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EVALUATION AND VALIDATION OF TECAN GENIOS MICROPLATE READER

FOR QUANTIFICATION AND NORMALIZATION

OF FAMILY REFERENCE DNA SAMPLES

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EVALUATION AND VALIDATION OF TECAN GENIOS MICROPLATE READER FOR QUANTIFICATION AND NORMALIZATION OF FAMILY REFERENCE DNA SAMPLES

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CHAPTER 1: INTRODUCTION

In 2001, the Texas State legislature established the Texas Missing Persons DNA Database (TMPDD) at The University of North Texas System Center for Human Identification laboratory. Texas was the first state to participate in the missing persons section of the federal (FBI) database titled COmbined DNA Index System or CODIS.[1] Two indices of CODIS include the Unidentified Human Remains index and the Relatives of Missing Person index. Medical specimens, such as bone marrow or blood, or personal items used only by the missing person, such as a toothbrush or hairbrush, are ideal for identifying human remains through comparison of DNA profiles; although, DNA samples can be taken from family members to help locate missing persons or identify remains.

DNA profiles from family reference samples, such as blood or buccal swabs from a close relative, are analyzed and uploaded into CODIS to allow federal, state, and local crime laboratories to exchange and compare profiles to missing persons electronically.[1] At the University of North Texas Health Science Center, family reference samples, missing person reference samples, and unidentified human remains are analyzed to obtain DNA profiles for comparison. This research project involves a method that is proposed to improve the efficiency of DNA analysis for family reference samples.

At the UNT System Center for Human Identification laboratory, the family reference samples are extracted in batches of 86 using the Tecan Freedom EVO® 100 extraction robot with the DNA IQ™ extraction kit from Promega Corporation. The DNA IQ™ extraction process is used in conjunction with the EVO® 100 robot in order to obtain a consistent amount of total extracted DNA; although, substantial variation has been detected in the output DNA quantity delivered. A considerable percentage (~20%) of samples exceed the optimal input template DNA amount required for successful amplification using the Applied Biosystems AmpF*l*STR® kits. A method of normalizing these samples was needed to bring the standard input DNA range within the optimal analytical range of the Applied Biosystems 3130 Genetic Analyzers and GeneMapper™ ID software.

The ultimate objective of this internship practicum was to improve the efficiency of DNA analysis for family reference samples by using the Tecan GENios microplate reader in conjunction with an OliGreen® assay to estimate DNA quantity with the aim of using the quantification values to normalize family reference samples into an ideal input range for genetic analysis.

CHAPTER 2: BACKGROUND

2.1 Quantification

The National Standards for Forensic DNA Testing Laboratories[2], which are quality assurance measures, require that each DNA sample be quantified. Standard 9.3 states that "the laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample where possible".[2] Quantification allows for the appropriate amount of DNA to be put into polymerase chain reaction (PCR) in order to optimize the DNA profile results. The ideal amount of input DNA can lead to higher quality results that are easier to interpret.

Typical DNA quantification methods include the measure of ultraviolet absorbance of nucleic acids at 260nm, visually comparing an unknown amount of DNA to known standards of DNA on a yield gel, and the measure of fluorescence from a dye that intercalates between the bases of the DNA like PicoGreen®.[3] Although, these methods are nonspecific for human DNA and therefore provide only a quick and general form of quantification for all DNA present in the sample. The use of human specific probes and primers in techniques such as slot blot hybridization and real-time PCR provide for human-specific DNA quantification methods, which are necessary to fulfill the quality assurance standards regarding evidentiary DNA samples. The drawback of

these DNA quantification methods is the high price of equipment and reagents and the time and labor necessary to perform each technique.

In recent years, human DNA quantification has moved toward new real-time quantitative PCR techniques and away from slot blot hybridization techniques. Real-time PCR combines DNA amplification, typically done by PCR, with real time amplified product detection. Real-time PCR offers a dynamic range in DNA quantification and is easily automated. It is also less time consuming than slot blot taking approximately 1 hour to set up and 2 hours to run. Other advantages of real-time PCR are that the results are quantitative as opposed to semi-quantitative or qualitative as in slot blot hybridization methods, and it can monitor PCR inhibition through an Internal Positive Control. The Internal Positive Control will only amplify DNA that will be able to be amplified with PCR.[4, 5] An example of an real-time assay that uses an IPC is the Quantifiler® Human DNA Quantification Kit supplied by Applied Biosystems.

The disadvantages of real-time PCR lie in the price of running high throughput samples. An Applied Biosystems 7500 Real-Time PCR System for Human Identification costs about $50,000, and a Quantifiler® Human DNA Quantification Kit is approximately $950 for 400 reactions[6], which becomes very expensive when batch plates of 96 samples are constantly being run. The cost and time consumption make the use of real-time PCR for quantification of batches of family reference samples inefficient and impractical. Because the family reference samples are part of the Texas Missing Persons

DNA database and not used in forensic investigations, quantification is not required by the national standards and therefore not performed at UNTHSC.

All of the human specific methods of quantification are adequate for DNA samples, but their prices make them unreasonable for batches of family reference samples. For the family reference samples of missing persons, human specific DNA quantification is not required, so for them, the nonspecific methods are more efficient and still provide the information needed, which is how much the DNA concentration needs to be adjusted in order to obtain optimal STR results.

2.2 Family Reference Samples

Family reference samples are typically blood or buccal samples submitted by relatives of the missing person. DNA is collected from the family reference samples for comparison to the DNA profiles obtained from unidentified human remains to allow for possible identification. Family reference samples are extracted in batches of 86 on the Tecan Freedom EVO® 100 with the DNA IQ™ extraction kit from Promega Corporation. The Tecan Freedom EVO® 100 automated liquid handler is one of the most adaptable instruments available for automation of the DNA IQ™ System.[8] Tecan scripts allow for much flexibility when extracting DNA. The EVO® 100 accommodates various types of samples, tubes or 96-well plates, and anywhere from 1 to 96 samples to be processed at one time.[8] Other advantages include two independent arms, one for liquid-handling and one for gripping, adjustable tip spacing with independent volume control on each of the 8 tips, and disposable-tip volumes of up to one milliliter.[9]

The DNA IQ™ System is a DNA isolation and quantification system designed specifically for the forensic and paternity community. This system employs an innovative technology with magnetic particles to prepare clean samples for short tandem repeat (STR) analysis easily and efficiently. The DNA IQ™ System can be used to extract DNA from stains, liquid samples such as blood, or DNA solutions. According to the Promega Corporation, "When working with larger samples found in paternity and databasing, the DNA IQ™ System automatically delivers a consistent amount of total DNA."[10] The Promega Corporation proposes that this consistent amount of total DNA gives reliable products; therefore, the customer presumes that DNA IQ™ extracts will give optimal results, which is not necessarily true.[10] Herein lays the problem.

For each sample that is extracted into a 96-well plate, 1uL of extract is added as template to a PCR reaction, which is then amplified and analyzed. Samples that do not produce interpretable STR results must be either reextracted, reamplified, reloaded, or diluted. With each family reference 96-well plate that is run, approximately 30% of the samples have to be altered in order to achieve optimal STR results, which means that the total amount of DNA from the DNA IQ™ System is not consistent. Approximately 10% of the samples are failing to give complete STR results and therefore have to be reextracted or reamplified with a higher volume. The other 20% of samples are too concentrated, which causes STR artifacts that make interpretation more difficult. These amplicons have to be diluted anywhere from a 1:5 to 1:30 dilution, and occasionally, the extracts are diluted and reamplified. These non-optimal STR results are present

throughout the batches, which is causing extra time and money to be spent on getting reasonable results. The percentage of samples having to be adjusted is significant enough to affect the analyst's workload; therefore, a solution was needed.

The Tecan GENios is a microplate reader that can quantify DNA by fluorescence, absorbance, or luminescence. The instrument is suitable to handle assays in multiple microplate formats from 96-well plates to PCR tubes and cuvettes. The GENios offers exceptional sensitivity and flexibility when quantifying DNA. For the quantification of DNA or protein samples you can select between 260/280 nm absorbance measurements or a fluorescence readout using appropriate fluorescence labels.[11]

The GENios has a measurement speed of less than 30 seconds for a 96-well plate and can shake the plate linearly or orbitally at three different speeds to mix the plate contents. Fluorescence is commonly used to quantify the DNA as a nonspecific method of DNA quantification. The GENios reads fluorescence from the top and bottom of the plate. With 200uL total well volume, a black microplate, and 100 flashes, the sensitivity is less than 3pg fluorescein (OliGreen®) per well with the wavelength range between 340 and 700 nm. Most importantly, for higher throughput, the GENios can be easily integrated into Tecan's whole range of robotic workstations.[11] Also, the GENios can be purchased for about $15,000[11], and the OliGreen® kit only costs about $300 for 2000 reactions (www.invitrogen.com). The speed and expenses that accompany the Tecan GENios and OliGreen® made them a reasonable solution for regulating the DNA concentrations of family reference samples.

The OliGreen® ssDNA quantification assay allows for detection of as little as 100 pg/ml of ssDNA, which is 10,000 times more sensitive than other nonspecific quantification methods like measurement of UV absorbance.[12] The extra sensitivity can save valuable sample. OliGreen® also has a linear detection range that spans over four orders of magnitude in oligonucleotide or ssDNA concentration.[13] OliGreen® is not as well published as PicoGreen®[3,5], but it has been useful for quantification of oligonucleotides in animal drug studies. One example is in drug research, circulating levels of therapeutic oligonucleotides have to be measured, and OliGreen® allowed for rapid quantification of oligonucleotide levels in plasma samples.[14] A very limited number of published articles involve the use of both the Tecan GENios and OliGreen®, but the combination has been used for quantification and normalization of cDNA, which is the DNA that is made from mRNA.[15]

Fluorescence is commonly used to quantify DNA not only because of its greater sensitivity over absorbance but because of its ability to function without the interference from nucleotides or contaminants from nucleic acid preparations.[13] The OliGreen® ssDNA quantification reagent intercalates into the bases of single stranded DNA and becomes intensely fluorescent upon binding single stranded nucleic acids. The assay has little sequence dependence, which allows for accurate measurement of DNA from many sources such as single stranded genomic DNA and viral DNA.[12]

For standard fluorescent measurements with the GENios, black 96-well plates are recommended in order to help minimize the background. According to the manufacturer,

it is not advised to use clear 96-well plates because the edge-well effect will occur where the wells around the edge of the plate will have different readings from those in the middle of plate.[16] It is important to choose the correct filter for each application. Fluorescent applications such as OliGreen® and fluorescein typically use 485 nm for the excitation filter and 535 nm for the emission filter.[16] The software provided with the Tecan GENios is Magellan™ v5.03. Magellan™ software assists all Tecan microplate readers and their detection techniques including fluorescence.[17]

The main objective of this internship practicum was to use the Tecan GENios microplate reader in conjunction with the PicoGreen®/OliGreen® Quantification kits from Molecular Probes to estimate the DNA concentration that was being extracted with the DNA IQ™ kit so that based on these estimated values, the DNA concentration of family reference samples could be adjusted into a typable STR range. In order to achieve this overall goal of normalizing the concentrations of family reference sample DNA extracts, some minor objectives had to be accomplished. First, human DNA standards of known concentrations had to be developed for use on each run of the GENios microplate reader to develop a standard curve. This standard curve and the GENios were used to evaluate DNA extracts with the OliGreen® system. The relative quantification values were then correlated with STR typing results with regard to RFU, pull up, etc. To allow for consistency, the Tecan MiniPrep was evaluated for its usefulness in preparing a GENios run plate by dispensing the OliGreen® master mix, the DNA extracts, and the standards. Once a model for establishing pass/fail criteria and dilution scheme was

developed, based on the GENios quantification values and their subsequent STR results, I was then able to achieve the ultimate goal of adjusting DNA samples to be within the acceptable range. By detecting and correcting this well established variation in concentration by quantitating and normalizing the DNA samples, the analysis process will be less expensive and less time consuming.

CHAPTER 3: MATERIALS AND METHODS

3.1 Samples

The samples used in this experiment were completed family reference samples submitted to the UNT System Center for Human Identification laboratory (Fort Worth, TX) for inclusion in the NDIS (National DNA Index System) family reference index. Family reference samples are extracted in two batches on one 96-well plate, which allows data analysis to be split between two DNA analysts. The four most recently completed plates (8 different batches) of DNA extracts were used as samples for testing the Tecan GENios Multi-Detection Reader (Tecan Inc., Research Triangle Park, NC). Batches 25, 26, 28, 29, 30, 31, 32, and 33 provided the nearly 350 family reference samples for analysis.

3.2 Standards

The DNA used to produce the standard curves for each GENios run was the Quantifiler® Human DNA Standard supplied in the Quantifiler® Human DNA Quantification Kit (Part# 4343895, Applied Biosystems, Foster City, CA). The Quantifiler® Human DNA Quantification Kit contains 120μL of ~200ng/μL Human DNA Standard. To check for accuracy, the quantity of DNA in the Quantifiler® Human DNA Standard (Applied Biosystems, Foster City, CA) was determined by quantifying a 1:10 dilution with the Quantifiler® Human DNA Quantification Kit on an Applied

Biosystems 7500 Real-Time PCR system (Foster City, CA).[18] The resulting quantity of 30ng/μL was used as a stock to dilute standards to known concentrations (0.025, 0.05, 0.1, 0.2, 0.4, 1, 2, and 3ng) with TE-4 (0.1mM EDTA, 10mM Tris-HCl) and stored at +4°C for repetitive use. Before being quantified with the Quant-iT™ OliGreen® ssDNA Assay Kit (Part# O11492, Molecular Probes Inc., Eugene, OR), the double stranded DNA standards had to be denatured. To make the standard DNA single stranded, 100μL was aliquotted into a new 1.5mL tube and put on a ~100°C heat block for 5 minutes, then immediately put on ice for 5 minutes.[19]

To evaluate the variation present among DNA quantities with the OliGreen® assay, each of the 9 standards, including the blank negative control, were run in 6 replicates on a 96-well plate and quantified 6 times on the GENios with one row as the standards and the other 5 rows as samples. The average, standard deviation, and coefficient of variation of the quantification values for the each of the standards were then calculated.

Each OliGreen® assay incorporated the creation of a standard curve produced by quantifying fluorescence of ssDNA produced with 0.125, 0.25, 0.5, 1, 2, 5, 10, and 15ng of human reference DNA template. These concentration estimates for the human standards were based on 5 μL of DNA addition to the GENios plate and were further evaluated by amplifying 5μL of standard DNA template with the AmpF*l*STR® Identifiler® PCR Amplification Kit (Part# 4322288, Applied Biosystems, Foster City, CA) according to recommendations from the supplier. For total concentration of standard

DNA, approximately 0.75ng to 5ng produced full STR profiles with allelic dropout present at the lower concentrations and artifacts such as extraneous peaks at the higher concentrations.

3.3 DNA Extraction

The DNA IQ™ System (Part# DC6700, Promega Corp., Madison, WI) of purification on a Tecan Freedom EVO® 100 liquid handler (Tecan Inc., Research Triangle Park, NC) was used for the DNA extraction of family reference samples for entry into the Relative of Missing Persons database.[20] The Promega DNA IQ™ System involves multiple steps: lysis of the sample with Lysis/DTT Buffer Working Solution, capture of the DNA using paramagnetic resin, washing of the resin with Wash Buffer, and elution of the DNA from the resin with Elution Buffer.[21] There are several advantages of this system over conventional DNA extraction techniques such as removal of inhibitors without hazardous chemicals or extensive washing/centrifugation, automation, and fairly consistent DNA yields because of the limited binding capacity of the resin.[21]

3.4 Quantification Using OliGreen®/GENios

Each assay included 198.5µL of 1xTE, which was a 1:20 dilution of the 20XTE (25mL of 200mM Tris-HCl, 20mM EDTA, pH 7.5) provided in the OliGreen® Kit, and 0.5µL Quant-iT™ OliGreen® ssDNA Reagent (Part# O7582, Molecular Probes, Inc., Eugene, OR). The assay followed the kit instructions provided by the manufacturer. A master mix of ~20mL can be made with 20.25mL of 1XTE and 51µL OliGreen® so that

199µL can be aliquotted into each well of a black flat bottom 96-well plate (Part# 655076, Greiner Bio-One, Monroe, NC). Black flack bottom plates are recommended in the GENios manual because they prevent fluorescence from passing between wells, and the flat bottom allows for the plate to sit flat in the instrument.

After the addition of the OliGreen®/TE master mix to each well on the GENios plate, 1µL of each sample extracted with the DNA IQ™ kit on the EVO® 100 from family reference samples is added to their respective wells. The DNA extracts are kept in the same order on the GENios 96-well plate as they are in on the EVO® 96-well extracted plate to allow for easy robotic transfer from the extract plate to the GENios plate. Following the addition of template, 5µL of each standard including a blank negative control is added to certain wells that are empty on the extracted plate (B1, C1, H2, A3, E5, G6, E7, H9, and B11). The plate is then temporarily covered with foil to keep it contaminant free and allow for the OliGreen® to bind the DNA. After 5 minutes, the foil is removed, and the plate is put on the GENios. The GENios is initialized by the Magellan™ software (Tecan Inc., Research Triangle Park, NC), which also collects the fluorescence data, forms a standard curve, and calculates the concentration of unknown samples from the standard curve.

In order to help define the optimal STR input range of GENios values, four 96-well plates for a total of 8 batches were quantified with the GENios and OliGreen®. Each plate was quantified 3 times, and the values for each sample were averaged to get one quantity to allow for a better estimation. The Identifiler® STR results were already

available for comparison to the GENios values and therefore did not have to processed. The most recently extracted plate, which included Batch 32 and 33, was quantified 3 times again with the GENios after the optimal range was defined. The proper dilutions of DNA extracts and additions of extra template to amplification reactions were made according to the GENios results using the range presented later in the results section. Then the samples were amplified with Identifiler® and loaded onto the ABI 3130xl for genetic analysis. The data was then analyzed to see if the range needed to be altered to achieve optimal STR results.

To allow for consistency between plates and within a plate, the Tecan MiniPrep (Tecan Inc., Research Triangle Park, NC) was considered and found to be useful. The 8 fixed tips on the right arm allow for column by column aliquots of first the 199µL OliGreen®/TE master mix from a trough and second the 1 µL of DNA template from an extract plate. The single tip on the left arm allows for 'cherry picking' of certain wells like with the 5µL of standards. After each column of template is added, the 8 fixed tips are soaked in 6% bleach, taken from the EVO® 100/DNA IQ™ protocol, and washed several times with water to remove excess DNA and help prevent cross contamination between templates. With the bleach steps and multiple water washes, the MiniPrep provides a consistent and automated way to set up a plate for quantification on the GENios with minimal room for human error. Each of these steps was programmed in the Gemini for MiniPrep software (Tecan Inc., Research Triangle Park, NC) and run as a script entitled **GENios Setup**.

3.5 Quantification Using Real-Time PCR

Estimates of DNA concentration in the stock standard as well as some random family reference samples used for comparison to the GENios were produced using real-time PCR with the Quantifiler® kit and 7500 Real-Time PCR System available from Applied Biosystems (Foster City, CA). Quantification reactions were prepared based on recommendations in the Quantifiler® kit User's Manual[18]. A total reaction volume of 25μL, including 2μL of template, was used. The standards for the OliGreen® assay were quantified in duplicate with Quantifiler® whereas only a single reaction was ran with the family reference samples. DNA quantities were calculated based on the standard curve using the recommended 7500 System manufacturer software, Sequence Detection Software (SDS) version 1.2.3 (Applied Biosystems, Foster City, CA). The highest concentration of quantitation standard contained 50ng/μL (Standard 1) of human genomic DNA supplied with the Quantifiler® kit. Serial dilutions were made of this DNA sample according to the manufacturer's instructions with the least concentrated sample corresponding to 0.023 ng/μL (Standard 8).

3.6 PCR Amplification and Genetic Analysis

Samples were amplified by PCR using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). AmpF*l*STR Identifiler® amplification reactions were set up following the instructions supplied with the STR typing kit, except only 10μL of PCR Reaction Mix and 5μL of the Primer Set were used instead of the recommended 10.5μL and 5.5μL respectively. In addition, sterile water was used in place of TE buffer for the

negative control and for dilutions of the samples. Positive control DNA (9947A) (Applied Biosystems, Foster City, CA) contained within the Identifiler® typing kit was amplified with every amplification at 28 cycles. For each sample, 1μL was added to amplification tube with 9μL of sterile water unless the GENios revealed a low concentration, then 5μL of samples with 5μL of sterile water was added.

For STR analysis, 1μL of amplification products produced by the AmpF*l*STR Identifiler® kits were mixed with 8.5μL Hi-Di™ Formamide (Part# 4311320, Applied Biosystems, Foster City, CA) containing 0.5μL GeneScan™ 500 LIZ® Size Standard (Part# 4322682, Applied Biosystems, Foster City, CA) according to the recommendations of the supplier with the exception of the decrease in Formamide from the recommended 24.5μL. The samples were then subjected to 10 seconds of electrophoretic injection and fluorescent analysis of 5 dyes (6-FAM, VIC, NED, PET, and LIZ) with the aid of an ABI Prism® 3100/3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and GeneMapper™ ID (Applied Biosystems, Foster City, CA) for STR analysis.

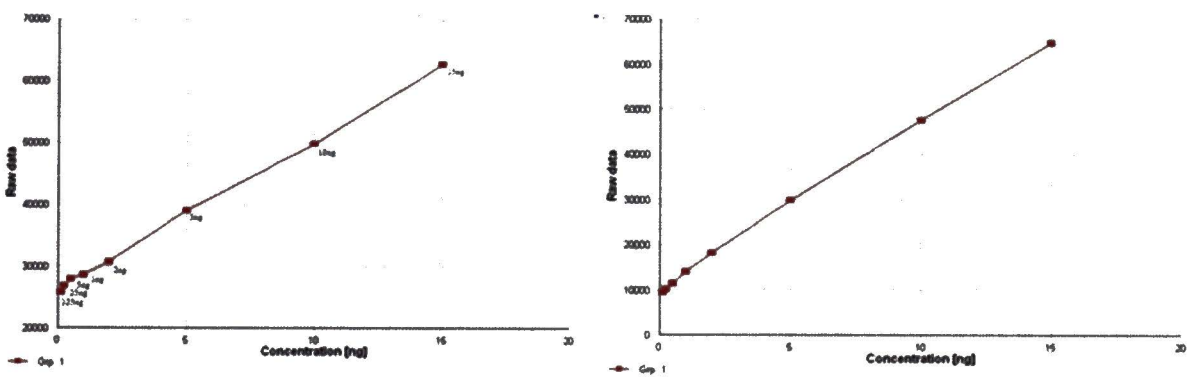
3.7 Data Analysis

For estimation of the DNA concentrations in unknowns, the relative fluorescence in the single-stranded DNA extracts was compared with the fluorescence in ssDNA from known amounts of reference DNA template using the GENios software, Magellan™. Magellan™ forms a standard curve and then calculates sample concentrations based on the fluorescent measurements of the standards. Once the GENios quantification values

were obtained for each sample, comparison of DNA quantities to relative fluorescence units (RFUs) of STR results from the sample plates could be carried out.

CHAPTER 4: RESULTS

The strategy for quantifying ssDNA in family reference sample extracts was to generate a standard curve of fluorescence with standards produce from known quantities of input template DNA. OliGreen® and the denatured standards produced a comparable standard curve to that of the double stranded DNA with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Part# P7589, Molecular Probes Inc., Eugene, OR).



OliGreen® PicoGreen®

Figure 1 – Standard Curves

The sensitivity of each assay was comparable also. The sensitivity of OliGreen® using the GENios seemed to be limited to the range of the standards, which is shown in Figure 1. The lowest concentration detected was ~0.13ng with the lowest standard being 0.125ng; any concentration below 0.125ng was quantified as <Min. The highest concentration detected was ~15ng with the highest standard being 15ng; any concentration above 15ng was quantified as >Max.

Each standard, represented in Figure 1, was amplified using 5μL of template and the Identifiler® STR typing kit on the ABI 9700 thermal cycler. After genetic analysis was performed on the ABI Prism® 3100, the STR data was analyzed. The call threshold is a relative fluorescent value that must be exceeded in order for an allele call to be made, and at 0.125ng, no alleles crossed the 100 RFU call threshold; therefore, no allele calls were made and no profile was retrieved. At 0.25ng, some allelic dropout was present at various loci throughout all dyes, but a partial profile was available. Allelic dropout was seen in just one locus in the red PET dye at 0.5ng (Figure 2a), and no allelic dropout was present from 1 to 5ng. Artifacts such as large stutter peaks and pull-up became obvious at 10 and 15ng of standard template (Figure 2b).

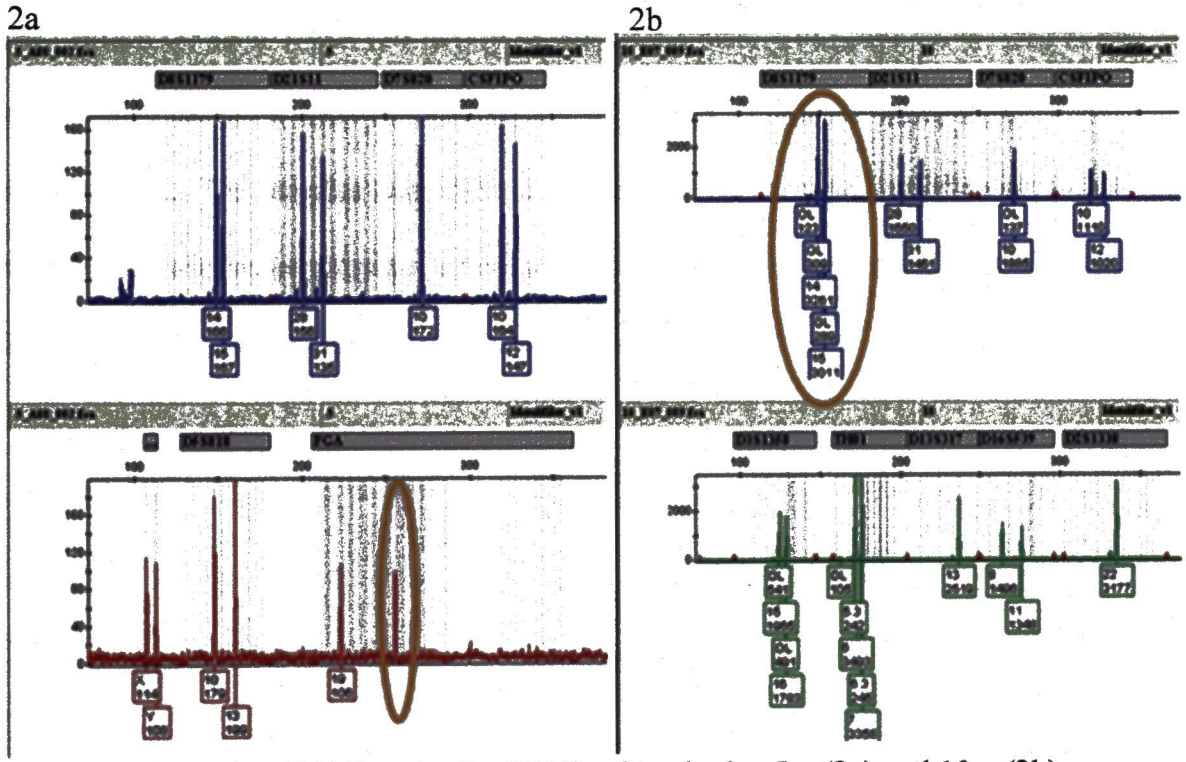


Figure 2 – STR Results for GENios Standards: .5ng(2a) and 10ng(2b)

The analysis of the standards gave a general idea of an acceptable range for DNA concentration to achieve optimal STR results, but the range was able to be further defined with GENios concentration estimates and STR results from the batches of family reference samples.

After GENios values were obtained for each of the four 96-well plates (8 batches with results shown in Appendix A), fifteen samples were randomly selected from each of the 8 batches (Batch 25, 26, 28, 29, 30, 31, 32 & 33) giving 120 samples for comparative analysis. AmpF*l*STR Identifiler® STR results from four loci of different sizes and different dyes were analyzed for each of these samples. The relative fluorescent unit (RFU) values for heterozygotes at each of these loci were graphed against the GENios (ng) values for each of the 120 family reference samples. The graphs were then color coordinated based on what the analysts did to alter the samples to make them interpretable. The samples were diluted, reextracted because the amplification failed, reamplified with more template, or not altered because there was good amplification. Figures 3a, 3b, 3c, and 3d display the results.

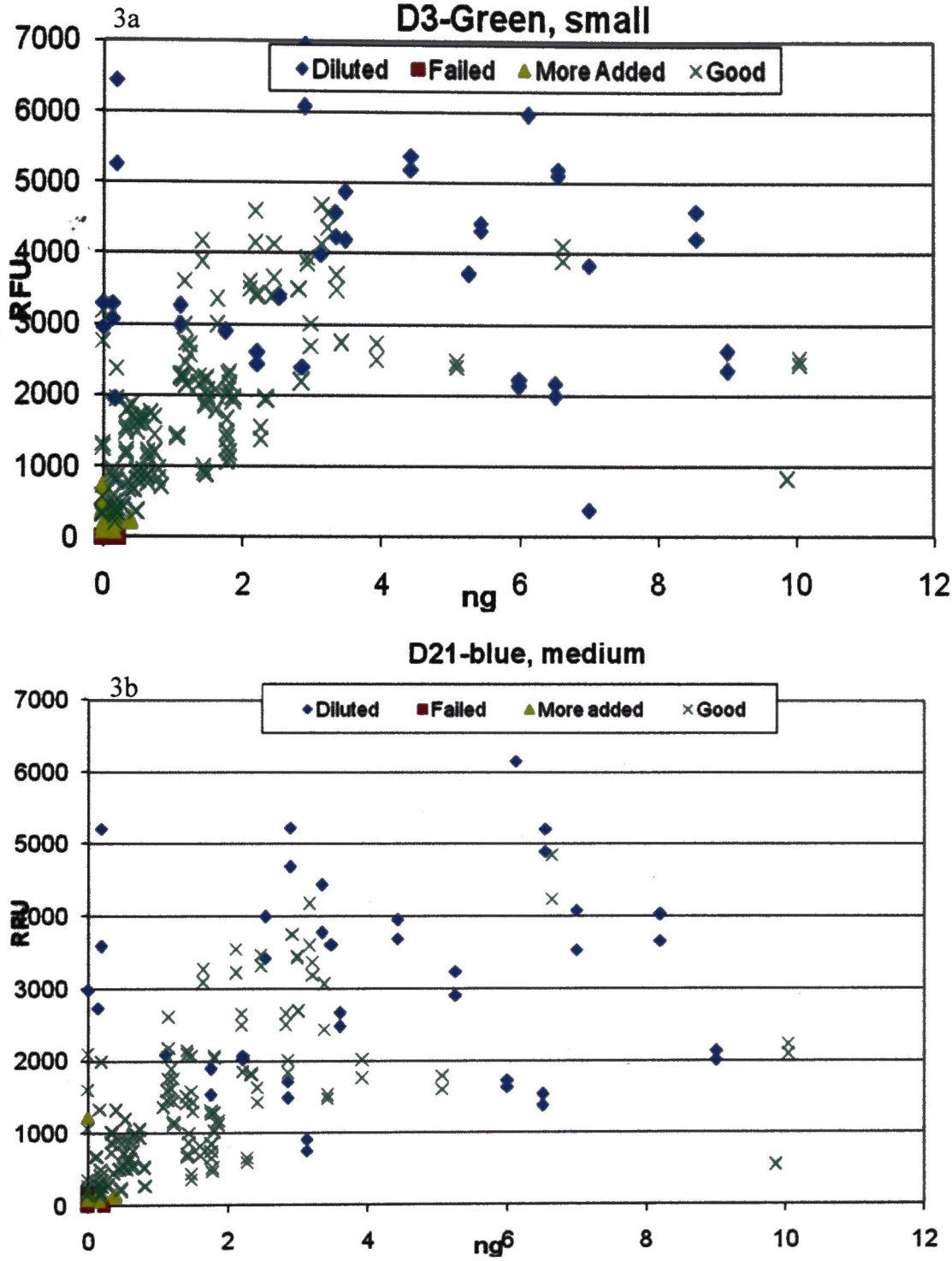


Figure 3 – Relative Fluorescence Units (RFU) vs. GENios Quant (ng) for Batch Samples

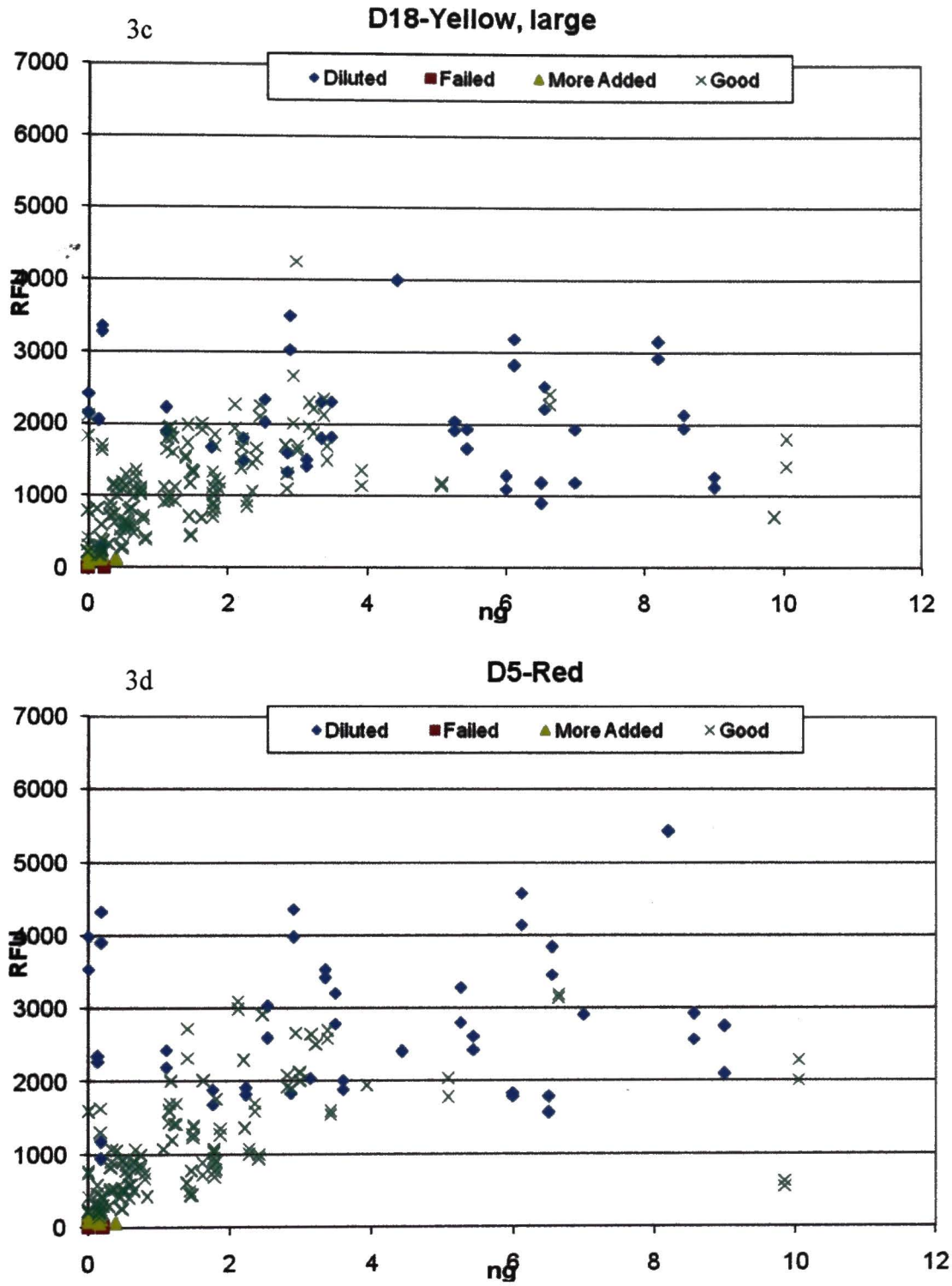


Figure 3 – Relative Fluorescence Units (RFU) vs. GENios Quant (ng) for Batch Samples

Ideally, a heterozygote peak, which represents one of two alleles at one locus, should not have a RFU over about 3000 or under approximately 500 RFU. Based on these analysis parameters, the majority of the acceptable samples fell between 0.5ng and 3ng. The following model criteria were established for the adjustment of Batch 32,33 quantities and achievement of optimal STR results: if less than 0.5ng then amplify 5μL of the extract; if greater than or equal to 0.5ng and less than 3ng then amplify 1μL extract; if greater than or equal to 3ng then dilute into ideal range with optimum being 1-1.5ng.

According to the GENios quantification values and the adjustment model, 6 samples were diluted 1 to 10, 5 samples were diluted 1 to 5, 16 samples were diluted 1 to 3, 7 samples were diluted 1 to 2, and 10 samples were amplified with 5μL of extract instead of the 1μL used with all diluted and unaltered samples (Chart shown in Appendix B). A total of 44 samples had to be normalized, and 42 samples did not have to be adjusted making a 51% revision rate.

For the Batch 32,33 plate that was corrected before AmpF*l*STR Identifiler® amplification according to the model mentioned in the previous paragraphs, the electropherograms revealed no overblown samples and no unacceptable artifacts such as excessive pull-up or stutter as can be seen in Figure 4.

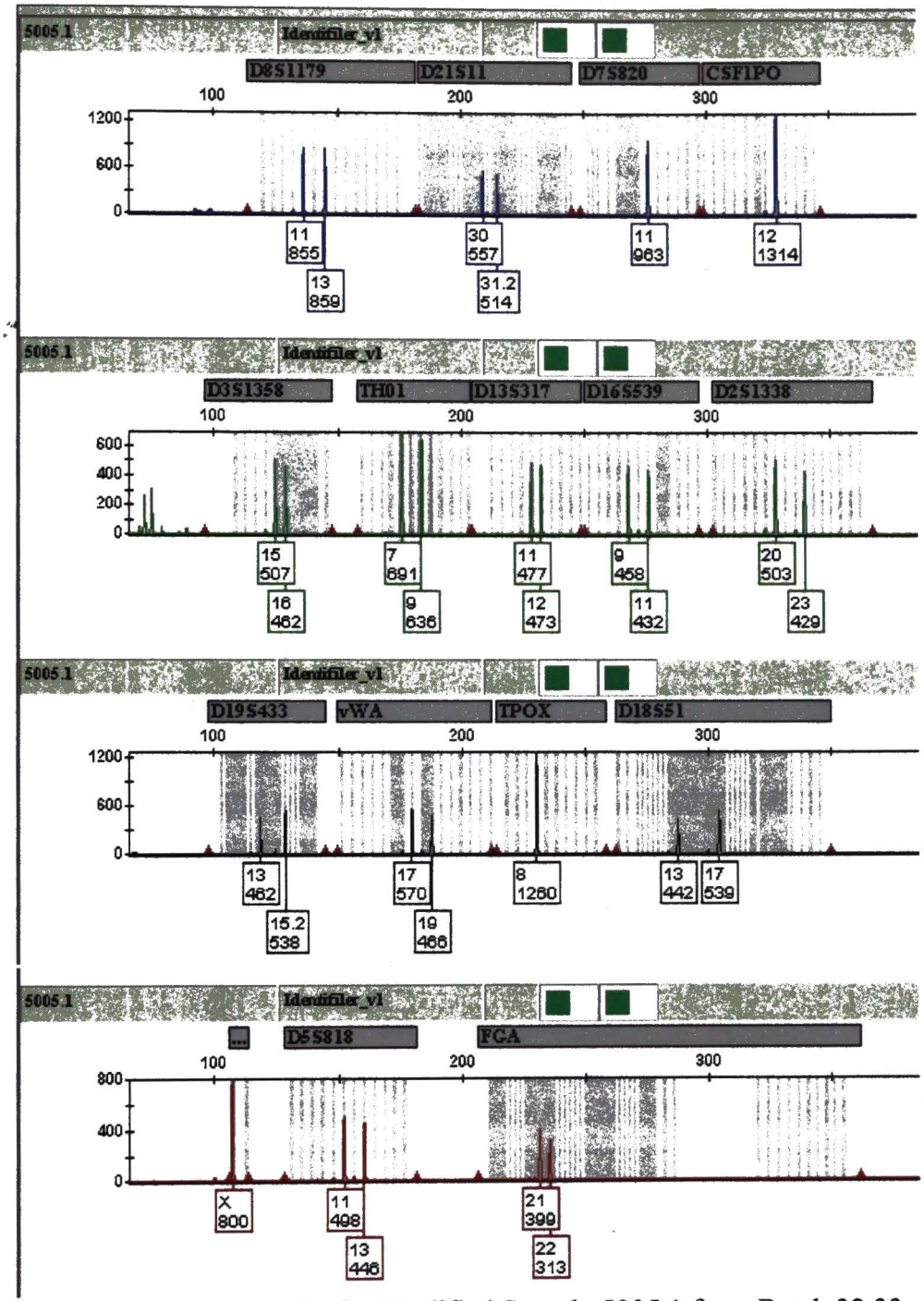


Figure 4 – STR Results for Modified Sample 5005.1 from Batch 32,33

When compared to the previous STR results for this sample, shown in Figure 5, the reduction in artifacts and peak heights is apparent.

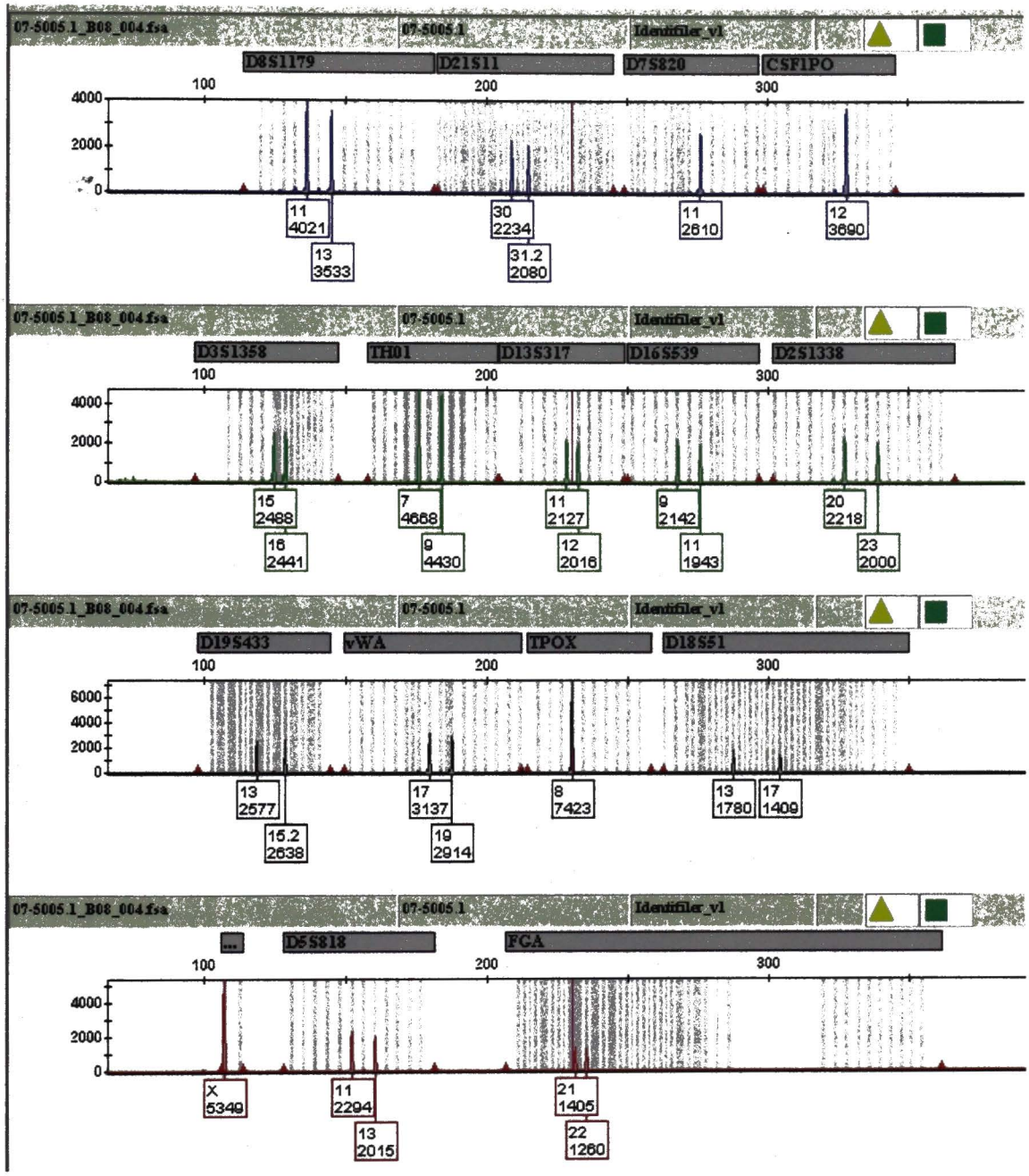


Figure 5 – Original STR Results for Sample 5005.1 from Batch 32,33

The dilution strategy appeared to be successful; however, the RFU values were under 1000 for most heterozygote samples showing that the dilutions may have been too extreme (Figure 6).

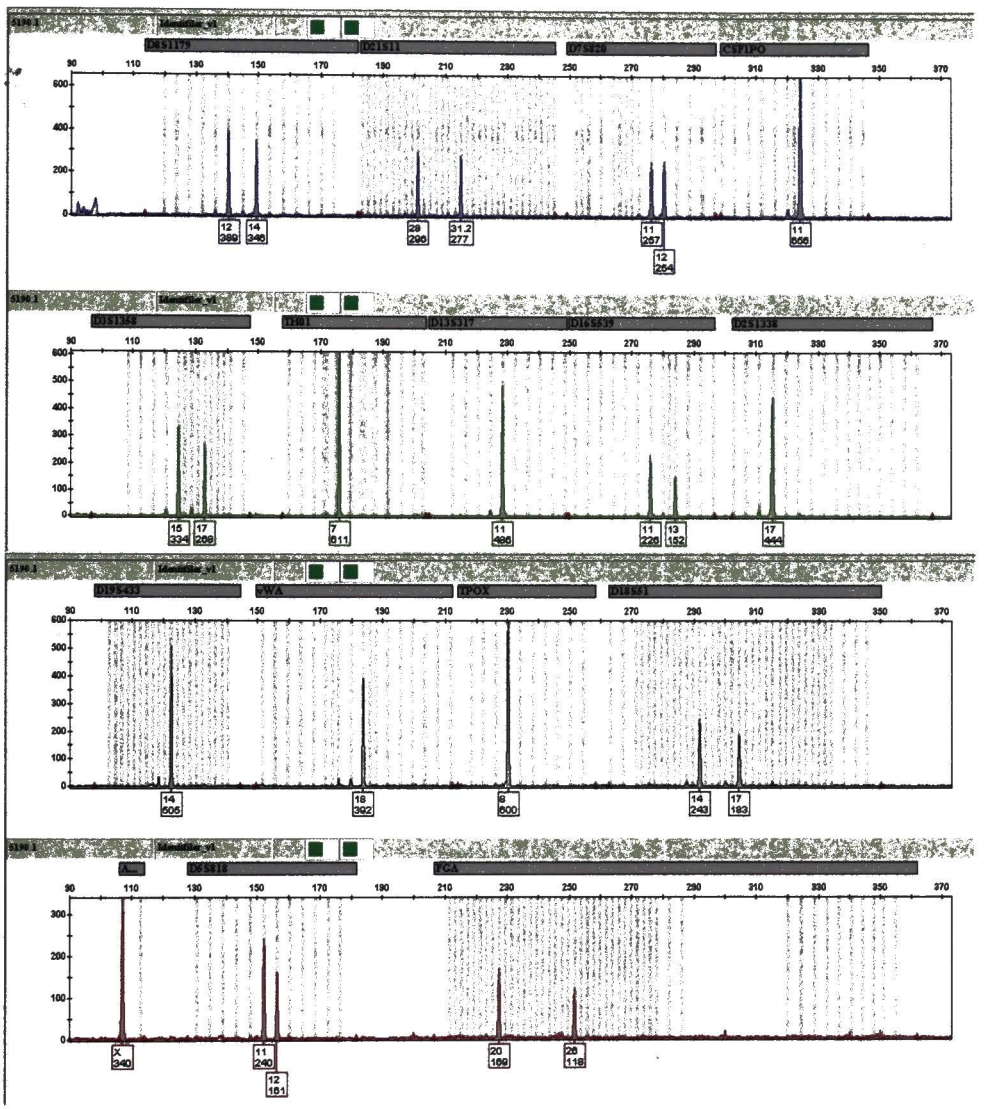


Figure 6 – STR Results for Modified Sample 5190.1 from Batch 32,33

Twelve of the 86 samples had some form of allelic dropout: either an allele was not amplified during PCR or the allele failed to be detected (Figure 7). Five of these samples had very low initial GENios quantification values and were amplified with 5μL of extract. These 5 were actually re-extracted by the analyst who originally analyzed these family reference samples and was not able to retrieve STR results. Most of the other 7 samples that had dropout were either diluted too much or not altered and should have been amplified with more template. Although, when Figure 7 and 8 are compared, the improvement in STR results for this sample is noticeable. Alteration to the optimal GENios range should fix most of the samples that had unnecessary dropout.

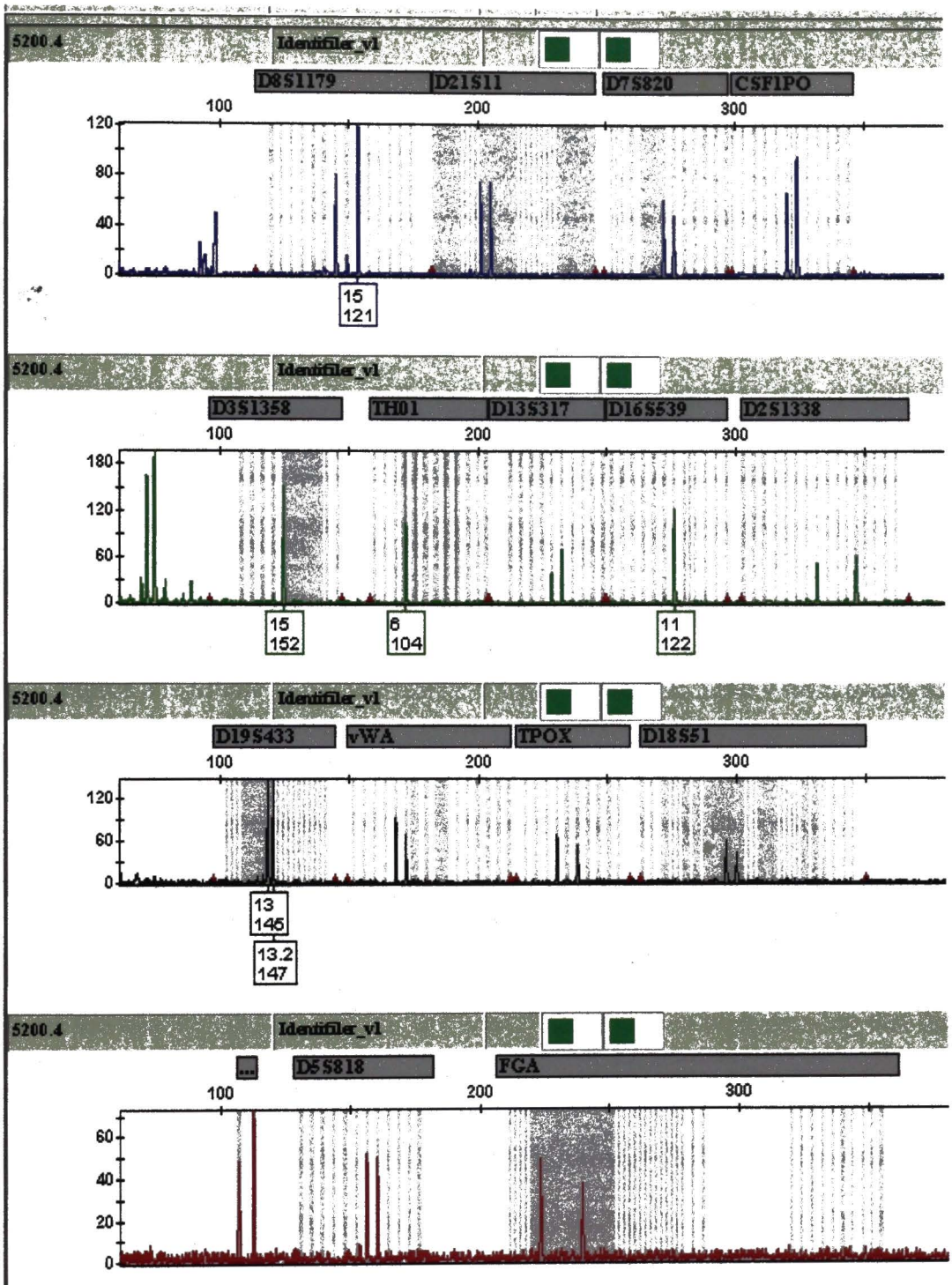


Figure 7 – Allelic Dropout Present in Modified Sample 5200.4 from Batch 32,33

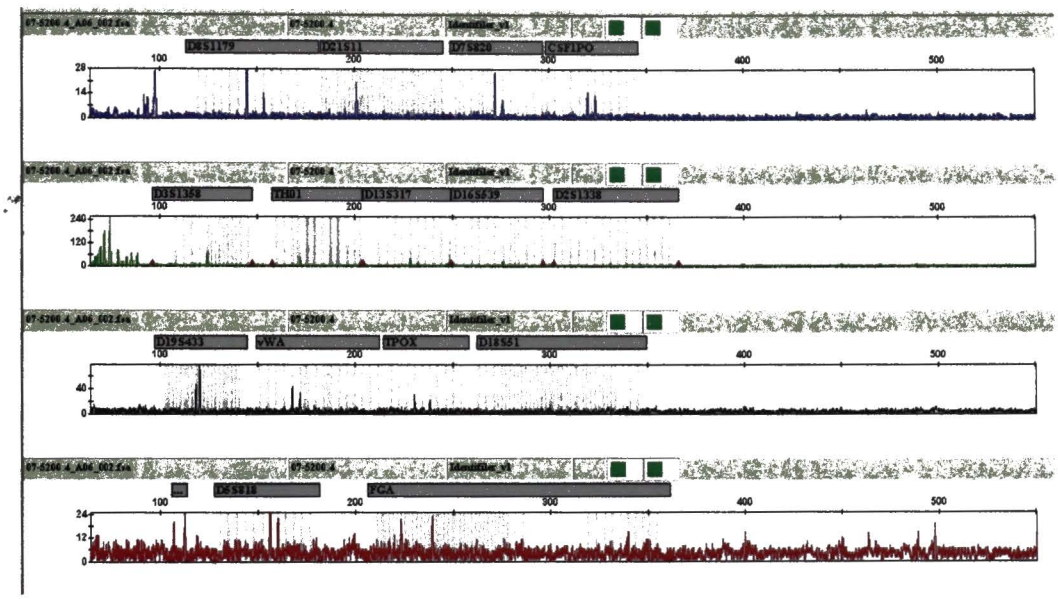


Figure 8 – Allelic Dropout Present in Original Sample 5200.4 from Batch 32,33

A table with approximate D8S1179 heterozygote values, the forms of modification, and the GENios values is located in Appendix C. The samples that had dropout are also labeled in the chart. The chart in Appendix C shows how some of the RFUs are too low for various samples, but none of them are too high showing that the dilution levels should be reconsidered. Also, a few of the samples that were not altered because of their GENios value resulted in low RFU values (<200 RFU) demonstrating their need for reassessment. In general, the optimal GENios range for ideal STR results needs to be re-evaluated and will be further mentioned in the discussion section.

The development of any new method involves comparison to existing, accepted methods. As part of the validation process, the GENios was used to quantify DNA in a set of samples that had been previously subjected to quantification with Quantifiler® during the course of their analysis because of unacceptable STR results. The comparison between DNA concentration estimates by the GENios and real-time PCR are shown as a scatter plot in Figure 9. Most samples did not show agreement in the estimates made by the two methods. Concentration estimates from the GENios and real-time PCR exhibited variation that demonstrated no obvious bias, which was unfortunate because an estimate that was consistently higher or lower with one method would allow for a correlation to be established.

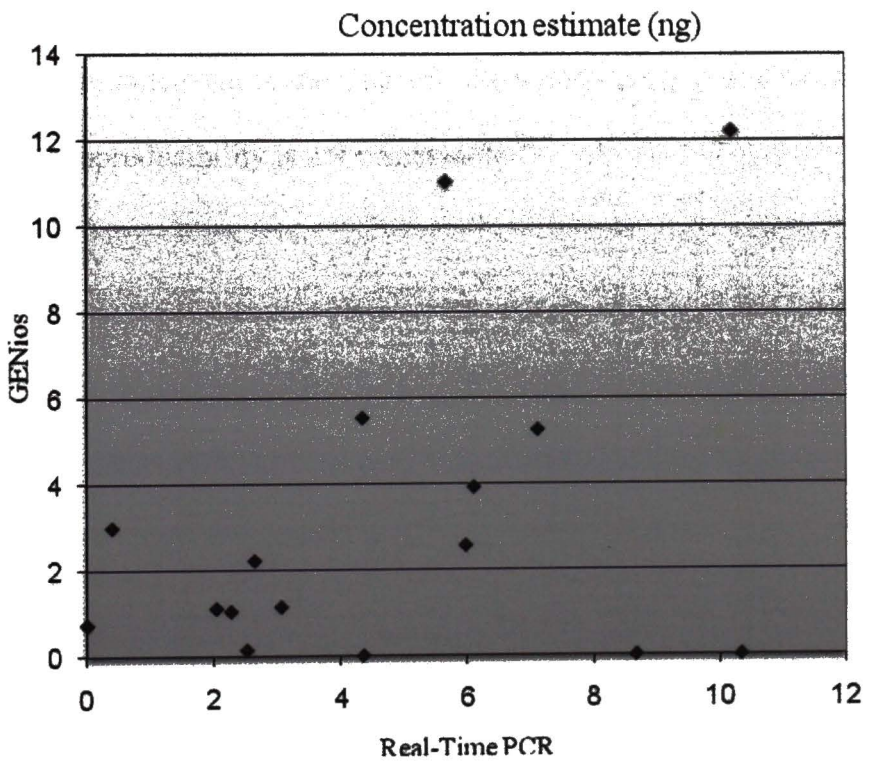


Figure 9 – GENios Quantification Values vs. Real-Time PCR Quantification Values

Typically, one might expect that very low or very high concentrations of DNA would exhibit the greatest disagreement between the two methods, but the discrepancies were seen throughout.

It is also imperative for any method used for DNA quantitation to be reproducible. The coefficient of variation calculated for thirty repeated measurements of DNA containing approximately 0.125–0.5 ng using the GENios was averaged to be around 43%. In the 1 to 2 ng range it was about 20%, and in the 5 to 15 ng range the CV was about 5% with a total average coefficient of variation around 23%. The highest percent coefficient of variation was seen in the lower concentrations such as 0.125ng and 0.25ng, and the lowest percent of variation (2%) was observed in the highest concentration of 15ng, which is expected. The overall coefficient of variation for the GENios with OliGreen® is over twice that of the Quantifiler® human assay which has an overall CV of 9.3%, so the reproducibility is not comparable.[22]

CHAPTER 5: DISCUSSION

For this project, the focus was on development of an OliGreen® assay for use with the Tecan GENios with less focus on validation due to time constraints. Since no one in the UNT System Center for Human Identification lab was familiar with the Tecan GENios or the Magellan™ software and little knowledge was available on the usefulness of OliGreen®, this project was informative; although, during its course, some limitations arose. One obstacle was obtaining a standard that was readily available to the lab in large quantities, inexpensive, and very concentrated, and a true limitation was understanding the standard curve and how the Magellan™ software interprets the standards and whether or not those methods are similar to Quantifiler®. Since the GENios had never been used in this lab, there ended up being a higher amount of variability than expected, which complicated the range definitions. Other areas of discussion are variation between quantification values of extracts, Quantifiler® values versus GENios values, Magellan™ software options, DNA IQ buffer and the switch from PicoGreen® to OliGreen®, lack of information on OliGreen®, drawbacks of OliGreen®, the MiniPrep setup, possible use of different standards, and adjustments to optimal GENios range.

Even though microplate readers have existed for a while, the Tecan GENios is a newer instrument. Very little information beyond an inadequate user's manual was available on the Tecan GENios and the Magellan™ software. Tecan customers could

benefit from manuals that are more user friendly; although, the Tecan Technical Support compensates for the manual's shortcomings.

In the beginning, the only DNA available in higher concentrations in the lab was 25µL of Promega 10ng/µL 9947A. After making a serial dilution of 8 standards including the 10ng/µL and using 2µL to achieve 20ng and running them in 6 replicates to observe the variation present, nearly all of the 25µL was consumed. With only 1 more tube available and it too expensive to constantly purchase, another form of standard DNA had to be acquired. Since large quantities of DNA can be obtained from buccal swabs, three buccal swabs were taken from an analyst, and the DNA was extracted and combined. Approximately 70ng/µL of DNA was extracted, but this DNA was not a permanent solution for standard DNA for the GENios because extraction takes time and DNA quantities will not always be consistent, so yet another type of standard DNA was needed.

For each Quantifiler® Human DNA Quantification kit consumed, approximately 100µL of the Quantifiler® Human DNA Standard is leftover. Since the Quantifiler® Human DNA Standard is approximately 200ng/µL, readily available to the lab in high quantities, and does not cost extra, it was an effective standard. A recommendation is to quantify a 1:10 dilution of the Quantifiler® Human DNA standard before making dilutions because the given concentration of 200ng/µL is not always an accurate representation of the true value. For example, after all of the GENios standards were diluted from the Quantifiler® standard, a 1:10 dilution was quantified with the ABI 7500

Real-Time PCR System and ended up quantifying at 30ng/μL, so dilutions for the standards were actually based off of a starting concentration of 300ng/μL not 200ng/μL. A 1mL stock of the 1:10 dilution from the Quantifiler® standard can be made and used for multiple GENios runs, but each time a new Quantifiler® Human DNA Standard is used, it must be quantified before being used to make GENios standards.

Within the Standard Curve dialog box under the Analysis Type tab, multiple analysis types are offered by which the standard curve will be calculated by the software. Two of the types listed are Point to Point and Linear Regression. For a fluorescent assay, generally the linear regression would be used, which would be where Magellan™ calculated the standards based on their fluorescence and the value selected and created a standard curve based on the best fit line, then would calculate the samples based on that curve. The key was to be consistent with ABI 7500 Real-Time PCR system and its software, and having Magellan™ calculate the standards was not consistent. Also, occasionally, linear regression will drop out the lower and upper standards by deeming them <Min and >Max respectively such as can be seen in C1 and B11 in Appendix D.

Point to point analysis allowed for the standards to be defined as a certain quantity like with the 7500 software, but the drawback is that the standard curve could end up distorted because the software does not correct for outliers. Although, the outliers can be manually removed by the analyst, and the concentration of the samples can be calculated based on the updated standard curve. The point to point analysis seemed to be the most relevant and accurate for this research.

The coefficient of variation of the OliGreen® assay with the GENios using replicates of the standards averaged to be approximately 23%, which is less than Quantiblot® (~35%) , but more than Quantifiler® (~10%). The most variation was present after the Batch 32,33 plate had been run for a second time to help narrow the optimal range. The GENios quantification values for each of the runs, which were both set up with the MiniPrep robot, are shown in Table 1 below. The shaded boxes are the only values that are identical between the two runs, and the darker lines outline the standards. The values from the second run are consistently higher than the values from the first run, but they are not uniformly higher. For example, one well has a quantification value of 1.798 on the first run and 2.231 on the second run, and then another well has 1.881 for the first and 5.101 for the second. The values from the first run are very close, but the quantities from the second run have a 2-fold difference. In the bottom right corner of Table 1, opposite quantification values are seen: <Min and >Max.

6/14/2007											
RB	0.699	0.500	2.328	0.493	<Min	0.850	5.101	3.052	3.681	3.476	3.498
Neg	0.236	1.480	0.657	1.425	<Min	5.337	>Max	3.186	1.146	15.000	3.441
0.125	0.397	1.825	9.252	1.566	0.926	7.722	3.356	5.610	1.597	1.109	0.874
0.298	0.659	<Min	13.800	1.344	0.834	1.353	10.803	0.996	0.813	3.280	3.898
0.723	1.201	1.024	0.740	1.000	0.763	5.000	1.993	0.943	1.940	3.089	3.874
0.480	0.494	2.742	0.490	1.547	0.939	2.409	2.966	EMPTY	3.547	2.202	1.162
0.588	0.663	<Min	2.385	2.231	2.000	11.715	1.725	3.177	1.507	2.798	5.058
0.841	Std 2-.25	0.192	<Min	0.298	1.267	3.764	1.563	10.000	3.061	1.610	>Max
5/30/2007											
RB	0.136	0.500	1.549	0.169	<Min	0.158	1.881	1.155	1.974	2.067	1.929
Neg	<Min	0.499	0.158	0.461	<Min	3.423	10.039	1.861	0.404	15.000	1.923
0.125	<Min	0.976	5.080	0.534	0.211	3.934	1.782	2.801	0.683	0.228	0.183
<Min	0.168	<Min	8.997	0.842	0.243	0.507	5.996	0.409	0.165	2.141	2.488
<Min	0.446	0.229	0.188	1.000	0.202	5.000	<Min	<Min	1.448	1.777	2.738
0.144	0.200	1.603	0.169	0.758	0.290	1.484	1.781	EMPTY	1.859	0.709	0.381
0.172	0.242	<Min	1.706	1.798	2.000	6.510	0.803	2.230	0.834	1.777	2.254
0.169	0.250	<Min	<Min	<Min	0.366	2.271	0.644	10.000	2.049	1.129	<Min

Table 1 – Comparison of GENios Quantification Values for Batch 32,33

This high level of discrepancy needs to be considered when deciding whether or not to use the GENios to quantify family reference samples.

qPCR-ng	GENios-ng
0.0184	0.73
0.404	2.99
2.04	1.11
2.27	1.04
2.52	0.14
2.64	2.22
3.06	1.14
4.34	5.54
4.36	<Min
5.66	11.04
5.97	2.56
6.1	3.92
7.11	5.26
8.67	<Min
10.18	12.21
10.33	<Min

Table 2 – Real-Time PCR Quantification Values and GENios Quantification Values

Comparison of GENios quantification values and real-time PCR quantifications values as shown in Figure 9 and again in Table 2 demonstrate no direct correlation. This lack of correlation not only prevents prediction of one value based on the other, but more importantly, this lack of relationship questions the accuracy of both quantifications methods because the quantities if not similar should at least follow a comparable trend. The levels of variation seen with OliGreen® and the GENios in this project provide evidence against the reliability of the GENios quantification, and the published Quantifiler® results offer verification for the consistency of the qPCR quantification.[22]

The Magellan™ software, provided with the Tecan GENios, can lead to the successful quantification of samples; however, not properly comprehending the options that must be selected for each type of assay could lead to failure, which is why the best options for this OliGreen® assay are discussed. First, the Tecan GENios has the ability to read the plate from the top or the bottom, so with the black opaque plates used in this experiment, it is essential that the plate be read from the top. No quantification values will be given if the plate is read from the bottom unless transparent plates are being used. Also, the proper plate definition file must be uploaded, which in this assay is GRE96fb.pdf to represent Greiner 96-well flat bottom black plates.

Since the GENios is capable of measuring fluorescence, absorbance, and luminescence, the fluorescence detector must be selected and the proper excitation and emission filters (485nm and 535nm) must be used. The resulting units of measurement are relative fluorescence units (RFU). Blank reduction is another option with the Magellan™ software. The amount of fluorescence coming from the blank or negative control is measured and that RFU value is considered background signal and is subtracted from the measurement of the standards to allow for more accurate calculations of the quantification values.

The most important measurement parameter that has to be defined is the gain, which is the amount of charge applied. The most commonly used detectors for fluorescence come in many varieties with different sensitivities in the wavelength ranges they cover. For instance, PicoGreen® had an optimal gain of 122, but when a gain of 122 was applied to an OliGreen® plate, the RFU values were very low which can be seen in Appendix E. The optimal gain for OliGreen® was determined to be 135. As the gain is increased, the signal will increase, but the relationship is exponential, so a small increase in the gain leads to a large increase in the signal.[16]

With Magellan™, there are multiple gain modes including manual, optimal, and calculated from well. Manual gain allows the gain to be chosen, which is useful when comparing raw data from different runs. Optimal gain is based on the sample with the highest signal on the plate, which becomes the maximal range of the instrument. When

the gain is calculated from a well, the instrument determines the optimal gain based upon that particular well.[16]

To determine the best gain for the OliGreen® assay or any assay, it is suggested to begin by selecting optimal gain. The well with the highest signal will have the largest RFU value with the ideal maximum RFU value being around 50,000. After 65,000 RFU, the instrument becomes oversaturated at that well, and the software considers that sample to be overflowed. When saturation occurs, the gain should be lowered until the most concentrated sample has approximately a 50,000 RFU value. After an optimal gain is achieved with no overflowing samples and a peak RFU value of 50,000, then that gain can be manually entered for the following runs. For the OliGreen® assay, a manual gain of 135 is used because that was determined to be optimal.

The majority of this project was conducted under the notion that the DNA extracted with DNA IQ™ was double stranded; therefore, the assay was originally designed with PicoGreen®, a dsDNA fluorescent reagent. The standards were developed, the GENios was tested, and Magellan™ options were set with PicoGreen®. Once the preliminary data was obtained and the standards were established, the first plate of DNA IQ™ extracts was tested with PicoGreen®, and although the standard curve was acceptable, no quantification values were given for the family reference samples as can be seen in Appendix A. The lysate from the most recent plate was reextracted with the EVO® 100 and DNA IQ™ to try quantifying a fresh plate and the quantification values were still low. After determining that the time since extracted and possible degradation due to the

DNA IQ™ resin or the 96-well plates were not factors in the lack of quantification results, the condition of the DNA IQ™ extracts was considered.

OliGreen®, the closely related reagent to PicoGreen®, was investigated because of its ability to bind single stranded DNA. Preliminary tests with OliGreen®, the GENios, and the DNA IQ™ extracts were successful, so the DNA IQ™ process was examined. The DNA IQ™ purification process involves a heating step with a Lysis Buffer, and the DNA extract is stored in Elution Buffer. Highly basic solutions can denature DNA[23], so the basicity of these buffers as well as the denaturing capabilities of the heating step was researched. The research revealed that the Elution Buffer used to store the DNA is the basic solution that is causing the DNA extracts to be single stranded. From there, the standards had to be made single stranded and the assay had to be reevaluated with OliGreen®.

Since OliGreen® is a relatively new dye compared to PicoGreen®, it is not very well characterized, especially for use in conjunction with the GENios. Published OliGreen® information is limited with most of the available data coming from the manufacturer, Molecular Probes.[5] There are disadvantages to using OliGreen®. The OliGreen® reagent exhibits fluorescence enhancement when bound to double-stranded DNA and RNA.[13] Also, the reagent has significant base selectivity showing large fluorescence enhancement when bound to poly dT sequences.[13] In addition to the enhanced fluorescence, OliGreen® is limited in its ability to differentiate intact DNA from partially

degraded strands of DNA.[14] Essentially, OliGreen® should only be used as a quick method to get a vague estimate of the quantity of DNA.

The Tecan MiniPrep was used for GENios plate setup. When the GENios script was originally written for the Tecan MiniPrep, there were only water wash steps, but more steps to prevent possible contamination of the extract or cross contamination between columns on the GENios plate had to be taken. A 10 second wash with 6% bleach was incorporated after each template addition in addition to a water wash before and after each bleach wash. The percent of bleach and length of wash was obtained from the Tecan EVO® 100 script for the DNA IQ™ extraction.

Since the standards developed for the GENios during this project were created with PicoGreen® as the fluorescent reagent, there is some doubt as to whether or not they are as effective with OliGreen®. The standard curve is comparable and the quantification values are similar, but the data appeared to be cleaner with PicoGreen®. The Quant-iT™ OliGreen® ssDNA Assay Kit includes 1mL of an oligonucleotide standard at a concentration of 100μg/mL or 100ng/μL. This standard was used by the manufacturer to acquire the available data on OliGreen®; therefore, it might be beneficial to try using the oligonucleotide standards with OliGreen® and the GENios to see if the results improve. The switch from PicoGreen® to OliGreen® seemed to cause some instability in the assay, so use of the provided standard could offer some consistency since the lab has no steady source of single stranded DNA readily available. Also, the current standards may

not stay denatured beyond 3 days[19], so in order to insure the stability of the GENios standards, DNA that is known to be single stranded like oligonucleotides should be used.

As mentioned previously in the results section, the method of normalization was based on the following terms: if less than 0.5ng then amplify 5μL of the extract; if greater than or equal to 0.5ng and less than 3ng then amplify 1μL extract; if greater than or equal to 3ng then dilute into ideal range with optimum being 1-1.5ng. The dilution scheme further broken down was: if greater than 2ng and less than 3ng then dilute 1 to 2; if greater than or equal to 3ng and less than 5ng then dilute 1 to 3; if greater than or equal to 5ng and less than 9ng then dilute 1 to 5; and if greater than or equal to 9ng then dilute 1 to 10. There were no overblown STR results with these ranges, which is a significant improvement compared to the previous genetic data from the family reference samples. All but 10 of the samples, which had dropout, produced acceptable genetics profiles, but the substantial number of RFU values between 100 and 500 with diluted and non-altered samples was an issue of concern. A simple solution is to adjust the optimal range and the dilution scheme to get superior STR results.

Based on the chart in Appendix C, which show RFU values relative to the GENios values and the level of alteration, if the GENios value is less than 0.4ng, then amplify 5μL of the extract, if it is greater than or equal to 0.4ng and less than 0.85ng then amplify 2μL, if greater than or equal to 0.85ng and less than 3ng then do not alter sample and amplify 1μL of extract, if greater than or equal to 3ng then dilute into ideal range with the optimum quantity being 2ng. A suggested dilution scheme would be if the GENios value

is greater than or equal to 3ng and less than 5ng then dilute 1 to 2, if the quantity is greater than or equal to 5ng and less than 8ng then dilute 1 to 3, if the value is greater than or equal to 8ng and less than 10ng then dilute 1 to 5, and if the quant is greater than or equal to 10ng then dilute 1 to 10.

CHAPTER 6: CONCLUSION

Each reagent, method, and instrument proved useful in this project. The Quantifiler® Human DNA Standard became an acceptable standard due to its availability, cost, and concentration. Magellan™ demonstrated its versatility as well as its powerful calculation and data reduction capabilities. The Tecan MiniPrep proved to be quick and accurate at setting up the GENios plate, which supports higher throughput.

Although this assay was originally designed for PicoGreen®, OliGreen® proved to be not only effective but necessary due to the single stranded nature of the DNA extracted with DNA IQ™. OliGreen® is only as useful as the other contributing factor, which is the GENios. Even though OliGreen® and the GENios do have nearly twice the coefficient of variation of Quantifiler®, it still has less variation than other quantification methods like Quantiblot® demonstrating its ability to effectively estimate DNA quantity. Quantifiler® and the ABI 7500 Real-Time PCR System each have their own levels of variation and are not flawless in their quantification, so even though there is no correlation between the GENios quantification values and the real-time PCR values, both systems still function as methods of DNA quantification.

The purpose of this project was to normalize the family reference samples' DNA extracts to allow for improved STR results. With only 12 samples having to be modified after genetic analysis of normalized Batch 32,33, there was almost a 3-fold decrease in

the number of samples that had to be adjusted following STR analysis. These results show that normalization of DNA concentrations based on GENios values can save time and money and allow for higher throughput of family reference samples. The Tecan GENios is not an exact quantification of the DNA, but it allows for an adequate estimate.

The method of normalization is not perfect yet, which was seen in the lack of normalization before STR analysis for some of the 12 unsuccessful samples in Batch 32,33, but it does work. Also, there is still some considerable variation between the Batch 32,33 runs on different dates. Having to aliquot out GENios standards and denature them before each run could be causing this date to date variability, so a single stranded set of standards could be beneficial.

The noticeable decrease in overblown samples shows that there was detection and correction of a well established variation in concentration of DNA IQ™ extracts through fluorescent quantification. For the purpose of making the analysis process of family reference samples less expensive and less time consuming, the normalization model adjusted the DNA concentration into an ideal input range, which allowed for higher throughput by preventing reanalysis of the family reference samples. The GENios and the normalization method should be used with caution, but do allow for improved STR results of family reference samples.

CHAPTER 7: SUGGESTIONS FOR FUTURE RESEARCH

Suggestions for future research include improvements on the GENios/OliGreen® assay for normalization of family reference sample DNA extracts as well as other potential uses for the Tecan MiniPrep and GENios. For the GENios/OliGreen® assay, the most room for improvement lies within the standards and the optimal GENios quantification range. Because of the difficulty and possibly inconsistency of using the double stranded Quantifiler® Human DNA Standard for a single stranded assay, it might be practical to switch to a single stranded standard such as the oligonucleotide standard provided in the OliGreen® kit. The lack of uniformity between the extracted family reference sample DNA and the oligonucleotide standard might be an issue, but the type of single stranded DNA shouldn't matter as long as it is quantifiable. Using a different standard to quantify is simply a suggestion, but in order to optimize the assay, it is necessary to adjust the optimal GENios quantification range. Suggested ranges for dilutions and addition of extra template as well as an ideal range and quantity to aim for are mentioned in the last paragraph of the discussion section.

When the normalization model is being utilized to correct the samples' quantities, a lot of hands on time is required to make the alterations necessary to the DNA IQ™ extracts. If the Tecan MiniPrep could be used to add a consistent amount of DNA extract

to a dilution plate and then be used to add varying amounts of water to make the correct dilutions, then the hands on time would be minimized.

Since the OliGreen® assay was the first research and utilization of the Tecan GENios by the UNT System Center for Human Identification, other possible uses should be explored. Tecan describes the GENios as a flexible instrument that is suitable for use in the quantification of nucleic acids such as PCR products, cDNA, and oligonucleotides.[24] Although the UNTSCHI lab rarely encounters cDNA or oligonucleotides, PCR products are generated on a regular basis. The ability to quantify mitochondrial DNA PCR products in order to eliminate quantification by an agarose gel or the Agilent Bioanalyzer would increase throughput for analyzing mitochondrial DNA. Detection of PCR products with the GENios plate reader has been published, but information on the use of the GENios to quantify mitochondrial DNA is limited.[25] Because of the time and money a mtDNA PCR product quantification assay would save, it would definitely be worth exploring.

APPENDICES

APPENDIX A

GENIOS QUANTIFICATION VALUES FOR BATCH FAMILY REFERENCE SAMPLES

5/30/2007 OliGreen®

25,26	1	2	3	4	5	6	7	8	9	10	11	12
A	RB	6.997	0.500	2.410	<Min	1.464	6.636	1.458	7.497	0.156	3.279	5.497
B	Neg	2.534	3.341	5.801	0.341	6.182	2.926	2.958	4.292	2.967	15.000	2.163
C	0.125	<Min	2.105	3.319	0.915	0.155	1.145	3.301	11.044	1.030	2.372	1.624
D	2.890	0.329	6.548	0.877	5.103	1.045	0.198	1.104	2.129	6.990	2.664	0.163
E	1.814	2.467	0.213	2.147	1.000	2.698	5.000	5.634	12.211	1.076	3.407	0.389
F	0.400	0.137	2.657	0.946	0.338	<Min	2.999	1.767	3.707	0.195	7.053	2.417
G	4.429	0.334	0.252	0.724	0.623	2.000	2.193	1.190	0.157	3.058	7.209	2.207
H	0.145	0.250	0.719	2.071	1.023	5.058	1.780	0.295	10.000	3.628	4.849	1.809

28,29	1	2	3	4	5	6	7	8	9	10	11	12
A	RB	0.188	0.500	5.319	8.555	3.822	0.618	1.638	0.308	3.801	0.633	2.640
B	Neg	<Min	4.209	1.579	1.709	2.685	1.143	2.993	4.445	1.463	15.000	2.986
C	0.125	0.578	1.018	0.792	0.312	0.170	5.444	3.224	<Min	2.486	1.373	3.610
D	2.822	<Min	5.542	0.609	1.163	1.616	3.157	1.492	0.750	2.506	3.634	3.938
E	3.599	0.509	1.647	0.286	1.000	1.416	5.000	6.125	3.576	1.923	1.355	1.639
F	1.393	3.483	1.130	2.354	0.506	<Min	1.192	0.526	3.103	2.801	3.619	5.742
G	1.436	2.503	5.497	0.517	3.921	2.000	3.368	0.144	2.121	1.749	0.515	1.389
H	0.176	0.250	<Min	1.764	0.195	0.217	0.449	0.193	10.000	1.801	0.967	1.575

30,31	1	2	3	4	5	6	7	8	9	10	11	12
A	RB	1.421	0.500	0.772	0.596	<Min	0.242	0.486	0.459	2.555	0.763	0.777
B	Neg	0.736	2.936	1.999	0.957	0.836	0.166	0.597	0.700	2.590	15.000	5.604
C	0.125	0.680	0.492	0.607	1.946	0.701	2.355	<Min	0.555	1.139	1.319	0.743
D	<Min	1.803	0.232	0.244	4.992	0.135	<Min	<Min	0.157	2.860	1.006	0.640
E	0.130	1.173	<Min	0.886	1.000	0.497	5.000	0.681	0.724	0.479	2.192	5.255
F	0.558	1.255	<Min	0.734	0.571	0.831	9.864	0.954	0.673	2.557	0.831	2.220
G	1.498	2.861	0.165	8.200	1.002	2.000	1.112	0.602	<Min	2.649	1.044	1.215
H	3.132	0.250	0.205	0.352	1.966	5.130	<Min	0.377	10.000	1.157	0.490	0.209

32,33	1	2	3	4	5	6	7	8	9	10	11	12
A	RB	0.136	0.500	1.549	0.169	<Min	0.158	1.881	1.155	1.974	2.067	1.929
B	Neg	<Min	0.499	0.158	0.461	<Min	3.423	10.039	1.861	0.404	15.000	1.923
C	0.125	<Min	0.976	5.080	0.534	0.211	3.934	1.782	2.801	0.683	0.228	0.183
D	<Min	0.168	#DIV/0!	8.997	0.842	0.243	0.507	5.996	0.409	0.165	2.141	2.488
E	<Min	0.446	0.229	0.188	1.000	0.202	5.000	<Min	<Min	1.448	1.777	2.738
F	0.144	0.200	1.603	0.169	0.758	0.290	1.484	1.781	<Min	1.859	0.709	0.381
G	0.172	0.242	<Min	1.706	1.798	2.000	6.510	0.803	2.230	0.834	1.777	2.254
H	0.169	0.250	<Min	<Min	<Min	0.366	2.271	0.644	10.000	2.049	1.129	>Max

5/14/2007 Batch 32,33-PicoGreen®

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	<Min	0.129	0.500	0.210	<Min	<Min	<Min	0.266	0.185	0.257	0.278	0.229
B	<Min	<Min	<Min	<Min	0.127	<Min	0.320	1.247	0.201	<Min	15.000	0.160
C	0.125	<Min	0.163	0.652	0.136	<Min	0.431	0.198	0.252	0.128	<Min	<Min
D	<Min	<Min	<Min	1.117	0.188	<Min	0.132	0.784	<Min	<Min	0.197	0.205
E	<Min	<Min	<Min	<Min	1.000	<Min	5.000	<Min	<Min	0.177	0.184	0.247
F	<Min	<Min	0.245	<Min	0.133	<Min	0.204	0.201	<Min	0.207	0.155	<Min
G	<Min	<Min	<Min	0.229	0.211	2.000	0.748	0.127	0.217	0.132	0.183	0.216
H	<Min	0.250	<Min	<Min	<Min	0.136	0.240	0.144	10.000	0.162	0.150	2.297

APPENDIX B

GENIOS RESULTS FOR BATCH 32,33 FAMILY REFERENCE SAMPLES

Alterations to Batch 32,33 Family Reference Samples Based on GENios Quantification Values and the Method of Normalization

Batch 32,33 NG

	1	2	3	4	5	6	
A	RB	0.699	0.500	2.328	0.493	<Min	
B	Neg	0.236	1.480	0.657	1.425	<Min	
C	0.125	0.397	1.825	9.252	1.566	0.926	
D	0.298	0.659	<Min	13.800	1.344	0.834	
E	0.723	1.201	1.024	0.740	1.000	0.763	
F	0.480	0.494	2.742	0.490	1.547	0.939	
G	0.588	0.663	<Min	2.385	2.231	2.000	Dilute 1:10
H	0.841	Std 2-.25	0.192	<Min	0.298	1.267	Dilute 1:5
							Dilute 1:3
	7	8	9	10	11	12	Dilute 1:2
A	0.850	5.101	3.052	3.681	3.476	3.498	Amp 5ul
B	5.337	>Max	3.186	1.146	15.000	3.441	Standards
C	7.722	3.356	5.610	1.597	1.109	0.874	Ignore
D	1.353	10.803	0.996	0.813	3.280	3.898	
E	5.000	1.993	0.943	1.940	3.089	3.874	
F	2.409	2.966	EMPTY	3.547	2.202	1.162	
G	11.715	1.725	3.177	1.507	2.798	5.058	
H	3.764	1.563	10.000	3.061	1.610	>Max	

Relative Fluorescent Units According to GENios

Batch 32,33 RFU

<>	1	2	3	4	5	6	7	8	9	10	11	12
A	19103	20060	19570	22901	19564	18572	20228	26513	23976	24588	24577	24258
B	18808	19146	21626	19844	21316	19043	27064	45285	24102	20876	44086	24367
C	19113	19243	22189	32011	21697	20307	30072	24183	27124	21668	20870	20480
D	19324	19752	18665	40735	21142	20147	21200	34743	20694	20101	24133	25000
E	20056	21025	20510	19913	20307	20048	26436	22488	20434	22419	23990	25038
F	19548	19656	23420	19463	21483	20304	23076	23699	18942	24586	22505	20762
G	19706	19904	18479	23186	22960	22189	36769	21995	23905	21517	23425	26623
H	20250	19731	18898	18504	19011	20871	24770	21552	32874	23874	21734	64402

APPENDIX C

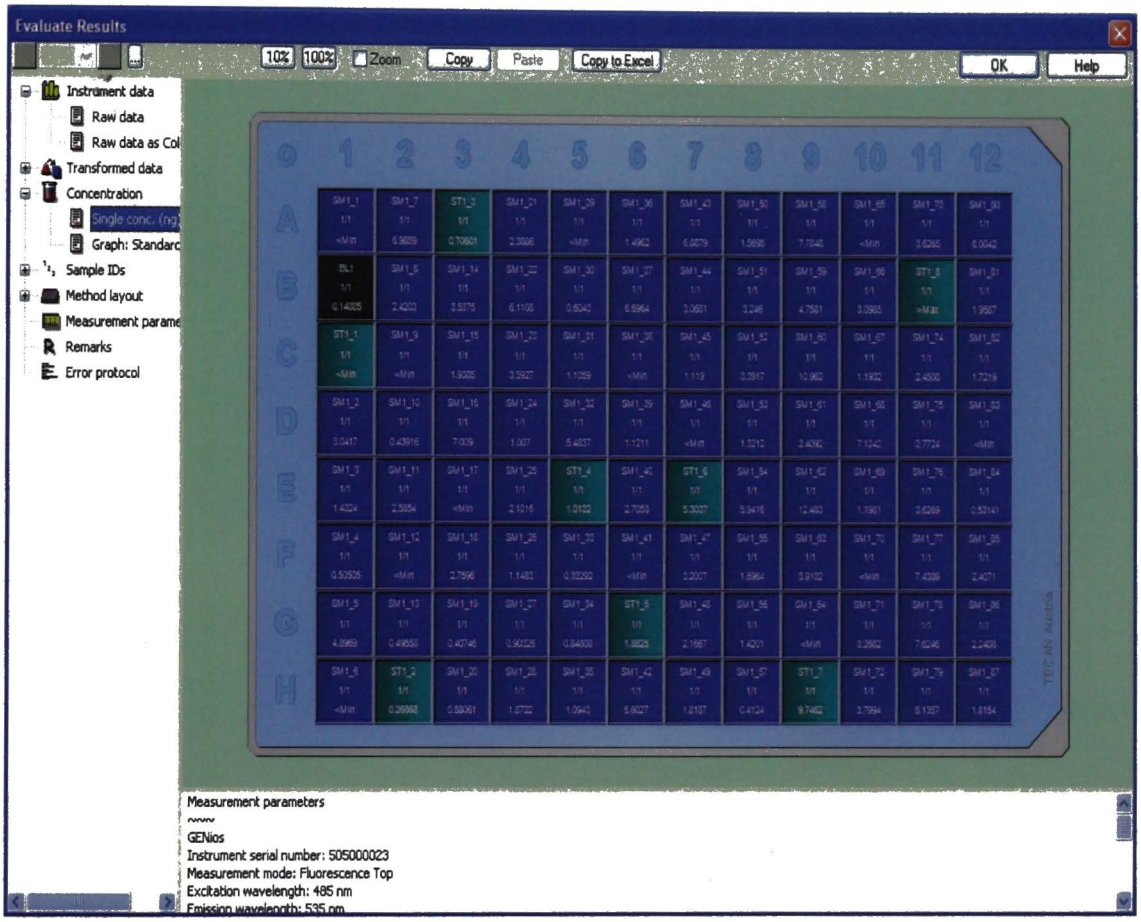
TABLE OF BATCH 32,33 AVERAGE RFU VALUES, MODIFICATIONS, AND GENIOS VALUES

Sample Name	RFU	Modification	ng/uL	
5212.1_H03_015.fsa	128	Amp 5ul	0.192	Mostly dropout
5197.1_B02_004.fsa	1100	Amp 5ul	0.236	
5189.1_D01_007.fsa	600	Amp 5ul	0.298	
5201.1_H05_015.fsa	800	Amp 5ul	0.298	
5198.1_C02_006.fsa	1400	Amp 5ul	0.397	
5211.1_G03_013.fsa	450	Amp 5ul	<min	
5200.3_B06_004.fsa	149	Amp 5ul	<min	Dropout all but 1
5200.4_A06_002.fsa	132	Amp 5ul	<min	Dropout half
5208.1_D03	0	Amp 5ul	<min	Nothing-too low
5200.3CC_H4	0	Amp 5ul	<min	Nothing-too low
4990.1_C04_006.fsa	800	Diluted 1:10	9.25	
5013.2_D08_008.fsa	405	diluted 1:10	10.8	
4989.1_G07_013.fsa	1050	diluted 1:10	11.72	
4993.1_D04_008.fsa	875	Diluted 1:10	13.8	
5005.1_B08_004.fsa	857	diluted 1:10	>max	
06-4048.2_H12_016.fsa	480	diluted 1:10	>max	
06-4341.1_G12_014.fsa	600	diluted 1:2	2.2	
5268.2_F11_011.fsa	700	diluted 1:2	2.23	
5203.2_G05_013.fsa	1000	diluted 1:2	2.32	
5253.4_A04_002.fsa	325	diluted 1:2	2.39	**-Should not have diluted**
5209.1CC_G04_014.fsa	565	diluted 1:2	2.41	
4982.1_F07_011.fsa	300	diluted 1:2	2.74	
5210.1_F03_011.fsa	1000	diluted 1:2	2.8	
5213.3_G11_013.fsa	700	diluted 1:3	2.96	
5247.1_F08_012.fsa	400	diluted 1:3	3.05	
5270.1_A09_001.fsa	420	diluted 1:3	3.06	
5285.1_H10_016.fsa	650	diluted 1:3	3.09	
5268.3_E11_009.fsa	815	diluted 1:3	3.18	
06-4341.3CC_G09_013.fsa	550	diluted 1:3	3.19	
5285.2_B09_003.fsa	505	diluted 1:3	3.28	
5270.2_D11_007.fsa	760	diluted 1:3	3.36	**-Should have diluted 1:2**
5006.2_C08_006.fsa	775	diluted 1:3	3.44	
5006.1_B12_004.fsa	340	diluted 1:3	3.48	
5270.4_A11_001.fsa	700	diluted 1:3	3.5	
5013.1_A12_002.fsa	265	diluted 1:3	3.55	
3255.2US_F10_012.fsa	750	diluted 1:3	3.68	Dropout larger alleles
4997.1CC_A10_002.fsa	1250	diluted 1:3	3.76	
4996.1_H07_015.fsa	800	diluted 1:3	3.87	
06-4341.3_E12_010.fsa	675	diluted 1:3	3.9	
06-4406.2_D12_008.fsa	480	diluted 1:5	5.06	
4997.1_A08_002.fsa	700	diluted 1:5	5.1	
06-4406.1_B07_003.fsa	505	diluted 1:5	5.34	**-Should have diluted 1:3**
3255.1US_C09_005.fsa	432	diluted 1:5	5.61	
4965.1_C07_005.fsa	625	diluted 1:5	7.72	

5191.1_F01_011.fsa	500	Ignore	0.48	
5193.1CC_F04_012.fsa	280	Ignore	0.49	
4994.1CC_A05_001.fsa	270	Ignore	0.493	Slight dropout at red
5203.1_F02_012.fsa	350	Ignore	0.494	
5192.1_G01_013.fsa	340	Ignore	0.588	
5254.2_B04_004.fsa	400	Ignore	0.657	
5200.1_D02_008.fsa	300	Ignore	0.659	
5204.1_G02_014.fsa	450	Ignore	0.663	**-Should have amped 2ul**
5194.1_A02_002.fsa	450	Ignore	0.699	
5190.1_E01_009.fsa	350	Ignore	0.723	
4994.1_E04_010.fsa	450	Ignore	0.74	
5196.1_E06_010.fsa	220	Ignore	0.763	Dropout all red
3690.2US_D10_008.fsa	200	Ignore	0.813	Droput half
5199.1_D06_008.fsa	325	Ignore	0.834	
5193.1_H01_015.fsa	800	Ignore	0.841	
06-4267.1_A07_001.fsa	300	Ignore	0.85	Dropout some red
4965.2_C12_006.fsa	615	Ignore	0.874	
5200.2_C06_006.fsa	700	Ignore	0.926	
5195.1_F06_012.fsa	575	Ignore	0.939	
3690.1US_E09_009.fsa	290	Ignore	0.943	Dropout some red
3568.1US_D09_007.fsa	400	Ignore	0.996	
5209.1_E03_009.fsa	465	Ignore	1.02	
5270.3_C11_005.fsa	436	Ignore	1.11	
5248.1CC_B10_004.fsa	1100	Ignore	1.15	
06-4341.2_F12_012.fsa	635	Ignore	1.16	
5202.1_E02_010.fsa	750	Ignore	1.2	
5188.1_H06_016.fsa	625	Ignore	1.27	
5253.2_D05_007.fsa	600	Ignore	1.34	
4979.1_D07_007.fsa	1000	Ignore	1.35	
5254.1_B05_003.fsa	650	Ignore	1.43	
5205.1_B03_003.fsa	450	Ignore	1.48	
06-4281.3_G10_014.fsa	945	Ignore	1.51	
5253.1_F05_011.fsa	1450	Ignore	1.55	
5268.1_H08_016.fsa	1000	Ignore	1.56	
5253.3_C05_005.fsa	565	Ignore	1.57	
06-4281.3CC_C10_006.fsa	1118	Ignore	1.6	
5213.2_H11_015.fsa	1000	Ignore	1.61	
5248.1_G08_014.fsa	1000	Ignore	1.73	
5206.1_C03_005.fsa	300	Ignore	1.83	
3568.2US_E10_010.fsa	1200	Ignore	1.94	
5213.1_E08_010.fsa	350	Ignore	1.99	Dropout larger alleles

APPENDIX D

GENIOS CONCENTRATION RESULTS – LINEAR REGRESSION ANALYSIS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10%
100%
Zoom
Copy
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OK
Help
Instrument data
Raw data
Raw data as Col
Transformed data
Concentration
Single conc. (ng)
Graph: Standard
Sample IDs
Method layout
Measurement parame
Remarks
Error protocol
1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H
Measurement parameters
GENios
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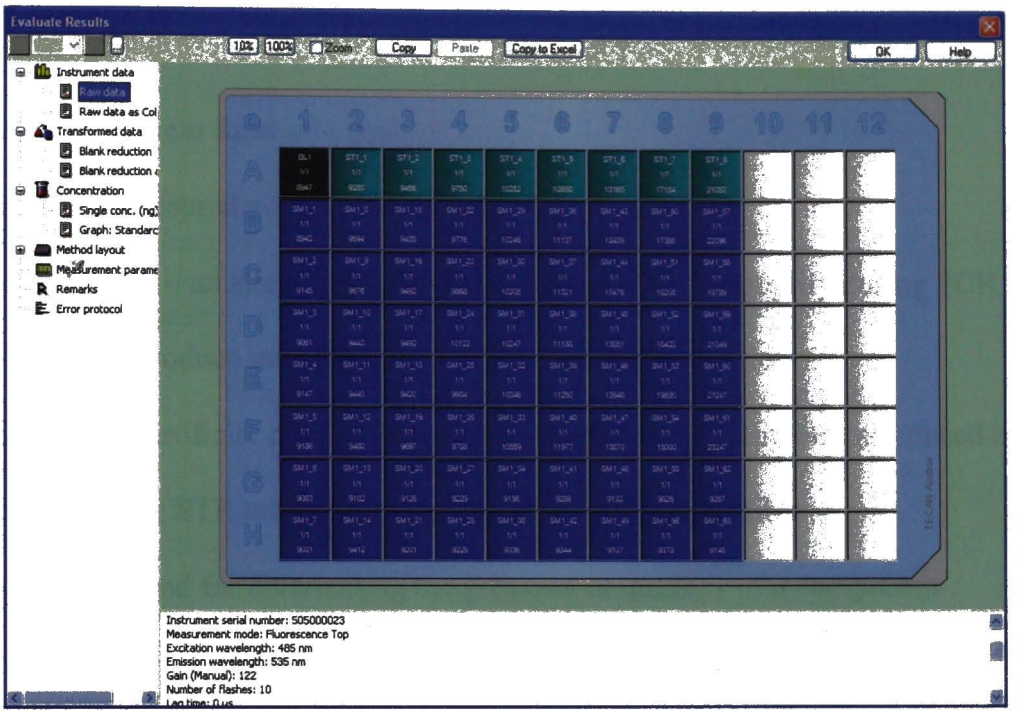
Emission wavelength: 535 nm

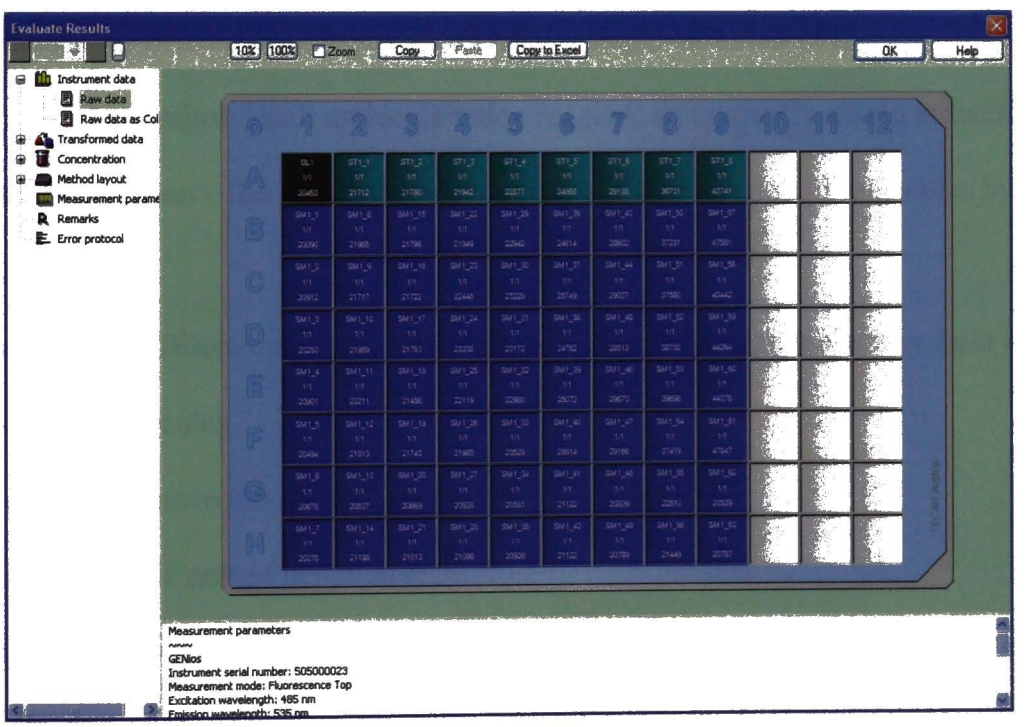
APPENDIX E

OLIGREEN® PLATE MEASUREMENTS

Gain of 122



Gain of 135



GLOSSARY

Allele – a different form of a gene

Aliquot – a measured portion of a sample taken for analysis

Allelic dropout – an allele is not detected; an allele fails to amplify during PCR

Amplify – to produce multiple copies of a specific DNA region

Artifact – any artificial product; anything not naturally present, but introduced by an external source; STR artifacts are recognizable and reproducible

Assay – a method for determining the presence or quantity of a component

Chromosome – the biological structure that carries hereditary information

CODIS – COmbined DNA Index System: a collection of databases of DNA profiles

Denature – to convert double-stranded DNA to single-stranded DNA

DTT – dithiothreitol: small molecule redox reagent; an unusually strong reducing agent

Electropherogram – the graphic representation of the separation of molecules by electrophoresis

Electrophoretic/Electrophoresis – a method of separating large molecules from a mixture of similar molecules

Extract – to remove the DNA from the cell

Gene – the basic unit of hereditary

Heterozygote – two different alleles present at one locus

Intercalate – to insert between the bases of a DNA molecule

Load – to put samples on a genetic analyzer for electrophoresis

Locus – the specific physical location of a gene on a chromosome

Lysate – the contents released from a lysed cell

Lysis – rupture and destruction of a cell

Microplate - a plate of standardized size, usually containing 96 containers (wells)

Nucleotide – a unit of DNA or RNA

Oligonucleotide – a molecule usually composed of 25 or fewer nucleotides

Overblown – when too much DNA is added to an amplification reaction

Paramagnetic – weakly attracted to magnetic fields

PCR – Polymerase Chain Reaction: a process where one or more specific small regions of DNA are copied

Pull up – a peak seen in one color that is not due to the presence of DNA but to the lack of separation of overlapping dyes

Resin

STR – Short Tandem Repeat: multiple copies of a short identical DNA sequence in series

Stutter – a minor peak that appears one repeat unit smaller than a primary STR

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