparable analysis was performed on mixtures in which the female contributor was the minor component (Figure 16). There is no significant difference between the samples composed of 1 female:1 male (3X/1Y) because the expected mean of 3.0 fell within the 95% confidence interval of 2.907 to 3.472. There is also no significant difference from expectation in the samples composed of 1 female:3 male (5X/3Y) because the expected mean of 1.670 fell within the 95% confidence interval of 1.582 to 1.761. Likewise, there is no significant difference in samples composed of 1 female:5 male ((7X/5Y); expected mean of 1.400 – 95% confidence interval = 1.254-1.485), and samples composed of 1 female:10 male ((12X/10Y); expected mean of 1.200 – 95% confidence interval = 1.193-1.376). Because females have two copies of the X chromosome, DNA samples consisting of minor female contributors are not as prone to inaccuracies resulting from low quantities of DNA as are samples composed of minor male proportions which only have one copy of the X gene.

Sample	Observed Average	Expected Ratio	Lower 95% Confidence Interval	Higher 95% Confidence Interval	Significant Difference
1F:1M (3X/1Y)	3.190	3.00	2.907	3.472	No
1F:3M (5X/3Y)	1.671	1.67	1.582	1.761	No
1F:5M (7X/5Y)	1.375	1.40	1.264	1.485	No
1F:10M (12X:10Y)	1.284	1.20	1.193	1.376	No
3F:1M (7X/1Y)	7.033	7.00	5.849	8.217	No
5F:1M (11X/1Y)	13.88	11.00	11.37	16.40	Yes
10F:1M (21X/1Y)	37.11	21.00	29.74	44.49	Yes

Table 4: All ratios are molar X/Y ratios comparing the average observed ratio to the expected ratio. The observed value is an average of all Q-TAT results produced for that mixture. The expected ratio is the molar ratio expected based on the input DNA. The 95% confidence intervals are based on the observed ratio and significance is determined based on whether or not the expected ratio falls within the confidence interval.

All the results are summarized together in Table 4. No significant difference from expectation was observed when mixtures of equal amounts of male and female DNA were produced, or when the amount of male DNA was high and the amount of female DNA was low. A significant difference was seen however when large amounts of female DNA and small amounts of male DNA are present. Results from mixtures indicating no significance difference of observed versus expected X/Y ratios means that the expected ratio fell within the 95% confidence interval described for the observed median. For those samples that are significantly different from expectation, the expected ratio fell outside the 95% confidence interval computed for the observed median. Although samples containing low proportions of male DNA in the presence of female DNA underestimate the proportion of male DNA in the mixture, Q-TAT still proves to be a reliable method for characterizing the male as a minor contributor in these samples.

IV.D.ii. Evaluation of Mixtures with Q-TAT and STR Analysis

The relative proportions of male and female DNA present in known mixtures and predicted by the Q-TAT assay were evaluated using standard STR typing procedures. The major and minor contributors of DNA were estimated from the peak areas of autosomal alleles visualized for loci revealing four alleles (Clayton et al., 1998; Butler, 2005). In this way, the accuracy of Q-TAT predictions of mixture proportions can be compared to the other "traditional" method in current widespread use.

For all of the mixtures, the RFUs in the X or Y peaks (whichever was greater) was divided by the sum of the alleles in the other peak. For samples containing excess female DNA, the RFUs corresponding to the amount of female DNA present was divided

by the RFU value corresponding to the amount of male DNA (as defined by the Y amplicon doubled). For samples containing excess male DNA, the RFUs calculated for the male proportion were divided by the resulting RFU value for the female proportion.

The major/minor peak ratio for X and Y amplicons produced using Q-TAT was compared to the major/minor peak ratio for autosomal STR alleles for selected loci amplified with the Profiler Plus multiplex kit. Only autosomal STR loci with four alleles were used to calculate the major/minor peak ratios (Figure 18). The ratio of autosomal allele peak areas for each selected locus containing four alleles was averaged to produce one value for each mixture of male and female DNA. The value for the expected ratio of RFU in the phenotypes was based on the known male/female ratio of amplified DNA template.

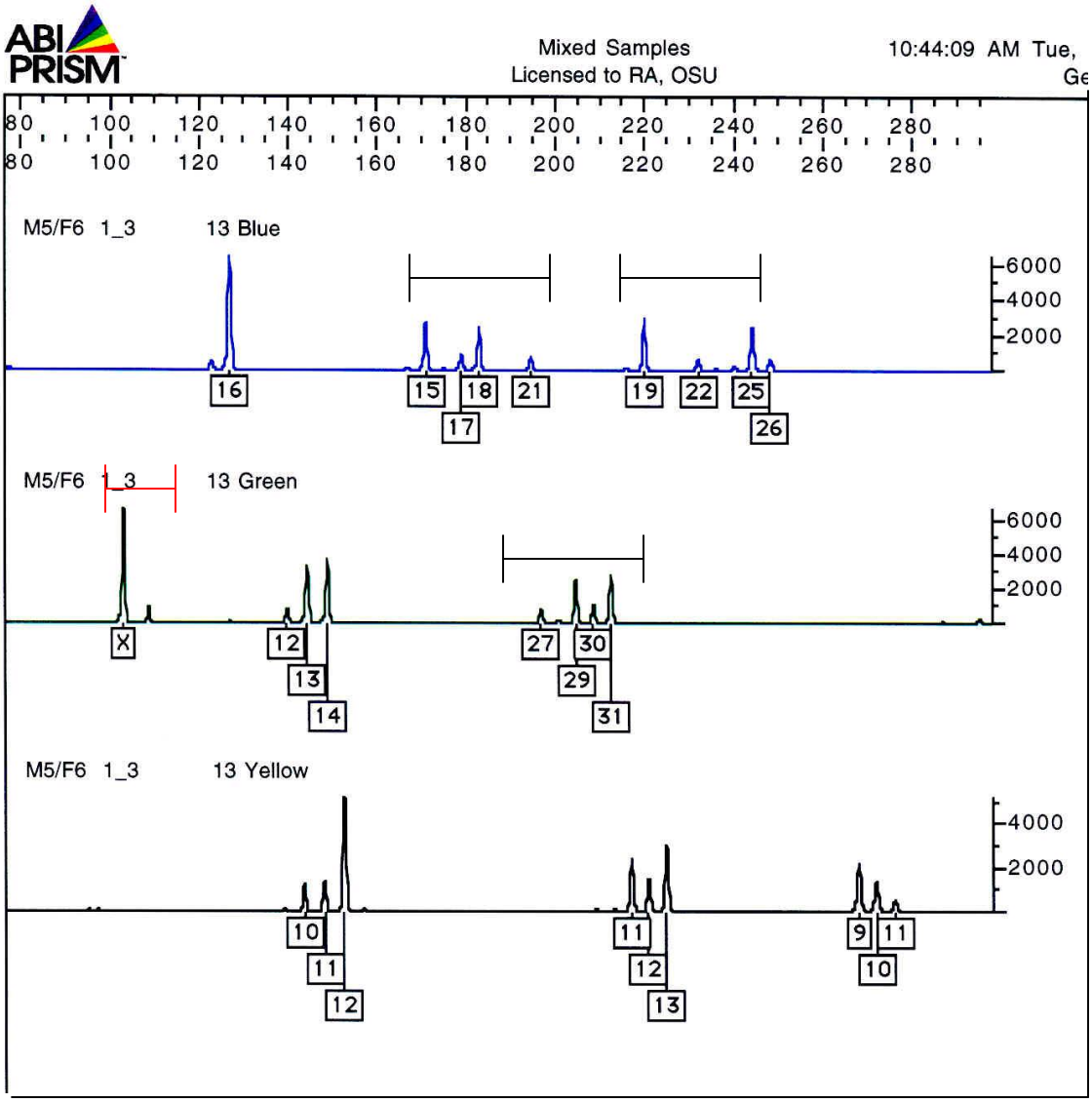

Figure 17: Example of Genotyper analysis for STR loci. For each locus showing four alleles, a ratio was calculated for the sum of the RFUs in each phenotype. The average ratio was computed for all loci containing four alleles at each concentration. The black brackets depict the four alleles for each heterozygous autosomal locus. The red bracket indicates the Amelogenin X and Y peaks which are also amplified by the Profiler Plus kit.

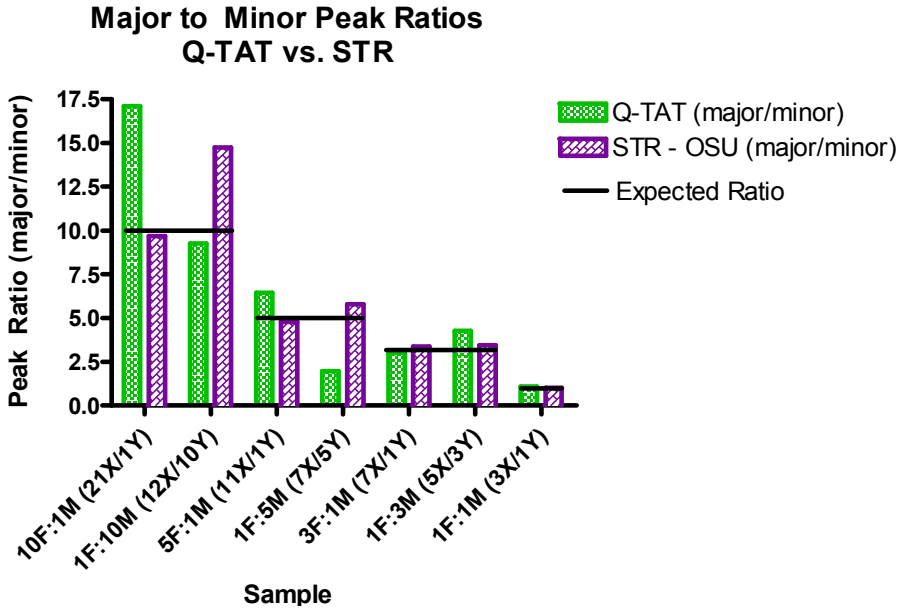

Figure 18: Analysis of major/ minor peak ratios for Q-TAT and STR analysis with varying proportions of male and female DNA. The black line shows the expected value for each mixture. STR major/minor peak ratios are averages of all loci containing four alleles for each known mixture. Q-TAT analysis was evaluated in duplicate.

Due to the nature of the sample composition, it was not possible to evaluate the data in this part of the study for statistical significance. However, for peak area ratios computed for samples with a minor male contributor in the presence of excess female DNA (10 female:1 male, 5 female:1 male) Q-TAT resulted in a higher then expected major/minor ratio. Previous results produced for allele imbalance assays (Figure 7) indicated that samples containing low amounts of male DNA suggested preferential dropout of the Y allele, producing ratios greater then the expect value. When converting X and Y peak data to male and female values, lower then expected Y values will result in an underestimate of the amount of male DNA and an overestimate of the female DNA component. Overall, similar amounts of variability are observed between the major/minor ratios produced with Q-TAT and the STR method of analysis.

IV.D. iii. Sample Comparison

As an important part of the investigation of Q-TAT as a method to estimate the relative proportion of male and female DNA in mixtures, the method was applied to actual sexual assault evidence obtained from non-probative cases received from the Tulsa Police Department (TPD) Laboratory. The evidence provided by the laboratory had been thoroughly investigated by STR analysis and the relative proportions of male and female DNA had been estimated by comparing RFU peak areas in autosomal STR alleles in the DNA profile as discussed previously.

The evidentiary samples received from TPD were composed of mixtures of different body fluids. However, most contained semen as the source of male DNA. In order to ensure that the DNA extracted from the blood samples used to create the known mixtures of male and female DNA would produce the same quantitation results with Q-TAT analysis as the evidentiary mixtures consisting of other body fluids, tests were first conducted to analyze male:female mixtures of DNA prepared from blood with mixtures of blood and semen. The X/Y ratio was calculated in these samples to determine whether differences in sample type had an effect on the balance of X and Y amplicons (Figure 19).

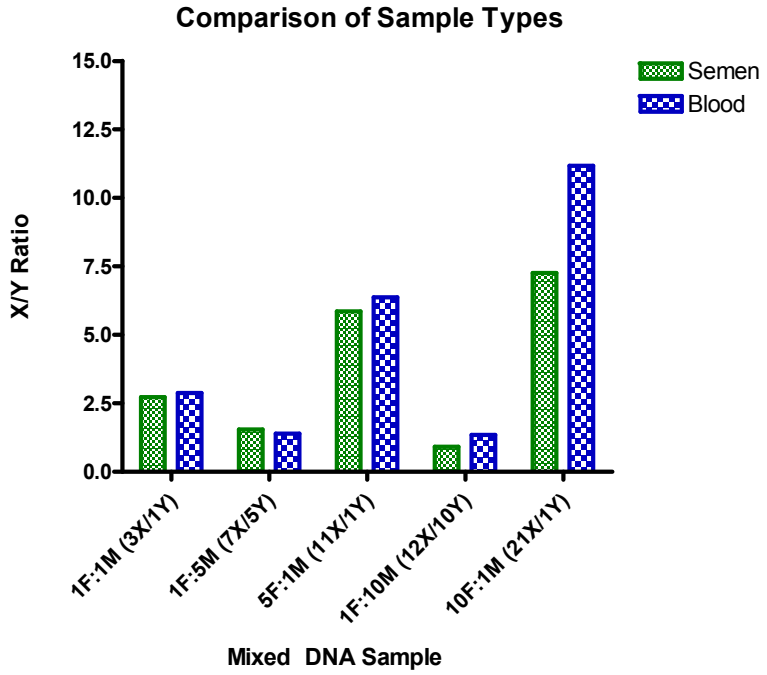

Figure 19: Comparison of X/Y amplicon RFU ratio produced with DNA from blood/semen mixtures from a common donor by Q-TAT. The molar X/Y ratios of each mixture were calculated and used for comparison. The bar represents the expected value for each set of mixtures. No significance was observed between the sample types using the Wilcoxon rank test (p=0.1250).

The Wilcoxon rank statistical test was used to evaluate the X/Y ratios produced for the different sample combinations consisting of varying female:male proportions of DNA. No significant difference was found between the two types of fluids analyzed at any of the concentrations produced (p=0.1250). The allele imbalance observed between samples composed of a major female DNA component extracted from blood and minor male DNA component extracted from blood were similar to those observed for the same female component combined with a minor male DNA component extracted from semen. The same was true for samples containing major amounts of male DNA in mixes with

minor female DNA. Therefore the results of known mixture proportions produced in this study can be applied to the unknown evidentiary samples received from TPD.

IV.E. Part III – Evaluation of Evidentiary Samples

IV.E.i. Quantitation of Evidentiary Samples with Q-TAT and QuantiBlot

QuantiBlot is the method used by TPD for the quantitation of human chromosomal DNA. QuantiBlot results give a two-fold range of possible DNA concentration whereas Q-TAT provides a more exact value for the quantitation. QuantiBlot and Q-TAT results have been previously compared for single source DNA samples, and a reliable correlation for the two methods has been reported (Allen and Fuller, 2006). As part of this study, Q-TAT and QuantiBlot were compared as quantitation methods when dealing with DNA samples consisting of male:female mixtures in non-probative evidence.

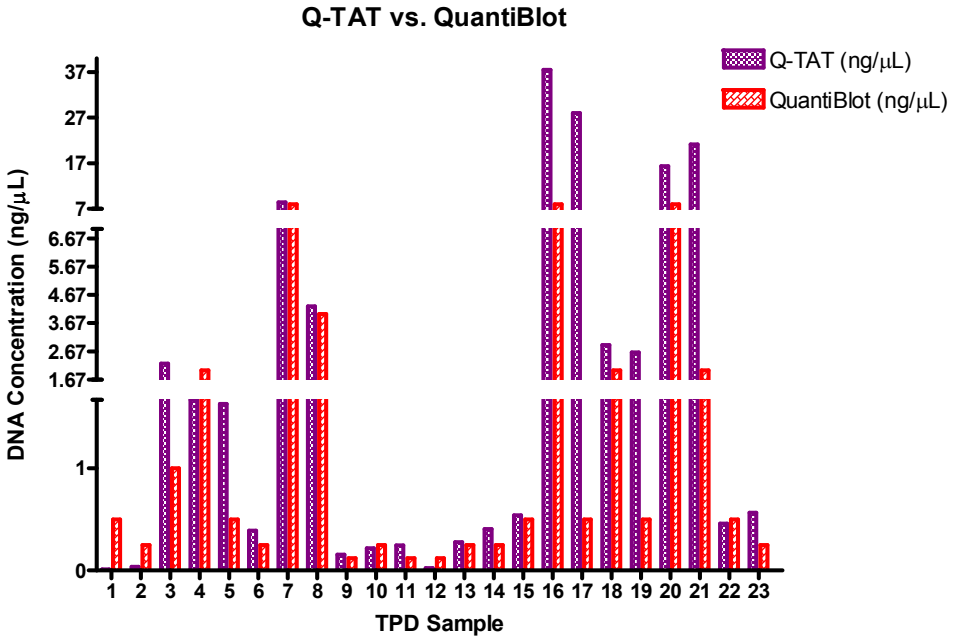

Figure 20: Comparison between Q-TAT and QuantiBlot for the quantitation of total DNA in mixed samples. TPD samples from casework and of unknown concentration were quantitated using Q-TAT. A significant difference was observed for the two quantitation methods using the paired t-test (p=0.0446).

When comparing the Q-TAT and QuantiBlot methods of quantitation, a slightly significant discrepancy in concentration estimates was observed for the two methods. Through a paired t-test analysis, a p-value of 0.0446 was observed (Figure 20). The majority of the samples produced similar results with both quantitation methods; however a few produced very different values. When differences were observed between Q-TAT and QuantiBlot, QuantiBlot usually provided a lower estimate. One contributing factor to the differences observed between Q-TAT and QuantiBlot is that Q-TAT provides an estimated value for the DNA quantity and QuantiBlot instead indicates a two-fold range of DNA concentrations. The method in which the quantities are reported could account for some of the variability seen between the two methods.

IV.E.ii. Analysis of Allele Imbalance with Q-TAT and STR Analysis

The X/Y ratios produced by Q-TAT were compared with the X/Y amplicon ratios produced with the Identifiler and the Profiler Plus STR typing kits (both kits include primers for the Amelogenin locus) (Applied Biosystems, Foster City, CA). STR analysis was performed by TPD using the Identifiler kit as part of their forensic analysis of the criminal case and repeat STR analysis was performed at OSU using the Profiler Plus kit for comparison purposes. The Amelogenin peak areas (RFUs) were used to produce the X/Y ratio for X and Y amplicons amplified by both Q-TAT and with the STR typing kit. However, the amount of input DNA template used for the profiling assays at TPD was determined by QuantiBlot whereas Q-TAT assays were the basis for input DNA amounts at OSU. Because of the differences observed between QuantiBlot and Q-TAT (Figure 20), the amount of input DNA used at TPD may therefore vary somewhat from the amount used by OSU.

Male DNA was not detected in all samples analyzed, as determined by absence of the Y amplicon. Twenty-three samples were analyzed and of those samples, Q-TAT was able to detect male DNA in 18 samples, TPD detected a minor male DNA contributor in 16 samples with the Identifiler kit, and OSU detected a minor contributor in 17 with Profiler Plus. Chi-square (χ^2) tests were conducted to determine if there was a significant difference in each method's ability to detect the presence of a male contributor. No significant difference was observed between the comparison of Q-TAT to TPD (χ^2 = 0.4510, p = 0.5019), Q-TAT to OSU (χ^2= 0.1195, p = 0.7296), or TPD to OSU (χ^2= 0.1072, p = 0.7433). Because no significant difference in peak detection was observed,

samples in which no male peak was detected were not included in the statistical calculations.

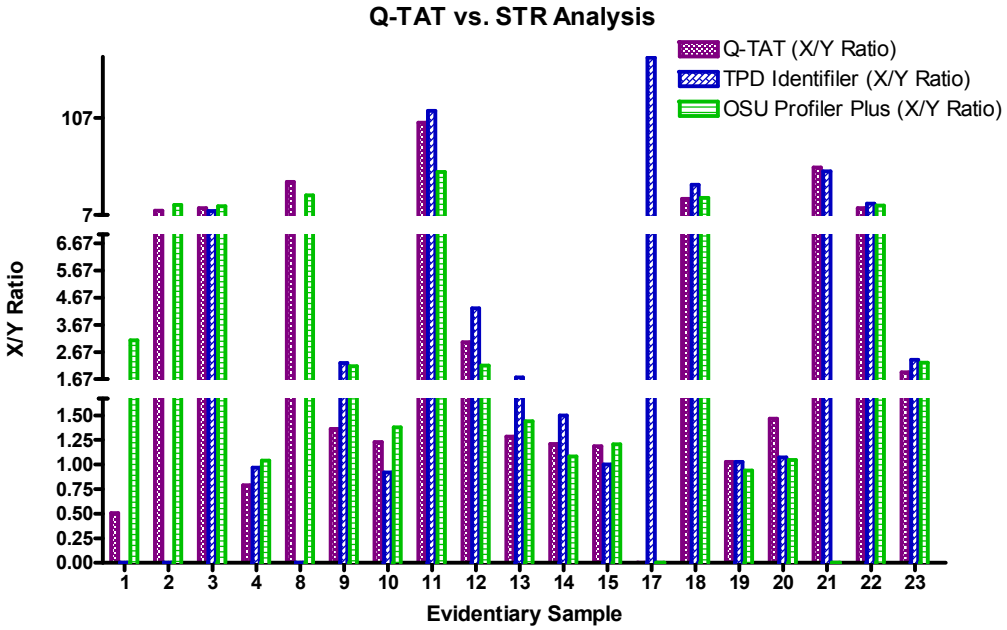

Figure 21: Comparison of the X/Y molar ratio produced by Q-TAT, the Identifiler multiplex STR typing kit by TPD, and the Profiler Plus multiplex STR typing kit by OSU. The samples used were non-probative evidentiary samples received from TPD. No significant difference was observed between the X/Y ratio produced with any of the three methods.

The nonparametric, paired, Wilcoxon ranked test was used to evaluate the X/Y ratio of Amelogenin products produced using Q-TAT, Identifiler and Profiler Plus STR typing methods performed by TPD and OSU, respectively. No significant difference was observed between Q-TAT and the Identifiler kit used at TPD ($p = 0.2293$), Q-TAT and the Profiler Plus kit used by OSU ($p = 0.3380$), or the two different STR typing kits ($p = 0.1531$). Based on these analyses, the X/Y ratio computed from peak areas representing Amelogenin amplicons was similar for Q-TAT and the commercially available STR typing kits. As a result, the differences in DNA quantitation estimates produced by the

Q-TAT and QuantiBlot methods used by OSU and TPD respectively had little to no effect on the X/Y ratios produced by the STR typing assays.

IV.E.iii. Evaluation of Evidentiary Samples with Q-TAT and STR Analysis

Finally, to validate the ability of Q-TAT to predict the autosomal STR typing characteristics of mixed samples, the relative proportions of male and female DNA estimated from the X and Y amplicon ratio produced with Q-TAT in real evidentiary samples was compared to proportions of DNA from donors using analysis of autosomal STR alleles peak areas. As described for the comparable analysis discussed above involving samples of known proportions, the Q-TAT results were analyzed based on an assumption that the X/Y allele ratios are balanced (Figure 7, 9). The female and male proportions were computed for the unknown samples just as they had been for the known mixtures discussed above. STR loci consisting of four alleles were used to compute the major/minor allelic ratio (Figure 17). Ratios were averaged to produce one value for each mixture. Samples containing at least one locus with four alleles were compared to Q-TAT major/minor ratios.

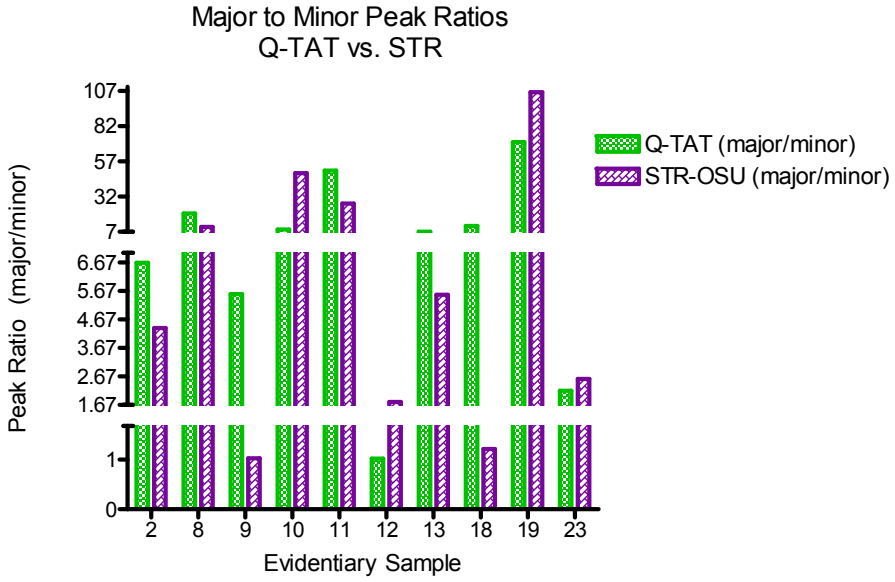

Figure 22: Comparison of major/minor peak ratios produced for the Amelogenin locus with Q-TAT and autosomal loci with Profiler Plus. STR major/minor peak ratios are averages of all loci containing four alleles for each known mixture. Q-TAT analysis was evaluated in duplicate.

Because of the nature of the sample composition, it was not possible to evaluate the data for statistical significance. Relatively minor differences in major/minor peak ratios are observed for Q-TAT compared to autosomal STR analysis. The small differences seen between the two methods may stem from a variety of causes. Some variations in the data may be attributed to Y allele dropout that affects the X/Y ratio as was seen in the analysis of samples consisting of minor amounts of male DNA in the presence of excess female DNA (Figure 7). Other discrepancies may result from Q-TAT's inability to distinguish between multiple same sex contributors. The evidentiary samples may in fact contain more then one male and/or female contributor.

V. DISCUSSION

Quantitating the amount of DNA present in biological evidence is an essential part of forensic DNA typing. Quantitation ensures that optimal amounts of human template are added to PCR reactions to produce STR profiles that are readily interpretable and free of artifacts produced when non-optimal amounts of template are amplified. In addition, quantitation of human genomic DNA is mandated by the FBI and accrediting agencies as part of the accreditation process. Quantitation is also important to preserve as much sample as possible in case confirmatory testing is needed. Often, only small amounts of sample are recovered from crime scenes and it is important to retain as much DNA for retesting if required.

Resolving DNA profiles produced from mixed biological samples add additional complexity to the analysis and interpretation of DNA typing results. Mixed samples can be recovered from virtually any crime scene, but sexual assaults represent perhaps the most common crime from which evidence consisting of mixed male and female body fluids will be produced. DNA Quantitation methods based on amplification of the Amelogenin locus have the potential to efficiently provide the most information about the amount of male and female DNA present in a sample.

By evaluating the X and Y amplicons produced through the amplification of the Amelogenin locus, an analyst may be able to characterize the composition of a sample before choosing an analysis strategy and initiating processing. Knowledge about the total amount of DNA in a sample and relative proportions of male and female DNA present in

a mixture could direct an analyst to choose autosomal STR typing rather than Y-STR typing (or visa versa) depending on the characteristics of the mixture. Such decisions will help ensure success in prosecuting sexual assailants by getting the most information possible, given the evidence available for testing.

Current methods for DNA quantitation and for the analysis of DNA mixtures do not provide information about the relative proportions of male and female DNA present in a mixture prior to STR analysis. **The purpose of this study was to determine whether the Q-TAT quantitation method (Allen and Fuller, 2006) has the ability to accurately estimate the relative contributions of male and female DNA to mixed biological samples.**

V.A. Quantitation Analysis

Current DNA quantitation methods widely used by DNA typing laboratories do not differentiate between male and female DNA. These methods therefore are incapable of identifying male:female mixtures that may be present in evidentiary samples. Quantitation methods such as UV spectrophotometry and yield gels can provide some information about the purity of the sample or the extent of degradation, but are not specific for human DNA. More commonly used methods such as slot blots (QuantiBlot) and real-time PCR methods (Quantifiler) are specific for human chromosomal DNA. While these methods provide a quantitative value for the total amount of DNA in a sample, they are unable to reveal a male:female mixture that may be recovered from a sexual assault and thus will tell an analyst nothing about the relative proportions of male and female DNA in the mixture.

The Q-TAT assay was designed to amplify the Amelogenin locus on the X and Y chromosomes (Allen and Fuller, 2006). By its very nature then, Q-TAT has the potential to be able to identify a mixed DNA sample and also to estimate the relative proportions of male and female DNA present during the quantitation process. Amplification of the Amelogenin locus on the X and Y chromosomes results in products of 210 and 216 bp, respectively. This six basepair difference allows for the separation of the amplicons by capillary electrophoresis. Given that the relative amount of fluorescence in the X and/or Y amplicons is proportional to the amount of PCR product present, and that the amount of amplicon produced is proportional to the amount of input template (Figure 6), the RFU contained within each amplicon can be used to estimate the relative proportion of male and female DNA in the sample. For the RFU contained with X and Y amplicons to be accurate predictions of male and female DNA however, two amplicons should be amplified with equal efficiency.

The efficiency with which the Amelogenin locus on the X and Y chromosomes is amplified was assessed in male DNA samples by analyzing the balance of fluorescence in X and Y amplicons (Figure 7, 9). Equal efficiency of PCR amplification would produce an X to Y ratio of 1.0. Repeated analysis of X/Y ratios among a cohort of single source male samples showed no significant difference in the efficiency with which the Amelogenin locus on the X and Y chromosomes is amplified (Figure 9) except at low levels of input DNA (Figure 7). These results are characteristic of the efficiency with which the alleles at heterozygous autosomal STR locus are amplified (Butler, 2005). Amplifying concentrations of male DNA below 62.5 pg results in increased imbalance of X and Y amplicons most likely due to dropout of the Y allele resulting from low amounts

of input Y chromosomal template (Figure 7). These results suggest therefore that the Amelogenin locus on the Y chromosome may not be as efficiently amplified as the locus mapping to the X chromosome. However, the difference in efficiency must be small and is revealed only at low concentrations of input genomic DNA.

Because the real-time PCR assay described by Alonso et al. (2004) is designed to amplify the Amelogenin locus as part of the quantitation process, it has the potential to be suitable for the analysis of male and female mixtures. However, a search of the literature reveals no published use of the real-time PCR quantitation method of Alonso et al. (2004) for the evaluation of male and female DNA in mixed samples. Moreover, unlike Q-TAT, the technology and instrumentation used for real-time PCR quantitation is different than that used for STR profiling assays. Also, the X and Y amplicons produced by this assay are smaller in size then the ones produced with the Q-TAT assay. Smaller amplicons are not able to predict the integrity of the sample as well as larger amplicons which are more representative of autosomal amplicons produced with STR typing kits. The differences in instrumentation and methodology, and amplicon sizes produced with the real-time Amelogenin quantitation assay and STR typing assays limits the amount of information that the real-time assay can predict about the STR typing results. Even though, real-time PCR has a greater dynamic range than Q-TAT, allele dropout was also observed in samples containing less then 60 pg of input DNA (Alonso, 2004).

The Quantifiler Y Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA) is a real-time quantitation method that targets the SRY locus on the Y chromosome and is designed to estimate the amount of male DNA in a sample (Applied Biosystems, 2005; Green et al., 2005). However, the Quantifiler Y kit it is not able to

simultaneously estimate the total amount of DNA in a mixed DNA sample. In order to obtain this information, two real-time PCR assays must be run; one to estimate total human DNA and a second to estimate the male DNA present (Applied Biosystems, 2005). Therefore, the Quantifiler Y method provides no information about the female component of the mixture or the relative proportion of male DNA in the mixed sample. Quantifiler Y only has the ability to evaluate the Y chromosome and therefore provide a quantitative estimate of the amount of male DNA present. Also, Quantifiler Y as well as all other real-time PCR methods require expensive, specialized additional instrumentation be purchased by a forensic DNA typing laboratory, additional training of analysts, allocation of space, and added quality control procedures.

As a quantitation method, Q-TAT has been previously shown to produce DNA concentration estimates comparable to quantitation methods widely used in forensic laboratories (Allen and Fuller, 2006). In this study, DNA quantitations produced by Q-TAT correlated well with yield gel and QuantiBlot estimates. Single source male and female samples characterized by yield gel and Q-TAT showed slight variation in concentration estimates but neither yield gels nor Q-TAT produced consistently higher or lower quantitation values. Non-probative, evidentiary samples quantitated with Q-TAT and compared to QuantiBlot estimates from the Tulsa Police Laboratory (TPD) indicated a subtle yet statistically significant difference between the two methods. However, most samples showed reasonable agreement. The variability observed between Q-TAT and QuantiBlot was most likely due to the fact that QuantiBlot reports quantitations as a two-fold range (Applied Biosystems, 2004) whereas Q-TAT provides a more precise quantitation estimate.

V.B. Mixture Analysis

Sexual assaults account for the majority of mixed biological samples recovered from crime scenes, and approximately 90% of all sexual assault cases include a male suspect and a female victim (U.S. Department of Justice, 2003). Often in sexual assault cases, the male suspect is the minor contributor and the challenge for an analyst becomes establishing a complete male profile for the evidentiary sample that can be compared to a suspect's reference DNA profile. The small amount of male DNA that may be present in such a case is often mixed with an excess of female DNA from the victim that can obscure the assailant's profile. Knowing that a small amount of male DNA exists in a mixed sample can cause other DNA typing methods to be used to obtain as much information as possible about the suspect.

Typing of STR loci mapping to the Y-chromosome is one such alternative strategy (Butler, 2005). Y-STR typing in such cases has the advantage of specificity in that the STR loci analyzed are restricted to the Y chromosome and therefore an excess of female DNA in a sample is ignored during PCR amplification (Prinz et al., 2001). However, Y-STR typing suffers from the limitation of discriminatory power relative to autosomal STR loci. Random match probabilities of one in 10,000 are seldom exceeded with Y-STR results as opposed to matching probabilities in the 1 in a quadrillion range that characterize autosomal STR multiplexes. In addition, Y-STRs are patrilinearly inherited (de Knijff 2003; Butler, 2005) which means all male descendants from a common male ancestor are equally likely as perpetrators of a crime involving Y-STR typing results. Thus, deciding upon an analytical approach to investigate a crime involving mixed male:female samples is important and can only be made after

discovering 1) a mixture exists, 2) the total amount of human DNA present, and 3) the relative proportions of male and female DNA present.

The initial step in the analysis of sexual assault evidence or other evidence containing a potential male:female mixture is to determine if a mixture is present. Based on the circumstances surrounding the collection of the sample combined with presumptive testing, the analyst can determine whether the sample should be treated as a potential mixed sample. For samples that indicate the presence of spermatozoa or seminal fluid based on a microscopic observation of sperm heads from slide smears or a positive P30 presumptive test, differential extraction can be used to attempt to separate the majority of DNA originating from the sperm from that recovered from epithelial cells. However, the detection of mixtures microscopically can be tedious and time consuming and moreover, male DNA may be present in a sperm-free sample due to epithelial cells present in an ejaculate from the male ductwork (unpublished observations). Even when sperm are present and visible microscopically, differential extractions are not always complete and can still result in mixed profiles.

Because the method of differential extraction is based on cellular structural differences that affect the ease of digestion of epithelial cells and spermatozoa, male epithelial cells that are present will not be separated into the "male" DNA fraction with the sperm cells. Rather, DNA from the male epithelial cells will partition with the female fraction and result in a mixed STR profile in the "female" fraction. In addition, if the sperm cells are not completely dispersed during the washing steps of the extraction process, epithelial DNA will not be completely removed from the "male" fraction and the male DNA will still be contaminated with female DNA. Thus, differential extraction is

not always complete. Identification of mixtures containing a minor male contributor is therefore one important step in forensic DNA analysis, especially in sexual assault investigations. Q-TAT is able to provide information as part of the routine DNA quantitation process, identifying male:female mixtures and estimating the relative proportions of male and female DNA present.

When mixtures consisting of known proportions of male and female DNA were evaluated, Q-TAT was able to 1) positively identify samples containing minor contributors of male DNA, and 2) within limits accurately estimate the relative proportions of male and female DNA in the sample. Thus, Q-TAT was able to characterize mixtures containing a minor male contributor in the presence of excess female DNA, mixtures containing equal amounts of male and female DNA, and mixtures containing a minor female contributor in the presence of excess male DNA (Figure 13, 14). For samples containing an excess of female DNA and a minor amount of male DNA (10 female:1 male, 5 female:1 male), the expected ratio fell slightly below the 95% confidence interval for the observed median (Figure 13). Even for these mixtures however, designation as mixtures containing a minor male contribution was accurate. For all other mixture combinations, the expected ratio fell within the 95% confidence interval for the observed median provided by Q-TAT (Figure 14).

The inability of Q-TAT to accurately estimate the proportion of DNA from minor male contributors in the presence of excess female may result from preferential Y allele dropout. This possibility is supported by studies on allele balance at low input amounts of DNA template (Figure 7) observed for the analysis of male DNA at varying concentrations. If Y allele dropout does in fact occur, the X/Y ratio would be expected to

artificially increase and result in X/Y ratios that are greater than the expectation for the mixture. This is exactly what was observed (Figure 18). However, these limitations do not hinder Q-TAT's ability to positively identify the presence of a minor male contributor in a mixed DNA sample. Moreover, allele imbalance observed for Q-TAT at low input template amounts is comparable to that seen for autosomal STR alleles amplified from comparable amounts of genomic DNA template (Butler, 2005; unpublished observations).

V.C. STR Mixture Interpretation

Once DNA quantitation has been performed for a sample, the appropriate amount of input template DNA is amplified in an STR multiplex typing reaction. With current methods for analyzing mixtures, a quantitation method such as QuantiBlot or Quantifiler would be used to determine the total amount of DNA in the sample. This quantitation value would then be used solely to determine the amount of input template DNA used for STR typing without regard for whether or not a mixture is present. Through the use of the Q-TAT method of quantitation, 1) the amount of DNA present, 2) the existence of a mixture, and 3) the relative proportion of male and female DNA in the sample are evaluated simultaneously.

By knowing the relative quantity of DNA from a minor male contributor, the amount of template DNA added to the PCR reaction can be altered to enhance the minor profile. Also, if the minor contributor is determined to be male and an inadequate amount of DNA from the minor contributor available for autosomal STR analysis, the analyst can instead use typing methods such as Y-STR analysis to produce a more complete male profile.

With the use of standard quantitation methods, an analyst does not know that a mixed DNA sample exists until the multiplex typing assay is complete. At that point, STR loci consisting of more than two alleles are visualized providing information about the number of possible contributors. For instance, if a maximum of four alleles are visualized at any STR locus, a minimum of two contributors of DNA is suggested. By evaluating all of the loci amplified with the multiplex STR kit, the analyst can get an idea of the number of individuals that contributed to the mixture.

Once a mixture has been detected, the next step in an analysis is to determine the relative proportions of DNA from the different donors. This information can have important consequences for the statistical interpretation of the DNA typing results (Clayton et al., 1998; Gill et al., 1998; Butler, 2005). The RFUs for the alleles at a locus consisting of four alleles are evaluated to determine if the sample contains a major and a minor contributor, usually defined by a 2-3 fold difference in RFUs for the deduced phenotypes. Major and minor phenotypes can be deduced from the four allele pattern by pairing the alleles in a manner consistent with comparable RFU quantities and assuming the proportionality of amplicon product with input amplified template DNA. A profile can then be generated for the major contributor and another for the minor contributor in the sample.

The Q-TAT quantitation method provides much of this information as part of the quantitation step before STR typing is performed. Thus, an analyst dealing particularly with sexual assault evidence will know before STR typing reactions are prepared that 1) a sample consists of male:female mixture, 2) the male (or female) is a minor (equal or

major) contributor to the sample, and 3) the sample is suitable for autosomal STR analysis rather than Y-STR typing.

The X and Y amplicons produced by amplification of the Amelogenin locus are used as the basis for determining the amount of male and female DNA present in each sample through the ratio of RFU in the X and Y amplicons. For example, in a sample consisting of 10 parts female and 1 part male DNA, the female is the major contributor and the male the minor. Therefore, the major/minor RFU ratio would be female/male. In comparing the X/Y peak ratios produced with Q-TAT as an estimate of the proportions of DNA in a mixture to the estimates produced using autosomal STR allele peak areas (ie the current standard method), samples known to contain high amounts of female DNA and low amounts of male DNA (10 female:1 male, 5 female:1 male), produced an X/Y ratio with Q-TAT that was slightly higher than expected (Figure 18). As discussed above, the imbalance may be due to a preferential dropout of the Y allele in samples containing minor amount of male DNA (Figure 7).

In the comparison of major/minor peak ratios between the two methods for non-probative evidentiary samples (Figure 22), similar results were produced for the majority of the samples with some minor variation. X/Y ratios in some evidentiary samples were imbalanced for reasons that do not seem to involve allele dropout with a minor male contributor. However, because only loci containing four alleles could be used to estimate the relative proportion of DNA from the contributors to the mixture using STR methods, the number of samples analyzed was limited. Also, the Q-TAT assay is not able to distinguish between same sex mixtures, and based on the number of alleles observed for

some samples from TPD, it is possible that some contained DNA from more then one male and/or one female.

V.D. Casework Processing Strategies

One of the earliest procedures performed by a DNA analyst investigating a crime involving DNA evidence is choosing an analytical strategy for evidence processing that will provide the greatest amount of probative evidence for the prosecution of a suspect. In making this decision, an analyst will incorporate knowledge of the crime as well as preliminary screening results (for semen, blood, or saliva detection) as to decide between testing strategies. In this regard, Q-TAT results can provide invaluable information about the DNA composition of evidentiary samples, particularly samples recovered from sexual assaults.

Based on Q-TAT analysis results, the analyst can determine the optimal method for typing and the best amount of input DNA to add to the reaction in order to produce the most complete profile of the assailant in a sexual assault case. If the quantitation results indicate that no mixture is present or that a sufficient amount of DNA exists for all components of the mixture, standard autosomal STR analysis can be preformed with its extremely high level of discriminatory power. However, if the quantitation results indicate that a mixture is present and that it contains a minor male contributor probably providing an insufficient amount of DNA template for autosomal STR typing reactions, the analyst can proceed with Y-STR analysis of the male component in the mixed sample. For such samples, Y-STR typing will allow the analyst to produce a more probative DNA profile of the male component even though the inherent power of the test method is lower

(deKnijff, 2003). Unlike autosomal STR typing, with Y-STR typing, excess female DNA does not interfere with a minimum amount of male DNA in the sample (Prinz et al., 2001). Examples of a possible decision making strategy and the role played by Q-TAT results in the flowcharts are diagrammatically summarized in Figures 23 and 24.

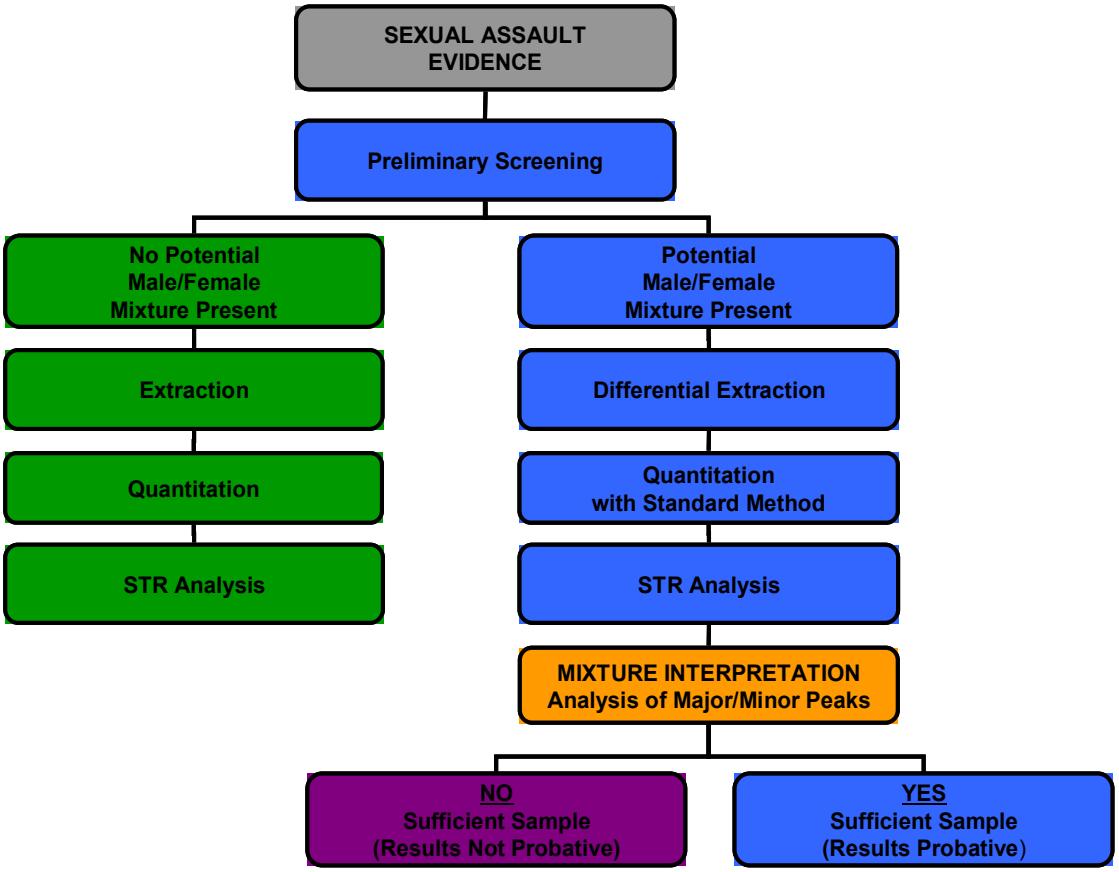

Figure 23: Flowchart for the processing sexual assault evidence by a crime lab when using standard quantitation methods.

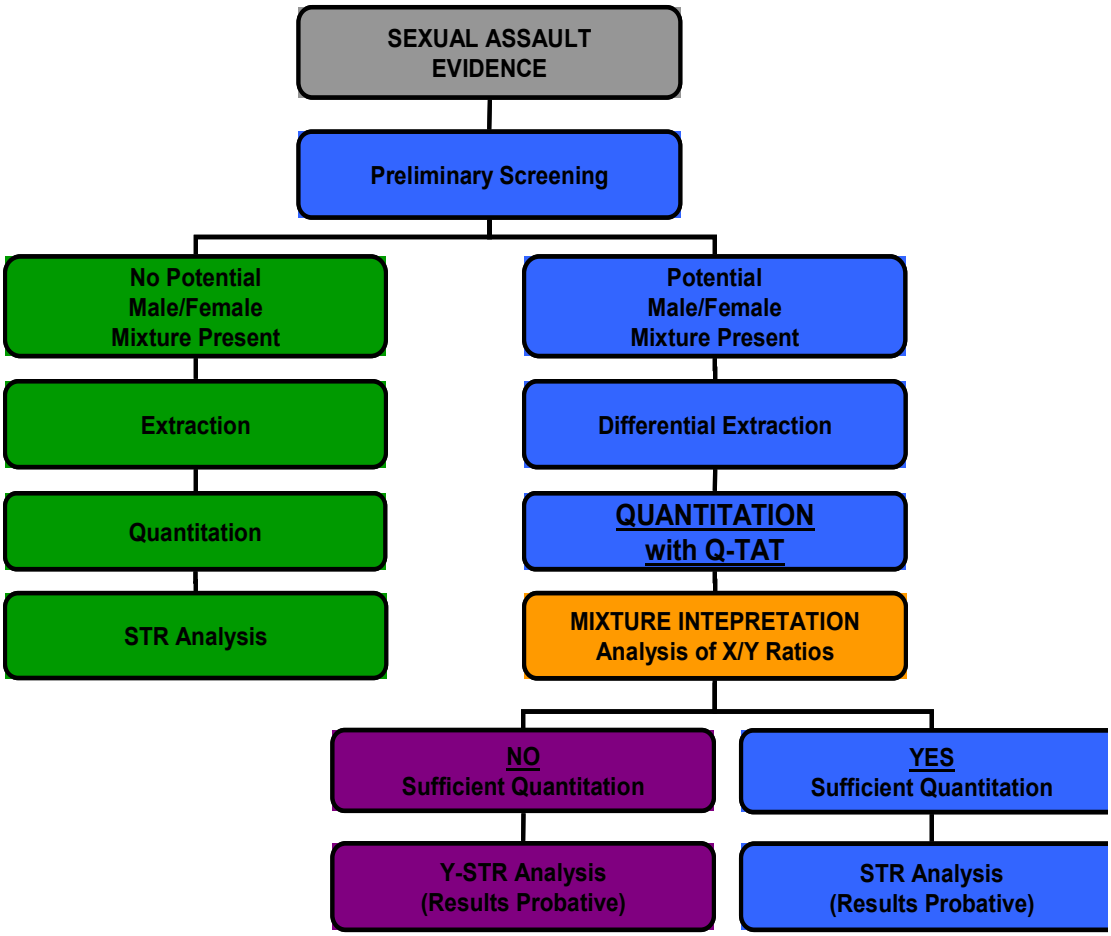

Figure 24: Flowchart for processing sexual assault evidence by a crime lab when using the Q-TAT quantitation method.

V.E. Additional Sample Types

Male and female mixtures can result from other types of evidence that are not composed of epithelial and sperm cells and therefore do not lend themselves to separation by differential extraction methods. Swabs are commonly collected in sexual assault cases and can contain mixtures of male and female epithelial cells. Sources of such samples include bite marks, breast swabs, and vaginal swabs from victims who have been orally assaulted. Male and female bloodstain mixtures may be collected from a homicide scene

and represent another example of evidentiary samples in which differential extraction methods provide no help in separating male and female DNA fractions. These samples must be extracted by standard extraction methods resulting in a final extract containing DNA from both donors.

Q-TAT is an especially important tool for evaluating samples such as these that are not amenable to differential extraction. Q-TAT analysis of samples consisting of blood and semen mixtures present in varying proportions produced results comparable to those obtained with mixtures prepared with DNA from blood (Figure 19). As discussed above for non-probative evidentiary samples from TPD, Q-TAT was also useful for resolving male and female proportions in a variety of evidentiary sample types (Figure 21, 22).

V.F. Overall Value of Q-TAT

This study emphasizes the fact that the implementation Q-TAT as a quantitation method into forensic laboratories will be useful for more than simply DNA quantitation. Q-TAT will provide additional information to a forensic DNA analyst that will assist with decision making during analysis so as to produce quality STR profile results. Because Q-TAT uses to the same instrumentation and methodology as STR typing, it does not require additional training for the analyst, the purchase of new equipment, or the allocation of additional space. Laboratories conducting multiplex STR typing reactions have all the instrumentation necessary to incorporate Q-TAT as a routine DNA quantitation method. The only additional investment would be primers designed to amplify the Amelogenin locus. Promega Corp. (Madison, WI) has a commercially

available sex typing kit designed for this purpose. Although the sex typing kit was not used for this study, it was used for much of the study of Allen and Fuller (2006) and the primers used here were identical in sequence to those provided with the Promega kit. In addition, both the upstream and downstream primers used in this study were labeled at the 5' end with fluorescein with no major difference in results.

Q-TAT also uses the same PCR amplification and DNA fragment detection technology as STR typing reactions. Any inhibitors that may affect the outcome of a typing reaction will also affect the outcome of the Q-TAT quantitation reaction. Components of the substrate of these samples, such as dyes, can interfere with differential extractions resulting in mixed male and female fractions, or interfere with PCR amplification resulting in the production of insufficient allelic products. Because Q-TAT and STR typing reactions share the same technology, most inhibitors of the typing reaction will also be inhibitory to the Q-TAT quantitation method. In this way, Q-TAT adds predictive value to the outcome of the PCR profiling assay. Other quantitation techniques, such as yield gel, QuantiBlot, and real-time PCR use different methodologies and therefore are not as likely to respond to inhibitors in the same way. In addition, the primers used to amplify the Amelogenin locus produce amplicons that are similar in size to alleles produced at STR loci (Applied Biosystems, 2005). This is important since evidentiary samples vary widely.

Q-TAT has the ability to produce quality quantitation results and provide predictive information for typing reactions. It is a cost effective procedure that could give a number of forensic laboratories the technology needed to predict the amount of male and female DNA present in mixed evidentiary samples recovered from crime scenes.

The main limitation to this procedure is its inability to distinguish between same sex mixtures. Because the Amelogenin amplification procedure is adapted to separation based on the amount of X and Y amplicons produced, multiple male or female contributors to samples also cannot be predicted. However, because sexual assaults account for the majority of mixed biological samples and a high percentage of these cases result from the assault of a single female by a single male, the Q-TAT method will be able to resolve most DNA mixtures recovered from a crime scene.

The Q-TAT method holds great promise for the analysis of mixtures. Current research in the laboratory is devoted to optimizing the conditions for the inclusion of an internal positive control that would provide additional information about each sample analyzed. The RFU value for the internal positive control would provide the analyst with information about possible inhibitors in the sample that could potentially interfere with the STR multiplex typing assay. This information would thus alert an analyst to the presence of inhibitors and cause additional "clean-up" steps to be taken to remove the inhibitor before consuming part of the sample through PCR.

Conclusions:

- Q-TAT will work within the context of typically encountered case work in a forensic laboratory.
- Q-TAT is able to accurately estimate the relative male and female contributions in mixed samples.
- For samples in which Q-TAT's accuracy is lacking, it still has the ability to identify the presence of a minor male contributor and provide a DNA analyst with enough information to choose the test that will provide the most complete profile.

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APPENDIX

Oklahoma State University
Center for Health Sciences
College of Osteopathic Medicine
Institutional Review Board
FWA # 00005037

MEMO

Date: August 31, 2005

8-31-05

To: Robert W. Allen, PhD
Chairman, Program Director, Associate Professor Forensic Sciences
OSU-CHS

From: Stephen Eddy, DO, MPH, CIM
Chairman, Institutional Review Board, OSU-CHS

exempt approval

Re: **Protocol IRB # 2005018**
Exempt Approval

Titled: **Quantitation of male and female DNA in mixed biological samples using quantitative amplification of the human Amelogenin locus**

Under the authority of Title 45, Subtitle A, Part 46, Subpart A (45 CFR 46) and the OSU-CHS Institutional Review Board (IRB), an exempt review was performed on Protocol – IRB # 2005018 (version Appendix B_Expanded (Juroske)_rev.8-31-05). It was determined the protocol meets exempted criteria under federal guidelines; therefore, you are free to begin the study.

This protocol will be submitted to the full IRB at the October 05, 2005 meeting. It is the IRB's prerogative to require additional information or changes in the protocol. You may be called before the board to provide additional information and answer questions. You will be informed if any changes are required.

Principal investigators, collaborating investigators, study coordinators and other personnel who have contact with **data or subjects** involved in human research are required to **receive training on human subjects** *before* beginning a research project. Please contact Steve Phillips at (918) 561-8488 about how you can fulfill these requirements if you have not documented previous training.

You are free to begin the study **once all persons involved with your study have completed the above-mentioned training and documentation of that training is received.**

As principal investigator of this protocol, it is your responsibility to insure that this study is conducted as approved. Any modifications to the protocol or consent form, initiated by you or

by the sponsor, will require prior approval. All study records, including copies of signed consent forms, must be retained for three (3) years after termination of the study.

If you have questions please contact Teri Bycroft, IRB Administrator at (918) 561-1243.

VITA

Denise Marie Juroske

Candidate for the Degree of

Master of Science

Thesis: QUANTITATION OF MALE AND FEMALE DNA IN MIXED BIOLOGICAL SAMPLES USING QUANTITATIVE AMPLIFICATION OF THE HUMAN AMELOGENIN LOCUS

Major Field: Forensic Science

Biographical:

Education: Graduated from Industrial High School, Vanderbilt, Texas in 1996; received Bachelor of Science degree with a double major in Biochemistry and Genetics from Texas A&M University, College Station, Texas in 2001; received Bachelor of Science degree in Molecular Genetic Technology from the University of Texas M.D. Anderson - School of Health Sciences, Houston, Texas in 2005. Completed the requirements for the Masters of Science degree with a major in Forensic Science at Oklahoma State University – Center for Health Sciences in July 2006.

Experience: Worked at the University of Texas M.D. Anderson Cancer Center in Houston, Texas for four years as a Research Assistant II; performed various methods of DNA extraction, amplification, runs on a 310 Gene Analyzer, and data analysis as a Graduate Assistant in the human identity laboratory led by research advisor Dr. Robert Allen during educational experience at OSU-CHS.

Professional Memberships: Member of the Association of Genetic Technologists for two years, Certified in Molecular Biology from the National Credentialing Agency and certified in Molecular Pathology from the American Society of Clinical Pathology for the past year.

Name: Denise Marie Juroske Date of Degree: July, 2006

Institution: Oklahoma State University – CHS Location: Tulsa, Oklahoma

Title of Study: QUANTITATION OF MALE AND FEMALE DNA IN MIXED BIOLOGICAL SAMPLES UING QUANTITATIVE AMPLIFICATION OF THE HUMAN AMELOGENIN LOCUS

Pages of Study: 96 Candidate for the Degree of Master of Science

Major Field: Forensic Science

Scope and Method of Study: The purpose of this study was to determine whether quantitative template amplification technology (Q-TAT) could be used to accurately evaluate the relative proportion of male and female contributors in evidentiary samples. Single source male and female DNA extracts were well characterized and then used to produce male and female mixtures of known proportions. Q-TAT was used to evaluate the male and female DNA mixtures through amplification of the Amelogenin locus. Amelogenin amplicons were analyzed on the 310 Genetic Analyzer, and quantitated using GeneScan software and Excel spreadsheets. Results from the analysis of the known mixtures were then applied to non-probative evidentiary samples obtained from the Tulsa Police Laboratory. STR analysis was used to ensure the quality of results produced from Q-TAT estimations.

Findings and Conclusions: Q-TAT provides useful information in the quantitative analysis of male and female mixed biological samples. With high amount of male DNA and a minor female contributor, no significant difference was observed between the observed and expected X/Y ratios. For samples containing high amounts of female DNA in the presence of a minor male contributor, the expected ratio was slightly lower than the observed ratio for ratios greater than 1 female:5 male. Major/minor peak ratios produced by STR typing were similar to those produced with Q-TAT for both known mixtures and non-probative evidentiary samples. The Q-TAT quantitation method is able to accurately estimate the relative male and female contributors in mixed samples. For samples in which Q-TAT's accuracy is lacking, it still has the ability to identify the presence of minor male contributor and provide a DNA analyst with enough information to choose the test that will provide the most complete profile.

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ADVISOR'S APPROVAL: Dr. Robert Allen