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EVALUATION OF APPLIED BIOSYSTEMS'

REAL-TIME HUMAN QUANTIFICATION

ASSAYS

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EVALUATION OF APPLIED SYSTEMS' REAL TIME HUMAN QUANTIFICATION ASSAYS

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CHAPTER I

INTRODUCTION

To perform the polymerase chain reaction (PCR) and downstream genetic analyses, it is critical to assess the quantity of deoxyribonucleic acid (DNA) present in samples. This ensures that amplification will proceed with the optimal input of genetic material while also minimizing excess consumption of the sample. Quantification in the realm of forensic science is especially important because the PCR required for subsequent genetic profiling performs within a limited range of quantities.

Quantification of forensic samples is a challenging task for the reason that these samples require high sensitivity and specificity. The DNA Advisory Board (DAB) was established by the Congress to develop national standards and guidelines by which all forensic laboratories throughout the country are to follow. In regards to quantification, the DAB established standard 9.3 that states, "The laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample where possible" (1). The critical factor in this standard is human quantification. Oftentimes forensic samples are contaminated with genetic material from bacteria or other species. Samples may also be mixtures of DNA from two or more individuals. In many cases, the DNA is present in severely limited amounts. For these reasons, it is important to be able to differentiate human from non-human DNA and to attempt to quantify the amount of DNA present, even if little yield is expected. Additionally, human quantification satisfies

legal issues in the courtroom. Current quantification methods fall short in properly detecting low-level amounts of DNA. Furthermore, no assays are available that can distinguish human male DNA that may be present alone or as a mixed sample of both genders.

Short tandem repeat (STR) analysis is used to generate individual genetic profiles. Although this type of analysis is carried out with well-validated kits, the multiplex PCR required to amplify numerous genetic loci performs within a limited range of DNA quantities. Moreover, the PCR can fail if inhibitors are present in DNA extracts. Heme, a common inhibitor, can usually be detected by visually inspecting the sample after extraction is completed. Other inhibitors, such as chloroform, EDTA, ionic detergents, and ethanol, just to name a few, are not easily detected (2). Rather, it is through the failure of PCR and/or STR analysis that inhibition is identified. This results in the consumption of expensive reagents and waste of valuable time. No methods exist that detect inhibitors and warn of impending analysis failure.

Currently, quantification methods include spectrophotometry, fluorometry, enzymatic and hybridization assays. Spectrophotometry is the most widely used quantification method. It measures the absorbance of DNA at 260nm and 280nm and has a sensitivity threshold of 500ng (3). This method is not human specific. In fact, spectrophotometry quantifies contaminants such as RNA, protein, and residual phenol. Spectrophotometry does not have the sensitivity and specificity that forensic samples require; therefore, it cannot be used for samples in which low yields of DNA are expected and when human quantification is necessary. With regard to aged forensic samples, Previderè et al. agree

that spectrophotometric determination is unreliable and often quantifications cannot be made on these types of samples (4).

Fluorometric quantification includes a variety of assays. The most common method is the yield gel that uses agarose gels stained with ethidium bromide to visualize and quantify DNA. The sensitivity of these gels is controlled by various factors such as the percentage of agarose, running buffers, volume of sample applied to gel, run conditions, and staining sensitivities. PicoGreen® (Molecular Probes, Inc., Eugene, OR), a cyanine dye, is another fluorescent molecule used for quantification purposes. It has increased sensitivity over other methods at 0.05ng (5) and can bind to double-stranded DNA (dsDNA). However, fluorescent dyes are not human specific and thus have limited uses in forensics. Additionally, the fluorometer used to detect fluorescence is only as accurate as its ability to discern concentration differences in the standard curve. The dynamic ranges achieved with a fluorometer do not possess the level of sensitivity that some forensic samples need.

The AluQuant™ Human DNA Quantitation System (Promega Corporation, Madison, WI) is an enzymatic assay designed to detect repetitive Alu sequences that occur frequently throughout the genome. What was once thought to be a promising method has been show to be unreliable and not reproducible (6). Therefore, it has yet to be embraced by the forensic community.

Hybridization assays, such as ACES™ 2.0^{+} Human DNA Quantitation System (Invitrogen, Carlsbad, CA) and QuantiBlot™ Human DNA Quantitation Kit (Applied Biosystems, Foster City, CA), provide good sensitivity (0.04ng and 0.15ng, respectively)

(7,8) and the human specificity that the forensic community needs. Unfortunately, the ACES™ quantification system has recently been discontinued. The forensic community has been left with few choices.

The QuantiBlot™ is the most widely used quantification method for forensic samples. This assay works by first spotting a DNA extract onto a membrane, then hybridizing a higher primate specific biotinylated probe which is complementary to DNA sequence at D17Z1 (9) to the DNA that may be present. After a series of washes, the blots are then developed by binding an enzyme conjugate: HRP-SA (horseradish peroxidase-streptavidin) to the D17Z1 probe and then developing the blot either colorimetrically or in a chemiluminescent fashion. Colorimetric development is most common. The oxidation of Chromogen: TMB, catalyzed by the horseradish peroxidase, produces a blue colored precipitate on the membrane. The intensities of the sample bands are visually compared to those of the standard curve. The problem with these assays is the subjective manner in which concentrations are determined. Expensive densitometry equipment could be used to avoid the subjectivity, but even the detection systems have limited dynamic ranges. Additionally, there are issues with the dynamic range of the standards. They are not adequate either on the high end (10ng in 5μl) or on the low end (0.15ng in 5μl). At times, the quantity of DNA exceeds that of the highest standard. In these cases, the analyst is forced to extrapolate the concentration above the highest tested range, or the assay must be repeated with a diluted sample. The QuantiBlot™ is a laborious assay, taking approximately two-and-a-half to four hours to complete, most of which is hands-on time. Additionally, the QuantiBlot™ cannot detect low-level amounts of DNA. In

spite of null results obtained with QuantiBlot™, most DNA analysts will proceed with STR analysis and very often successfully generate a genetic profile. This means that the QuantiBlot™ does not have the same sensitivity as the methods of STR analysis. Applied Biosystems has determined that the sensitivity of the QuantiBlot™ is 0.15ng in 5μl or 0.03125ng/μl (8), although this is not always achieved. Hybridization efficiency of samples may be affected by inhibitors present in the extract. This causes a decrease in the sensitivity of the assay.

Clearly, quantification has become both a technical and legal challenge to forensic scientists. In 2001, Duewar et al. published a study evaluating interlaboratory comparison of DNA analysis that included all aspects from the extraction through the evaluation of STR data. They concluded that DNA amplification anomalies are associated with, among other factors, inaccurate DNA quantification and that "STR systems may well require improved DNA quantification technology" (10). Previderè et al. go so far as to say that hybridization assays are not good quantification methods when dealing with degraded samples. Degraded DNA may not be able to bind to the specific hybridization probe. They point out that this topic is crucial, but "so far has not received the attention it deserves" (4).

To aid the forensic community with its quantification issues, Applied Biosystems is currently developing human specific and human male specific quantification assays using Real-Time PCR (RT-PCR) and TaqMan® probes. The human specific assay amplifies an autosomal specific gene, located on chromosome five, while the human male specific assay amplifies a region on the Y chromosome. The purpose of this project was to

evaluate the assays with forensic samples to determine if the use of these kits would be appropriate for the forensic community. These kits are not commercially available at the time of this writing. Therefore, several details have been omitted to protect the patent and legal issues that are still pending.

It is expected that these assays will surpass the sensitivity and specificity of current methods. This will not only meet, but also exceed the standard set forth by the DAB. By providing additional information such as human male DNA quantification and PCR inhibitor detection, these kits can provide what the forensic community has been lacking. The human male DNA detection and quantification is valuable in providing proof that male DNA was present in an intimate sample from a sexual assault case. This would be especially important in a case in which the offender was a vasectomized male, and for resolving mixtures of the victim and offender's genetic profiles. The detection of PCR inhibitors for the elimination of futile genetic analysis is a novel component that would provide additional advantages. These kits will offer means for proper quantification to allow for minimal sample waste, and allow for successful multiplex PCR within its optimal range. Today, STR analysis will proceed, and is often successful, even if no quantification results are obtained with current methods. The legal system questions this approach. The ability of the autosomal specific and Y-chromosome specific RT-PCR quantification assays to assess low level DNA would provide the justification for subsequent analysis that would quiet the legal system's arguments concerning human quantification.

CHAPTER II

BACKGROUND

Real-Time PCR and TaqMan® probe concepts

To understand RT-PCR, it is important to understand what happens during PCR. Three phases comprise the reaction (Figure 1). The first phase of PCR is the exponential phase. Reagents are readily available, kinetics drive the reactions forward, and the amplicons are truly doubling with each cycle (assuming 100% efficiency). In the linear phase, reagents are being consumed and thus are not as readily available. Therefore, the reactions are slowing and are no longer doubling with each cycle. By the plateau phase, amplification has ceased because reagents are completely consumed. It is at this plateau phase that traditional gel-based PCR is detected, typically on agarose gels (11).

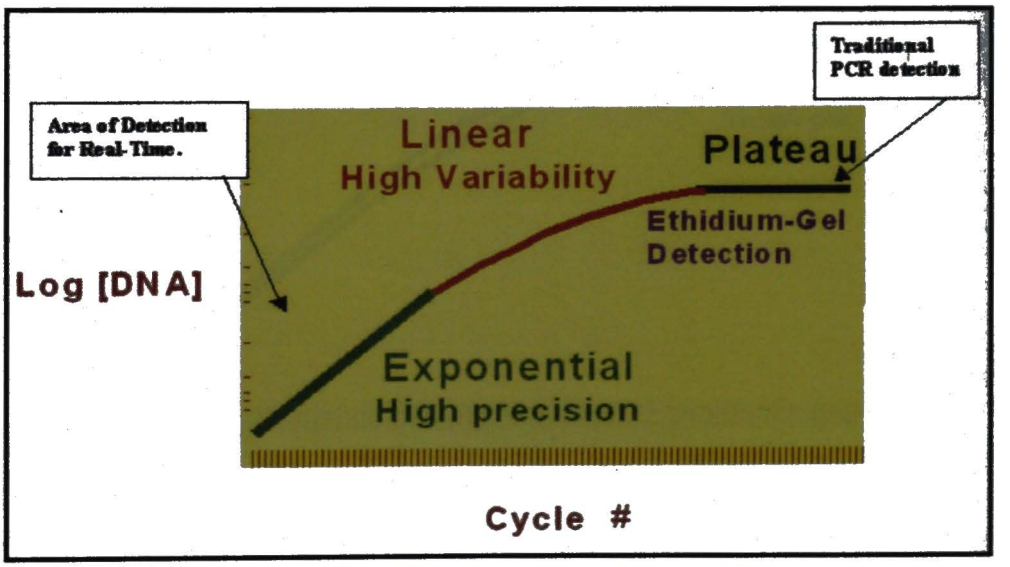

Figure 1. Phases of PCR. (11).

Figure 2 shows three replicates of the same sample. They begin PCR with the same quantities, but due to variations among reactions, the end results show variable amounts of products. In traditional gel-based PCR, it is at this final plateau step that measurements are taken. However, it would be more accurate to take measurements at the exponential phase when the reactions are truly equal. This fact is further substantiated by Figure 3. This shows a five-fold dilution series. At the plateau phase, the amplification lines merge and give the appearance of similar quantities. On the other hand, the exponential phase lines clearly illustrate that each dilution crossed the fluorescent threshold at different cycle numbers and therefore contain different quantities of input DNA (11).

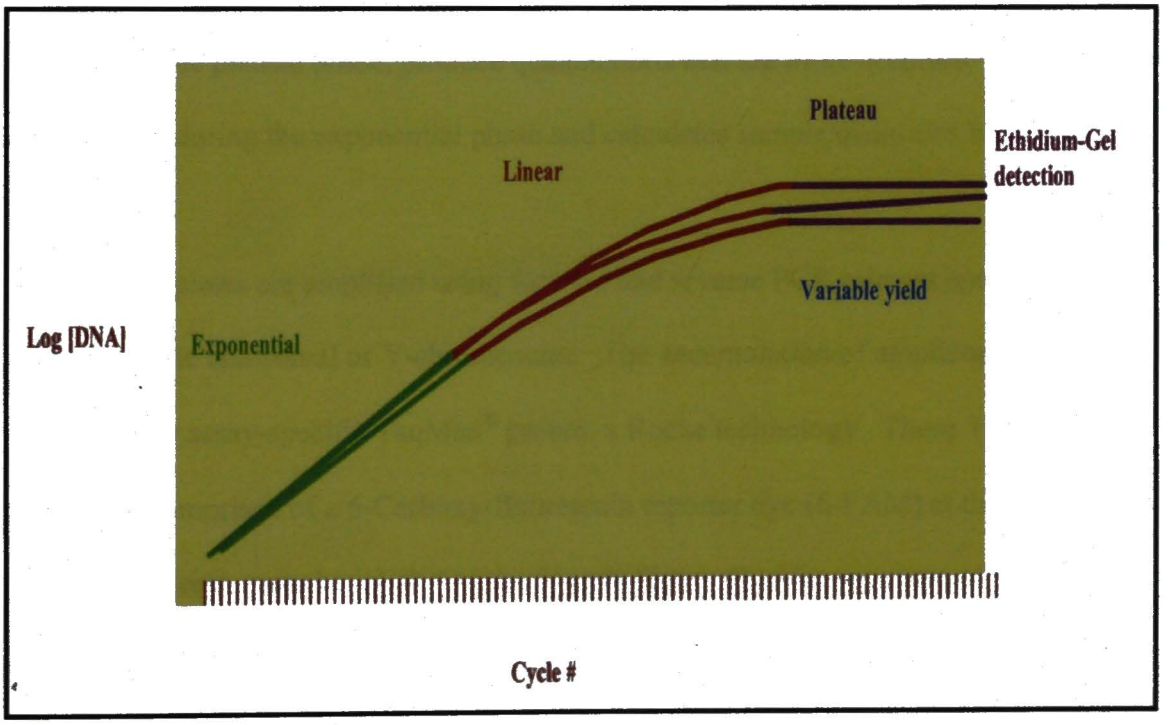

Figure 2. PCR of three replicates (11).

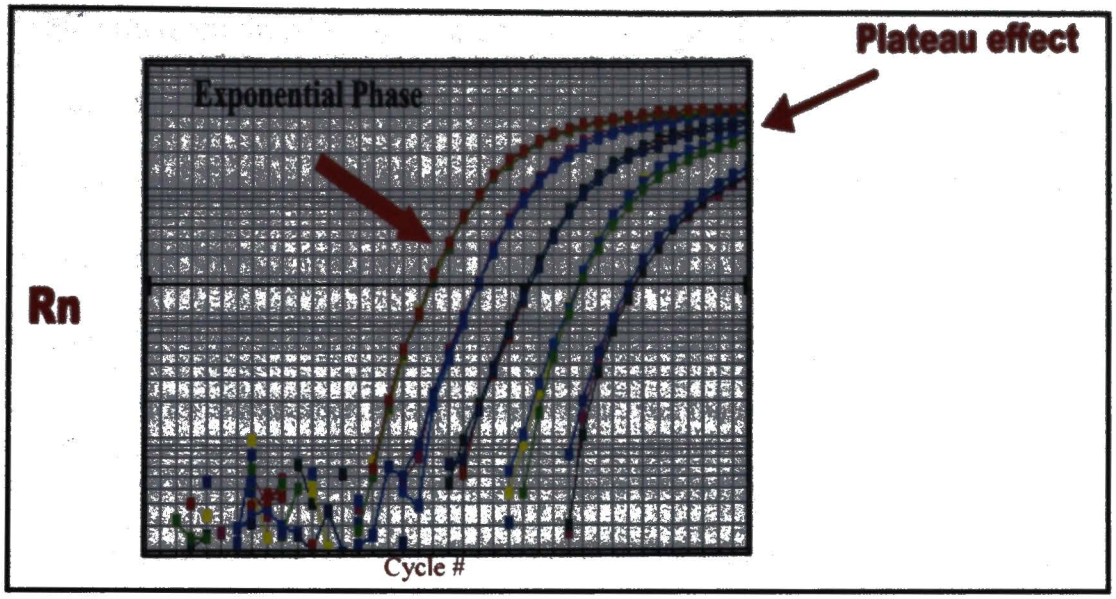

Figure 3. PCR of five-fold dilutions (11).

These series of figures show that measurements taken during the exponential phase of PCR, not at the plateau phase, produce quantitations that are more accurate. RT-PCR collects data during the exponential phase and calculates sample quantities based on this information.

DNA templates are amplified using forward and reverse PCR primers specific for each assay, the autosomal or Y-chromosome. The accumulation of amplicons is monitored by assay-specific TaqMan® probes, a Roche technology. These TaqMan® probes are comprised of a 6-Carboxy-fluorescein reporter dye (6-FAM) at the 5' end and a non-fluorescent quencher (NFQ) at the 3' end (Figure 4). The sequence of the probe is designed to anneal to a region on the amplified product of interest (Figure 5). TaqMan® probes take advantage of fluorescent resonance energy transfer technology (FRET). This means that when a high energy dye, the 6FAM dye, is in close proximity to an energy

transfer acceptor, the NFQ, there will be an energy transfer from high to low. As PCR products are formed, the specific TaqMan® probes will anneal to the amplified templates. Then as AmpliTaq Gold DNA Polymerase® moves across the template to synthesize a new strand of DNA, it will use its 5' nuclease activity to cleave the TaqMan® probe that has annealed to the template (Figure 6). This causes the reporter and quencher to be separated from one another, FRET cannot occur, and therefore the fluorescence of the reporter dye is no longer suppressed. The fluorescence of the reporter dye, which is proportional to the number of amplicons produced, is detected and used to calculate the quantities of the genetic material present in the reaction. Data are collected during the exponential phase of PCR where measurements are more accurate than with traditional gel-based PCR (11).

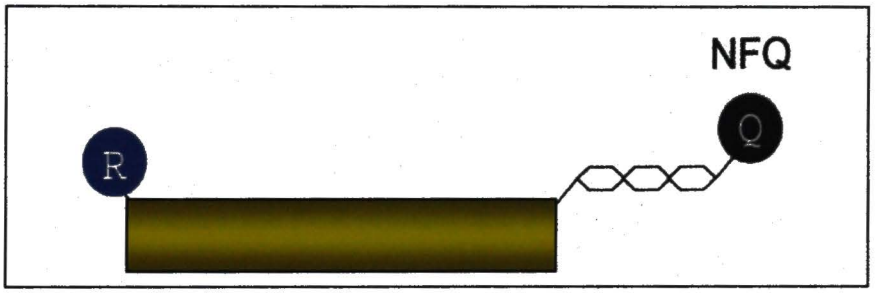

Figure 4. TaqMan™ probe (12).

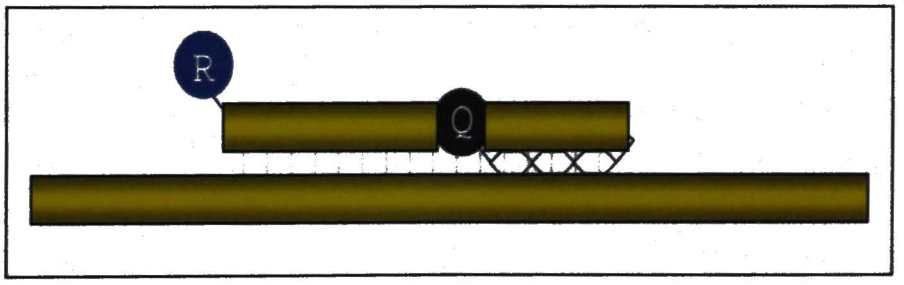

Figure 5. TaqMan™ probe annealed to template (12).

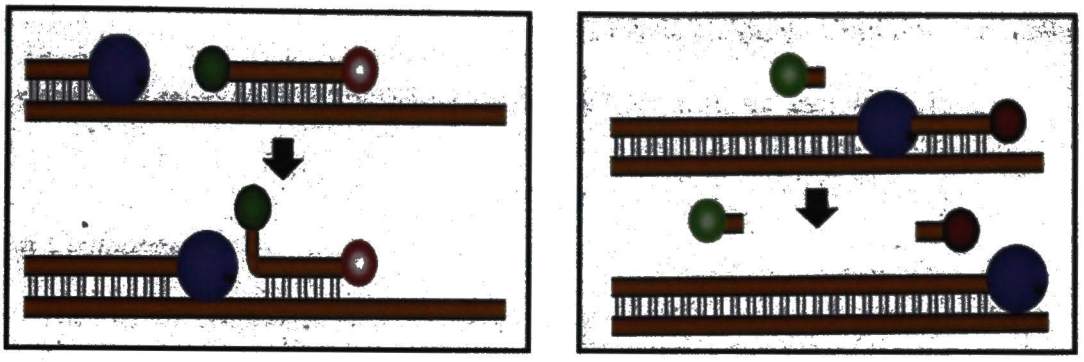

Figure 6. TaqMan™ probe annealing to template (left). 5' nuclease activity cleaves TaqMan™ probe (right) (11).

Internal Positive Control

The autosomal specific and Y-chromosome specific RT-PCR quantification assays are actually duplex assays, which contain two independent sets of PCR primers and TaqMan® probes. One of the assays is the target specific assay, either the autosomal or Y-chromosome, that consists of the PCR primers and the 6FAM-labeled TaqMan® probes as just described. The other assay is known as the internal positive control, or IPC, and is identical in each assay. The IPC is comprised of 10,000 copies per reaction of synthetic template not found in nature, the PCR primers, and a VIC labeled TaqMan® probe for its amplification and detection. The purpose of the IPC is to monitor the working conditions of the reactions. Proper amplification and detection of the IPC indicates that all conditions are operational, both chemistry and instruments, even if DNA is not present. If the IPC fails to be amplified and detected, this indicates the existence of a problem or error, most likely due to inhibition of the PCR. This is an extremely useful predictor of impending failure of the multiplex PCR step of STR analysis. This would prevent the

consumption of expensive reagents and waste of valuable time. Until now, no methods existed that detected PCR inhibitors and warned of imminent analysis failure.

Kit Components

The kits are comprised of three components: human male DNA standard, the Primer/Probe mix, and the TaqMan® 2X Universal PCR Master Mix. The human male DNA standard is provided at a concentration of 200ng/µl, which is diluted in a four-fold fashion to obtain standards of 50, 12.5, 3.12, 0.78, 0.195, 0.049, and 0.012ng/µl. The Primer/Probe Mix contains all the PCR primers, and the FAM- and VIC-labeled TaqMan® probes as previously described. The TaqMan® 2X Universal PCR Master Mix contains the reagents necessary for PCR (AmpliTaq Gold DNA Polymerase®, dNTPs, magnesium chloride, and buffers) and a passive reference dye, ROX (carboxy-X-rhodamine). The passive reference dye serves to normalize the emission of the reporter dye detected throughout the assay plate or tubes.

ABI PRISM® 7000 Sequence Detection System

All samples are assayed on the ABI PRISM® 7000 Sequence Detection System, a 96-well RT-PCR unit (Figure 7).

During PCR, light from a halogen lamp is focused on each of the 96 wells of the amplification plate (Figure 8). This light excites the fluorescent dyes. The resulting fluorescence emission is collected between 500 and 660nm. The emissions are focused onto a charge-coupled device (CCD) camera via lenses, filters, and a dichroic mirror.

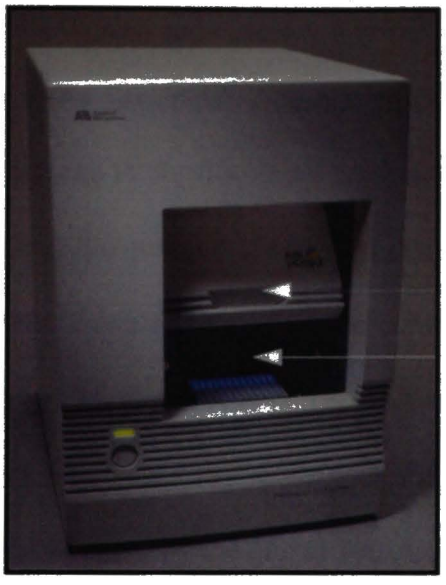

Figure 7. ABI PRISM ® 7000 Sequence Detection System (13).

The filters separate the light based on wavelength across the surface of the CCD camera. The ABI PRISM ® 7000 software collects the fluorescent signals and uses algorithms to convert the signals into quantification values (14).

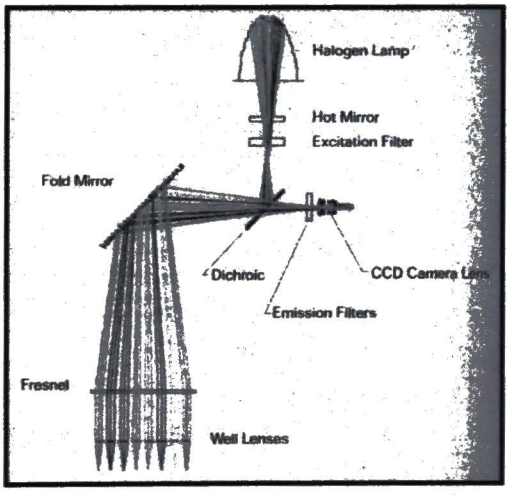

Figure 8. Fluorescence Detection of ABI PRISM ® 7000 SDS (14).

Software Analysis

The quantities of the genetic target are determined by monitoring cycle-to-cycle changes in fluorescence during the PCR. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the quantity of starting material.

The ABI PRISM ® 7000 software distinguishes the contribution of each fluorescent dye in the reaction. These include the 6FAM and VIC reporter dyes on the TaqMan® probes and ROX, the passive reference. The reporter signals, emitted from the reporter dyes, are normalized by dividing them by the signal detected from the passive reference dye, referred to as R_n (normalized reporter). Since the passive reference is a component of the PCR master mix, it is present in equal concentrations in all wells of the PCR plate. By normalizing the data, the software can account for minor variations in signal strength caused by pipetting differences (14). Figure 9 shows a typical display of the contributions of each component (actual data, 3/25/03).

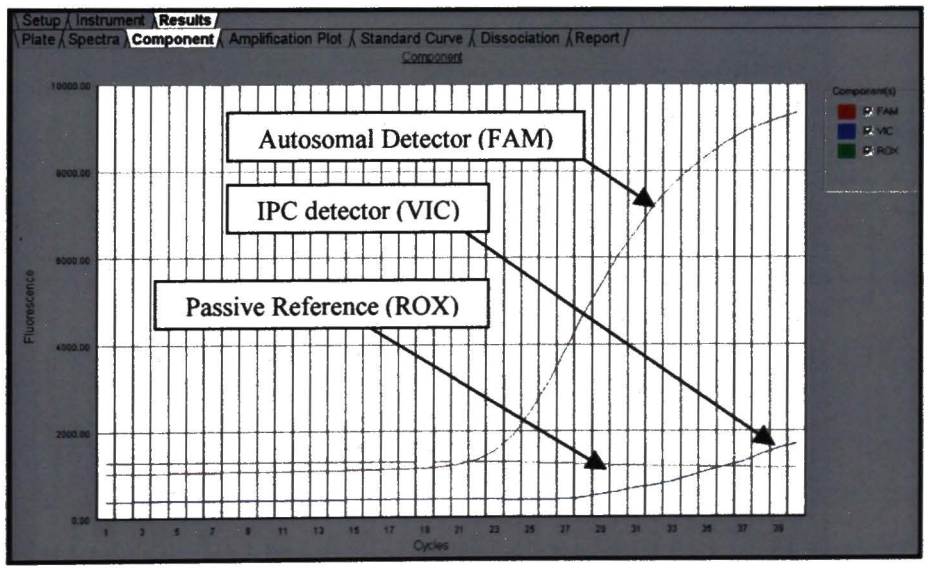

Figure 9. Contributions of each dye. 50ng/µl standard, 3/25/03.

Normal amplification of PCR products generates a curve like the one shown in Figure 10 (actual data, 3/25/03). The three phases of PCR can be distinguished. Initially, ΔR_n is below detection limit (R_n minus the R_n value of an unreacted sample). As PCR products increase, signal is detected. The amplification continues and the ratio of polymerase to products decreases. When template concentration reaches 10^{-8}M, products cease to grow exponentially, which signals the plateau phase. The software calculates the C_T value (point at which fluorescent signal crossed threshold setting) based on data collected from a range of PCR cycles (defined as the baseline). C_T values are dependent on starting template copy number and efficiency of PCR (14). After the run is analyzed, results are displayed in an amplification plot and in a spreadsheet report showing the C_T value, standard deviation of C_Ts (if performed in duplicate), quantity of sample, mean quantity, and standard deviation of quantity (if performed in duplicate). The quantifications are based on the quantities of the samples that make up the standard curve.

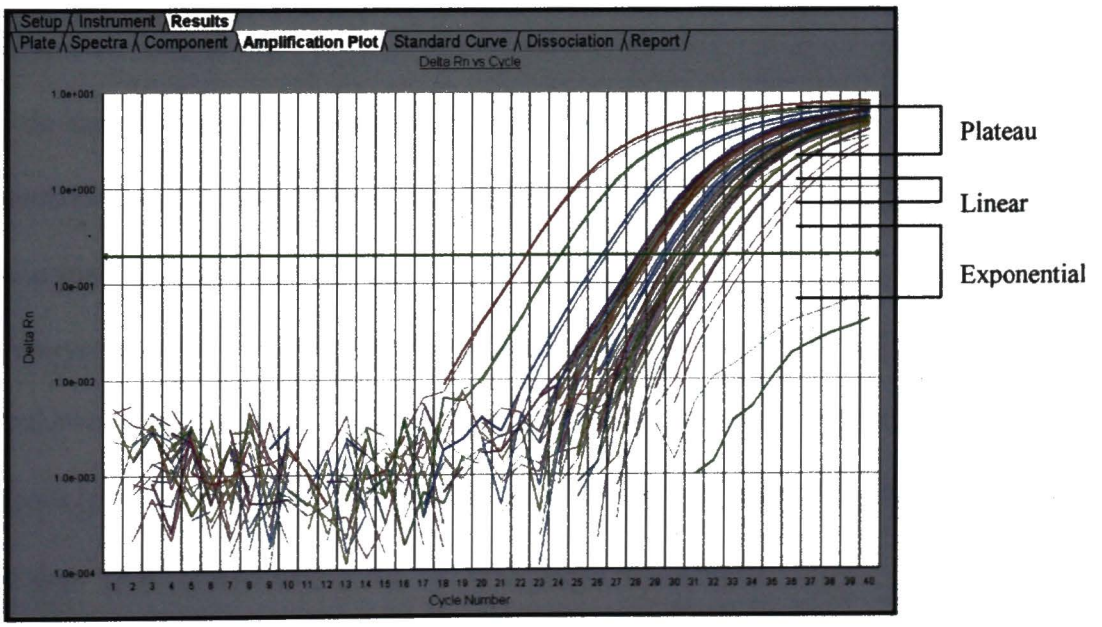

Figure 10. Amplification plot of an autosomal assay (3/25/03).

CHAPTER III

RESEARCH DESIGN

The purpose of this study is to evaluate Applied Biosystems RT-PCR human quantification assays with forensic samples to determine if the use of these kits would be appropriate for the forensic community. There are six objectives to this study.

Objective 1

Comparisons will be made between the autosomal specific RT-PCR quantification assay and current quantification methods: spectrophotometry, fluorometry, and hybridization assays. Spectrophotometry is the most common quantification method, although it does not have a good level of sensitivity and it is not human specific. Fluorometric assays, while having better sensitivity, are not human specific. However, they do have limited uses in forensics and paternity. The hybridization assay, QuantiBlot™, is the most commonly used quantification method in forensics today. It is human specific but there are issues with its sensitivity.

Assayed samples will include those isolated from a variety of tissue sources (blood, buccal swabs, fetal material, and sexual assault samples) extracted using an array of methods (phenol/chloroform, chelex, DNA IQ™ (Promega Corporation, Madison, WI), PrepMan™ (ABI, Foster City, CA), and differential extraction).

Objective 2

STR data of several samples, first quantified with QuantiBlot™ and then with the autosomal specific RT-PCR quantification assay, will be compared. Applied Biosystems AmpFℓSTR® Identifiler™ PCR Amplification Kit will be used to conduct the STR analysis. Factors such as stutter (peaks that are one repeat unit smaller than the true allele), off ladder alleles (peaks that are not consistent with a nominal allele size), peak balance (equal heterozygous allele peaks), and relative fluorescent units (RFUs) will be gauged.

Objective 3

Female and male DNA extracts will be combined in various ratios (50:50, 75:25, 80:20, 90:10, 95:5, 99:1, and 99.5:0.5) to determine the sensitivity of the detection of male DNA present in mixed samples. Both female and male DNA will be obtained from fresh blood and extracted with the phenol/chloroform method. Samples will first be quantified using the autosomal specific RT-PCR quantification assay, diluted to the proper ratios, and then analyzed using both assays: autosomal and Y-specific RT-PCR quantification assays.

Objective 4

Samples that are suspected of containing carry-over male DNA in what would essentially be a female DNA extract will be analyzed with the Y-chromosome specific

RT-PCR quantification assay. The ratios of total DNA, determined by using the autosomal specific RT- PCR quantification assay, to male DNA will be calculated and subsequent STR analysis will be examined to determine if these ratios are beneficial in resolving mixtures. Samples will include those from sexual assault cases and fetal material used for determining paternity.

Objective 5

Sensitivity experiments will be performed to establish the detection levels of each quantification assay. DNA isolated from blood, using the phenol/chloroform method, will first be quantified using the autosomal specific RT-PCR quantification assay. The DNA will then be diluted in concentrations ranging from 1ng/μl to 0.0005ng/μl then assayed using both the autosomal specific and Y-chromosome specific RT-PCR quantification assays.

Objective 6

Low copy number samples, or extracts suspected of containing trace amounts of DNA, are tested using the autosomal specific RT-PCR quantification assay. Samples will be collected from doorknobs, telephones, and soda cans in a controlled manner and then extracted using a silica extraction technique. Attempts will be made to utilize the C_T values generated during the RT-PCR quantification assay (defined as the cycle number at which fluorescence of PCR reaches threshold) to determine if increasing the number of PCR cycles in the STR multiplex amplification step will improve the ability of obtaining

a genetic profile of an individual. Applied Biosystems AmpFℓSTR® Identifiler™ PCR Amplification Kit will be used to conduct the STR analysis. Additionally, these low copy number samples will be quantified using the current QuantiBlot™ method and compared to the autosomal specific RT-PCR quantification assay.

CHAPTER IV

MATERIALS AND METHODS

All autosomal specific and Y-chromosome specific RT-PCR quantification reagents were provided by Applied Biosystems, Inc., Foster City, CA.

RT-PCR Human Quantification Protocol

Each assay was performed with the same volume of reagents and under the same universal cycling parameters as put forth by the provided protocol (unpublished and proprietary). All reactions, including the standards, were performed in duplicate. The protocol is as follows:

1. Dilute human male DNA standard in a four-fold fashion. Into seven 0.5ml tubes, place 15µl of sterile water. Thaw and vortex the human male DNA standard. From the human male DNA standard tube (200ng/µl), pipet 5µl into the first standard tube containing 15µl of sterile water. Vortex. This creates the 50ng/µl standard. Change tips and pipet 5µl from the 50ng/µl standard into the second tube containing 15µl of sterile water. Vortex. This creates the 12.5ng/µl standard. Change tips and continue performing serial dilutions until all standards are made. Concentrations of standards are 50,

12.5, 3.12, 0.78, 0.195, 0.049, and 0.012ng/µl. It was found that making standards fresh before each assay worked best.

2. Create the plate layout using a 96-well template worksheet. Plate format is flexible. Throughout this project, the standards were assayed in wells A1-A8 and B1-B8. See chart below. It is acceptable to run both assays on one plate; however, it is recommended that each assay be analyzed with standards run under the appropriate assay (autosomal or Y-chromosome). All samples and standards were run in duplicate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	12.5	3.12	0.78	0.195	0.049	0.012	NEG				
B	50	12.5	3.12	0.78	0.195	0.049	0.012	NEG				
C												
D												
E												
F												
G												
H												

3. Create and save a new session on the ABI PRISM® 7000 SDS Software.

4. Calculate volumes of Primer/Probe Mix and TaqMan® 2X Universal PCR Master Mix for the master mix. Each reaction receives 10µl of Primer/Probe Mix and 12.5µl of TaqMan® 2X Universal PCR Master Mix. Make enough master mix for all samples plus a few extra to account for loss during pipetting.

5. Thaw reagents, vortex, and dispense as calculated. Vortex again. Limit the Primer/Probe Mix's exposure to light.
6. Aliquot 22.5µl of master mix into appropriate wells of a MicroAmp Optical 96-well Reaction Plate (P/N N801-0560).
7. Pipet 2.5µl of sample into each reaction well. Ensure that each sample is as homogenous as possible (vortex if needed). Each sample will require a total of 5µl to perform in duplicate.
8. Seal the plate with an Optical Adhesive Cover (P/N 4311971) with the sealing tool.
9. Centrifuge plate briefly at 2600rpm.
10. Place plate into ABI PRISM® 7000 Sequence Detection System with plate oriented so that well A1 is in top left corner.
11. Run assay(s) under universal cycling parameters.
    a. 95°C for 10 minutes
    b. 40 cycles of 95°C for 15 seconds/60°C for 60 seconds.
    c. The assay takes 1.75 hours to complete
12. Analyze run. Analysis settings should be threshold at 0.20000, baseline start cycle at 6, and baseline end cycle at 15.
13. Examine standard curve. A PCR with near 100% efficiency should have a slope of –3.2 and an R^2 value of 0.99 or greater.
14. Click on 'report' tab to see numerical data formatted in a spreadsheet. Data are automatically saved.

DNA Samples

Throughout this project, hundreds of samples have been assayed. Archived forensic and paternity case samples were requested from other analysts for analysis. Many other samples originated from a current research project ongoing in the laboratory. DNA was extracted from a variety of tissue sources using an assortment of isolation methods, Table 1.

Sources	Extraction Method
buccal swabs	phenol/chloroform
blood, fresh & old	chelex
vaginal swabs	DNA IQ™
fetal material	PrepMan™
blood on FTA	Differential
contact samples	Silica

Table 1. Sources and extraction methods of DNA.

Some samples were extracted by other analysts and therefore, protocols will not be listed in their entirety. Definitions of sample sources are as follows. Buccal swabs are scrapings from the inside of the cheek. Vaginal swabs are scrapings from the vaginal wall where both vaginal epithelial cells and spermatozoa can be collected. Fetal material are separated into mother and fetus portions if possible. FTA paper is a filter paper that is designed to trap and entangle DNA and is impregnated with antifungal and antibacterial agents to prevent degradation of the DNA (used for archiving purposes). Contact samples include skin cells that have been deposited onto inanimate objects.

Phenol/chloroform, chelex, and differential extractions were performed according to UNTHSC DNA Identity Lab protocols. Phenol/chloroform a the standard method of

organic extraction for forensic samples. Chelex is a simple extraction technique that uses an ion exchange resin to isolate DNA as single strands. A differential extraction is used on vaginal swabs/sexual assault samples. It consists of first lysing the female vaginal epithelial cells while leaving the sperm heads intact. Once the female fraction is separated from the male fraction, the sperm heads are then lysed using DTT (dithiothreitol) that breaks the disulfide bridges within the sperm head. DNA from both portions of the sample is then extracted using phenol/chloroform.

DNA IQ™ (Promega Corporation, Madison, WI) is an extraction technique designed to extract a fixed amount of DNA from samples (100ng) using a magnetic resin. Extractions were performed according to manufacturer's instructions, but some variations of the protocol were experimented with at times, such as the addition of Proteinase K. PrepMan™ (ABI, Foster City, CA) is the second generation of the chelex extract. Extractions were performed according to manufacturer's protocol. Silica extractions are used to isolate low copy number (LCN) samples, or trace DNA. Little DNA yield is expected from these samples. They include contact samples such as skin cells from inanimate objects. Low copy number samples were collected in a controlled manner specifically for this project. Therefore, extraction details are presented here.

A doorknob and a telephone were cleaned thoroughly with Clorox® wipes and allowed to dry. Two persons volunteered to participate in this study. Each person rotated the doorknob twice or handled a telephone (receiver and earpiece) once. Objects were cleaned between the handling of each volunteer. A cotton swab was dipped into sterile water and then used to thoroughly swab each inanimate object. Swabs were stored in a

cool, dark place, in original swab packaging for 24 hours until extraction. These same persons provided soda cans for collection and extraction of skin cells and saliva. The cans were swabbed in the same manner as the other inanimate objects, paying particular attention to areas that would be exposed to the lips, tongue, and saliva. Extractions proceeded as follows:

1. In a clean hood, swabs were removed from packaging and cut away from stem into 2ml tubes. Another swab that had not encountered any biological sample was carried throughout the extraction process to serve as a negative control.
2. 1000µl of L6 lysis buffer (10ml of 0.1M Tris-HCl, pH 6.4; 12g guanidinium thiocyanate; heated to 60°C; 2.2ml 0.2M EDTA, pH 8; 250µl Triton X-100; 750µl of 0.10gm/ml size fractionated silica) was added to each tube/swab.
3. Tubes were vortexed and incubated at 60°C for 2 hours, with vortexing at 20 minute intervals.
4. Sample tubes were centrifuged at 14,000 rpm for 30 seconds. 700µl of supernatant was transferred to a fresh tube.
5. 600µl of L6 lysis buffer and 60µl size fractionated silica (0.10gm/ml) were added. Tubes were closed and vortexed.
6. Samples are incubated at room temperature for 15 minutes to allow DNA to bind to silica.
7. Tubes were vortexed and centrifuged for 30 seconds at 14,000 rpm.
8. Discard supernatant.
9. Pellets were washed as follows (add, resuspend, centrifuge, discard supernatant)

a. 2 times with 1ml L2 buffer (10ml Tris-HCl, pH 6.4; 12g guanidinium thiocyanate; heated to 60°C; 750µl of 0.10gm/ml size fractionated silica)
b. 2 times with 1ml 70% ethanol
c. 1 time with 1ml acetone

10. Pellets were dried at 60°C for 8 minutes.
11. 65µl of sterile water was added, vortexed to resuspend silica.
12. Samples were incubated at 60°C for 9 minutes.
13. Centrifuged, moved supernatant to fresh tube. Centrifuged again and removed supernatant to fresh tube. Stored samples at -20°C.

Quantification Methods

Samples were quantified using spectrophotometry, PicoGreen® fluorescent assay, QuantiBlot™, and the human autosomal specific and Y-chromosome specific RT-PCR quantification assays.

Six samples expecting of yielding large amounts of DNA (blood/phenol-chloroform extraction) were quantified using a spectrophotometer. Additionally, 20 samples in which the yield was expected to be 1ng/µl or less (old blood and blood on FTA paper/DNA IQ™ extraction) were quantified in the same manner. 10µl of DNA extract were added to 490µl of sterile water (1:50 dilution). Samples were quantified by measuring the absorbance at 260nm and 280nm on the BioMate™ 3 Series by Thermo Spectronic.

Eight high-yield DNA samples (buccal swab/phenol-chloroform extraction) and the same 20 low-yield samples were quantitated using the PicoGreen® assay as follows:

1. PicoGreen® dye was diluted 1:200 and 50µl were aliquoted into each assay well on a 96-well plate.
2. Standards were made according to manufacturer's instructions to arrive at final concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.0156ng/µl. 50µl of standards were added to the appropriate wells.
3. One well consisted of a blank (50µl of diluted PicoGreen® dye and 50µl of 1X TE buffer). Another well, designated as zero, contained 100µl of 1X TE buffer.
4. Each DNA sample was diluted five-fold by combining 10µl of DNA extract with 40µl of 1X TE buffer. The entire 50µl of diluted sample was added to the appropriate well with 50µl of diluted PicoGreen® dye.
5. Plate was allowed to sit in the dark for 5 minutes before scanning.
6. Plate was scanned using a Hitachi FMBIO image scanner. Image Quant® for Macintosh software was used to calculate the concentrations.

QuantiBlots™ were performed, using the colorimetric detection, by various analysts on a large number of samples according to manufacturer's instructions. Samples included those in which both large and small DNA yields were expected. These included differentially extracted vaginal swabs, blood, buccal swabs, silica extracted LCN samples, and blood on FTA paper. The procedure is briefly outlined here.

1. QuantiBlot™ standards were made according to manufacturer's protocol and were at concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125ng/ul.
2. 5ul of sample and standard were added to 150ul of spotting solution (0.4N NaOH, 25mM EDTA, 0.00008% Bromothymol Blue). The entire 155µl were spotted and vacuumed onto a nylon membrane.
3. Prehybridization wash with hybridization solution (0.9M NaCl, 50mM ($NaH_2PO_4 \bullet H_2O$), 5mM EDTA, 0.5% w/v SDS) at 50°C for 15 minutes in a hybridization oven.
4. Hybridize the D17Z1 probe to membrane in hybridization solution at 50°C for 20 minutes in a hybridization oven.
5. Wash membrane with warm wash solution (0.27M NaCl, 15mM ($NaH_2PO_4 \bullet H_2O$), 1.5mM EDTA, 0.5% w/v SDS).
6. Wash membrane with warm wash solution and the enzyme conjugate HRP: SA for 10 minutes at 50°C in hybridization oven.
7. Rinse membrane two times in warm wash solution.
8. Rinse membrane one time in citrate buffer (0.1M sodium citrate, pH 5).
9. Add Color Development Solution, shake for 30 minutes at room temperature.
10. Wash three times with water to stop color development, 10 minutes each.
11. Visually determine concentrations by comparing samples to standards.

Quantifications using the autosomal specific and Y-chromosome specific RT-PCR quantification kits were performed as previously described.

STR analysis

Twenty-four samples were analyzed using Applied Biosystems AmpFℓSTR® Identifiler™ PCR Amplification Kit according to manufacturer's instructions. Comparisons were made between samples quantified with different methods, samples containing mixed profiles of two individuals, and LCN samples that were assayed using 28 and 33 PCR cycles. Particular attention was paid to peak heights (RFUs), peak balances, off ladder alleles, and stutter.

Female:Male DNA Mixtures

Two DNA extracts (fresh blood/phenol-chloroform), one from each gender, were quantified using the autosomal specific RT-PCR assay. Samples were then diluted to 1ng/µl based on those quantifications. Table 2 shows the manner in which the female and male DNAs were mixed to achieve various ratios.

Ratio F:M	% male	ul F DNA sample	ul M DNA sample
50:50	50	10	10
75:25	25	15	5
80:20	20	16	4
90:10	10	18	2
95:5	5	19	1
99:1	1	99	1
99.5:0.5	0.5	99.5	0.5

Table 2. Volumes of female:male DNA to achieve various ratios.

Samples were vortexed several times throughout the mixture process. After mixtures were made, samples were vortexed once again and assayed using both the autosomal

specific and Y-chromosome specific RT-PCR quantification assays (described previously).

Three vaginal swabs processed by differential extraction and one fetal sample in which the fetal material was separated from the maternal tissue, were quantified using both the autosomal specific and the Y-chromosome specific RT-PCR quantification assays. The purpose was to determine if low-levels of carry-over male DNA would be detected in what should be essentially a female DNA extract.

After male DNA detection, the quantification value of the Y-chromosome specific RT-PCR assay (denoted as Y in the equation) is adjusted to account for the fact that the assay is starting with one chromosome per cell (as opposed to the two chromosomes per cell of chromosome five in the autosomal assay). This is done by dividing the Y-chromosome quantification value by two, or the assay is reanalyzed by halving the standard control values (this is discussed further in Results and Discussion section). This adjusted value is then subtracted from the autosomal specific RT-PCR quantification value (denoted as A in the equation). This attempts to adjust for the male DNA detected in the total autosomal specific assay (an effort to separate the female portion). The adjusted autosomal quantification value was then divided by the adjusted Y-chromosome quantification value. Thus the simplified equation: [A-(Y/2)]/(Y/2) was used estimate of the ratio of female to male DNA.

Next, the STR data from these samples were examined for mixed profiles (samples analyzed with Applied Biosystems AmpFℓSTR® Identifiler™, Profiler™, and COfiler™

PCR Amplification Kits). The peak height ratios of non-shared alleles were calculated to validate the ratios of female to male DNA quantities.

Sensitivity Experiments

Sensitivity experiments were performed to establish the sensitivity levels of each RT-PCR quantification assay. A male DNA extract (blood/phenol-chloroform) that was first quantitated using the autosomal specific RT-PCR quantification assay, was diluted to concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.008, 0.004, 0.002, 0.001, and 0.0005ng/μl. Serial dilution to create this range of samples were performed as follows:

1. Placed 20μl of a 2ng/μl dilution into a 0.5ml tube containing 20μl of sterile water to create the 1ng/μl concentration. Tube was vortexed.
2. Pipet tip was changed and 20μl was removed and placed into next tube that contained 20μl of sterile water.
3. Serial dilutions were continued in this manner.

Autosomal specific and Y-chromosome specific RT-PCR quantification assays were performed on this dilution series as described previously.

Utilization of C_T values

Low copy number (LCN), or trace, samples were collected and extracted as previously described. Quantifications were determined using the autosomal specific RT-PCR quantification assay.

An attempt was made to utilize the C_T values to determine by how many cycles the STR multiplex PCR should be increased. An optimal STR analysis will amplify 10µl of a DNA extract that has been diluted to 0.1ng/µl (amplification of 1ng). Examining previous RT-PCR quantification assays, a standard of 0.12ng/µl (a standard concentration used in the early stages of the kit development which has since been modified) always had a C_T value of 31 cycles. This became the "standard" C_T value with which to work. Reasoning that this perhaps equated to the 28 traditional gel-based PCR cycles used in STR analysis, the C_T values of the LCN samples were adjusted in this manner. The range of C_T values of the LCN samples were 35-37, the average 36. Since this value differed from the 31 C_Ts of the 0.12ng/µl standard by five, the multiplex PCR for STR analysis was increased by five cycles. LCN extracts were analyzed with Applied Biosystems AmpFℓSTR® Identifiler™ PCR Amplification Kit using both 28 PCR cycles and 33 PCR cycles.

CHAPTER V

RESULTS AND DISCUSSION

Comparisons of quantification methods

Comparisons were made between the autosomal specific RT-PCR quantification assay and spectrophotometry, fluorometry, and hybridization-based quantification methods.

In general, the spectrophotometer quantitated the high-yield samples (blood/phenol-chloroform) higher than did the autosomal specific RT-PCR quantification assay (Figure 11) by a factor of 1.9. This is likely due to contaminants such as proteins, RNA, residual phenol, and bacterial DNA that the spectrophotometer, but not the autosomal specific RT-PCR quantification assay, can measure. The results of the RT-PCR quantification assay provide a clear representation of the amount of amplifiable human DNA present in a sample, rather than the total amount of DNA that may be present.

Samples in which a low DNA yield was expected (old blood and blood on FTA paper/DNA IQ™) were also quantitated using spectrophotometry. The results were not consistent among samples (Figure 12). Some extracts had high quantification values, while others had little, or even negative, values (negative values are not shown). This demonstrates the unreliability of spectrophotometry measurements when assaying forensic samples. Values obtained from the autosomal specific RT-PCR quantification assay shows similar human DNA concentrations in all samples. This is understandable given the samples were of similar nature (old blood or blood on FTA paper) and the

fact that they were extracted using the same method (DNA IQ™). Although difficult to discern in Figure 12, the values ranged between 2.43 and 0.0361ng/µl.

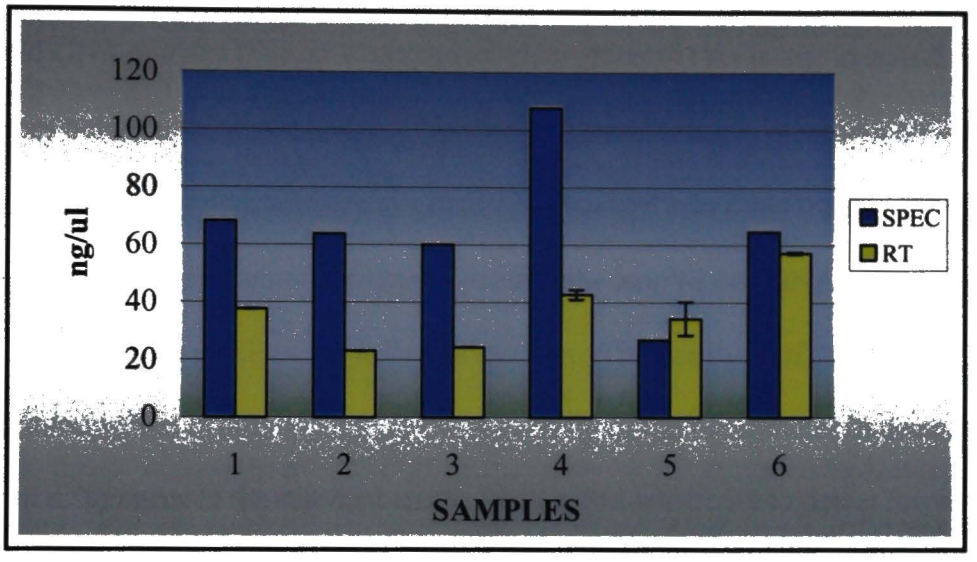

Figure 11. Comparing spectrophotometry and autosomal specific RT-PCR quantification results. DNA samples extracted from blood using phenol/chloroform.

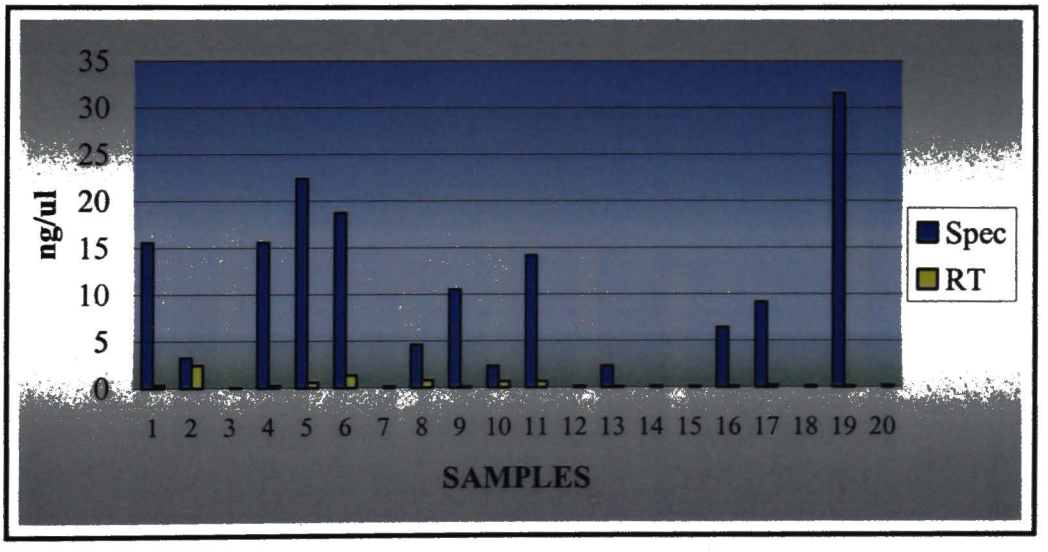

Figure 12. Comparing spectrophotometry and autosomal specific RT-PCR quantification results. Samples 1-10 extracted from old blood using DNA IQ™. Samples 11-20 extracted from blood on FTA paper using DNA IQ™.

The autosomal specific RT-PCR quantification assay's values were higher than those obtained from the PicoGreen® assay by an average of 1.7 fold (Figure 13) when assaying high yield DNA extracts (buccal swab/phenol-chloroform). This is due to sensitivity differences between these two assays. PicoGreen® fluorescence is measured on an image scanner that then uses densitometry to calculate a standard curve and the quantity of the samples. Densitometry cannot accurately quantify the amplifiable DNA present in a sample; rather it uses pixel values in its calculations. The accuracy of these quantification values is only as good as the sensitivity of the fluorometer in detecting saturation differences in the standard curve. In fact, the sensitivity to detect low-level DNA is not adequate as Figure 14 shows. Again, this method is not human specific, as the PicoGreen® dye will bind to any dsDNA.

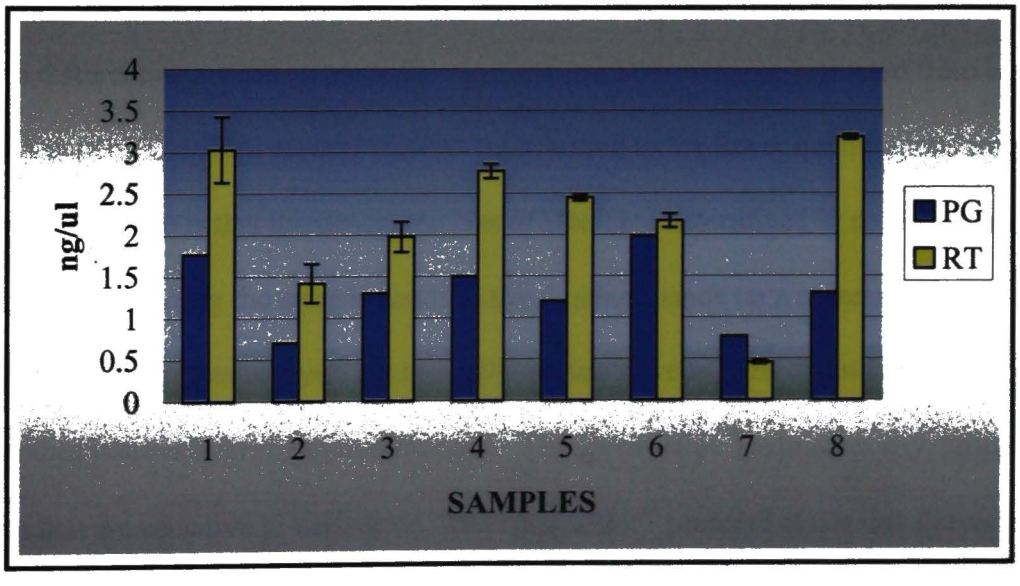

Figure 13. Comparing PicoGreen® assay to autosomal RT-PCR quantification assay. Samples are DNA extracts from buccal swabs extracted using phenol/chloroform.

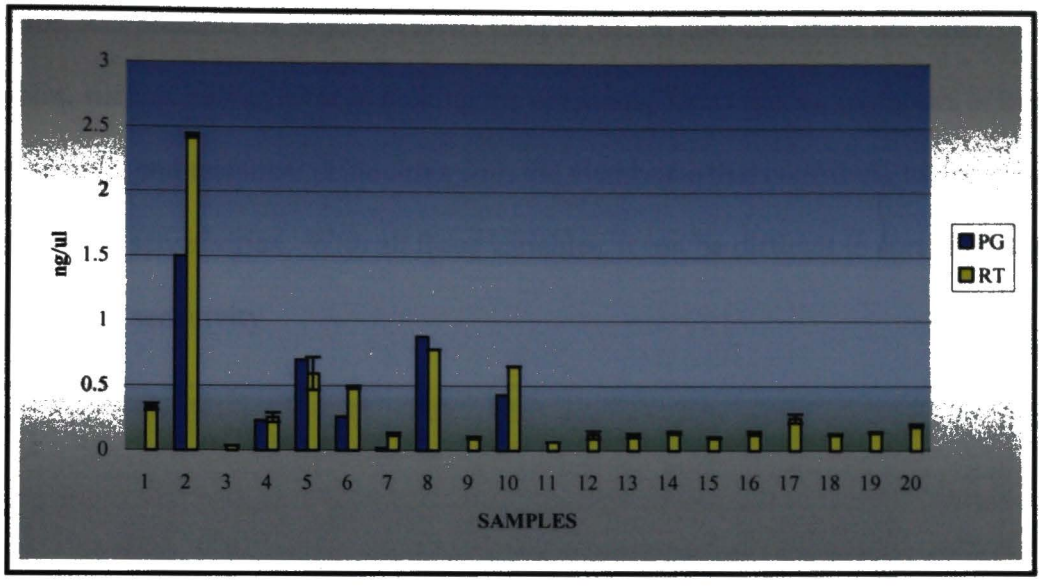

Figure 14. Comparing PicoGreen® assay to autosomal RT-PCR quantification assay. Samples 1-10 extracted from old blood using DNA IQ™. Samples 11-20 extracted from blood on FTA paper using DNA IQ™.

The autosomal specific RT-PCR quantitations were on average 3.5 fold higher than QuantiBlot™ quantitations (Figure 15). Four of the eight DNA samples were buccal swabs extracted with chelex. Three are male DNA extracts isolated from vaginal swabs using a differential extraction procedure and one is a DNA sample extracted from blood on FTA paper. There are several reasons contributing to the lower quantitations from the QuantiBlot™. One is that the concentrations of DNA are determined in subjective manner by visually comparing the sample bands to the standard bands on a nylon membrane. These results show that analysts are typically conservative in their quantity estimations.

Another issue is hybridization efficiency. The QuantiBlot™ requires many stringent washes that are sensitive to variations. The QuantiBlot™ protocol states that decreased sensitivity could be caused by a variety of reasons: incorrect NaOH or EDTA concentrations in spotting solution, hybridization temperature too high, inactive hydrogen

peroxide, and presence of $MgCl_2$ in DNA sample (8). In addition, there are other variables, such as human error in making the solutions, bad reagents, inhibitors in the DNA sample, and technical difficulties with the membrane that contribute to decreased hybridization sensitivities. With all these variables, it can be difficult to sort out the root cause for low sensitivity.

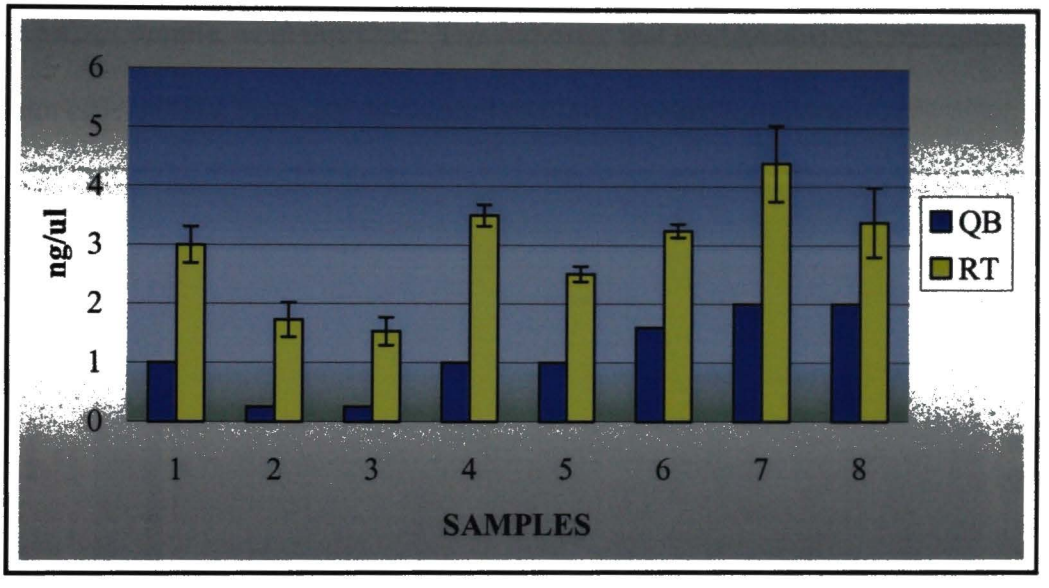

Figure 15. Comparing QuantiBlot™ to autosomal RT-PCR quantification. DNA samples from a variety of sources, extracted using several methods.

The range of the QuantiBlot™ standards is not sufficient. At times, DNA concentrations exceed the highest tested standard (2ng/µl). This forces the analyst to extrapolate the concentration beyond the standard curve, or repeat the assay with a diluted DNA sample. The QuantiBlot™ is a laborious assay, taking two-and-a-half to four hours to complete.

As the next two figures show (Figures 16 and 17), the QuantiBlot™ is not sensitive to detect low-levels of DNA. Figure 16 shows 20 samples that were extracted using the DNA IQ™ method. Concentrations were obtained for 13 of the 20 samples (65%) using the QuantiBlot™. According to the autosomal specific RT-PCR quantifications, all samples were greater than 0.03125ng/μl, the detection limit of the QuantiBlot™ when testing a 5μl sample, as in this case. This indicates that the QuantiBlot™ hybridization was not efficient and cannot truly detect 0.03125ng/μl concentrations.

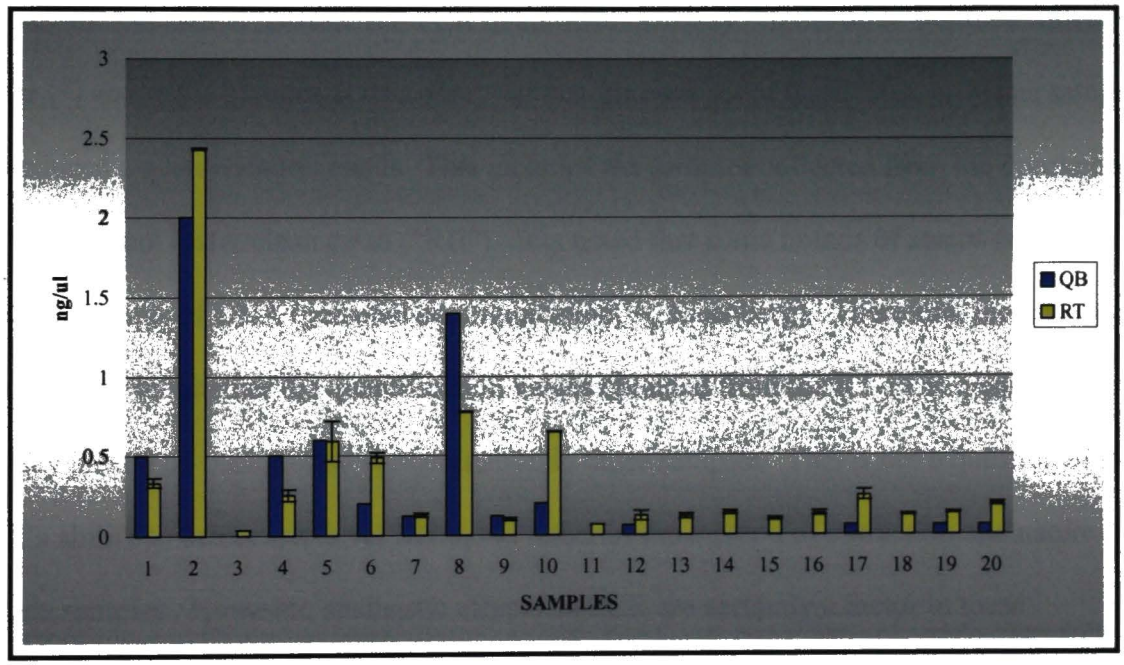

Figure 16. Comparing QuantiBlot™ to autosomal RT-PCR quantification. Samples 1-10 extracted from old blood using DNA IQ™. Samples 11-20 extracted from blood on FTA paper using DNA IQ™.

Figure 17 compares the quantification results of the LCN DNA extracts using the QuantiBlot™ and the autosomal specific RT-PCR quantification assay. Obviously, the QuantiBlot™ is not adequate for quantitating these trace DNA samples as no results were obtained. The contact samples from the soda cans (denoted as "C" on Figure 17) were within the detection limits of the QuantiBlot™, yet decreased hybridization sensitivity made these samples impossible to quantify. The other samples ("K" = doorknob, "P" = phone) fell below QuantiBlot™'s detection limit, although quantitations were obtained using the autosomal specific RT-PCR quantification assay. In the three samples with a star (*) above the column, it was observed that for each set of duplicates, only one sample produced a quantification result. This included the samples collected from the doorknobs and a reagent blank/clean swab ("RB"). It is noted that some brands of swabs contain trace amounts of DNA (personal communication, A. Eisenberg). Therefore, there exists a concern as to whether the quantification values obtained from the RT-PCR quantification assay picked up background contamination in those samples. This is not a question of the kit's ability to detect low-level DNA, but rather of the method of extraction and nature of trace samples. However, stochastic effects on PCR are certainly a factor in these situations and these quantification values should be used cautiously.

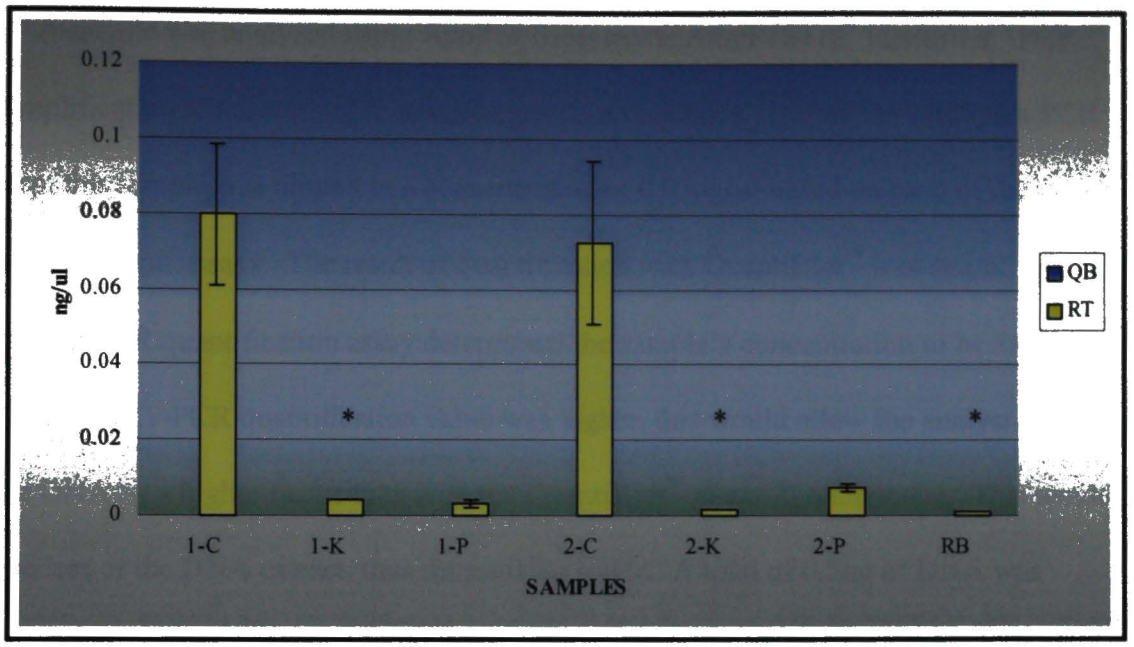

Figure 17. Comparing QuantiBlot™ to autosomal RT-PCR quantification of LCN samples. 1 = volunteer 1; 2 = volunteer 2; C = soda can, K = doorknob, P = telephone, RB = reagent blank/clean swab; * = only one of the two samples in duplicate set produced a quantitation result.

STR Analysis of Different Quantification Methods

The multiplex PCR used for STR analysis works within a narrow DNA quantity range. If too much template DNA is added, artifacts, such as stutter (which occurs when a polymerase slips past a tandem repeat and the resulting allele appears as one repeat unit smaller), noisy baselines, and pull-up (a fluorescent dye 'bleeds' over into next dye due to spectral overlap) will occur. These artifacts could cause the resulting genetic profile to appear as a mixture of two or more individuals. If too little template DNA is added to the reaction, the PCR will not be efficient; alleles will fail to be detected (allelic dropout) and a full genetic profile will not be obtained.

A sample was analyzed using Applied Biosystems AmpFℓSTR® Identifiler™ PCR Amplification Kit according to manufacturer's instructions. Before the multiplex PCR step, this sample was diluted to a concentration of 0.05ng/µl based on each of the quantification assays. The result of quantification with QuantiBlot™ was 1ng/ul, while the RT-PCR quantification assay determined the sample's concentration to be 3ng/µl. Since the RT-PCR quantification value was higher, this would allow the analyst to dilute the DNA by a higher factor than with the QuantiBlot™ quantification value. This would use less of the DNA extract, thus minimizing waste. A total of 0.5ng of DNA was amplified, well within working range for STR analysis. Figure 18 shows the STR profile obtained based on the QuantiBlot™ quantification value and Figure 19 is the corresponding STR result obtained when the RT-PCR quantification value was used.

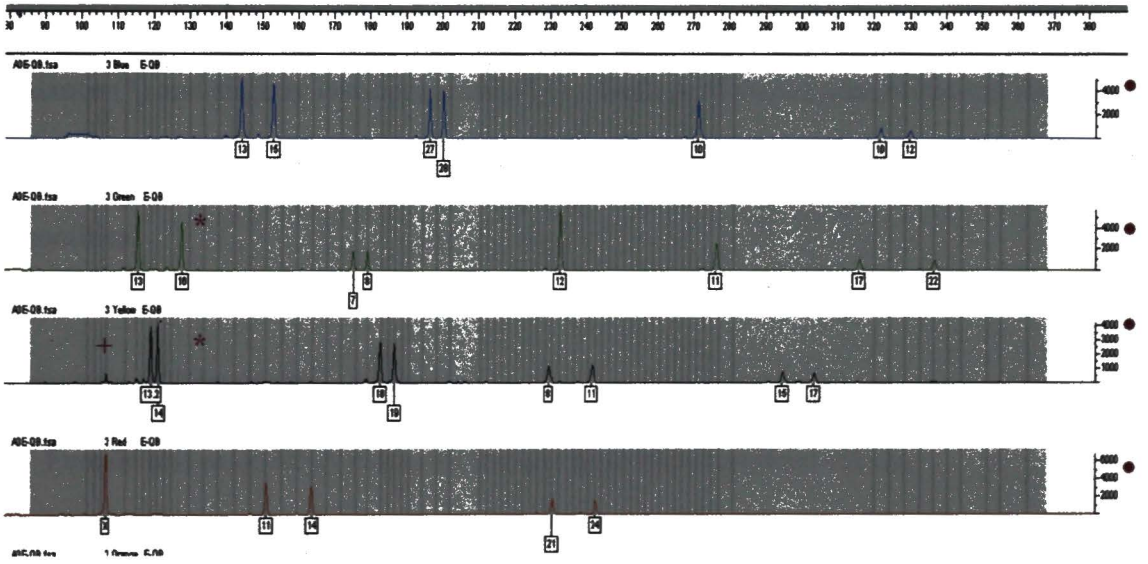

Figure 18. STR analysis of Sample E diluted to 0.5ng/µl according to QuantiBlot™ quantification results.

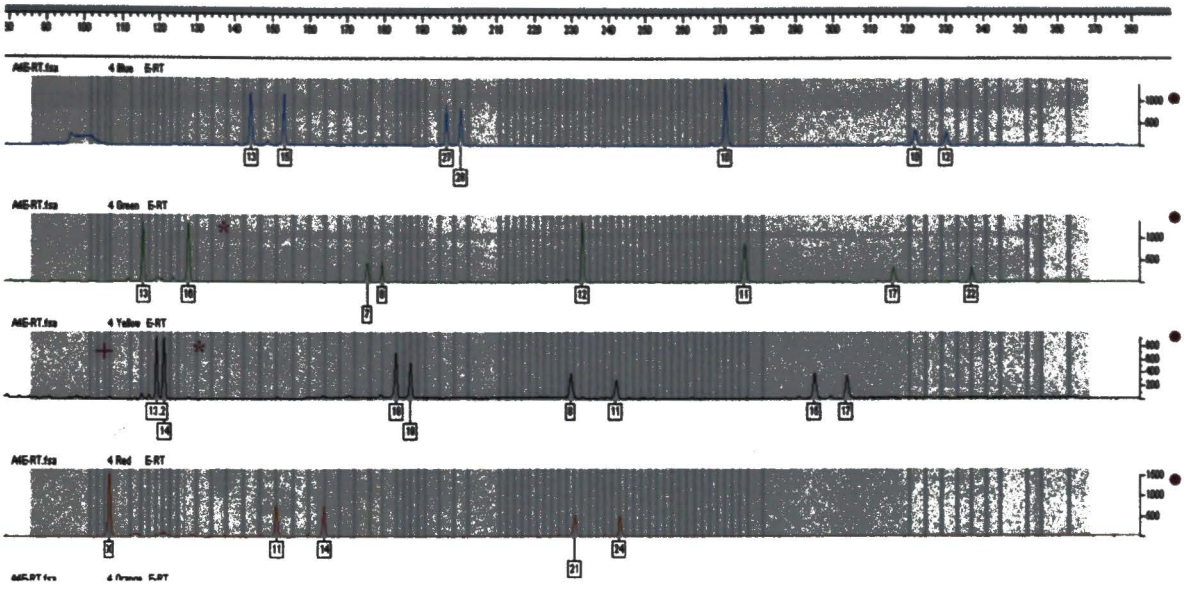

Figure 19. STR analysis of Sample E diluted to 0.5ng/µl according to RT-PCR quantification results.

The differences are not remarkable. However, the sample analyzed using the RT-PCR quantification value showed lower RFUs, 4,000 versus 1,000 (•), slightly better peak balance (*) and slightly less background (+) . Both profiles are acceptable but these results do show that the use of less DNA is just as effective, if not preferable than higher quantities.

Detection of Female:Male Mixtures

Female and male DNA extracts (1ng/µl) from blood isolated via the phenol/chloroform method were combined in the following ratios of female to male DNA: 50:50, 75:25, 80:20, 90:10, 95:5, 99:1, 99.5:0.5. Each mixture was then quantitated using both the autosomal specific and Y-chromosome specific RT-PCR assays. Figure 20 shows that the male DNA was detected in all samples, including the

mixture in which the male DNA only constituted 0.5% of the entire extract. The quantification value of that male DNA was 2.6pg/µl. This is roughly equivalent to one sperm head/µl.

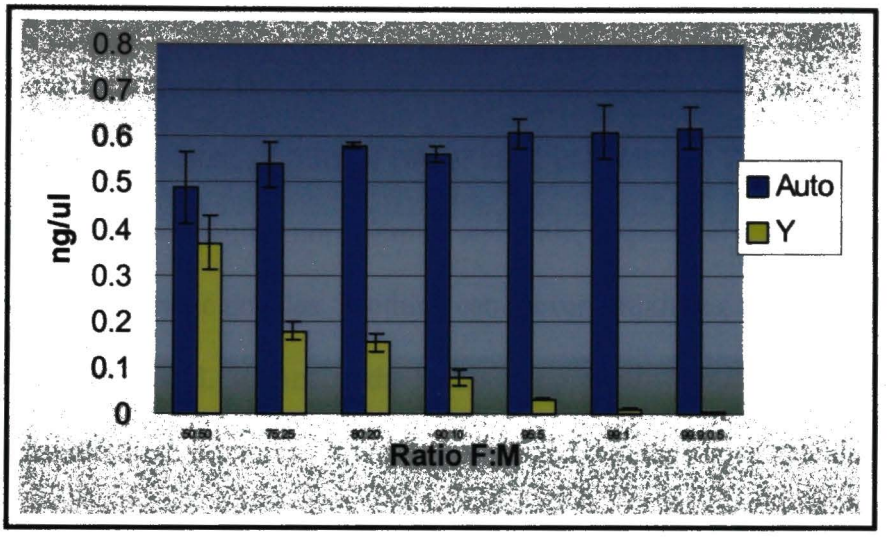

Figure 20. Detection of male DNA via the Y-chromosome specific RT-PCR quantification assay in female:male mixtures at fixed ratios.

Carryover Male DNA in Female DNA Extracts

Intimate samples from sexual assaults and fetal material are inherent mixtures of two or more individuals. Although procedures exist to separate the individual portions and thus the DNA, these methods are not 100% efficient at completely separating the genetic material contributed by different individuals. This carryover of genetic material is discovered when analyzing STR data. It was speculated that the Y-chromosome specific RT-PCR quantification assay could be used as a tool for discovering carryover male DNA in what was essentially a female DNA extract. Three vaginal swab differential extracts and two extracts of the same fetal sample were analyzed. Figure 21 shows total DNA concentration as determined by the autosomal specific RT-PCR quantification

assay and male DNA that was detected via the Y-chromosome specific RT-PCR quantification assay. The number above the column represents the ratio of female to male DNA (total DNA quantitations were corrected for male contribution, Y-chromosome values were adjusted for a one chromosome/cell amplification).

As mentioned, carryover DNA can be discovered during STR analysis. These samples create mixed genetic profiles of two or more individuals. The analyst is left to sort out major and minor contributors (female and male, respectively, in these cases) to decipher individual genetic profiles. Nothing can prevent mixtures; they are a part of the nature of the forensic field. It remains the forensic community's burden to determine how best to handle these mixtures. It was hypothesized that the above calculated ratios of female to male DNA could be used to determine if subsequent STR analysis will result in mixed profiles that are difficult to separate into individual genetic profiles. This was

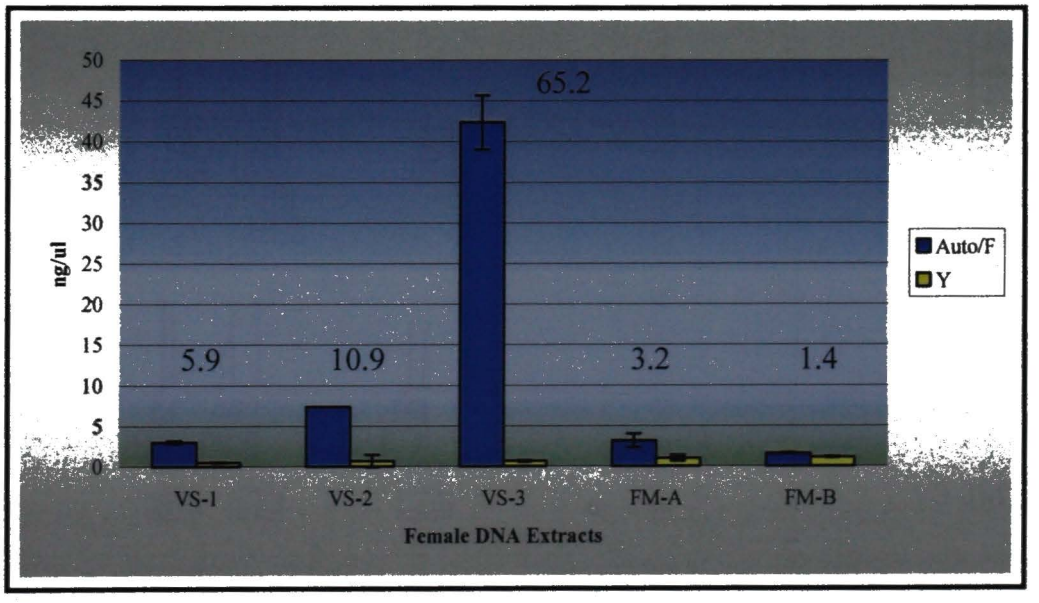

Figure 21. Detection of male DNA in a female DNA extract via the Y-chromosome specific RT-PCR quantification assay. Numbers above columns represent F:M ratios.

accomplished by using allele peak heights from the STR electropherogram. The allele peak heights attributable to the victim or mother were divided by the allele peak heights attributable to the offender or fetus. No calculations were made for alleles shared except in the case of amelogenin, the sex determining alleles. For brevity, the entire table of calculations is not included here, but an example is demonstrated in Table 3. Figures 22-24 show the electropherograms for VS-1, VS-3, FM-A, and FM-B.

VS-1	Locus		
	D8	D21	CSF
F profile	13,15	27,28	10,12
M profile	10,13	28,30	11,12
F allele peak height	3840	4585	845
M allele peak height	1776	1018	250
Ratio	**2.2**	**4.5**	**3.4**

Table 3. An example of calculating allelic ratios of female to male using allele peak heights.

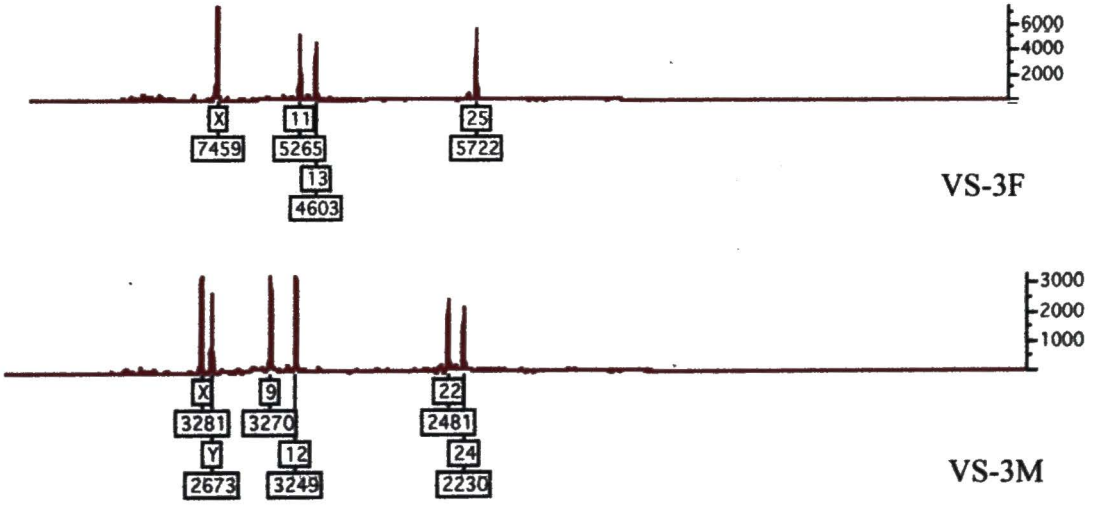

Figure 22. VS-3, partial genetic profile of female and male portions of vaginal swab.

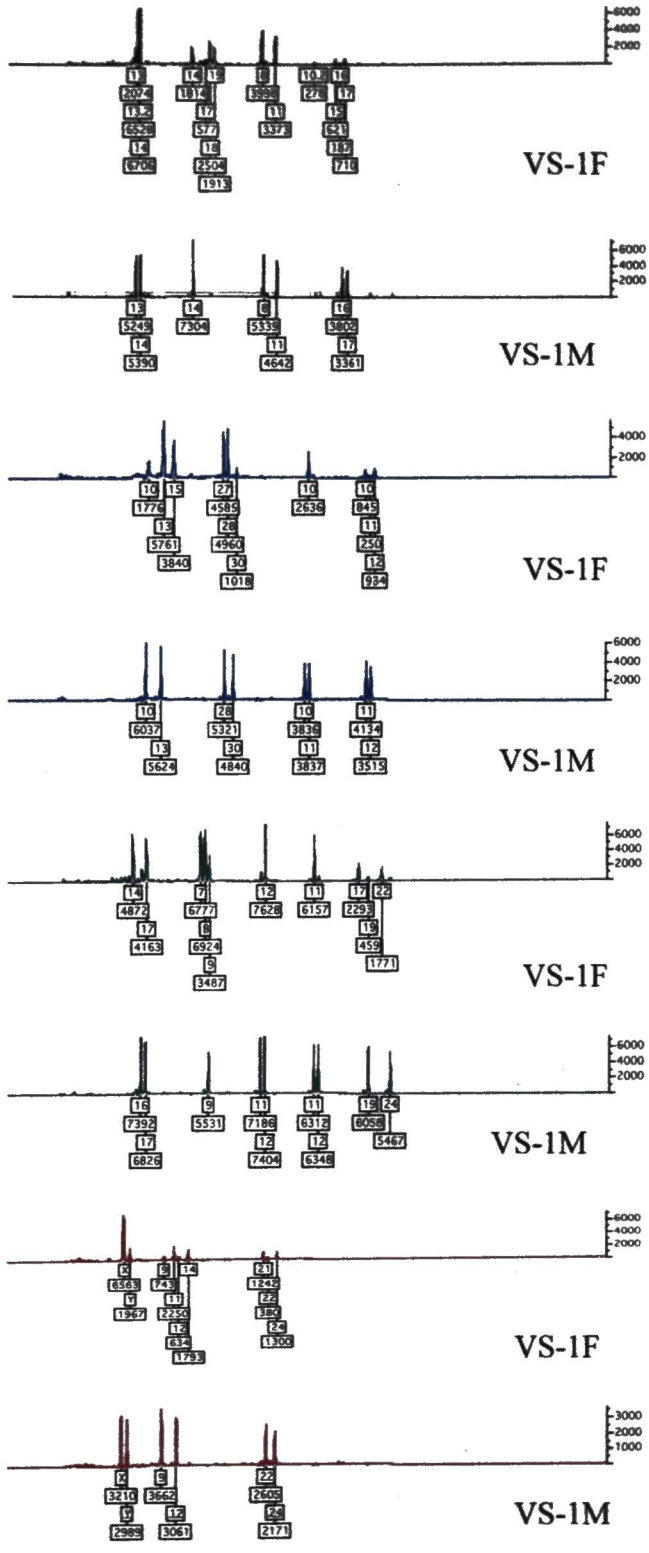

Figure 23. VS-1, genetic profiles of female and male portions of vaginal swab.

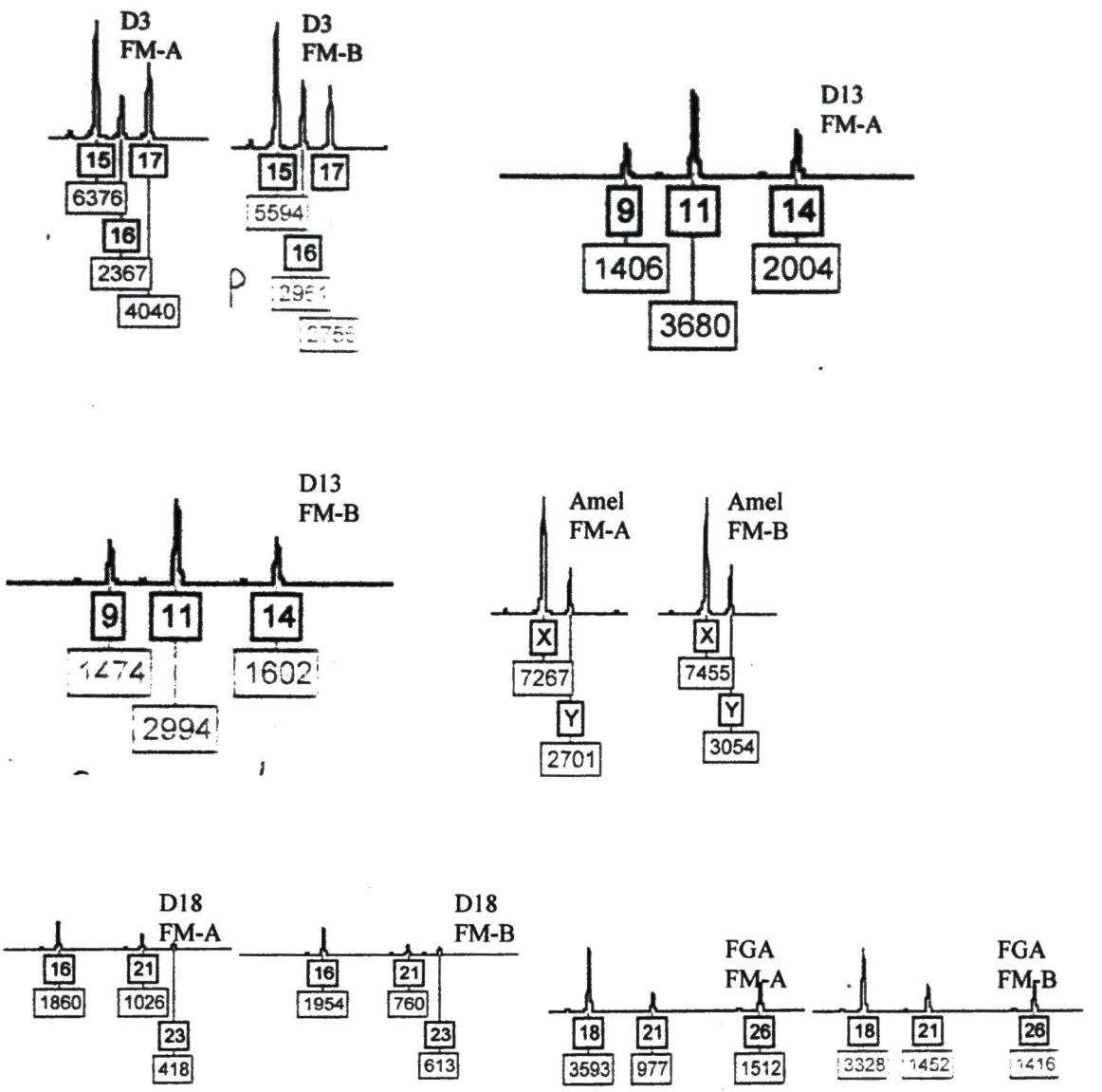

Figure 24. Partial genetic profile of two extracts (FM-A, FM-B) of fetal material.

The genetic profiles for sample VS-1, both female and male, are shown in Figure 23. It is obvious that a mixture exists in the female fraction. Examining the male profile, all alleles can be accounted for in the mixed profile originating from the female portion of the vaginal swab. Data from eleven loci were used for female to male peak height ratios.

The ratios ranged from 1.4 to 5.0, with the average being 3.1 (Table 4). This value is lower than the calculated female to male ratio obtained using the RT-PCR quantification values. This could be due to the inability to examine each locus of the genetic profile. Ratios of shared alleles were not calculated in this study because it can be difficult to discern how much of the peak is attributable to each person in the mixed profiles. Additionally, there are other contributing factors to the peak heights; none of which have to do with the ratio of female to male DNA. Rather, the conditions of the capillary electrophoresis (matrices, polymer, voltage, denaturation of samples, amount of PCR product analyzed, etc) can cause variations in peak heights. Nevertheless, the ratio of female to male DNA as established using the RT-PCR quantification values remains a helpful figure to anticipate the level of mixtures.

Only two loci, plus amelogenin, from sample VS-3, both female and male, are shown in Figure 22. The ratio of female to male as determined by the RT-PCR quantification was quite high. There is little evidence of mixed profiles in this female epithelial DNA extract when examining the STR data and as seen with the amelogenin X and Y alleles (Figure 22); therefore, not all data is shown. The high female to male ratio obtained via the RT-PCR values provided a clear indication that a mixed genetic profile would be unlikely. At some loci, very small peaks are seen but not called as alleles by the software since they fall below the threshold value of 100 RFUs. Note as well, that these data (including VS-1) show that too much DNA had been amplified in the multiplex PCR. This is indicated by the high RFUs and the amount of baseline noise and stutter. These

samples were originally quantitated, amplified, and analyzed, based on a value acquired using the QuantiBlot™ quantification method before this project took place.

Figure 24 displays the electropherograms of samples FM-A and FM-B (two extracts of the same fetal material). These mixed profiles are of maternal and fetal origin, therefore a locus will never show more than three alleles. The peak heights of the non-shared allele were used to calculate female to male ratios (fetus was of male origin). Data from nine loci were used for female to male peak height ratios for both FM-A and FM-B. The ratios ranged from 1.4 to 3.3 for FM-A and from 0.97 to 2.0 for FM-B, with the average being 2.3 and 1.3, respectively (Table 4). Note that in FM-B, some of the peak heights attributed to the male fetus were higher than peaks of maternal origin. The RT-PCR quantification values for FM-B indicated an almost 1:1 ratio of female to male DNA. This signifies an equally mixed sample that was confirmed by STR analysis.

Sample	No of loci used for peak height ratios	MIN	MAX	AVG	RT-PCR F:M
VS-1	11	1.4	5	**3.1**	**5.9**
FM-A	9	1.4	3.3	**2.3**	**3.2**
FM-B	9	0.97	2	**1.3**	**1.4**

Table 4. Female to male peak height ratios calculated from the STR analysis of three mixed samples. The average value is compared to the F:M ratio obtained from RT-PCR quantifications.

The observation was made that the female to male peak height ratios were smaller for those of shorter PCR fragments. Additionally it was noticed that the differences between the female to male ratios acquired from RT-PCR quantification and from allele peak height data decreased with smaller female to male ratios. In other words, the greater the mixture of female and male DNA (ratio approaching 1), the stronger the ratios agreed

between RT-PCR quantifications and allele peak height data. These observations reveal that the Y-chromosome specific RT-PCR quantification assay can be used as a tool for predicting the complexity of mixed genetic profiles in subsequent STR analysis. At some point, the ratio of female to male DNA is so high that a mixture will not be detected with STR analysis (as seen with VS-3). It is not known what this cut-off ratio is, but knowing this value would be beneficial.

Sensitivity Experiments

Both the autosomal and Y-chromosome specific quantification assays were tested for sensitivity. A male DNA extract (blood/phenol-chloroform) was first quantitated with the autosomal specific quantification assay. Based on those calculations, samples were diluted in a two-fold fashion to achieve concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.008, 0.004, 0.002, 0.001, and 0.0005ng/μl. One diploid cell contains approximately 6pg and a single sperm head contains approximately 3pg. Obviously, these tested concentrations are extremely dilute and do not necessarily represent encountered concentrations. Quantities above 1ng/μl were not directly tested since it is very evident these RT-PCR quantification kits are capable of determining concentrations well above 1ng/μl (highest quantity determined during this project was 450ng/μl, from a cell line). Furthermore, a 1ng/μl concentration is within a range that works well with forensic samples.

The level of detection of the autosomal specific RT-PCR quantification assay is around 0.006ng/μl (~6pg or 1cell). The Y-chromosome specific RT-PCR quantification

assay is sensitive to roughly 0.03ng/μl (~30pg or 10 sperm heads). Results are shown in Figure 25. The sensitivity levels were determined to be the last concentration at which the two-fold dilutions were stable (denoted by a * on chart, color coded).

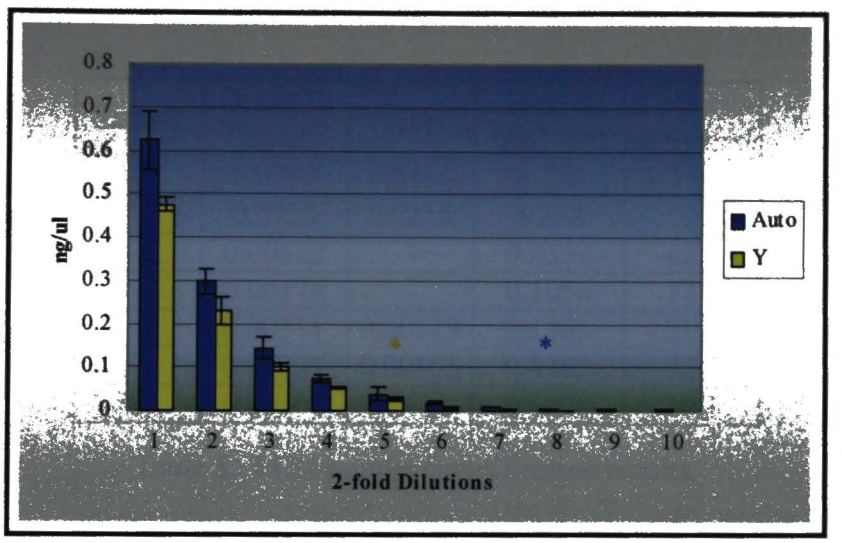

Figure 25. Sensitivity experiments of both assays.

LCN Analysis

LCN samples were quantified using both the autosomal specific RT-PCR quantification assay and the QuantiBlot™. Data were presented in previous section. The QuantiBlot™ failed to render quantification results while the autosomal specific RT-PCR quantification assay showed low-level concentrations of DNA. Results are displayed in Table 5 and Figure 17.

An attempt was made to utilize the C_T values to determine by how many cycles the STR multiplex PCR should be increased. It was determined, as described above, that 28 (normal) and 33 cycles of multiplex PCR would be compared. The STR data are displayed in Figures 26-32, one Figure for each sample. Each Figure shows comparisons

between the 28 and 33 cycles of PCR. Figures 33 and 34 are the reference profiles from each of the volunteers. No attempt was made to adjust the data for off ladder alleles or stutter.

Sample	Quantity ng/ul	SD	Total in 60ul (ng)	# of cells equivalent
1-C	0.0796	0.0186	4.776	796
2-C	0.0723	0.0216	4.338	723
1-K	0.00422	-	0.2532	42.2
2-K	0.00176	-	0.1056	17.6
1-P	0.00311	0.00119	0.1866	31.1
2-P	0.00727	0.00105	0.4362	72.7
RB	0.00101	-	0.0606	10.1

Table 5. LCN sample quantification results.

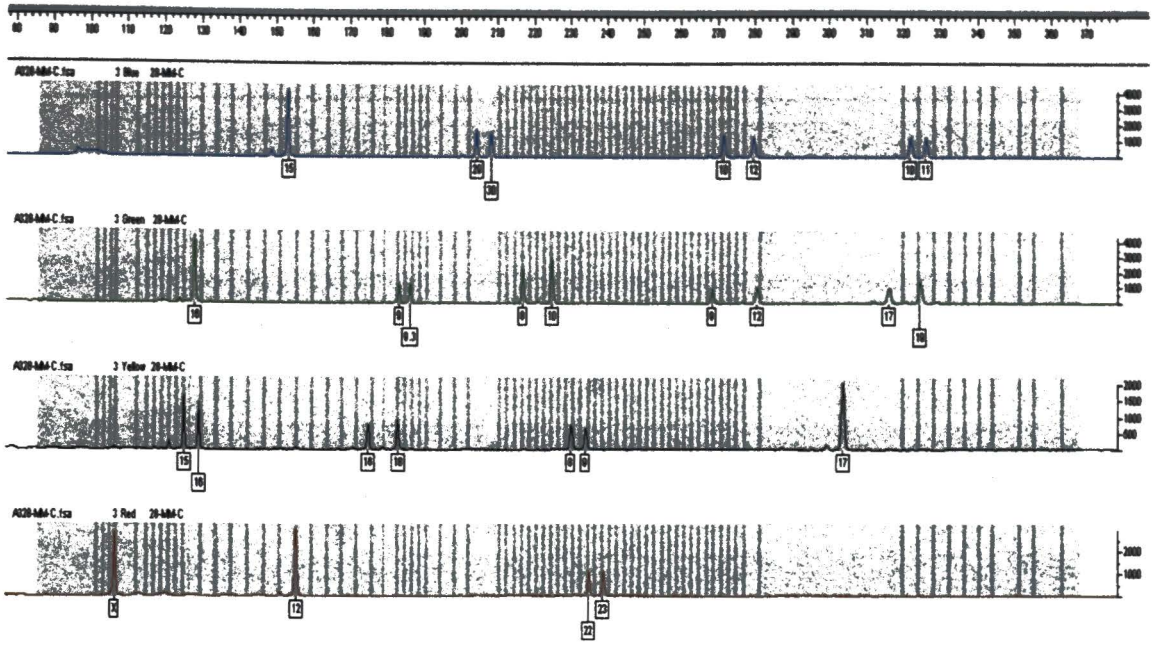

Figure 26A. 1-C, sample from soda can. 28 PCR cycles.

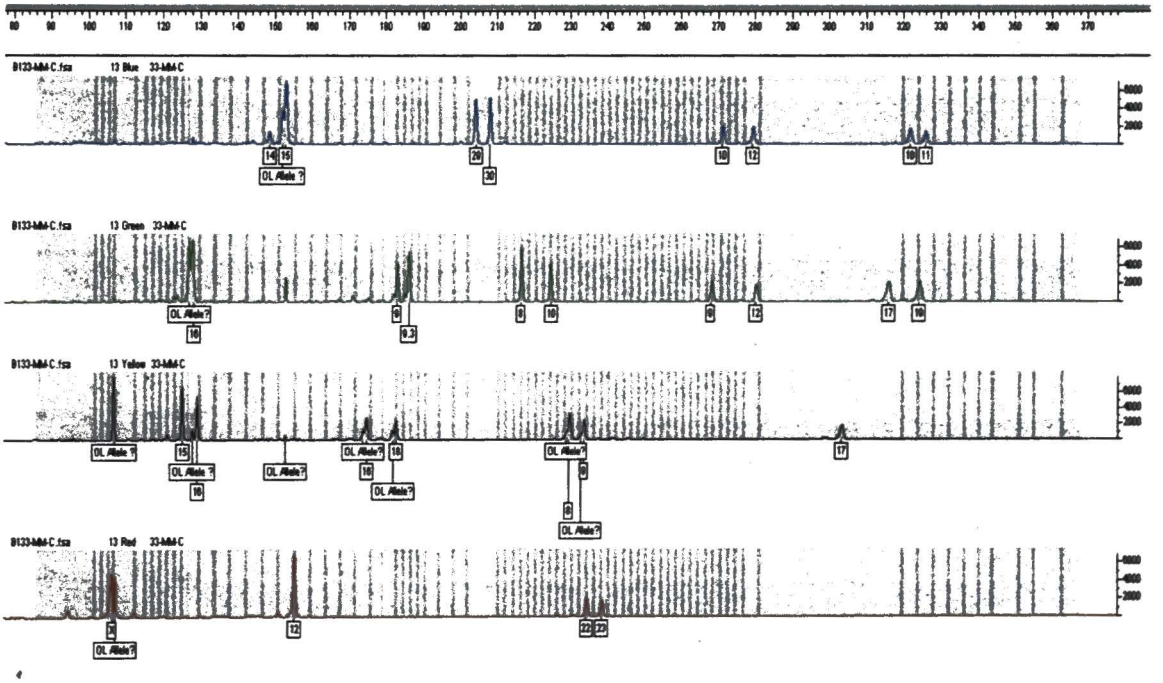

Figure 26B. 1-C, sample from soda can. 33 PCR cycles.

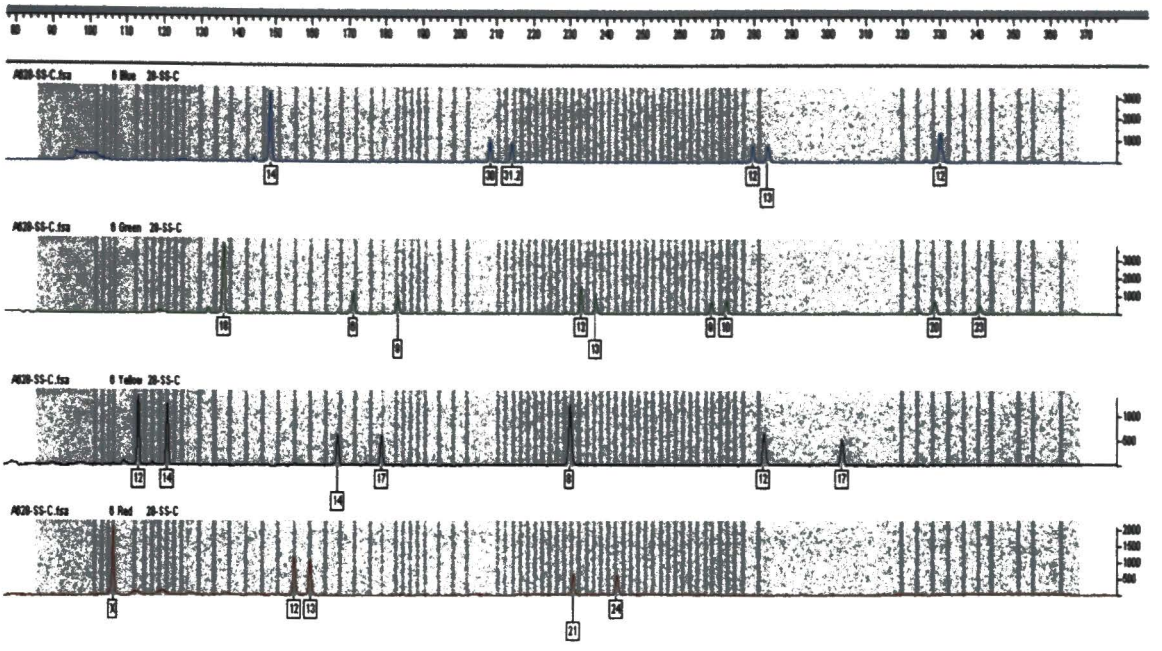

Figure 27A. 2-C, sample from soda can. 28 PCR cycles.

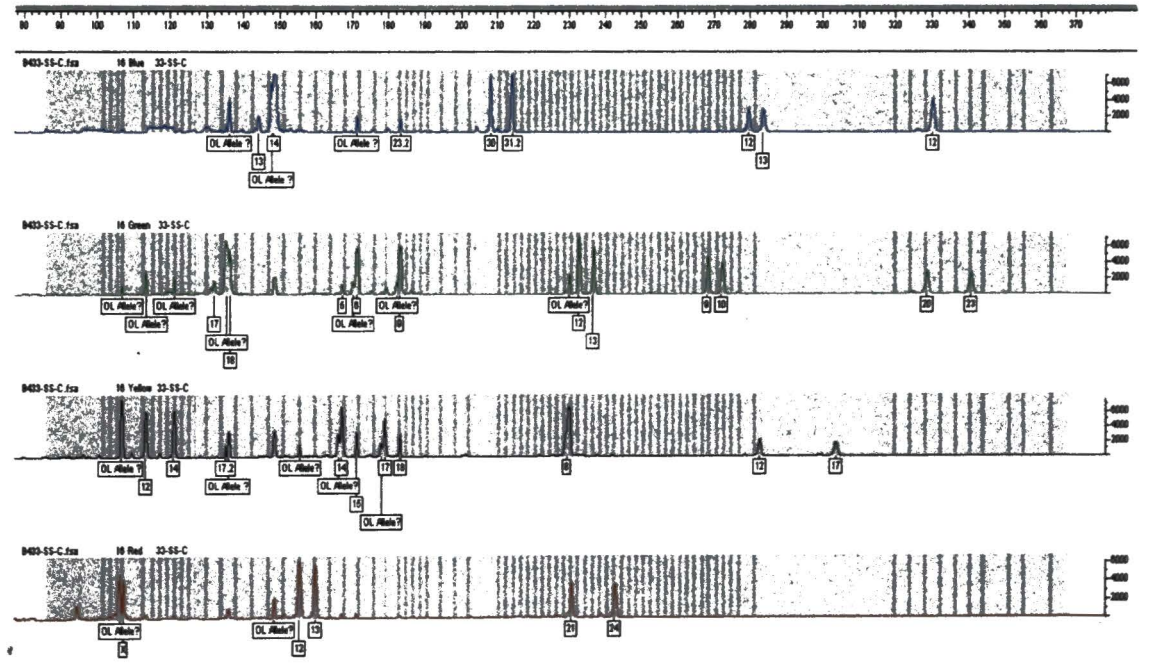

Figure 27B. 2-C, sample from soda can. 33 PCR cycles.

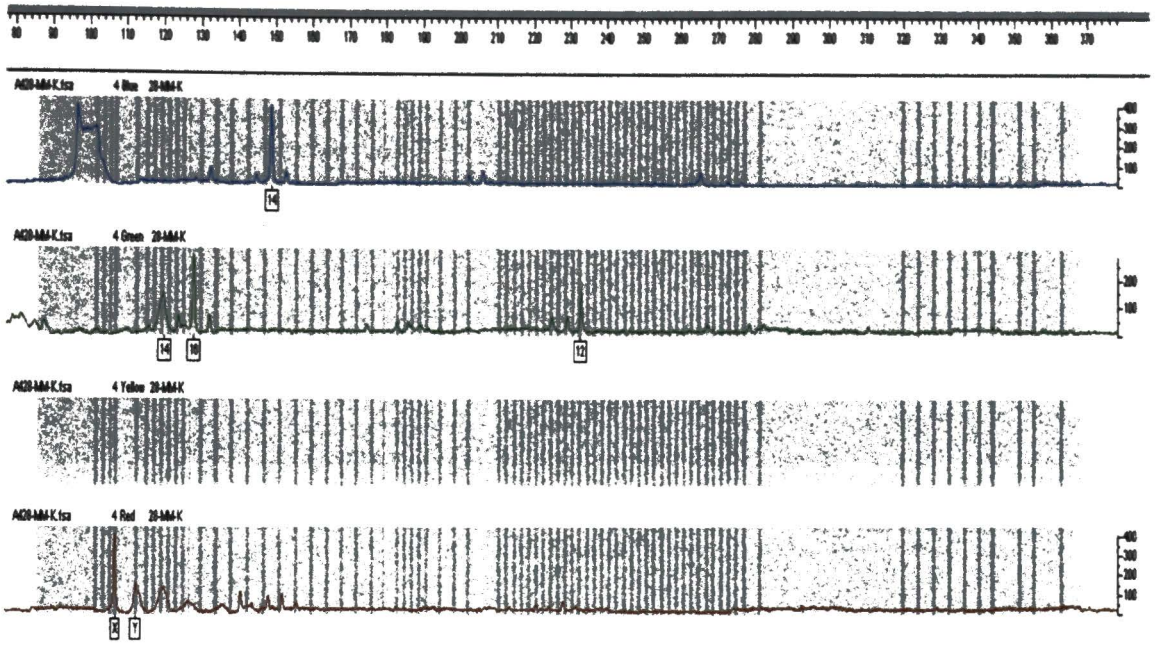

Figure 28A. 1-K, sample from doorknob. 28 PCR cycles.

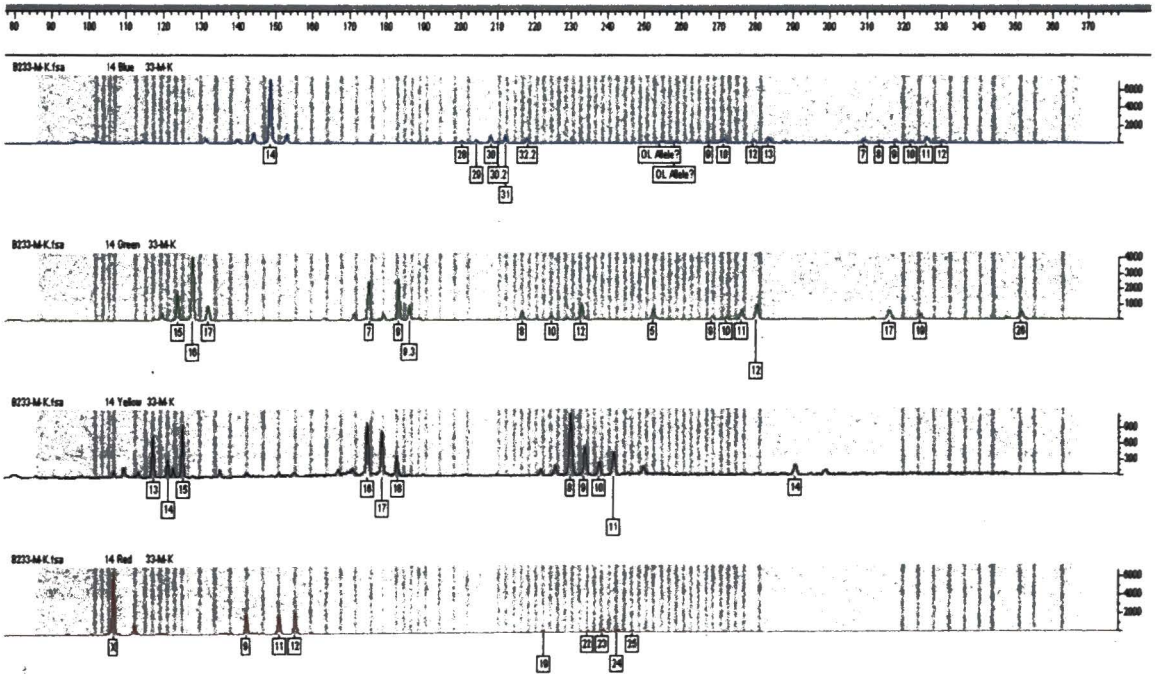

Figure 28B. 1-K, sample from doorknob. 33 PCR cycles.

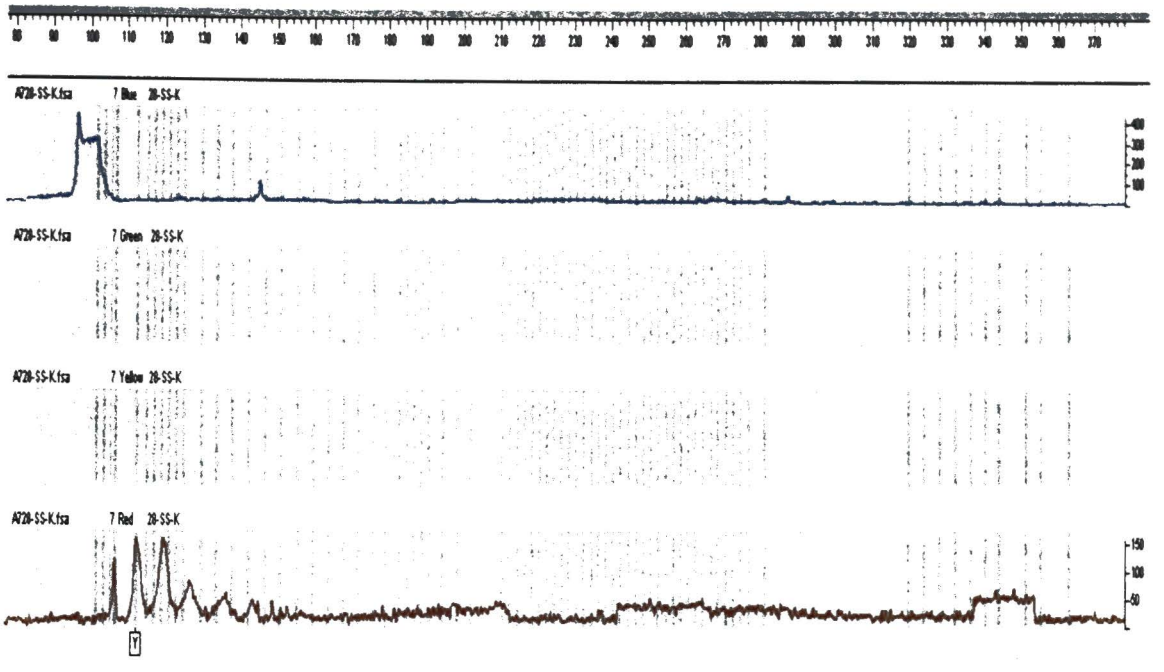

Figure 29A. 2-K, sample from doorknob. 28 PCR cycles.

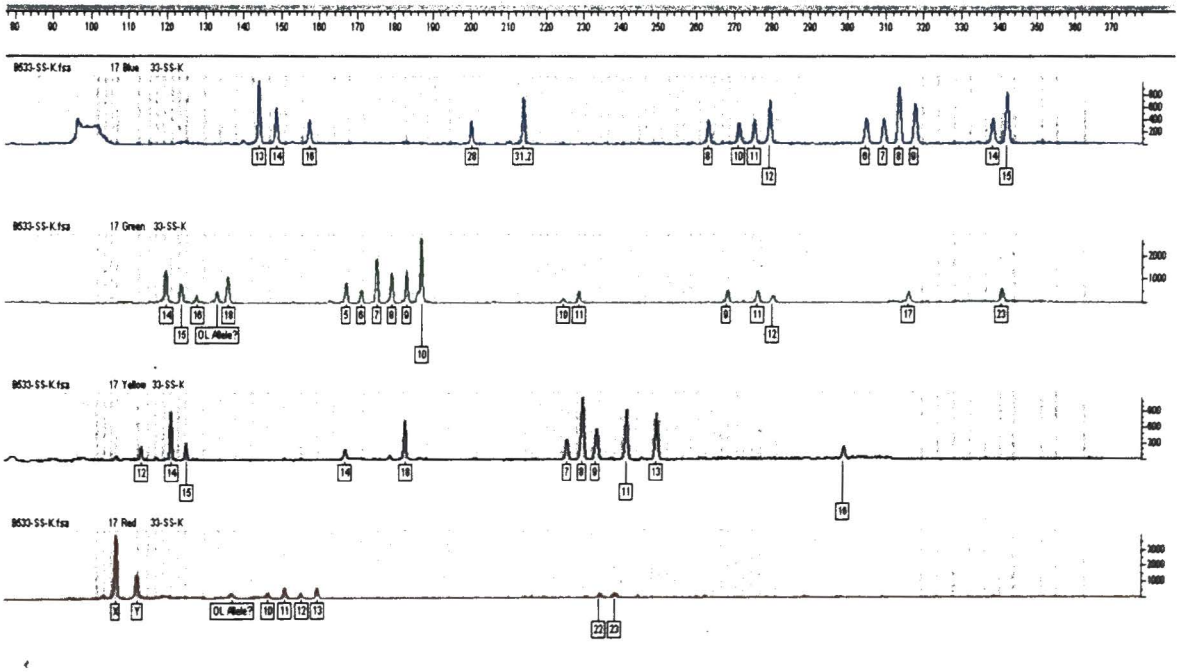

Figure 29B. 2-K, sample from doorknob. 33 PCR cycles.

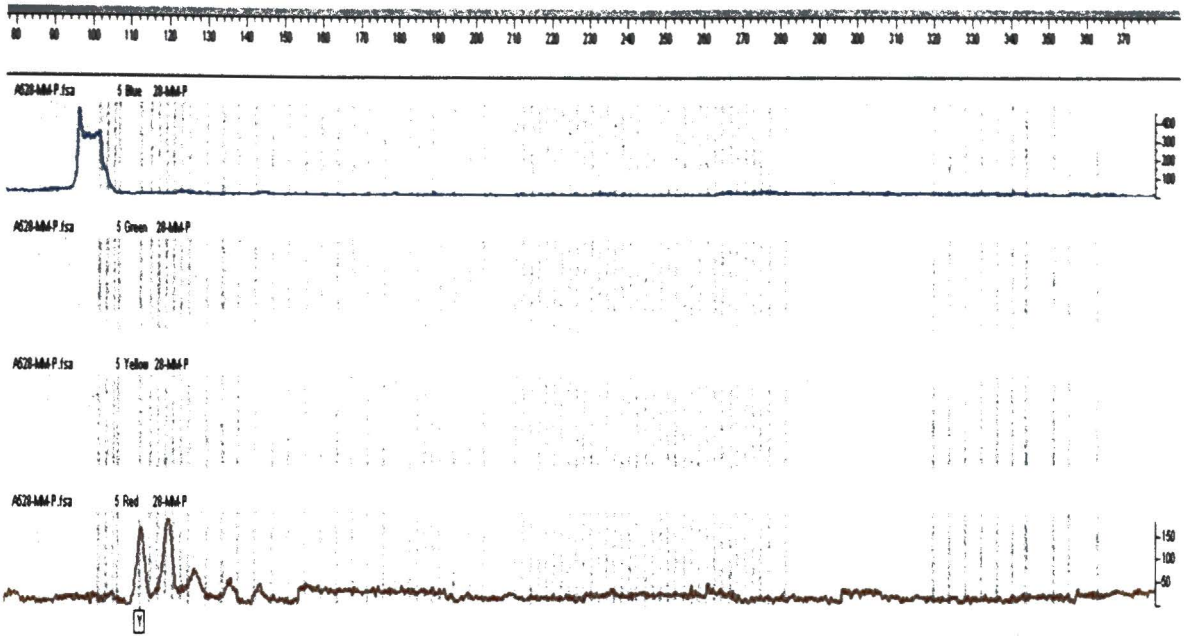

Figure 30A. 1-P, sample from telephone. 28 PCR cycles.

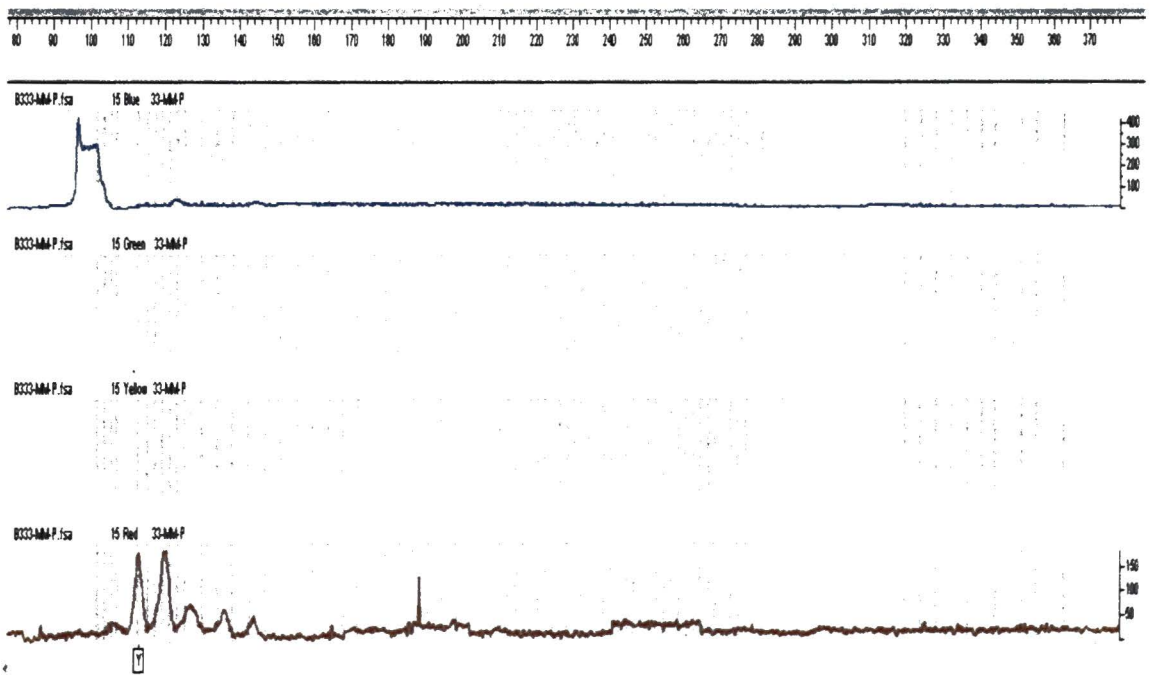

Figure 30B. 1-P, sample from telephone. 33 PCR cycles.

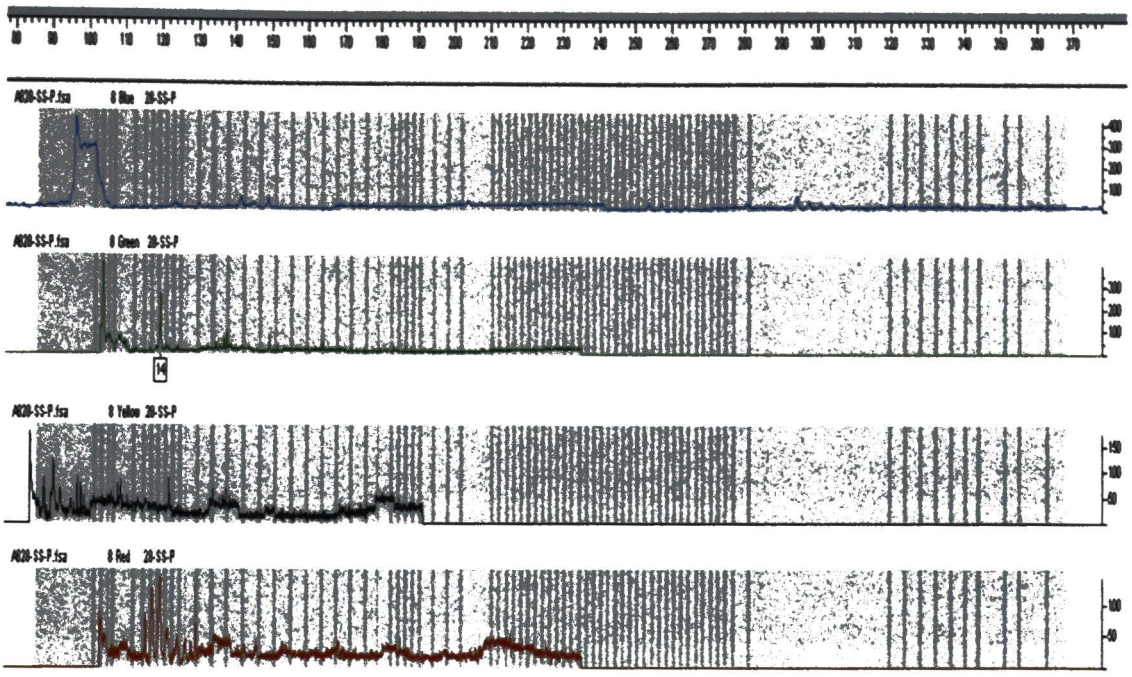

Figure 31A. 2-P, sample from telephone. 28 PCR cycles.

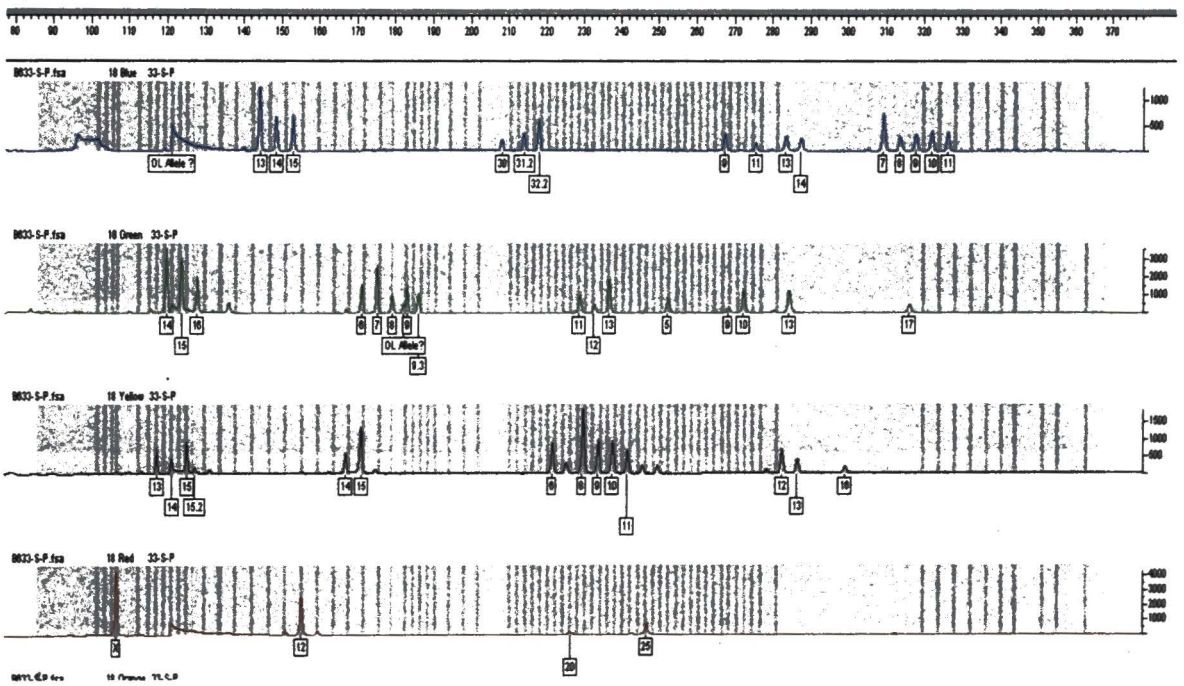

Figure 31B. 2-P, sample from telephone. 33 PCR cycles.

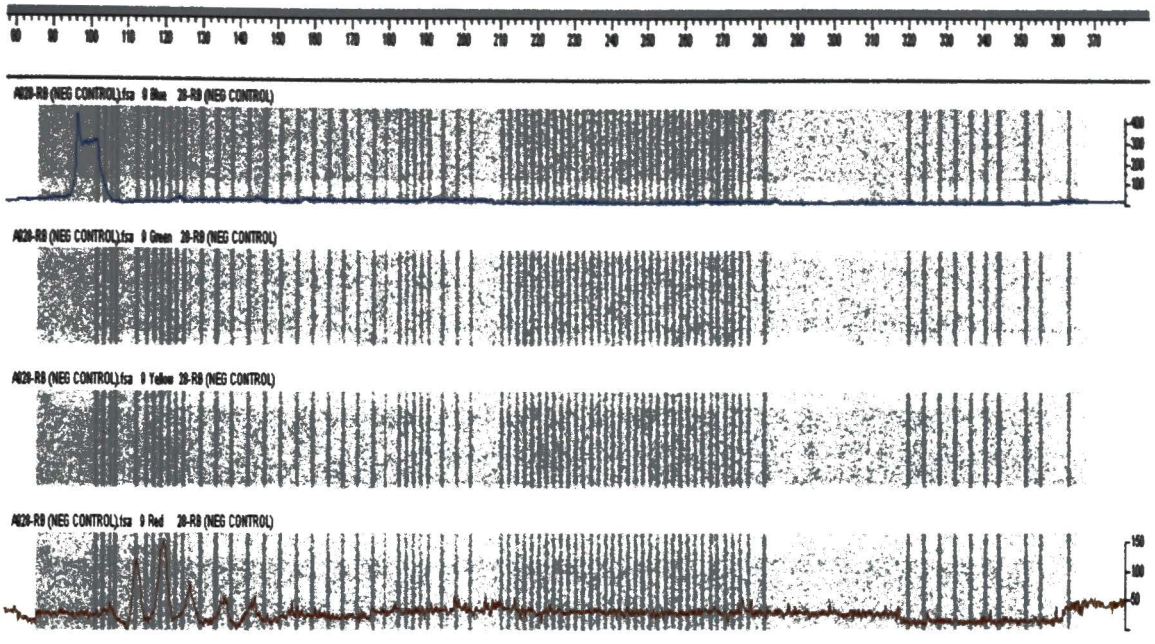

Figure 32A. Reagent blank/clean swab. 28 PCR cycles.

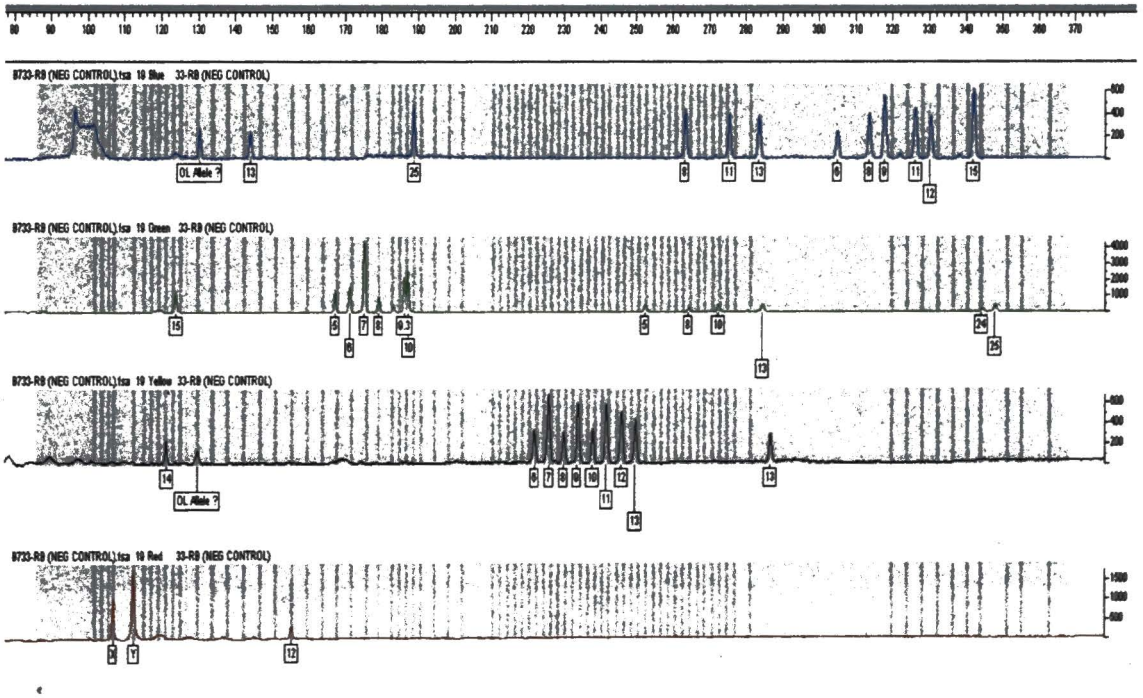

Figure 32B. Reagent blank/clean swab. 33 PCR cycles.

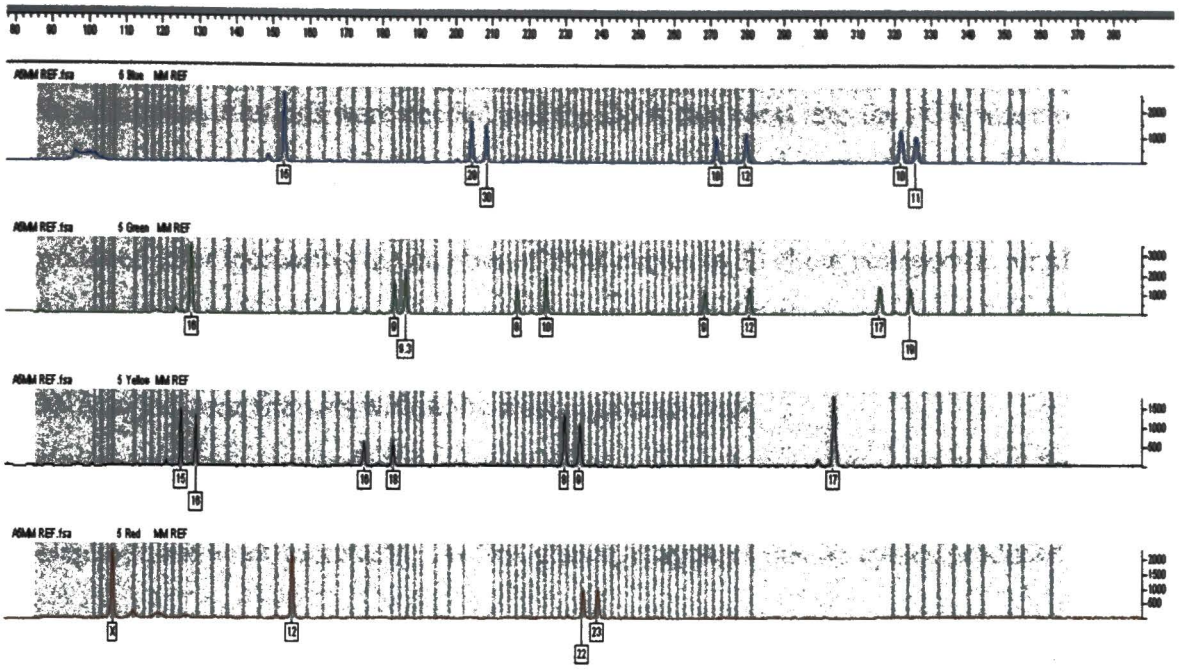

Figure 33. Reference profile of volunteer 1.

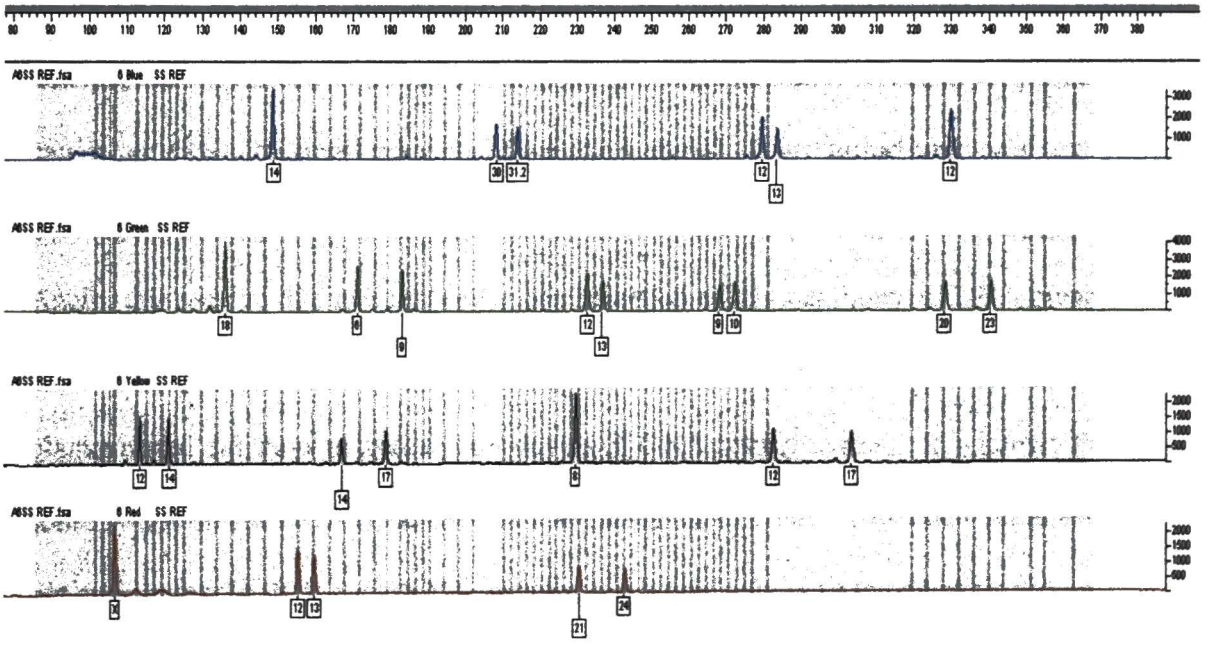

Figure 34. Reference profile of volunteer 2.

Genetic profiles from the samples collected from the soda cans were easily analyzed. No additional dilutions were needed and the DNA that went into the PCR was close to 1ng (800pg). This is well within the working range of the STR kits. The genetic profiles produced after increasing the number of PCR cycles to 33 show nonspecific amplification and increased stutter. This should be avoided as the silica extraction yielded plenty of DNA to perform analysis within the working parameters.

The STR analysis of the DNA extracted from contact samples off the doorknob and telephone were not successful. At first glance, it appears that 33 cycles produced a genetic profile from some samples, but upon comparing these samples with the appropriate references, the alleles are not the same. In addition, at most loci, there are many alleles, as if there is a large mix of individual profiles. This may have been due to nonspecific amplification during PCR. Stochastic effects and preferential amplification can occur with such small amounts of genetic material. It is interesting to note that even the reagent blank (or clean swab) produced allelic peaks after 33 cycles of amplification. Again, this may be due to nonspecific amplification of trace amounts of DNA present. According to the autosomal specific RT-PCR quantification assay, one of the two reagent blanks tested did show extremely low-levels of DNA contamination. It is known that some swabs do show contamination of trace amounts of DNA. This trace amount was not seen in the 28 PCR cycle STR analysis, but it was only until the amplification was pushed to its limits that the contamination could be detected.

The purpose of this objective was not to determine how best to perform STR analysis on low copy number DNA, rather it was designed to test if the autosomal specific RT-PCR would be able to quantitate the extracts. Some value was generated, however, due to the background contamination of the blank swab, it is likely the RT-PCR quantification detected this low-level DNA. This is certainly not a downfall of these kits, but is an asset since they are sensitive even to minute amounts of DNA. These kits could be used to identify challenging samples and allow the analyst to apply the best techniques for their analysis. To analyze LCN samples, other extraction methods and genetic profiling techniques could be employed.

It is not advisable to correlate the C_T values to other PCR methods. Real-time PCR operates under very different parameters than do the traditional gel-based PCR methods with which most laboratories are familiar.

Degraded DNA

During the course of this project, the RT-PCR quantification kits have been used in another project for determining the effectiveness of newer DNA extraction procedures. It was noticed that a particular batch of samples, which gave substantial quantification values via the autosomal specific RT-PCR quantification assay, was not able to generate a full genetic profile when performing STR analysis. Figures 33 and 35 are extracts from the same individual but extracted with different methods (phenol/chloroform versus PrépMan™, respectively). Shown in Figure 33, a full genetic profile is obtained yet in Figure 35, a less than optimal profile is seen. This points out an important fact

discovered about these RT-PCR quantification kits: degraded DNA is capable of being quantified using these kits, however, successful quantification does not guarantee that STR analysis will be fruitful.

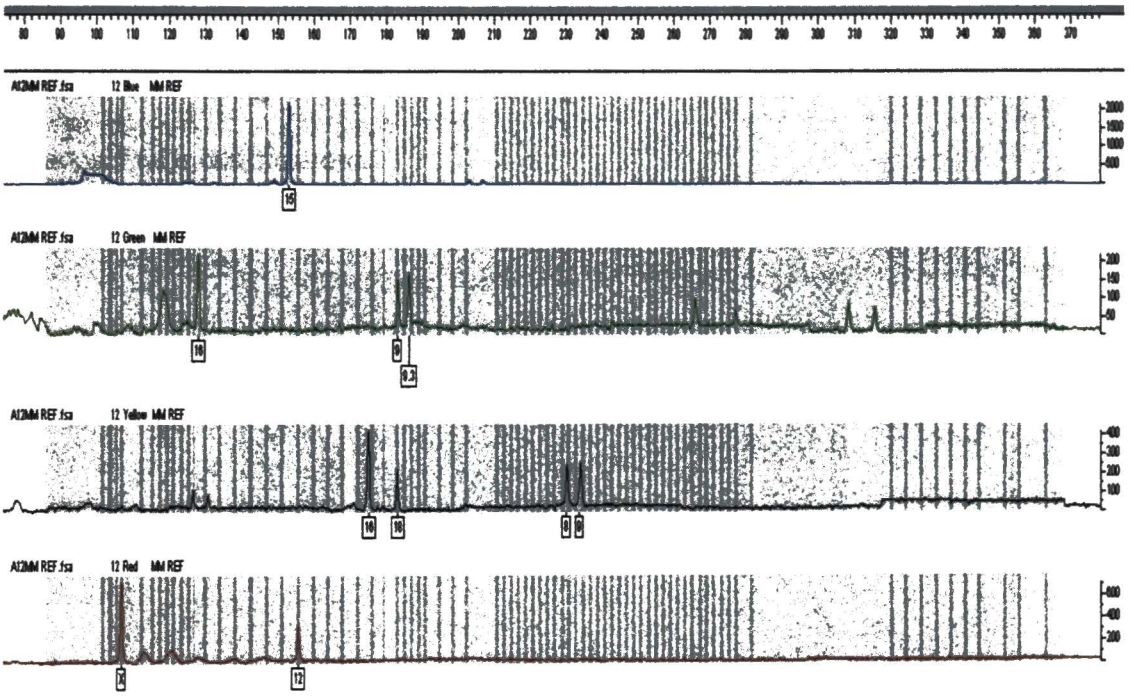

Figure 35. STR analysis of a degraded DNA sample that was successfully quantitated via autosomal specific RT-PCR quantification assay.

Autosomal versus Y-chromosome Quantification

Another interesting observation concerns the comparison of the autosomal specific to the Y-chromosome specific RT-PCR quantification assays. DNA extracts that were known to originate from a single source male were quantified with both the autosomal and Y-chromosome specific RT-PCR quantification kits. The results are shown in Figure 36 and 37. On average, the Y-chromosome quantifications were two times higher than the autosomal quantifications.

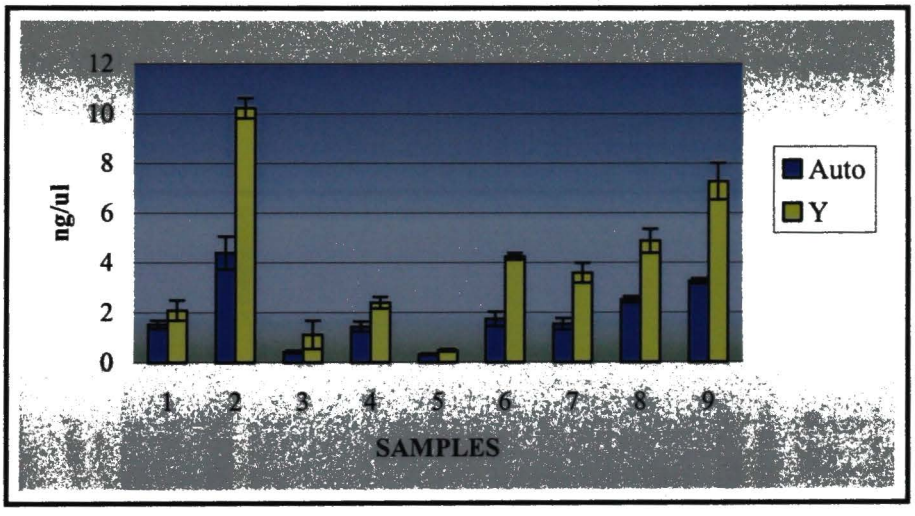

Figure 36. Comparison of the autosomal and Y-chromosome specific RT-PCR quantifications performed on male DNA samples.

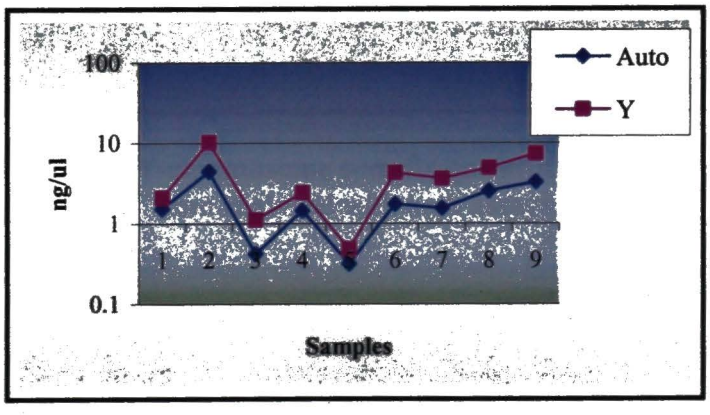

Figure 37. Data from Figure 36 plotted logarithmically.

The starting material per cell for each assay is different; a haploid cell versus a diploid cell. The protocol, provided by an ABI scientist, makes no mention how to allow for this disparity. It was reasoned that since the concentrations of the standards are based on a diploid cell, then the concentrations regarding the Y-chromosome are half the amount. Upon reanalysis of some assays by adjusting standard control concentrations, the quantifications between both assays were essentially equal (Figure 38). It is unknown at this time if Applied Biosystems will include a modification of this type in the final version of the Y-chromosome specific quantification assay, but this inequality should be addressed.

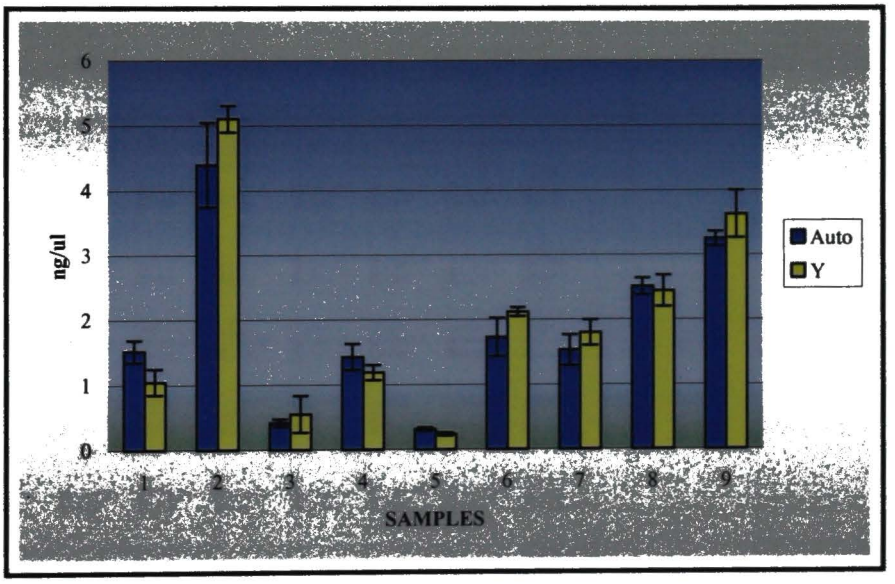

Figure 38. Reanalysis of Y-chromosome specific quantification assay. Same samples were analyzed with both assays.

Software Analysis

The next several figures are screen shots of the ABI PRISM™ 7000 Sequence Detection System analysis program. After a run is complete, analysis is as simple as clicking the "analyze" button. There are a number of ways to review to the data. The first screen shows the "plate" view (Figure 39). This displays the layout of the plate, where samples are located, and which assay was run in those wells. After analysis, this view will also display the quantities of the samples. Standards are located in wells designated with an "S". "U" represents either a test sample, or the Internal Positive Control. The pink boxes represent those samples being assayed with the autosomal specific quantification assay and the blue boxes represent those assayed with the Y-chromosome specific quantification assay. The IPC is represented by the green boxes.

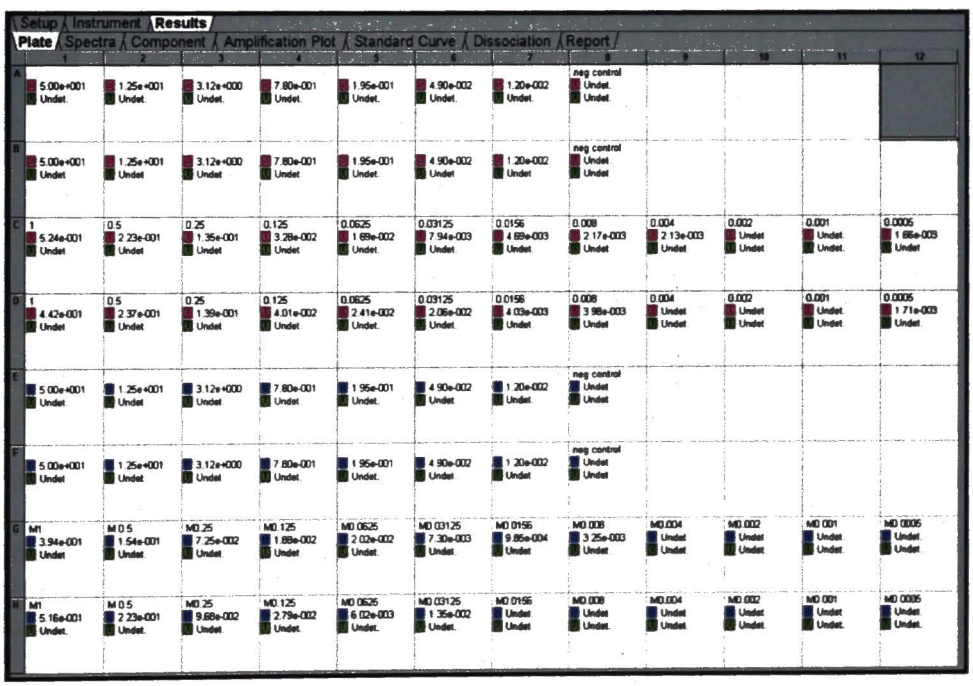

Figure 39. Screen shot of Plate layout.

The "amplification plot" layout shows the samples as they cycled through the RT-PCR assays. When running multiple assays on one plate, it is possible to examine each individual component alone (Figure 40) or together (Figure 41). Figure 40 shows the autosomal standards in the amplification plot view. The green horizontal bar represents the fluorescence threshold (set at 0.200000). The C_T value is defined as the cycle number at which the fluorescence crossed this threshold. The 50ng/µl and the 0.012ng/µl standards are labeled.

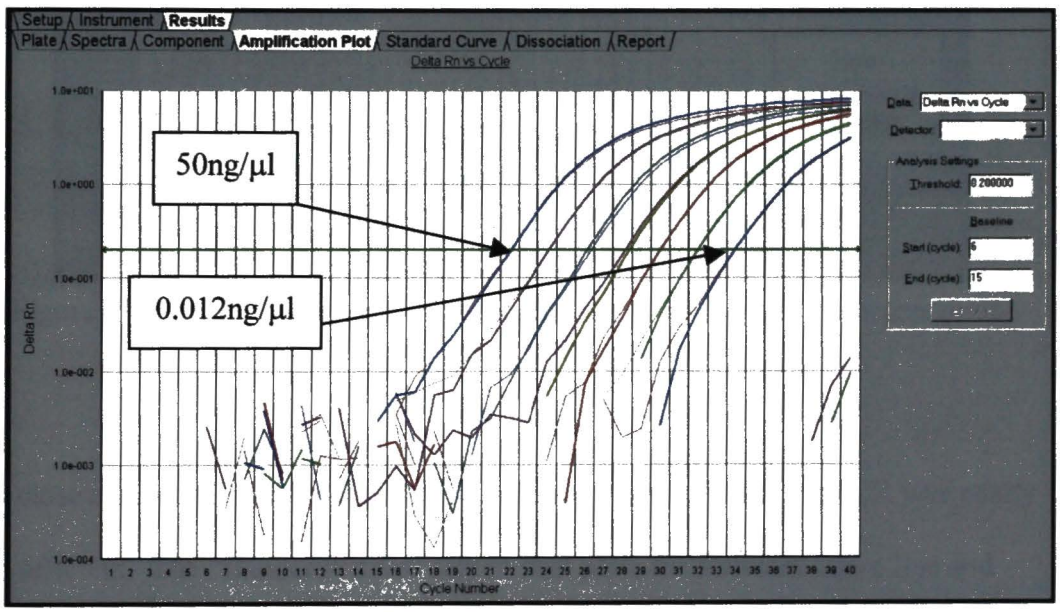

Figure 40. Amplification plot showing the autosomal specific RT-PCR quantification standards.

Figure 41 shows the multiple assays that were analyzed on one plate: autosomal, Y-chromosome and the IPC assayed in both. When working optimally, the IPC will have a C_T value near 28. The amplification plot makes it clear the samples that did not cross the fluorescence threshold (negative controls and samples containing no DNA). The very bottom of the screen shows the plate layout in a grid fashion. Each well can be selected,

alone or together in any combination, or the entire plate can be viewed at once (as in Figure 41).

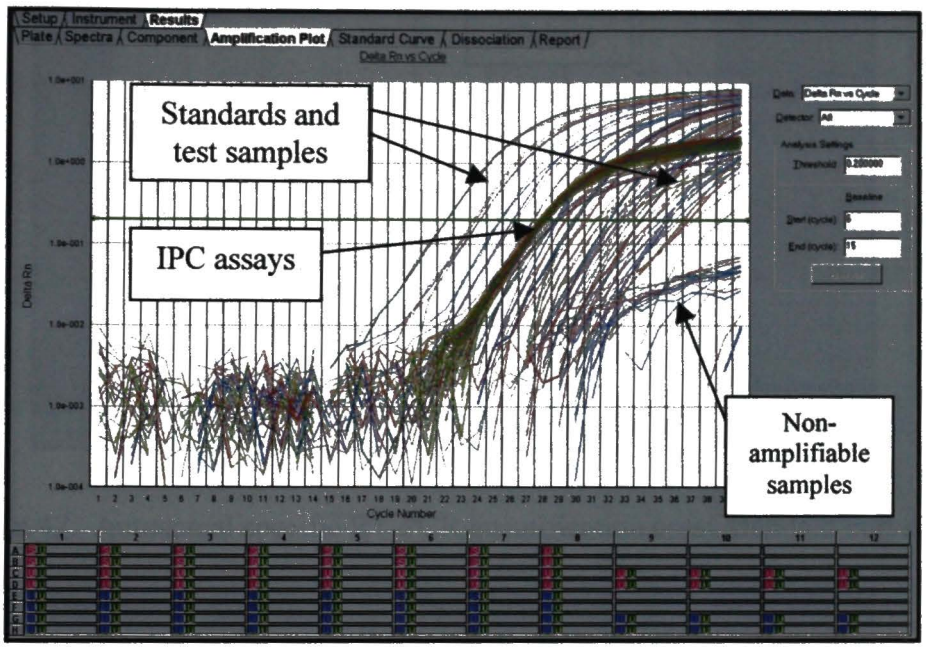

Figure 41. Amplification plot showing all assays (autosomal, Y-chromosome, and IPC) analyzed on the entire plate.

A close examination of the standard is necessary to verify that the PCR was nearly 100% efficient. The "standard curve" screen shot plots the standard curve line and displays slope, intercept, and R^2 values. To indicate near 100% amplification efficiency, the slope should be close to –3.2 and the R^2 value should be greater than or equal to 0.99. Again, if both assays were performed, the "standard curve" plot display one or both standard curves (Figures 42 and 43, respectively).

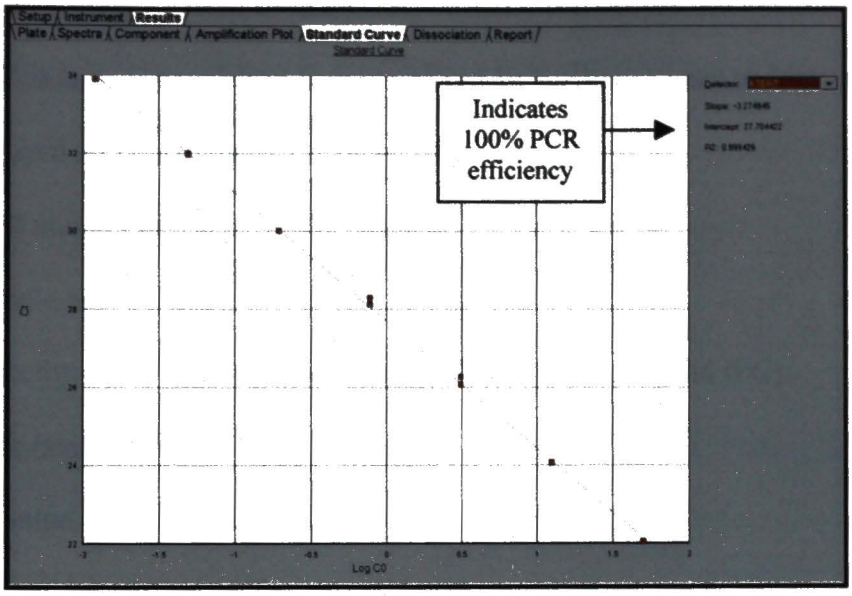

Figure 42. Autosomal specific RT-PCR quantification assay standard curve plot.

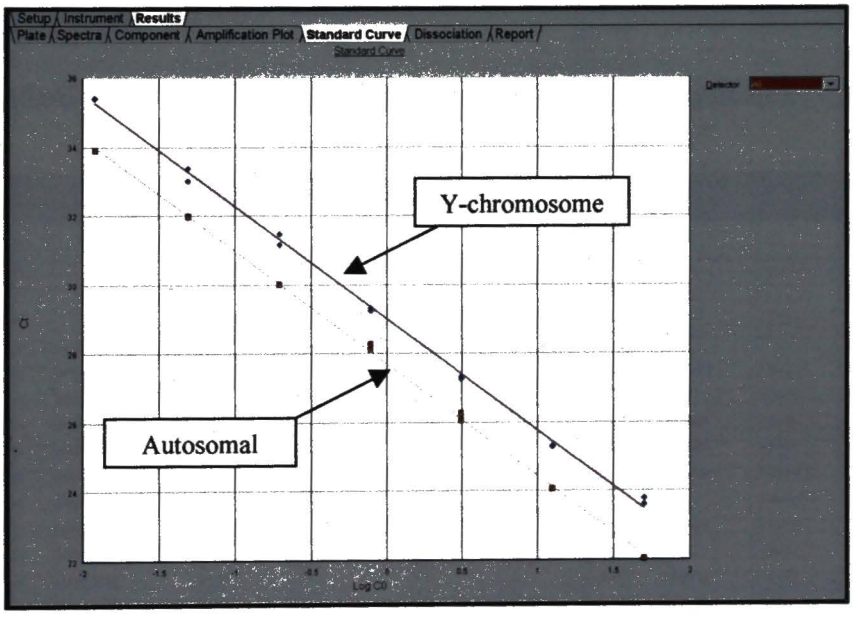

Figure 43. Standard curves of both assays.

The "report" screen shot displays the numerical results of the standards and the test samples. This is in a spreadsheet format and can be easily exported to Microsoft® Excel. Items displayed are as follows (see Figure 44):

1. Well number of sample
2. Sample name
3. Detector (assay specific: autosomal or Y-chromosome, and IPC)
4. Task (standard or unknown test sample)
5. C_T value
6. Standard deviation of C_T values (if performed in duplicate)
7. Quantity of sample (reported in the same units as entered for the standards)
8. Mean quantity
9. Standard deviation of those quantities (if performed in duplicate).

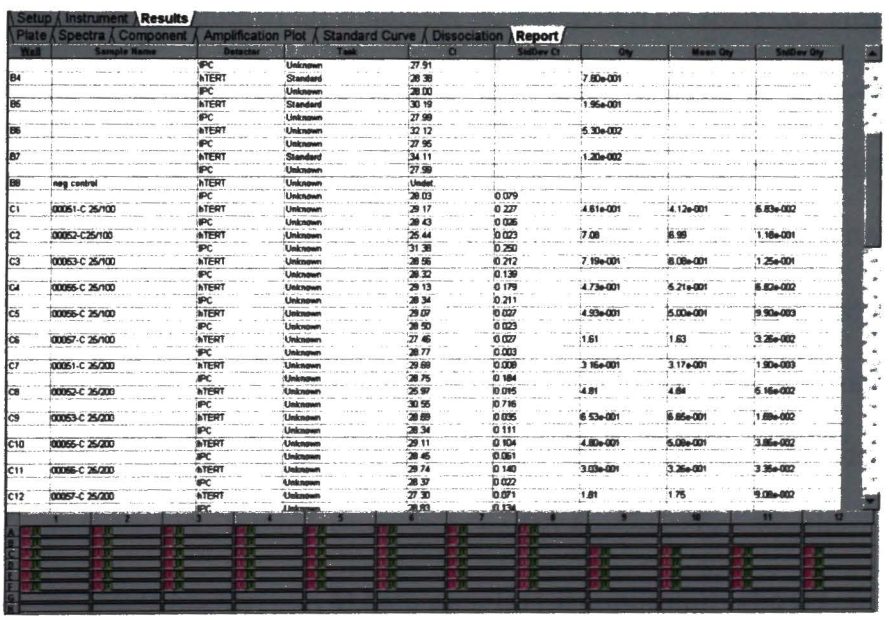

Figure 44. Example of a report displaying an assay's numerical values.

Internal Positive Control

As previously mentioned, the autosomal specific and Y-chromosome specific RT-PCR quantification assays are actually duplex assays, which contain two independent sets of PCR primers and TaqMan® probes. One of the assays is the target specific assay, either the autosomal or Y-chromosome. The other assay is known as the internal positive control, or IPC, and is identical in each assay. The IPC is comprised of 10,000 copies per reaction of synthetic template not found in nature, the PCR primers, and a VIC labeled TaqMan® probe for its amplification and detection. The purpose of the IPC is to monitor the working conditions of the reactions. Proper amplification and detection of the IPC indicates that all conditions are operational, both chemistry and instruments, even if DNA is not present. If the IPC fails to be amplified and detected, this indicates the existence of a problem or error, most likely due to inhibition of the PCR. This is an extremely useful predictor of impending failure of the multiplex PCR step of STR analysis. This would prevent the consumption of expensive reagents and waste of valuable time. Until now, no methods existed that detected PCR inhibitors and warned of imminent analysis failure.

The series of figures below show an example of the use of the IPC to determine the status of the test sample. In Figure 45, the duplicates of two unknown samples are shown. It is clear that the replicates from one test sample cross the fluorescence threshold and thus was able to be amplified and quantitated, while the other test sample did not cross the threshold. Is this a situation of no DNA present or inhibition? Figure 46 shows the IPC amplification plot of both samples (in duplicate). The IPC in both sets

of replicates cross the threshold at cycle number 28, the IPC's normal C_T value. Thus, the sample truly lacked DNA because the IPC was able to amplify correctly.

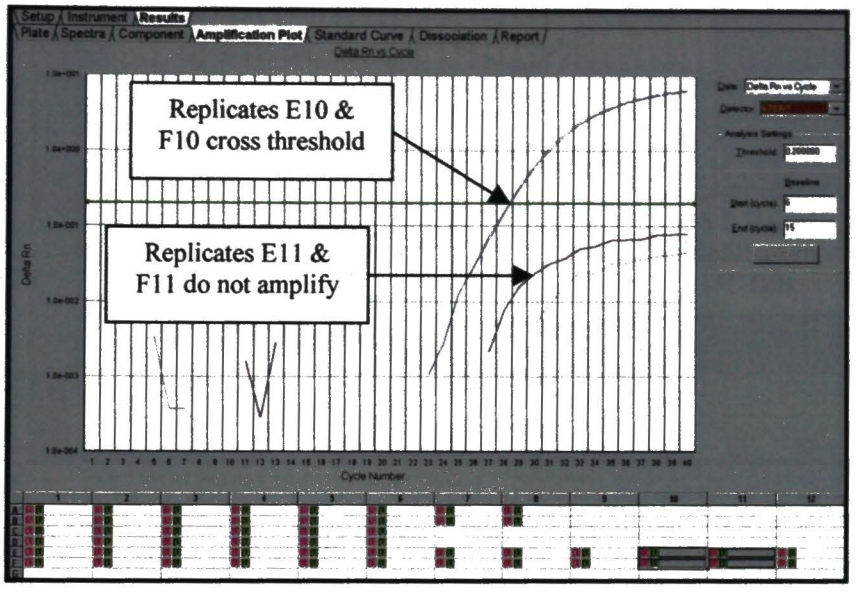

Figure 45. Replicate test samples. One set amplifies, the other does not.

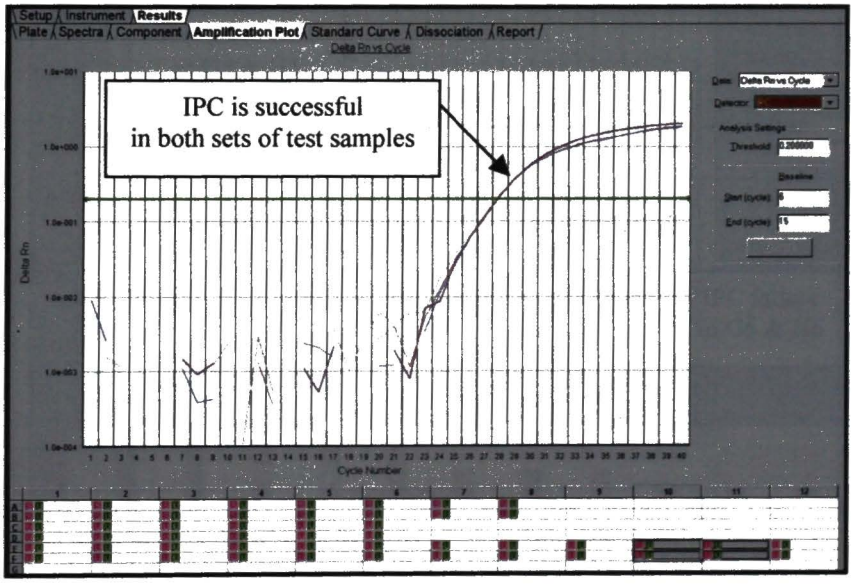

Figure 46. IPC amplification in each set of replicates is successful.

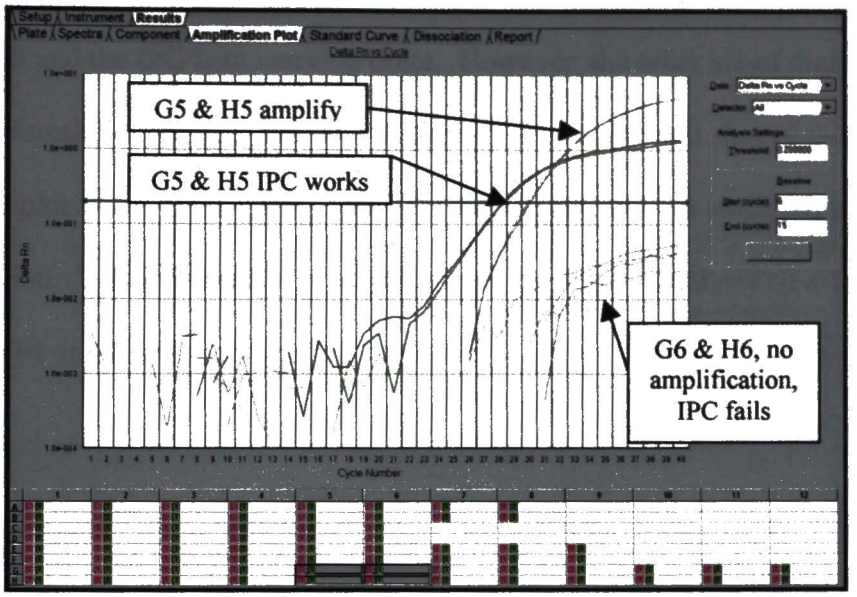

Figure 47. Autosomal amplification: IPC failure in test samples.

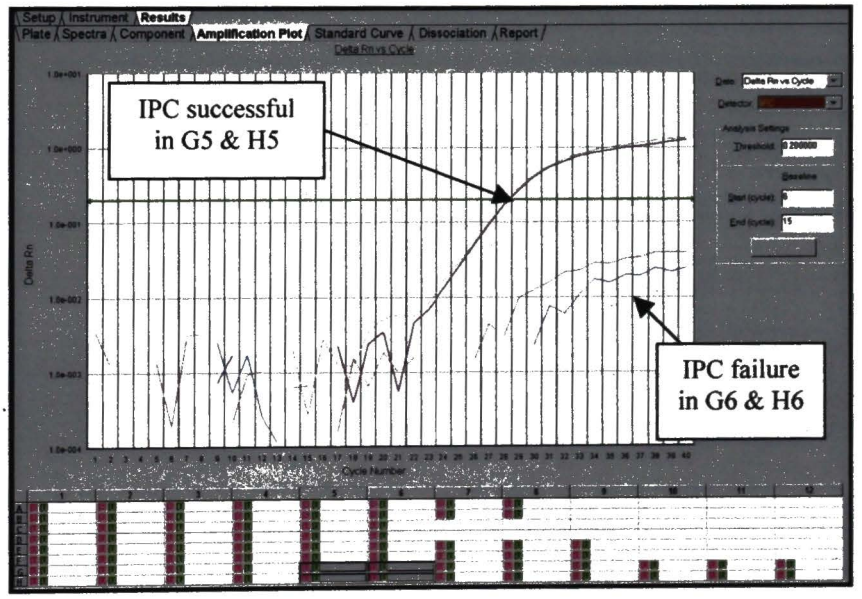

Figure 48. Display of the IPC amplification plot alone.

A test sample that shows inhibition is displayed in Figure 47. One set of replicates amplified well and the IPC is in working order. However, the other set of duplicates did not cross the threshold, nor does the IPC. Figure 48 shows a screen shot of the IPC amplification plot alone. This visibly confirms that the IPC did not amplifying properly due to inhibition. This information is helpful to prevent further analysis on a sample that is unlikely to be amplified successfully.

CHAPTER VI

CONCLUSIONS

Applied Biosystems' autosomal specific and Y-chromosome specific RT-PCR quantification assays can impart increased levels of sensitivity and human specificity needed to assess the genetic material in samples. These assays are more sensitive and more specific than current quantification methods because of the use of the polymerase chain reaction. PCR has proven to be a sensitive and robust method to produce a large quantity of targeted genes. Real-time PCR has the added benefit of monitoring changes early in the PCR, during the exponential phase. This allows for accurate quantification that is sensitive to initial starting material. RT-PCR is being widely used for the quantifications of bacterial and viral pathogens and gene activity changes.

By providing additional information such as human male DNA quantification and PCR inhibitor detection, these kits will furnish what the forensic community has been lacking. The human male DNA detection and quantification is valuable by offering proof that male DNA was present in an intimate sample from a sexual assault case. This would be especially important in a case in which the offender was a vasectomized male, and for resolving mixtures of the victim and offender's genetic profiles. The IPC assay presents a novel method for detecting PCR inhibitors present in a DNA extract. This is an extremely useful predictor of impending failure of the multiplex PCR step of STR

analysis, thus preventing the consumption of expensive reagents and waste of valuable time.

Today, STR analysis will proceed, and is often successful, even if no quantification results are obtained with current methods. The legal system questions this approach. The autosomal specific and Y-chromosome specific quantification assays will be able to quantify low level DNA and therefore provide the justification for subsequent analysis that would quiet the legal system's arguments concerning human quantification.

The autosomal specific RT-PCR quantification assay has been shown to be more sensitive in detecting low-level DNA over current methods. This assay has better sensitivity and specificity that exceeds spectrophotometry, the PicoGreen® assay, and the QuantiBlot™ hybridization assay. These assays allow for objective and automated quantification, unlike the QuantiBlot™. In comparing STR data between samples quantitated with both the autosomal specific RT-PCR quantification assay and the QuantiBlot™ hybridization assay, remarkable differences were not seen. However, using the RT-PCR assay, less sample was amplified and small improvements were noted. These samples do not represent the more challenging types of samples commonly encountered in the forensic field.

The Y-chromosome specific RT-PCR quantification assay will be a huge boon to the forensic community. This project has shown that male DNA can be detected in mixtures down to 0.5% of the total DNA present. Additionally, the detection of male DNA in what is essentially a female DNA extract, such as those from sexual assault cases, proved useful in gauging the level of mixture of female to male DNA as seen in the STR data.

This will allow an analyst to anticipate samples that will be difficult to interpret due to a low ratio of female to male DNA (approaching a value of 1). Furthermore, these challenging mixtures of male DNA could possibly be analyzed for Y-STRs or Y-SNPs (single nucleotide polymorphisms) to single out a male genetic profile.

The autosomal specific RT-PCR quantification assay was capable of detecting trace DNA samples, down to 10 cells (or 20 copies of chromosome five). Although the use of the C_T values were not successful in generating a full genetic profile, the assessment of the samples was remarkable nonetheless. This certainly exceeds any quantification method currently used in forensics.

Due to the differences in one-chromosome versus two-chromosome amplification, it is recommended that the Y-chromosome specific assay be used with caution. Allowances will have to be made to account for these disparities (such as reanalysis with standards that account for a haploid chromosome or dividing quantification values by two). It is unknown how, or if, Applied Biosystems will address this issue.

It is also important to realize the limitations of these kits when working with degraded samples. Due to small amplicon size (less than 70bp), the quantification of the samples is robust and proceeds in spite of degradation that may have occurred. This remains extremely helpful information even if STR analysis fails.

The autosomal specific and Y-chromosome specific RT-PCR quantification assays can offer superior advantages over current methods of forensic sample quantification. These include a wide dynamic range, high sensitivity, low standard deviation between replicates, no post-PCR steps, higher throughput, ease of use, less hands-on time, and

better sensitivity via detection of human male DNA. Furthermore, the sensitivity levels of these assays exceed the sensitivity limits of STR analysis. Since the dynamic range is so high, assays will not need to be repeated with diluted samples. However, as with any technology, there are limitations.

The most obvious limitation of the use of these assays is the equipment that is needed for detection. Most forensic labs do not have the funds to purchase a RT-PCR unit, or specifically the ABI 7000 Sequence Detection System. Although moderately priced, the $50,000 price tag would prove prohibitive for many forensic labs to take advantage of this technology. There are other uses for RT-PCR detection units, such as allelic discrimination assays and a variety of absolute quantification applications. Unless the forensic community can benefit from these other applications, RT-PCR will only be used in private or well-funded public laboratories.

Although these assays have yet to be released commercially and prices are unknown at this time, it is likely that these kits may not offer a cost savings over current methods. The cost may be nominal however when considering the price of repeat STR analysis, an analyst's time, and loss of a sample that cannot be recovered.

The major limitations of RT-PCR technology include the probe's sensitivity to mismatches in the binding region, and standardization of reagents, instrumentation, and data analysis across laboratories should be equal (15). However, these limitations are eliminated due to the development, manufacturing, and quality control measures taken by the research and development scientists at Applied Biosystems in designing these RT-PCR quantification kits. The limitations listed here are relevant only when developing a

RT-PCR quantification assay and do not apply to the end user of these kits, the forensic analyst.

These kits do require excellent pipetting techniques, which are controlled by not only that analyst, but also the calibration of the pipettors. Proper pipetting is essential for preparing the standards and aliquoting the samples into the reactions. The homogeneity of the samples is of great concern. Homogeneity is necessary for obtaining accurate quantifications of a representative sampling of the extracts.

Finally, it is unlikely that information, other than the quantification value, generated from RT-PCR analysis can be used for improving the performance of traditional gel-based multiplex PCR that is used for subsequent genetic profiling. RT-PCR operates under universal conditions that are not comparable to traditional gel-based PCR methods. This certainly does not mean that these RT-PCR quantification kits do not improve subsequent STR analysis. Since STR kits are manufactured by various companies, the forensic analyst has little control over the PCR process and its various parameters (concentration of primers, taq, dNTPs, $MgCl_2$, etc). A forensic analyst does have control over the number of PCR cycles used for amplification and the amount of template DNA added to the reactions. Using the optimal input of DNA into these multiplexed assays will only serve to improve all aspects of genetic profiling. It is likely that the forensic community will welcome these assays, as an improved quantification method is long overdue.

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