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This study evaluated the AluQuant™ Human DNA Quantitation System (Promega Corporation, Madison, WI) using the 96-well plate format for possible implementation by Orchid Cellmark Dallas. The importance of human DNA quantitation in forensics is two-fold. First, the Quality Assurance Standards set forth by the DNA Advisory Board requires human DNA in forensic samples be quantitated. Also, the highly sensitive PCR multiplex assays used in forensics have been optimized for a narrow range of template DNA, thus requiring accurate and consistent quantitation.

This evaluation consisted of three general goals: examination of the Reporter™ Microplate Luminometer (Turner BioSystems, Sunnyvale, CA), alteration of the assay variables to obtain optimal performance, and characterization of the assay. The Reporter™ produces reproducible results and is sensitive to at least 4.88×10^{-9} moles ATP. Of the variables tested, quick centrifugation of the incubation plate had the most noticeable effect on the results obtained. The assay did not perform as characterized by Promega. AluQuant™ is not reproducible, nor does it consistently produce results within a two-fold accuracy range. Therefore, Orchid Cellmark Dallas will not be implementing the AluQuant™ assay.

EVALUATION OF THE ALUQUANT™

HUMAN DNA QUANTITATION SYSTEM

USING THE 96-WELL PLATE FORMAT

Uvonna Faye Lewallen Alexander, B.S.

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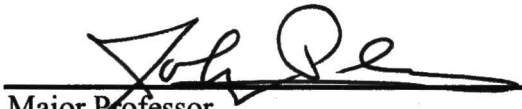

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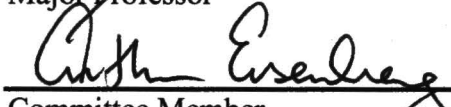

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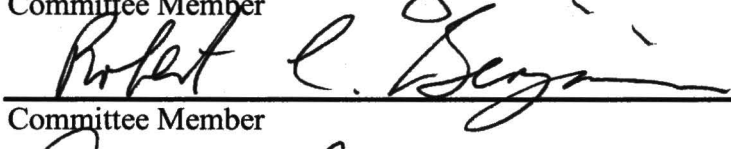

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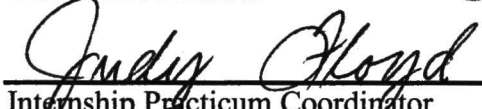

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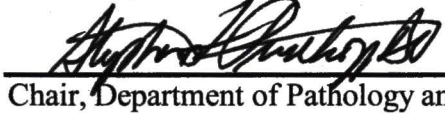

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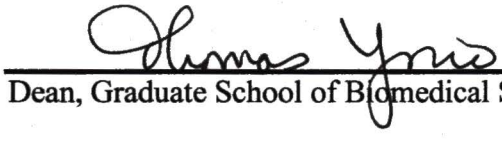

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EVALUATION OF THE ALUQUANT™ HUMAN DNA QUANTITATION SYSTEM USING THE 96-WELL PLATE FORMAT

INTERNSHIP PRACTICUM REPORT

Presented to the Graduate Council of the

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University of North Texas
Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

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ABBREVIATIONS

ABI 3100, ABI Prism® 3100 Genetic Analyzer

ADP, adenosine diphosphate

AluQuant™, AluQuant™ Human DNA Quantitation System

AMP, adenosine monophosphate

ART™, Aerosol Resistant Tips

ATP, adenosine triphosphate

CycleFoil®, Robbins Scientific CycleFoil® plate cover

CycleSeal®, Robbins Scientific CycleSeal® plate cover

DNA, deoxyribonucleic acid

dNTP, deoxyribonucleoside triphosphate

5'-overhang end, five prime overhang end

HCl, hydrochloric acid

L/L, Luciferase/Luciferin

MM, Master Mix

MMC, Master Mix Control

NaOH, sodium hydroxide, denaturation solution

PCR, polymerase chain reaction

PMT, photomultiplier tube

Quantiblot®, Quantiblot® Human DNA Quantitation Kit

RNA, ribonucleic acid

Reporter™, Turner Reporter™ Microplate Luminometer

RFU, relative fluorescent unit

RLU, relative light unit

SINEs, short interspersed nuclear element

STR, short tandem repeat

TE^{-4}, tris EDTA pH 8.0-> 10mM Tris and 0.1 mM EDTA (ethylenediaminetetraacetic disodium salt)

3'(A), three prime adenylation

3'-terminal bond/base, three prime terminal bond/base

CHAPTER I

INTRODUCTION

In forensic human DNA identification, quantitation of extracted DNA is performed prior to amplification of the DNA for subsequent short tandem repeat (STR) analysis. The ideal human DNA quantitation system for forensic purposes would possess the following characteristics: robustness, reproducibility, accuracy, sensitivity, human specificity, a low level of labor intensity with minimum manipulation, and adaptability to high throughput. A quantitation system with these features would result in a decreased need for reanalyzing samples by analysts. Therefore, resulting in a lowered cost per sample with respect to time, reagents, and labor savings. The importance of having a human DNA quantitation system that would meet the above criteria is two-fold. First, the Quality Assurance Standards for forensic DNA testing laboratories set forth by the DNA Advisory Board in October of 1998 states, "Standard 9.3 - The laboratory shall have and follow a procedure for evaluating the quantity of the human DNA in the sample where possible"(1).

Second, due to the highly sensitive polymerase chain reaction (PCR) multiplex assays that have been optimized for a narrow range of template DNA, accurate and consistent quantitation of human DNA for forensic work is critical. If too much DNA is added into the PCR reaction, amplification artifacts such as stutter and incomplete three

prime adenylation (3'A) occur. Stutter is characterized by a smaller peak one repeat unit less than the corresponding main allele peak. Incomplete 3' (A) nucleotide addition results in split peaks where the allele of interest is represented by two peaks one base pair apart. These artifacts can interfere with the clear interpretation and genotyping of alleles present in the DNA template of any sample. These problems are especially evident in those samples containing mixtures of DNA (2). The addition of an insufficient quantity of DNA into a PCR reaction most often results in an incomplete or absent profile.

Currently, various slot blot hybridization procedures, such as the Quantiblot® Human DNA Quantitation Kit (Applied Biosystems, Foster City, CA), are the predominant methods used for DNA quantitation in forensic DNA analysis. However, there are some aspects of quantitation by slot blot that fail to meet the complex needs of the forensic community. For example, slot blot quantitation requires the immobilization of target DNA on a solid support followed by hybridization of a human specific probe to the DNA, and finally a series of stringent washing steps to remove any unbound probe. Then, chemiluminescence, fluorescence, or colorimetric detection is used to record the bound probe on either x-ray film or nylon membranes. Clearly, quantitation by slot blot hybridization can be time consuming and does not easily lend itself to automation. Furthermore, these methods can produce variable results due to the subjective visual comparison of band intensities between sample extract DNA and a series of DNA standards. Although the use of expensive digital imaging systems to capture the image can address the subjectivity of visual examination, the use of film still has a limited dynamic range due to saturation thresholds (3). As a result, current methods often

produce variable results that can necessitate the need to reamplify and reanalyze samples, translating to increased cost per sample. In light of these observations, the forensic DNA community seeks an alternative quantitation system.

The need for a more accurate quantitation system by the forensic community is discussed in a report by the National Institute of Standards and Technology. The study focused on the performance and importance of DNA extraction and DNA quantitation technologies. Their report states: "All allele measurement failures (true alleles not called, stutter called as an allele) are attributable to inefficient extraction of DNA from the samples, inaccurate estimation of the amount of DNA used in the PCR mixture, and/or analytical threshold policies. To the extent that simultaneous amplification of multiple alleles demands better control of initial conditions as the complexity of the system increases, highly multiplexed STR systems may well require improved DNA quantification technology" (4).

Promega Corporation's (Madison, WI) proposed solution to the quantitation dilemma is the AluQuant™ Human DNA Quantitation System. According to Promega, the system has several salient features that address the needs and concerns of the forensic community. The system is a solution based hybridization format that can be read on Turner BioSystems' (Sunnyvale, CA) luminometers. Therefore, it does not require gel electrophoresis, blotting steps, x-ray film, or amplification of DNA sequences, as do current competitor systems. Unlike membrane-based formats, the AluQuant™ System has been reported to more accurately quantitate degraded DNA. Degraded DNA is often found in forensic samples due to environmental insults such as nucleases, sunlight, and

pH changes. In addition, AluQuant™ uses a human-specific DNA probe to highly repeated sequences in chromosomal DNA. These repeated sequences are also found in other higher primates. The system is unaffected by DNA from other species, such as bacteria or yeast, that may be present in forensic samples. Additional specificity is provided by the fidelity of DNA polymerase in recognition of perfect hybrids that are either blunt ended or have a 5'-overhang end. Promega claims that the optimal quantitation range of this system is between 0.02-4.0 ng/μl; the signal to DNA quantity relationship remains proportional above this range but accuracy decreases. The system is reported to give precise and reproducible results with a two-fold range of accuracy. The AluQuant™ Calculator, a Microsoft® Excel program, uses luminometer numeric outputs to calculate the amount of human DNA in a sample, therefore eliminating subjectivity (5,6).

The AluQuant™ Human DNA Quantitation System utilizes two incubation steps. In the first incubation, a coupled enzymatic reaction takes place following an initial denaturation of the DNA. The first reaction is known as a depolymerization reaction in which the DNA polymerization reaction is reversed. A DNA polymerase, READase™ Polymerase, catalyzes the addition of a pyrophosphate across the 3'-terminal bond of double-stranded DNA. This addition results in the release of the 3'-terminal base from the DNA strand as a deoxyribonucleoside triphosphate (dNTP), and the terminal phosphate of the released dNTP is then transferred to ADP to form ATP using the second enzyme in the reaction mixture, the READase™ Kinase. During the second incubation, ATP production from the first incubation is quantitated by measuring the amount of light

produced from a bioluminescent reaction involving the cleavage of pyrophosphate from the ATP with luciferase, luciferin, and molecular oxygen as principal biochemical components. Background "noise" is determined by concurrent analysis of the samples without the human-specific probe. The quantity of DNA can then be calculated by comparison of the signal to standards of known DNA quantity using the AluQuant™ Calculator (5,6).

The human-specific DNA probe employed by the AluQuant™ System is a form of highly repetitive DNA called an *alu* insertion sequence. *Alu* insertion polymorphisms have been examined extensively in evolutionary studies and are classified as short interspersed nuclear elements (SINEs). They are named for an *Alu*I restriction endonuclease site typical of the consensus sequence for the element. These polymorphisms are believed to be one of the most successful mobile genetic elements due to their wide dispersion via a process known as retro-transposition. *Alu* elements are approximately 300 base pairs in length and are thought to be ancestrally derived from the 7SL RNA gene. These repeats comprise 5-10% of the human genome and are considered stable genetic markers that do not appear to be subject to loss or rearrangement once inserted (7,8,9).

The AluQuant™ System utilizes READIT® Technology that employs a luciferase reaction to generate a light signal proportional to the amount of human DNA contained in a sample. The luciferase reaction is a bioluminescent reaction that requires considerable amounts of energy. By definition, bioluminescence is a type of chemiluminescence in which a chemical reaction is catalyzed by an enzyme. In the firefly, ATP is employed in

a set of reactions that converts chemical energy into light energy. The principal biochemical components in the reaction are luciferin, a complex carboxylic acid, and luciferase, an enzyme. The luciferin is activated by an enzymatic reaction involving the cleavage of pyrophosphate from the ATP followed by the transfer of the adenylate moiety to luciferin to form luciferyl adenylate. In the presence of molecular oxygen and luciferase, the luciferin undergoes a multi-step oxidative decarboxylation to oxyluciferin, inorganic pyrophosphate, AMP, and light. Therefore, the amount of light given off is proportional to the amount of ATP produced that is proportional to the amount of DNA in a sample. In addition, firefly luciferase is a commonly used bioluminescent reporter because the luminescence assay is rapid, convenient, and sensitive (10,11).

A luminometer reads the light given off by the luciferase reaction, and this is then used to calculate the concentration of human DNA present in a sample. The amount of ATP and light produced are proportional to the amount of DNA present in the sample. The analytical technique used to measure chemi- and bioluminescent reactions, luminometry, has several advantages over other analytical techniques. These advantages include: sensitivity, wide dynamic range, inexpensive instrumentation, and low background. Luminometry is up to 100,000 times more sensitive than absorption spectroscopy and is at least 1,000 times more sensitive than fluorometry (12). According to Turner BioSystems, "A well-designed luminometer can detect as little as 0.6 picograms of adenosine triphosphate (ATP) or less than 1.0 femtogram of luciferase, two common luminescent analytes" (12).

Promega designed the AluQuant™ Human DNA Quantitation System to be performed in a single-tube or to be extended into a 96-well plate format. In this study, the system was evaluated using the 96-well format. In examining and evaluating the AluQuant™ Human DNA Quantitation System, attention was given to those features claimed by Promega to be prominent characteristics of the assay. In so doing, the criteria for the ideal quantitation system were explored as well.

CHAPTER II

MATERIALS AND METHODS

Experiments Performed:

The AluQuant™ Human DNA Quantitation System (Promega Corporation, Madison, WI) was evaluated by performing experiments that can be classified in at least one of the following categories:

- Reproducibility of the Reporter™ Microplate Luminometer (Turner BioSystems, Sunnyvale, CA)
- Sensitivity of the Reporter™ Microplate Luminometer
- Reagent preparation
- Importance of constant 55°C temperature during the one-hour incubation
- Flash spinning of the incubation plate
- Accuracy and sensitivity of the assay
- Reproducibility of the standard curve
- Acceptability of the standard curve
- Consistent amplification

Protocol for the AluQuant™ Human DNA Quantitation System:

In general, the steps in performing the 96-well assay include:

- Human genomic DNA standard preparation
- Luciferase/Luciferin (L/L) reagent preparation
- Master Mix preparation (one with probe and one without)
- Denaturation of standard and sample DNA
- Incubation of standard and sample DNA with Master Mixes for one hour at 55°C
- Combine contents of Master Mixes with L/L reagent in luminometer plate
- Immediately place luminometer plate into Reporter™
- Calculation of DNA concentrations using AluQuant™ Calculator 2.0 (a Microsoft® Excel Program)

Promega Corporation's Technical Bulletin No. 291 for the AluQuant™ Human DNA Quantitation System (Appendix A) outlines the above mentioned steps. However, the evaluation of the assay progressed, it was deemed necessary to incorporate the following additions and clarifications in an attempt to obtain reproducible and acceptable results.

1. Preparation of the human DNA dilution series: After thawing, the 20 ng/µl DNA solution was vortexed for 1½ minutes prior to pipetting 15 µl of the DNA solution into a 1.5 ml microcentrifuge tube containing 60 µl of water. This created the 4 ng/µl standard dilution concentration. Then, the DNA and water mixture was vortexed for 30 seconds, allowed to sit at room temperature for four minutes, and vortexed again for 30 seconds before proceeding to the

next dilution. This scheme was repeated between each subsequent dilution. The remaining dilutions were created by adding 40 µl of the previous dilution standard into 40 µl of water to create the remaining standards of 2, 1, 0.5, 0.25, 0.125, and 0.063 ng/µl. (addition and clarification to Technical Bulletin No. 291, III. A.)

2. The Luciferase/Luciferin (L/L) reagent was allowed to sit at room temperature for at least two hours prior to use whether it was newly reconstituted or simply thawed. It was never vortexed. (clarification to Technical Bulletin No. 291, III. B.)
3. All reagents, other than the L/L reagent, were vortexed for 30 seconds before use. (addition to Technical Bulletin No. 291)
4. Just after both Master Mixes were prepared and placed on ice, and prior to exposing the Denaturation solution to air, all standards and samples were vortexed for 15-30 seconds. (addition to Technical Bulletin No. 291)
5. A multi-channel pipette was used to place the Denaturation Solution (NaOH) into each of the denaturation wells. Water (9 µl) was added to sample wells before sample DNA was added. Standard and sample DNA solutions were added to the wells, and each mixed five times by pipetting. (addition and clarification to Technical Bulletin No. 291, III. E. 2 & 3)
6. Master Mixes were vortexed for 30 seconds before using a multi-channel pipette to place them into their appropriate wells in the incubation tray. (reminder needed in Technical Bulletin No. 291, III. E. 5)

7. A multi-channel pipette was used to place and mix, (five times by pipetting), the hydrochloric acid, HCl, into each of the denaturation wells. A multi-channel pipette was then used to transfer and mix, (five times by pipetting), 10 µl from the denaturation tray to the Master Mixes on the incubation tray. The tray was incubated immediately. (addition to Technical Bulletin No. 291, III. E. 6 & 7)
8. The 96-well tray was incubated at 55°C for one hour in a heat block. (alternative to Technical Bulletin No. 291, III. E. 8)
9. Just before the end of the one-hour incubation, the multi-channel pipette and non-ART tips were used to place the L/L reagent into the luminometer plate. In addition, the luminometer was prepared to read the plate. (addition to Technical Bulletin No. 291, III. E. between steps 8 & 9)
10. After the 55° C incubation for one hour, the plate was flash-spun in a centrifuge without allowing the plate to sit for any length of time before proceeding. (addition to Technical Bulletin No. 291, III. E. 9)
11. The contents of the Master Mix incubation tray were mixed five times using a multi-channel pipette, 25 µl was then transferred to the corresponding wells in the luminometer plate and mixed five times. (addition to Technical Bulletin No. 291, III. E. 11)
12. The "Calculate" button at the top of the page was selected every time any data was imported into the AluQuant™ Calculator even though trend lines already appeared. (addition to Technical Bulletin No. 291, IV. 5)

13. After the computer program calculated the DNA concentrations of the samples, any dilution factor used was taken into account by manually moving the decimal point one place to the right. (addition to Technical Bulletin No. 291)

Most samples used had been organically extracted and precipitated with ethanol. A few samples had been washed using Microcon™ 100 filter devices. Two different Turner Reporter™ Microplate Luminometers and versions of the Reporter™ Software were used; both luminometers were Model # 9600-002. The first instrument used software version 2.2 and had a serial number of 960011, and the second instrument used software version 2.3 and had a Serial # of 960031.

Protocol for the Amplification of Unknown Samples:

The AmpFLSTR® Profiler Plus™ PCR Amplification kit (Applied Biosystems, Foster City, CA) was used along with a Perkin Elmer GeneAmp 2400 PCR System Thermocycler (Applied Biosystems, Foster City, CA) to amplify 15 samples including a positive and negative control. The amplification program used is as follows: 95° C for 11 minutes, {94° C for 1 minute, 59° C for 1 minute, 72° C for 1 minute} for 28 cycles, 60° C for 60 minutes, and a 4° C hold. The PCR Master Mix included the following: 10.5 µl of reaction mix + 2.5 units (or 0.5 µl of 5 U/µl) Ampli*Taq* Gold™ DNA polymerase + 5.5 µl of primer solution for a total of 16.5 µl. The reaction mix comes already made by Applied Biosystems, and it includes magnesium chloride, dNTPs, bovine serum albumin, and 0.05% sodium azide in buffer and salt. Final concentrations were considered proprietary by Applied Biosystems and were not provided. Then, 10 µl

of template DNA diluted to 0.125 ng was added to 15 µl of Master Mix for a total PCR volume of 25 µl. In effect, 1.25 ng of sample DNA was added into the PCR reaction. However, 10 µl of 0.1 ng/µl 9947A positive control DNA was added into the PCR reaction, and 10 µl of water was added into the negative control reaction.

Protocol for the Analysis of Unknown Samples:

Due to the sensitivity of the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), the samples including positive and negative controls were diluted 1:25, vortexed, and collected by centrifugation. Then, 1 µl of the diluted samples and ladder was added to 9 µl of a ROX 500 and formamide 1:100 mixture, vortexed, and collected by centrifugation. The loading tray was then denatured for five minutes at 95°C followed by five minutes on ice. Next, the loading tray was placed on the instrument and allowed to analyze the samples, which took about 45 minutes for the 16 samples (13 samples, 1 positive control, 1 negative control, and 1 ladder). Finally, GeneScan® and Genotyper® software (Applied Biosystems, Foster City, CA) were used to analyze the data the ABI Prism® 3100 had collected.

CHAPTER III

RESULTS AND DISCUSSION

In evaluating the AluQuant™ Human DNA Quantitation System using the 96-well plate format, not only were attributes of the assay itself examined, but also several variables in the assay were adjusted in order to optimize the quantitation system. First, the reproducibility and sensitivity of the Reporter™ Microplate Luminometer were tested. In addition, reagent preparation was assessed in order to maximize performance. The importance of a constant, precise 55°C temperature during the one-hour incubation was examined as well as the effects of flash spinning the tray after incubation. The accuracy and sensitivity of the assay was also investigated. Likewise, the standard curve's reproducibility and the characteristics of an acceptable standard curve were determined. Finally, the ultimate goal of consistent amplification was scrutinized using all of the adjusted variables to give optimal efficacy.

1. Reproducibility of the Reporter™ Microplate Luminometer:

During this study two different Reporter™ Microplate Luminometers were used. The original luminometer gave questionable results; therefore, four experiments were performed in which an empty white plate was scanned to troubleshoot the Reporter™ to determine if it had internal moisture. It was determined, by the manufacturer, that the

original luminometer had contamination on the photomultiplier tube (PMT), as seen by the four scans that gave readings sporadically from zero RLUs to 250 RLUs. According to Turner BioSystems, each well should read zero RLUs with an empty white plate. Therefore, the luminometer was returned to Turner BioSystems for cleaning. With the second Reporter™, empty white luminometer plates were also scanned using version 2.2 of the Reporter™ software already installed as well as with version 2.3 sent with the new Reporter™. Both versions produced zero readings in all 96-wells of the plate.

In order to test the reproducibility of both luminometers used throughout the evaluation, experiments were performed in which the luminometer was set to scan the same plate three times. Two experiments were performed with the original Reporter™. Chart 1 shows an example of one standard curve dilution series (DNA concentrations from 0 ng/μl to 4 ng/μl) in which the net relative light units or RLUs (readings from wells with probe minus wells without probe) were averaged over the three scans made by the luminometer. Two additional experiments were performed using the second Reporter™. Chart 2 shows an example of one standard curve dilution series in which the net RLUs were averaged over the three scans made by the luminometer. Although the original Reporter™ had internal contamination, results from these experiments suggest that both luminometers were reproducible.

2. Sensitivity of the Reporter™ Microplate Luminometer:

The sensitivity of the luminometers was examined through a series of experiments. The first experiment was designed to determine whether the extra sensitivity option in the Reporter™ software would improve the instrument's detection

limit. According to Turner BioSystems, selecting the extra sensitivity option improves the detection limit, thus giving higher RLUs; but, the test time will increase. As seen in Table 1, comparing the relative light units, between wells that contain the same DNA concentration and within the same column (with or without probe), reveals that the first luminometer did not give readings that were consistently higher with the extra sensitivity option selected. However, the net RLUs, both with and without the extra sensitivity option selected, reached the same maximum reading at approximately 400,000 RLUs. With the second Reporter™ Luminometer (Table 2), the RLUs were higher, with one exception, on an individual well basis with the extra sensitivity option selected. Again, whether or not the extra sensitivity option was on or off, the net RLUs reached a maximum reading of approximately 300,000 RLUs.

It is possible that the first Reporter™ did not consistently produce higher RLU values with the extra sensitivity option selected because it had internal contamination causing sporadic readings. Regardless, the extra sensitivity option was not routinely used throughout the evaluation for the following two reasons: 1) Luciferase/Luciferin only has a 15-minute half-life; therefore, the increased time to read a plate with the extra sensitivity option selected proved to be disadvantageous; 2) with both luminometers the net RLUs obtained were very similar regardless of the extra sensitivity status.

Additional experiments were performed to further examine the sensitivity of the luminometers. A serial dilution with ATP demonstrated that the photomultiplier tube (PMT) in the Reporter™ becomes saturated by the light given off due to the ATP-Luciferase/Luciferin reaction between 1.95×10^5 and 1.95×10^7 moles of ATP. This is

consistent with Turner BioSystems' claims that the Reporter™ becomes saturated at 1.5×10^{6} RLUs. It was also determined that the ENLITEN® Luciferase/Luciferin (L/L) reagent, as well as the luminometer, was able to detect at least 4.88×10^{-9} moles of ATP. Promega Corporation claims that the L/L reagent can detect as little as 10^{-11} to 10^{-16} moles of ATP. Also, Turner BioSystems claims that the Reporter™ Luminometer engineered for the AluQuant™ System has a detection limit not more than ten times different than that of the normal Reporter™ of 10^{-19} moles luciferase. Variation can occur with each type of luminometer and luciferase.

In order to determine if cross-talk would occur between adjacent wells, an experiment was performed using water, L/L reagent, and ATP dilutions that would both saturate and not saturate the PMT in the Reporter™ (Table 3). Cross-talk occurs when light given off from one well interferes with the PMT taking light readings in adjacent wells. Cross-talk did occur in all four adjacent wells to those with a saturated dilution of ATP regardless of whether the adjacent wells contained water, L/L, or a combination. The cross-talk values were between approximately 350 RLUs and 750 RLUs. The wells containing a dilution of ATP that would not saturate the PMT did not cause cross-talk when surrounded by water or L/L. However, when a mixture of water and L/L surrounded the non-saturated dilution, each well to the right and left of the non-saturated well gave readings of approximately 57 RLUs and 108 RLUs. In addition, one well containing only L/L, surrounded by empty wells and one well with water, gave a reading of 99 RLUs. Turner BioSystems indicated that the L/L reagent could radiate light

naturally. However, because RLU readings were obtained from wells to the right and left of one non-saturated well and in a well containing only L/L, it does not appear that ATP contamination in the wells can be confidently excluded as a possible explanation.

3. Reagent Preparation:

Various reagents were prepared and handled in different ways at the request of Promega in order to attempt to obtain acceptable and reproducible standard curves with the AluQuant™ assay. There was no observable difference between the standard curves created when the standard dilution series was generated with nuclease-free water versus TE^{-4} (pH 8.0). The acceptability and reproducibility of the standard curves created from the standard dilution series did not improve due to variations, such as the vortexing and incubation times, in the actual scheme for generating the standard dilutions. Altering the handling of the L/L reagent did not positively influence the type of standard curves obtained either.

Throughout the evaluation, inconsistent results were obtained both before and after reagent preparation and handling parameters were altered. Therefore, any alterations made in reagent preparation were not mandatory to obtain acceptable results. Similarly, the reproducibility of the assay did not appear to be considerably affected by any of the reagent preparation and handling parameters modified. Variations in the preparation of reagents were maintained to determine if reproducibility and acceptability would be obtained under a particular set of combined treatments. Unfortunately, no particular combination of reagent preparations resulted in a noticeable difference in the standard curves obtained.

4. Importance of Constant 55°C Temperature During the One-Hour Incubation:

Due to the fluctuations from 55°C to 57°C in the water bath, a 96-well heat block set at 55°C was also used for the one-hour incubation. However, both acceptable and unacceptable curves were obtained using a water bath and a heat block, and the reproducibility of the assay also varied from experiment to experiment. Therefore, it does not appear that one or the other is best to use due to the inconsistent results obtained. The 96-well plate cover used with the heat block versus the water bath did affect the results obtained though. Optimal results were obtained with the heat block and Robbins Scientific (Sunnyvale, CA) CycleFoil® plate cover. However, when using the water bath, Robbins Scientific CycleSeal® obtained better results. When the CycleSeal® cover was used with the heat block, the edges of it curled up allowing complete evaporation in the corner wells and some evaporation in all other wells.

5. Quick Centrifugation of the Incubation Plate:

Even though Promega's protocol does not give any instructions on centrifuging the 96-well plate before or after the one-hour incubation, it was postulated that removing any condensation or bubbles could be critical because of the very small quantities of DNA present. Therefore, quick centrifugation of the incubation plate for approximately 10 seconds both before and after the one-hour incubation was attempted. However, centrifugation both before and after the one-hour incubation did not prove to be advantageous for the reproducibility and acceptability of the standard curve. This approach was unsuccessful due to the time interval before the plate was placed at 55°C.

However, quick centrifugation of the incubation plate only after the 55°C incubation proved to be beneficial. Figures 1 and 2 display the standard curves obtained by quick centrifugation of the incubation plate only after the one-hour incubation period. Both curves are considered acceptable (see Section 8 for the characteristics of an acceptable curve). These curves are the best representation of reproducibility within an experiment obtained in the evaluation of the assay. The "samples" at the bottom of Figures 1 and 2 represent the standard points of the curve made to be samples as a way of testing the standard curve obtained.

6. Accuracy and Sensitivity of the Assay:

Four different standard DNA concentrations (2 different 10 ng/µl standards, 0.1 ng/µl standard, and 2.5 ng/µl standard) were run in duplicate from three different sources (Applied Biosystems, Promega Corporation, and American Type Culture Collection) to investigate the accuracy of the assay. In a separate experiment, three of the above mentioned standard DNA concentrations were processed again to examine the accuracy of the kit. The DNA concentrations given by AluQuant™ ranged from 4.5 ng/µl to 11.5 ng/µl for the 10 ng/µl standards. The 0.1 ng/µl DNA standard yielded results from 0.2 ng/µl to 0.7 ng/µl. The 2.5 ng/µl DNA standard yielded results from 2.7 ng/µl to 11.1 ng/µl. Curves 1 and 2 on Chart 3 represent the average of two DNA concentration values as determined by the AluQuant™ System for each of the four different manufacturer's standard DNA concentrations. Curve 3 on Chart 3 represents one quantity obtained from only three of the standard DNA concentrations processed in a different experiment. Chart 3 reveals the variation obtained in the accuracy of the DNA standards. It appears

that the assay is more accurate at lower concentrations, coinciding to the 0.1 ng/μl standard, than at the higher concentrations of 2.5 ng/μl and 10 ng/μl. Promega affirms the assay is accurate within two-fold. For example, if the standard is known to be 10 ng/μl, then Promega claims the AluQuant™ to be accurate between 5 ng/μl and 20 ng/μl. However, the data obtained during this evaluation was not consistent with this claim.

The assay performs optimally between 0.02 ng/μl and 4.0 ng/μl according to Promega; but, because there is often very little DNA in forensic samples, the sensitivity of the assay was examined at the lower end of the range. In fact, the sensitivity of the assay was tested by extending the standard serial dilution scheme beyond the lowest point, 0.063 ng/μl, by four serial dilutions (0.0313 ng/μl, 0.0156 ng/μl, 0.0078 ng/μl, and 0.0039 ng/μl). The dilutions were then quantitated in duplicate as though they were unknowns. Chart 4 shows the relationship between the DNA concentrations obtained with the assay versus the actual dilution standards. Below the 0.0156 ng/μl dilution standard, which corresponds to the lower end of the optimal range according to Promega, no signal was detected. However, at the 0.0156 ng/μl point, the assay yielded concentrations between 0.4 ng/μl and 0.9 ng/μl. Perhaps the dilution standards did not contain the amount of DNA expected. Nevertheless, the assay is sensitive but not necessarily accurate.

7. Reproducibility of the Standard Curve:

The reproducibility of the standard curve was surveyed each time an experiment was performed, therefore allowing for intra- and inter-experimental inspection. Figures 1 and 2 are the best representation of intra-experimental reproducibility obtained

throughout the entire evaluation. The experiment performed subsequent to the one seen in Figures 1 and 2 proved to be the best reproducibility between successive experiments with very similar standard curves. However, neither of these situations was typical of the results obtained during the AluQuant™ evaluation. Figures 3 and 4 are more representative of the overall examination where the curves are nothing alike. Due to the inconsistency of the assay it was necessary to perform two standard curves per plate in order to obtain one that could be used to analyze samples. Overall, the standard curves were not reproducible either within one experiment or between two different experiments.

8. Acceptability of the Standard Curve:

All experiments in this evaluation were used to determine the features of an acceptable curve. The following is a list of those characteristics: all *R* statistics must be >0.99, the trend line must be very close to the actual standard curve, and a single point may be taken out (if possible avoid taking out the 0 ng/µl or 4 ng/µl points because without them the quantitation range becomes narrower). The *R* statistics indicate the linear regression within a sample. The R^2 statistic is referred to as the sample coefficient of determination, and it falls between zero and one. If R^2 equals zero, there is no correlation between the raw data and the expected data. If R^2 equals one, the raw data and the expected data are perfectly correlated. Therefore, a straight line would have a R^2 value of one. Basically, both *R* curve fitting statistics give an indication of how close the raw values are to the expected. If a curve possesses the above mentioned features, then it

will follow that the "curve" is fairly straight and that analyzing the standard points as unknowns will produce quantities very similar to their known standard dilution values.

Figure 5a and b represent an example of an unacceptable standard curve resulting from an erroneous reading at 2 ng/μl. The known dilution samples were analyzed as unknowns at the bottom of Figure 5a. Figure 5b shows the 13 samples analyzed with this particular curve. The computer program for the assay assumes ten microliters of a DNA extract is quantitated; however, in this evaluation per the protocol only one microliter of unknown samples was quantitated to preserve sample extract. Therefore, sample DNA concentrations will need to be corrected for the ten times dilution factor used. The AluQuant™ Calculator does not have a column to allow for the correction of any dilution factors. Due to small sample extracts often encountered with forensic samples, the fact that one microliter can be used to quantitate is desirable; however, the computer program does not allow for dilution corrections.

Figure 6a and b represent the same standard curve and samples as seen in Figure 5a and b, but the erroneous point has been removed. Removing the 2 ng/μl point gives the curve *R* statistics of >0.99 and a smooth straight appearance with the trend line superimposed on the raw data curve. The DNA standards used as unknowns also reflect the acceptability of the standard curve because the values calculated are very similar to the "known" values for those points. If an erroneous point is removed from a curve to make the curve acceptable, the DNA concentrations calculated for both standards and unknown samples changes from the data produced when the erroneous point is included.

9. Consistent Amplification:

Thirteen samples previously quantitated with Quantiblot® were quantitated with AluQuant™. As seen in Table 4, both systems produced similar DNA concentrations. Refer to Figure 6a and b for the standard curve used to analyze the 13 samples. The values obtained by AluQuant™ were used to amplify and analyze the samples with Profiler Plus™ on the ABI Prism® 3100 Genetic Analyzer in order to determine if consistent amplification would result. All parameters were kept identical including the amount of DNA amplified, 1.25 ng. Figure 7a and b shows an example of the consistent amplification resulting between the two systems using identical platforms. In fact, the AluQuant™ amplification produced slightly better heterozygote peak balance. Both Figures 7a and b and 8a and b are representative of the AluQuant™ amplifications having sometimes slightly higher, but more commonly comparable relative fluorescent units (RFUs) to those quantitated with Quantiblot®. Overall, the AluQuant™ quantitation values used produced consistent amplification as well as comparable results to those of Quantiblot®.

CHAPTER IV

CONCLUSIONS

The AluQuant™ Human DNA Quantitation System using the 96-well format does not meet the criteria for the ideal quantitation system. The AluQuant™ System is not robust; in other words, the system does not perform adequately when conditions are less than optimum. The assay is unforgiving of slight variances in time, pipetting, and mixing. Despite Promega's insistence on the critical importance of a constant 55°C temperature during the one-hour incubation, the only linear standard curve was obtained with the water bath varying from 55°C to 57°C. Perhaps, that particular curve was an anomaly.

Although the Reporter™ Microplate Luminometer is reproducible, the standard curve necessary for quantitating samples with the assay is not. In fact, the standard curve had to be run in duplicate with each experiment in an attempt to obtain one acceptable curve. Due to the lack of reproducibility, the acceptability of the standard curve becomes a critical issue that each forensic laboratory would have to address. Individual validation studies would be required to define the characteristics of an acceptable standard curve in each laboratory. As a result of Promega's lack of guidelines for determining the parameters of an acceptable curve, a certain amount of ambiguity between labs would occur. In that respect, the AluQuant™ assay has no advantage over the currently used

subjective slot blot methods. Also, because standard curves had to be run in duplicate on each experiment, fewer samples per plate could be quantitated which is an important aspect to remember when considering cost analysis. Unacceptable curves were obtained for a variety of reasons. Pipetting variances, incomplete mixing, evaporation, and condensation were the most plausible reasons why an acceptable curve was not obtained. However, even with these issues corrected, unacceptable curves were obtained which implies a stochastic problem with the chemistry of the reaction itself.

The assay and luminometer do appear to be sensitive, since they are able to detect very low amounts of DNA. However, the quality of the DNA sample may effect the rate of the enzymatic reactions due to the sensitivity of the assay resulting in non-reproducible, unacceptable standard curves even with all parameters of the assay optimized for peak performance. In addition, Promega claims the system has a two-fold range of accuracy, but this evaluation revealed that the assay did not always produce results within two-fold. It is questionable that two-fold accuracy is worthy of mentioning as a prominent feature of the assay due to its very broad range. The protocol is labor intensive and not adaptable to high throughput in the current 96-well format due to several critical timing steps.

Despite the failure of the assay to possess all the characteristics of the ideal quantitation system, the assay with modifications can produce comparable quantitation to Quantiblot® (Applied Biosystems, Foster City, CA). As seen in this evaluation, it is obvious that the 96-well plate format needs to be modified to include a flash spinning of the plate after the one-hour incubation to force all condensation off the plate cover. As a

result of the unforgiving nature of the assay, automation of the pipetting and mixing might produce more consistent results. There are liquid handling systems, such as the Beckman-Coulter Biomek® 2000 Liquid Handling System, that are available that could eliminate human error due to those aspects. One laboratory has even used a different type of luminometer that has incorporated into it an incubator, shaker, and dispenser. This lab claims the Fluoroscan Ascent FL luminometer speeds up the entire AluQuant™ System while keeping the reaction conditions constant for all samples (13). The AluQuant™ System should also incorporate known standards to be processed with each plate as part of the kit that would not require any manipulation by an analyst to serve as controls. Also, it is advantageous to quantitate a sample extract using only one microliter of DNA extract; however, Promega needs to incorporate into the AluQuant™ Calculator program the capability to correct for dilutions made. Finally, the Turner Reporter™ Microplate Luminometer should come with a calibration 96-well plate that would give certain relative light units (RLUs) in particular wells; therefore, allowing the user to have confidence that the luminometer is functioning properly.

If Orchid Cellmark Dallas Forensic Laboratory were to implement the AluQuant™ Human DNA Quantitation System using the 96-well format, several additional studies would need to be performed that were not performed in this evaluation due to the difficulty obtaining reproducible and acceptable curves. First, the specificity of the assay should be examined using a variety of different species' DNA that might be present in forensic samples. Even though some of the samples tested in this evaluation were highly degraded, additional degraded samples should be tested. In addition,

different sample types (blood, swabs, etc.) should be quantitated to ensure the quantitation is not affected by sample type. Similarly, samples that have been extracted with different methods should be evaluated in order to determine if the assay will be inhibited by components in the extract. Also, experiments to shorten the amount of time to make the DNA standard dilution series should be performed because it took approximately 45 minutes to make the standard dilutions in this evaluation. As a result, it took approximately three hours to perform the entire assay when it could possibly take only two hours.

In order to implement the AluQuant™ Assay as examined in this evaluation, Orchid Cellmark Dallas would have to buy the Reporter™ Luminometer that costs $10,000. Due to inconsistencies observed with both the water bath and heat block, they could choose either one. However, they would probably use the water bath they already own. If they chose to purchase a heat block similar to the one used in the evaluation, it would cost approximately $750. Orchid would also need to purchase a centrifuge with a 96-well plate adapter that could be placed in a pre-polymerase chain reaction (PCR) room; a centrifuge similar to the one used in this evaluation costs approximately $2,500. Then, the AluQuant™ assay itself costs $595 for 400 determinations ($1.49 per determination). On the other hand, the quantitation system currently in place at Orchid, Quantiblot®, costs $200 for 480 tests ($0.42 per test). There are additional supplies needed with each Quantiblot® kit such as x-ray film and the chemiluminescent detection reagent. One hundred sheets of eight-inch by ten-inch x-ray film can be purchased for approximately $130, and Orchid typically places two blots per sheet of film. The

chemiluminescent detection reagent costs approximately $175. Therefore, with all costs considered for both systems, the two are more similar in price than it first appears with the exception of the initial start-up costs associated with the AluQuant™ System. In conclusion, although Quantiblot® certainly has disadvantages, AluQuant™ in the 96-well format appears to be a costly proposition that produces only comparable quantitations and offers too few advantages to become Orchid Cellmark's quantitation system of choice.

CHAPTER V

CHARTS, TABLES, AND FIGURES

Chart 1 Reproducibility of the First Reporter™ Microplate Luminometer

Mean net RLUs and standard deviation bars obtained from a standard curve dilution series scanned in triplicate.

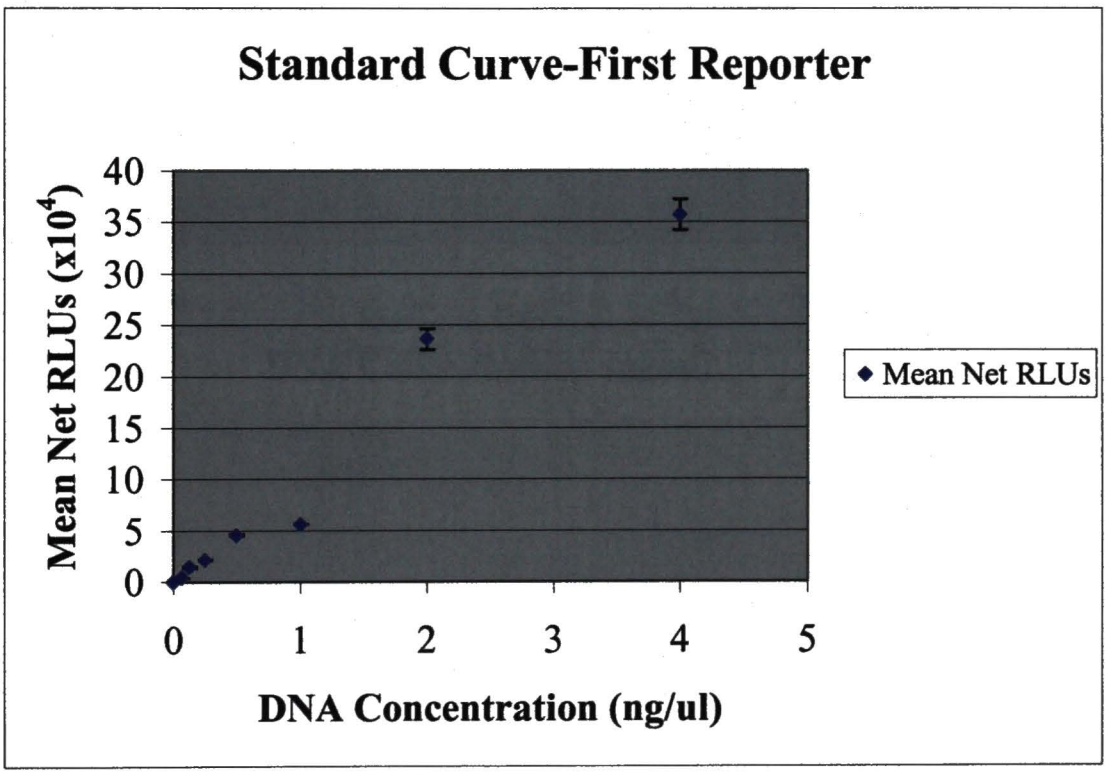


Chart 2 Reproducibility of the Second Reporter™ Microplate Luminometer

Mean net RLUs and standard deviation bars obtained from a standard curve dilution series scanned in triplicate.

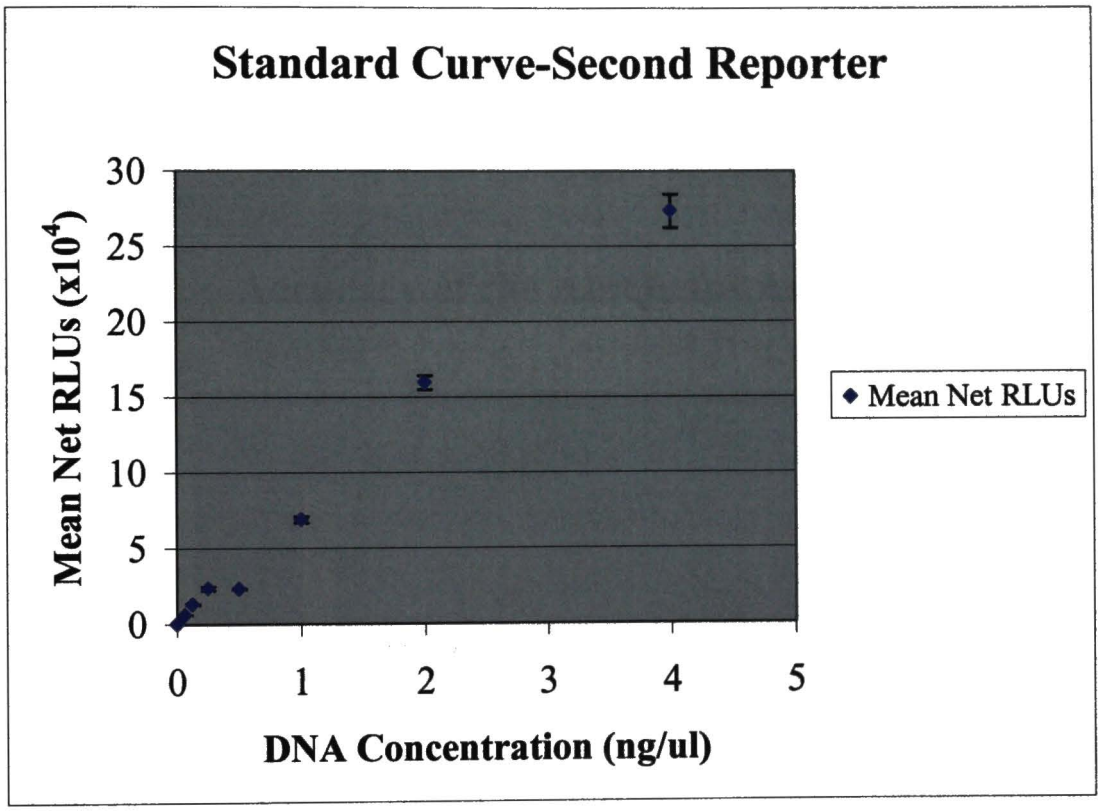


Chart 3 Accuracy of the AluQuant™ Assay

Three different manufacturer's standard DNA of different concentrations was used.

1. Applied Biosystems, K562, 10 ng/µl
2. Promega Corporation, 9947A, 10 ng/µl
3. Promega Corporation, 9947A, 0.1 ng/µl
4. American Type Culture Collection, 45514, 2.5 ng/µl

DNA concentrations charted from curves 1 and 2 represent the average of the standards quantitated in duplicate. DNA concentrations from curve 3 were run only once except for Promega's 9947A 10 ng/µl that was not run on curve 3.

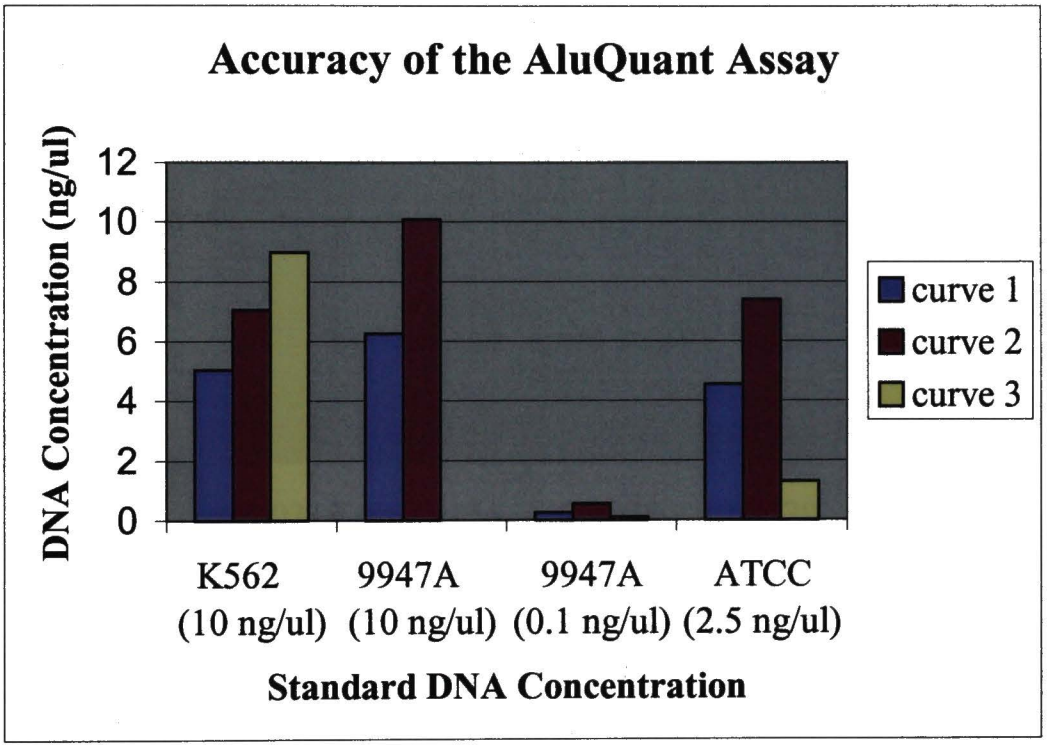


Chart 4 Sensitivity of the AluQuant™ Assay

The DNA standard dilution series created for the standard curve was extended from the lowest dilution by four more doubling dilutions that included 0.0313 ng/µl, 0.0156 ng/µl, 0.0078 ng/µl, and 0.0039 ng/µl to test the sensitivity of the assay.

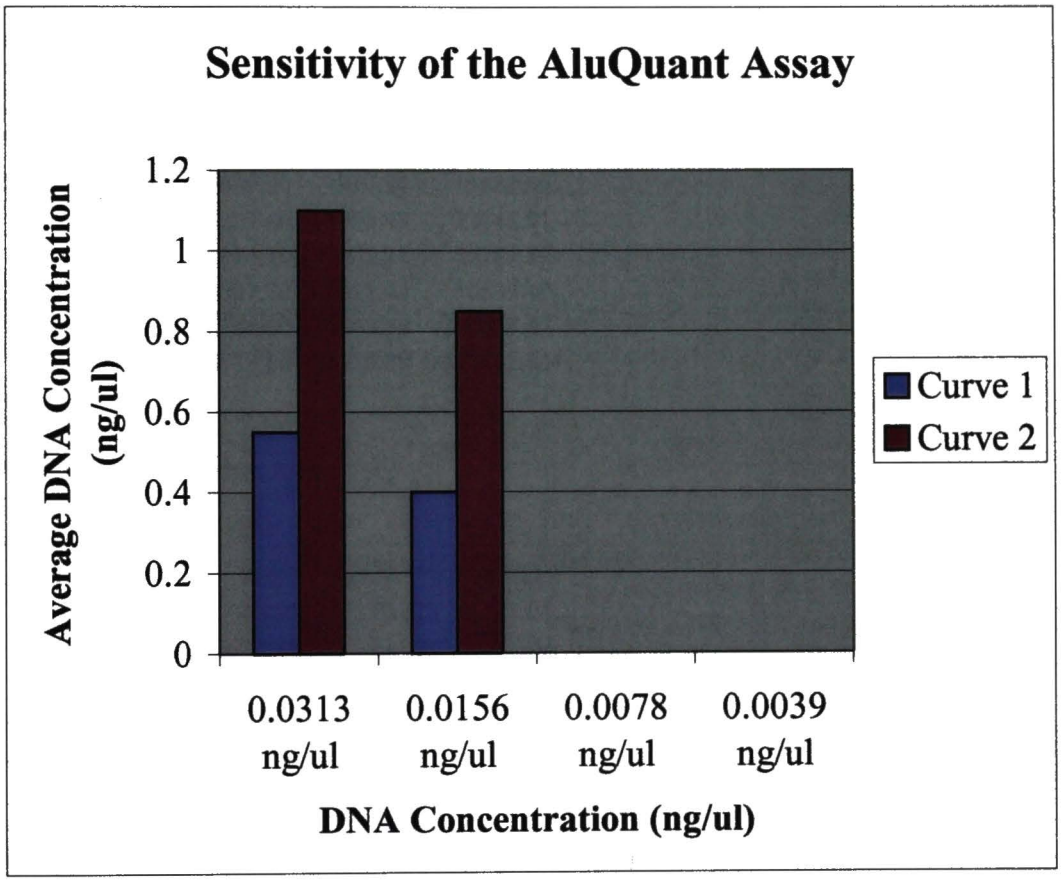


Table 1 Sensitivity of the First Reporter™ Microplate Luminometer

A. A standard curve dilution series with the extra sensitivity option selected.
B. A standard curve dilution series without the extra sensitivity option selected.
The numbers represent relative light units or RLUs.

A.

Extra Sensitivity Option: ON

DNA concentration (ng/µl)	MM*	MMC**	Net RLU
0	640.34	525.91	114.44
0.063	4636.27	465.00	4171.27
0.125	16679.30	997.34	15681.96
0.25	23237.40	1095.49	22141.91
0.5	47975.10	1187.21	46787.89
1.0	58027.70	1518.22	56509.48
2.0	252146.00	4668.53	247477.47
4.0	404973.00	32120.20	372852.80

B.

Extra Sensitivity Option: OFF

DNA concentration (ng/µl)	MM*	MMC**	Net RLU
0	548.03	170.02	378.01
0.063	5029.94	332.00	4697.94
0.125	14885.60	304.35	14581.26
0.25	31364.50	888.14	30476.36
0.5	49207.00	1048.69	48158.31
1.0	124703.00	1993.17	122709.83
2.0	241194.00	2869.91	238324.09
4.0	396294.00	5758.20	390535.80

*MM represents those columns containing Master Mix with probe.
**MMC represents those columns containing Master Mix Control without probe.

Table 2 Sensitivity of the Second Reporter™ Microplate Luminometer

A. A standard curve dilution series with the extra sensitivity option selected.
B. A standard curve dilution series without the extra sensitivity option selected.
The numbers represent relative light units or RLUs.

A.

Extra Sensitivity Option: ON

DNA concentration (ng/µl)	MM*	MMC**	Net RLU
0	1042.14	903.90	138.24
0.063	7394.79	994.79	6400.00
0.125	14556.10	1071.65	13484.45
0.25	25676.70	1051.15	24625.55
0.5	23767.60	470.73	23296.87
1.0	71482.00	967.91	70514.09
2.0	165250.00	1262.04	163987.96
4.0	285457.00	1729.35	283727.65

B.

Extra Sensitivity Option: OFF

DNA concentration (ng/µl)	MM*	MMC**	Net RLU
0	464.63	474.90	-10.27
0.063	4931.65	371.26	4560.39
0.125	9726.79	426.07	9300.72
0.25	23885.10	487.60	23397.50
0.5	25145.90	680.96	24464.95
1.0	58745.90	722.17	58023.73
2.0	149997.00	946.11	149050.89
4.0	294682.00	1306.56	293375.44

*MM represents those columns containing Master Mix with probe.
**MMC represents those columns containing Master Mix Control without probe.

Table 3 Sensitivity of the Reporter™ Microplate Luminometer-ATP Cross-talk

A. Represents layout of water, L/L, and ATP dilutions on 96-well plate.
B. The numbers are the RLU values given by the luminometer for the respective wells.

A.										
	water				L/L				water + L/L	
water	saturated ATP dilution	water		L/L	saturated ATP dilution	L/L		water + L/L	saturated ATP dilution	water + L/L
	water				L/L				water + L/L	
		water	L/L	water		L/L	water	L/L		
	water				L/L				water + L/L	
water	unsaturated ATP dilution	water		L/L	unsaturated ATP dilution	L/L		water + L/L	unsaturated ATP dilution	water + L/L
	water				L/L				water + L/L	

B.										
	433.78				415.93				649.44	
620.36	sat	469.28		407.59	sat	391.77		486.93	sat	751.19
	435.41				342.94				515.98	
		0	0	0		99.25	0	0		
	0				0				0	
0	14079.10	0		0	16115.90	0		108.73	18438.90	57.44
	0				0				0	

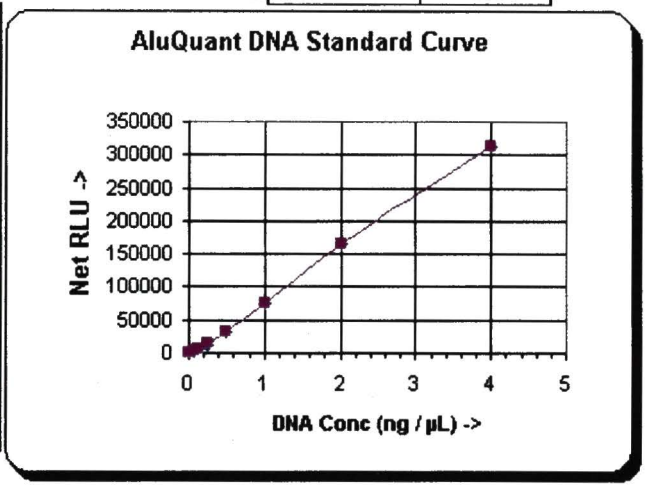
Table 4 AluQuant™ Concentration Versus Quantiblot® Concentration

Sample Name		AluQuant Concentration (ng/ul)		Quantiblot Concentration (ng/ul)
2594		2.200		1.250
2597		0.900		0.625
2622		2.300		2.500
2626		0.200		0.156
2627		1.200		1.250
2628		0.400		0.156
2633		2.000		5.000
2637		1.000		0.312
2676		1.100		3.000
2687		2.200		3.000
2691		1.100		5.000
2709		0.800		0.312
2735		0.000		0.000

AluQuant™
Human DNA Quantitation

Curve Fitting Statistics	Lower Curve	Full Curve
Std Error	1061.85	1002.49
RSquare	0.9972	0.9999
R	0.9986	1.0000

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	89.22	236.57	-147.35
0.063	3609.17	315.95	3293.22
0.125	4890.67	415.50	4475.17
0.25	12480.80	318.51	12162.29
0.5	34321.60	1117.73	33203.87
1	78704.50	2087.57	76616.93
2	168172.00	3808.97	164363.03
4	319972.00	6171.70	313800.30



Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	89.22	236.57	-147.35	0.00		
0.063 ng/ul	3609.17	315.95	3293.22	0.09		
0.125 ng/ul	4890.67	415.50	4475.17	0.12		
0.25 ng/ul	12480.80	318.51	12162.29	0.25		
0.5 ng/ul	34321.60	1117.73	33203.87	0.50		
1.0 ng/ul	78704.50	2087.57	76616.93	1.01		
2.0 ng/ul	168172.00	3808.97	164363.03	2.00		
4.0 ng/ul	319972.00	6171.70	313800.30	4.00		

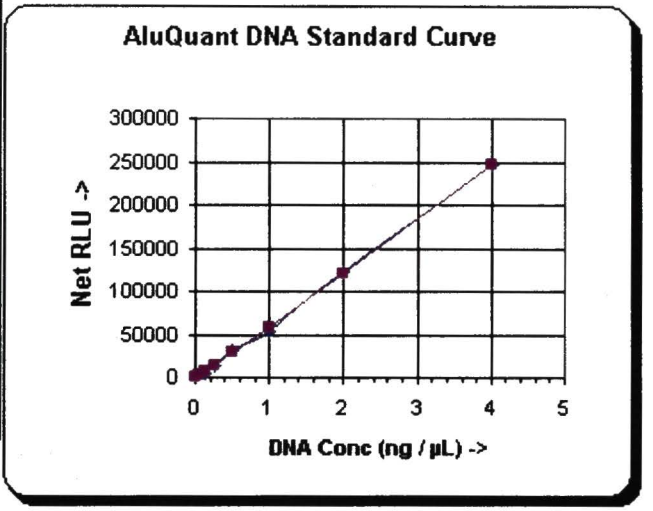
Figure 1 Quick Centrifugation of the Incubation Plate After One-Hour Incubation

Standard Curve 1 (Standard curve performed in duplicate on same plate.)

AluQuant™
Human DNA Quantitation

Curve Fitting Statistics	Lower Curve	Full Curve
Std Error	1273.42	3074.66
RSquare	0.9958	0.9991
R	0.9979	0.9996

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	268.45	364.58	-96.13
0.063	4155.76	401.71	3754.06
0.125	5003.95	395.76	4608.19
0.25	14753.00	667.96	14085.04
0.5	33231.10	1027.15	32203.95
1	55139.60	1664.69	53474.91
2	124334.00	2299.01	122034.99
4	249803.00	4228.13	245574.87



Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	268.45	364.58	-96.13	0.00		
0.063 ng/ul	4155.76	401.71	3754.06	0.09		
0.125 ng/ul	5003.95	395.76	4608.19	0.10		
0.25 ng/ul	14753.00	667.96	14085.04	0.26		
0.5 ng/ul	33231.10	1027.15	32203.95	0.50		
1.0 ng/ul	55139.60	1664.69	53474.91	0.92		
2.0 ng/ul	124334.00	2299.01	122034.99	2.03		
4.0 ng/ul	249803.00	4228.13	245574.87	4.00		

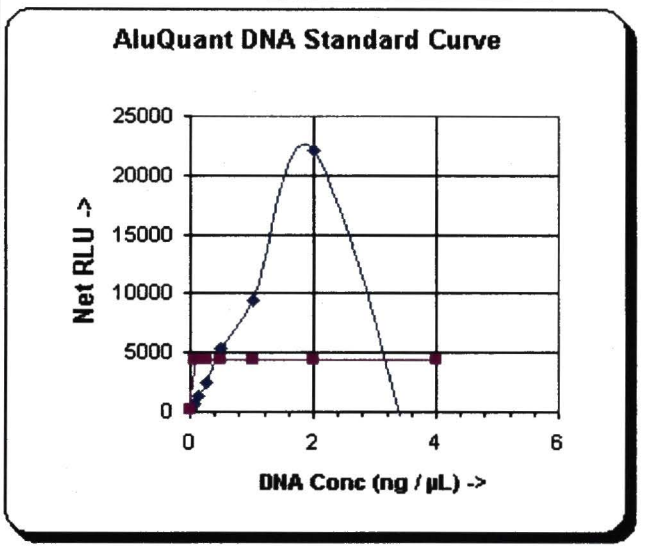
Figure 2 Quick Centrifugation of the Incubation Plate After One-Hour Incubation

Standard Curve 2 (Standard curve performed in duplicate on same plate.)

AluQuant™
Human DNA Quantitation

Curve Fitting Statistics	Lower Curve	Full Curve
Std Error	106.84	10950.73
RSquare	0.9989	0.0449
R	0.9994	0.2119

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	318.07	117.74	200.34
0.063	1416.62	766.02	650.60
0.125	3337.60	2077.23	1260.37
0.25	7232.19	4739.13	2493.06
0.5	17669.90	12294.70	5375.20
1	37798.70	28313.90	9484.80
2	77485.50	55410.90	22074.60
4	132669.00	143529.00	-10860.00



Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	318.07	117.74	200.34	0.01		
0.06 ng/ul	1416.62	766.02	650.60	0.07		
0.125 ng/ul	3337.60	2077.23	1260.37	0.13		
0.25 ng/ul	7232.19	4739.13	2493.06	0.25		
0.5 ng/ul	17669.90	12294.70	5375.20	4.05		
1.0 ng/ul	37798.70	28313.90	9484.80	4.05		
2.0 ng/ul	77485.50	55410.90	22074.60	4.05		
4.0 ng/ul	132669.00	143529.00	-10860.00	0.00		

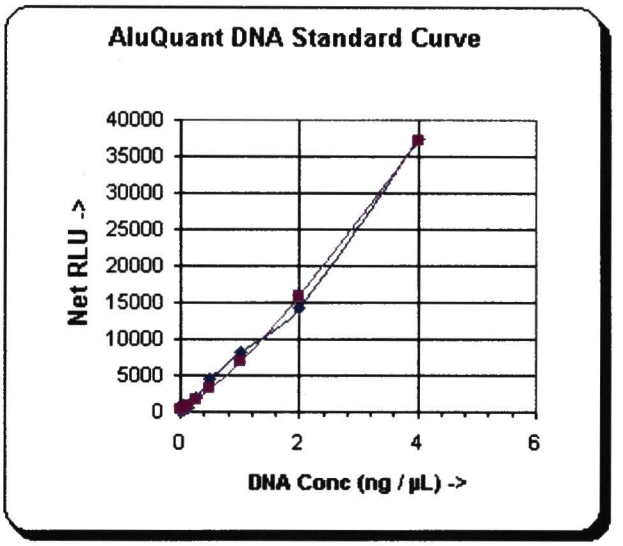
Figure 3 Typical Reproducibility of the Standard Curve Within an Experiment

Standard Curve1 (Standard curve performed in duplicate on same plate.)

AluQuant™
Human DNA Quantitation

Curve Fitting Statistics	Lower Curve	Full Curve
Std Error	59.88	1157.22
RSquare	0.9995	0.9944
R	0.9998	0.9972

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	333.93	312.79	21.14
0.063	853.27	717.35	135.92
0.125	2015.91	1361.71	654.20
0.25	4947.38	3225.21	1722.17
0.5	11600.00	7232.82	4367.18
1	25535.80	17318.10	8217.70
2	42615.30	28425.70	14189.60
4	96458.40	59030.10	37428.30



Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	333.93	312.79	21.14	0.02		
0.06 ng/ul	853.27	717.35	135.92	0.05		
0.125 ng/ul	2015.91	1361.71	654.20	0.13		
0.25 ng/ul	4947.38	3225.21	1722.17	0.25		
0.5 ng/ul	11600.00	7232.82	4367.18	0.50		
1.0 ng/ul	25535.80	17318.10	8217.70	1.15		
2.0 ng/ul	42615.30	28425.70	14189.60	1.82		
4.0 ng/ul	96458.40	59030.10	37428.30	4.00		

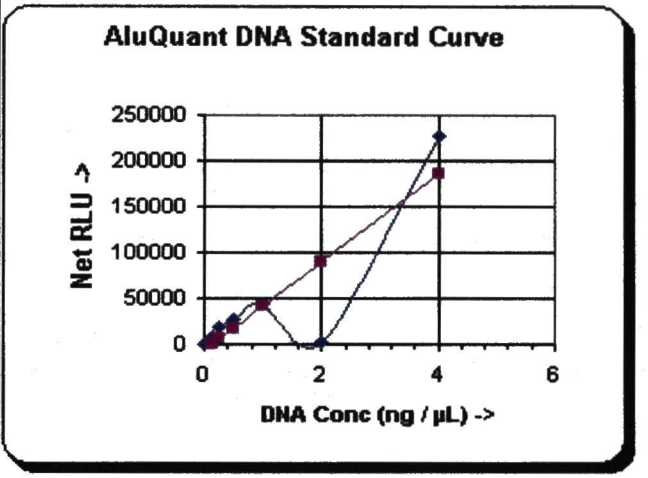
Figure 4 Typical Reproducibility of the Standard Curve Within an Experiment

Standard Curve (Standard curve performed in duplicate on same plate.)

AluQuant™
Human DNA Quantitation

Curve Fitting Statistics	Lower Curve	Full Curve
Std Error	39969.20	
RSquare	0.7639	
R	0.8740	

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	635.05	560.76	74.29
0.063	4314.25	569.67	3744.58
0.125	7792.82	468.62	7324.20
0.25	19343.40	497.27	18846.13
0.5	28804.80	2001.75	26803.05
1	47775.60	3315.19	44460.41
2	2871.34	34.30	2837.04
4	227069.00	1015.71	226053.29



Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	635.05	560.76	74.29	0.13		
0.063 ng/ul	4314.25	569.67	3744.58	0.21		
0.125 ng/ul	7792.82	468.62	7324.20	0.29		
0.25 ng/ul	19343.40	497.27	18846.13	0.53		
0.5 ng/ul	28804.80	2001.75	26803.05	0.69		
1.0 ng/ul	47775.60	3315.19	44460.41	1.06		
2.0 ng/ul	2871.34	34.30	2837.04	0.19		
4.0 ng/ul	227069.00	1015.71	226053.29	4.84		

Figure 5a Acceptability of Standard Curve

Unknowns						
Sample Name	**+ Probe**	**- Probe**	**Net RLU**	**DNA Conc. (ng / uL)**	**uL / PCR***	**Total Yield (ng)****
2594	17036.00	889.90	16146.10	0.47		
2597	6199.36	1315.74	4883.62	0.23		
2622	19204.30	1968.11	17236.19	0.49		
2626	1158.75	511.93	646.82	0.15		
2627	9525.65	1594.86	7930.79	0.30		
2628	1910.73	652.10	1258.63	0.16		
2633	15207.70	617.20	14590.50	0.44		
2637	7379.61	1620.78	5758.83	0.25		
2676	7476.54	1023.26	6453.28	0.27		
2687	17452.90	1229.05	16223.85	0.47		
2691	7932.49	1279.21	6653.28	0.27		
2709	5375.27	915.93	4459.34	0.23		
2735	711.77	468.19	243.58	0.14		
9974A 0.1 ng/ul	847.39	444.88	402.50	0.14		
ATCC 2.5 ng/ul	10020.70	1374.97	8645.73	0.31		
K562 10 ng/ul	45621.70	1569.46	44052.24	1.05		

Figure 5b Acceptability of Standard Curve

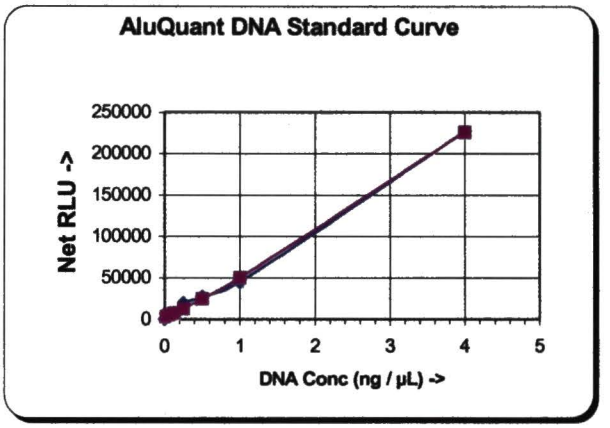
DNA concentrations of samples were calculated using the standard curve in Figure 5a. Sample DNA concentrations given above should be moved one decimal place to the right to correct for the ten-fold dilution made during the quantitation process by only quantitating 1 µl of the DNA extracts instead of 10µl.

AluQuant™
Human DNA Quantitation

ng / PCR Reaction	1.25
Max Template (uL	25
Elution Vol (uL)	100

Curve Fitting Statistics		
	Lower Curve	Full Curve
Std Error	1044.50	4676.19
RSquare	0.9964	0.9979
R	0.9982	0.9989

Standard Curve			
DNA Conc. (ng / uL)	+ Probe	- Probe	Net RLU
0	635.05	560.76	74.29
0.063	4314.25	569.67	3744.58
0.125	7792.82	468.62	7324.20
0.25	19343.40	497.27	18846.13
0.5	28804.80	2001.75	26803.05
1	47775.60	3315.19	44460.41
4	227069.00	1015.71	226053.29



squares represent calculated trendline
triangles represent data points

Unknowns						
Sample Name	+ Probe	- Probe	Net RLU	DNA Conc. (ng / uL)	uL / PCR*	Total Yield (ng)**
0 ng/ul	635.05	560.76	74.29	0.00	25.0	0.0
0.063 ng/ul	4314.25	569.67	3744.58	0.08	16.5	7.6
0.125 ng/ul	7792.82	468.62	7324.20	0.12	10.8	11.6
0.25 ng/ul	19343.40	497.27	18846.13	0.26	4.9	25.7
0.5 ng/ul	28804.80	2001.75	26803.05	0.49	2.5	49.2
1.0 ng/ul	47775.60	3315.19	44460.41	0.91	1.4	90.9
4.0 ng/ul	227069.00	1015.71	226053.29	4.00	0.3	400.0

Figure 6a Acceptability of Standard Curve

Unknowns						
Sample Name	**+ Probe**	**- Probe**	**Net RLU**	**DNA Conc. (ng / uL)**	**uL / PCR***	**Total Yield (ng)****
2594	17036.00	889.90	16146.10	0.22	5.8	21.7
2597	6199.36	1315.74	4883.62	0.09	14.0	8.9
2622	19204.30	1968.11	17236.19	0.23	5.4	23.2
2626	1158.75	511.93	646.82	0.02	25.0	2.4
2627	9525.65	1594.86	7930.79	0.12	10.2	12.2
2628	1910.73	652.10	1258.63	0.04	25.0	3.9
2633	15207.70	617.20	14590.50	0.20	6.4	19.6
2637	7379.61	1620.78	5758.83	0.10	12.6	9.9
2676	7476.54	1023.26	6453.28	0.11	11.7	10.6
2687	17452.90	1229.05	16223.85	0.22	5.7	21.8
2691	7932.49	1279.21	6653.28	0.11	11.5	10.9
2709	5375.27	915.93	4459.34	0.08	14.8	8.4
2735	711.77	468.19	243.58	0.00	25.0	0.0
9974A 0.1 ng/ul	847.39	444.88	402.50	0.01	25.0	1.4
ATCC 2.5 ng/ul	10020.70	1374.97	8645.73	0.13	9.6	13.0
K562 10 ng/ul	45621.70	1569.46	44052.24	0.90	1.4	90.1

Figure 6b Acceptability of Standard Curve

DNA concentrations of samples were calculated using the standard curve in Figure 6a. Sample DNA concentrations given above should be moved one decimal place to the right to correct for the ten-fold dilution made during the quantitation process by only quantitating 1 µl of the DNA extracts instead of 10µl. In addition, the decimal point must be moved one place to the left to determine the amount of DNA to place into the PCR reaction. Finally, assuming 100 µl was the elution volume of each sample the Total Yield in ng must be moved one decimal place to the right to again correct for the dilution.

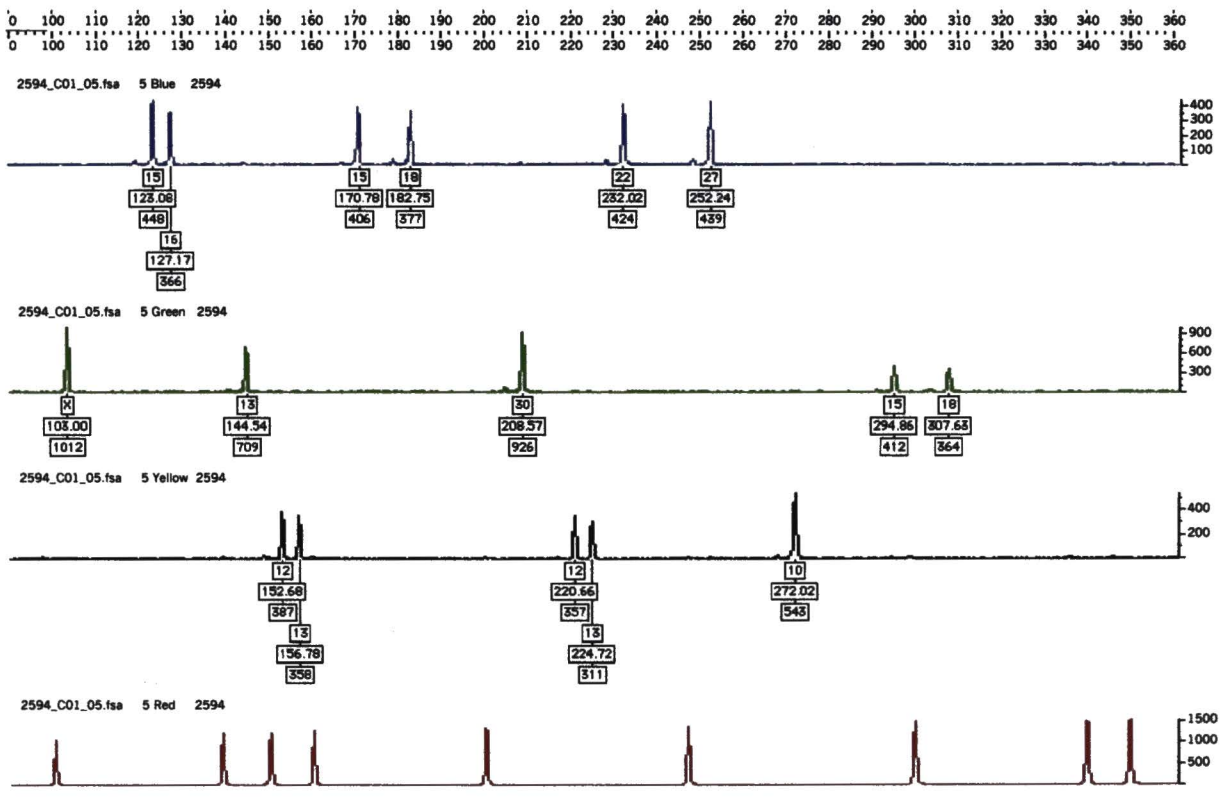


Figure 7a Example of Consistent Amplification

This sample yielded 2.2 ng/µl with AluQuant™.

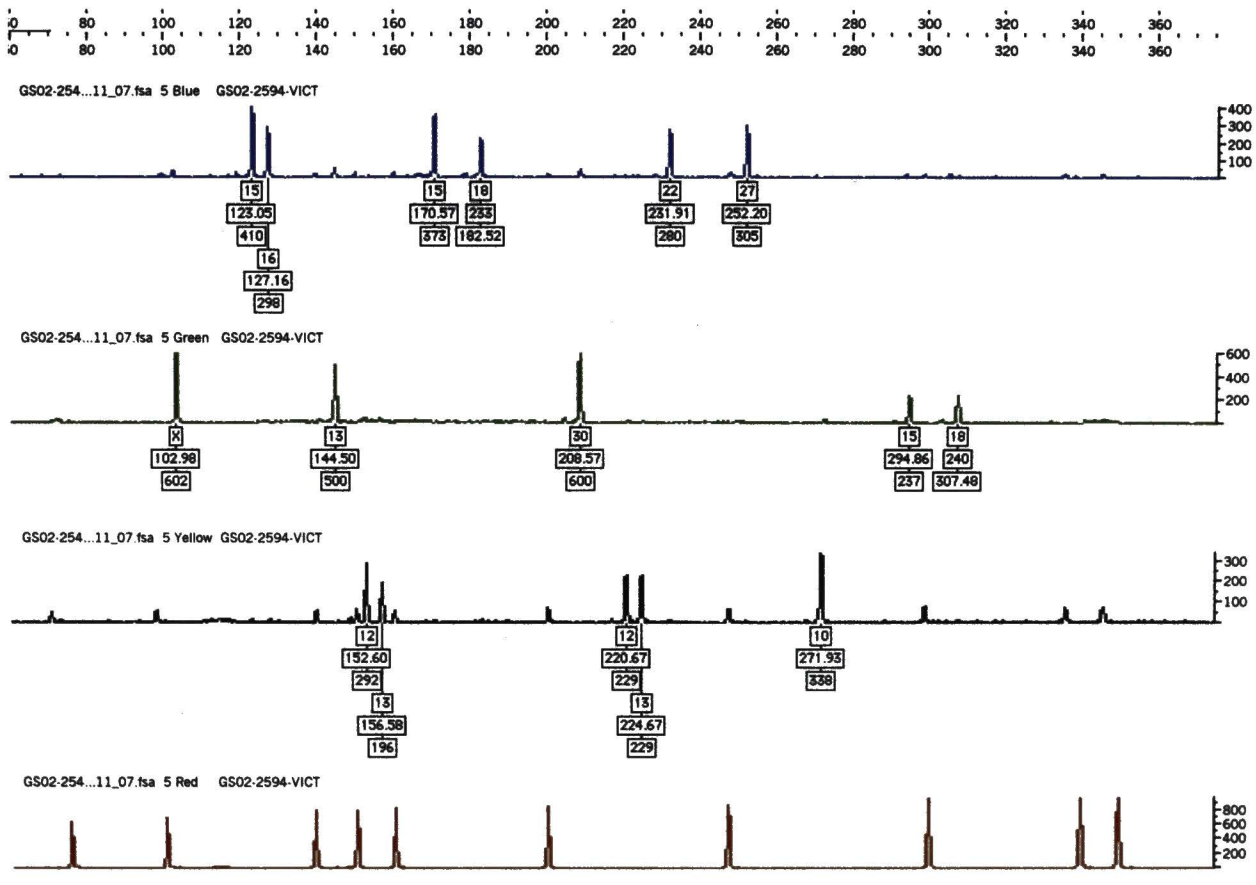


Figure 7b Example of Consistent Amplification

This sample yielded 1.25 ng/µl with Quantiblot®.

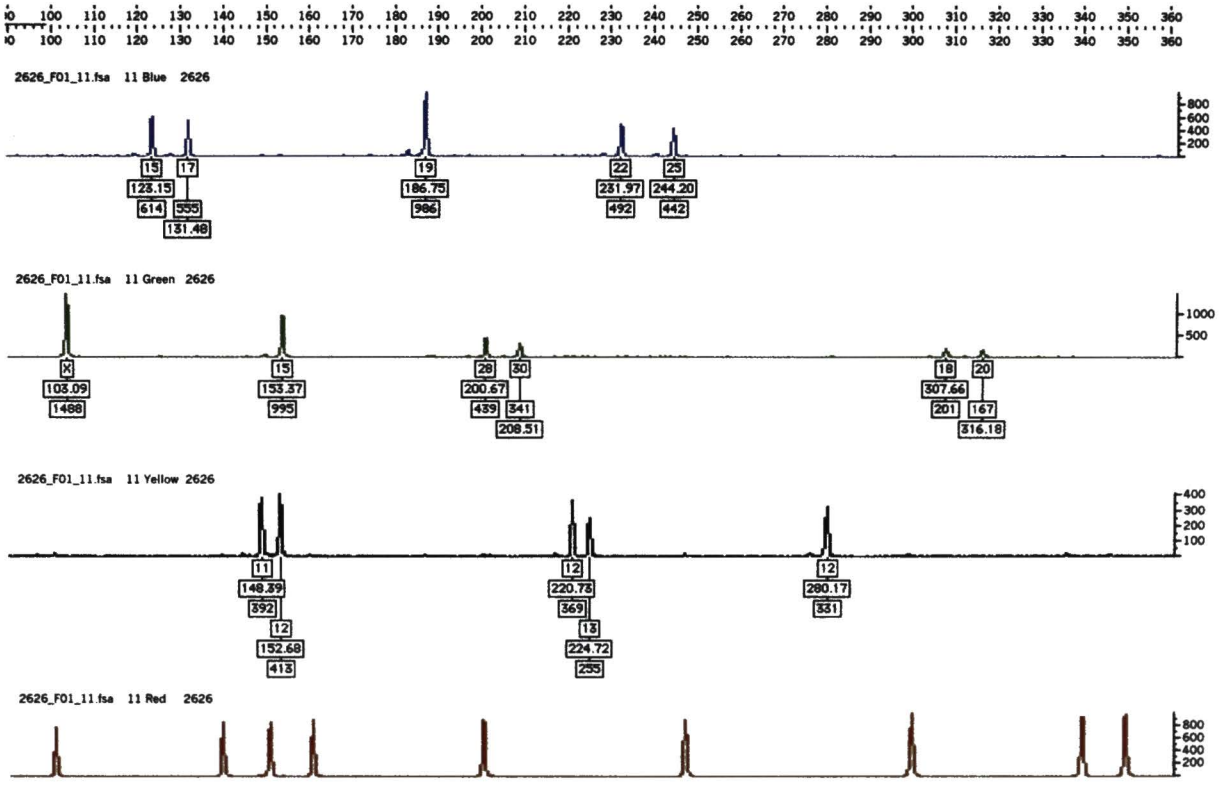


Figure 8a Example of Consistent Amplification

This sample yielded 0.20 ng/µl with AluQuant™.

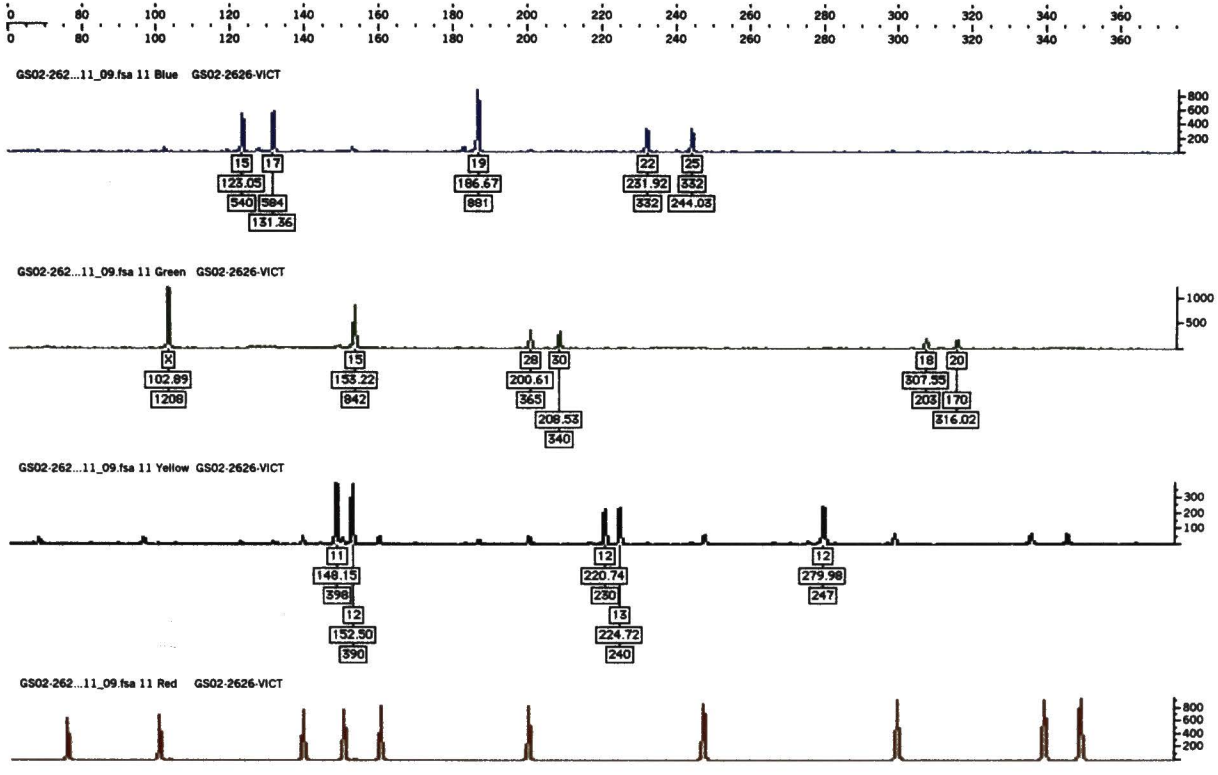


Figure 8b Example of Consistent Amplification

This sample yielded 0.156 ng/µl with Quantiblot®.

APPENDIX A

TECHNICAL BULLETIN NO. 291

AluQuant™ Human DNA Quantitation System

Promega

Technical Bulletin No. 291

INSTRUCTIONS FOR USE OF PRODUCT DC1010 and DC1011. *PLEASE DISCARD PREVIOUS VERSIONS.*
All technical literature is available on the Internet at www.promega.com
Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

I. Description

In human forensic identification, highly sensitive PCR multiplex assays are commonly used for genotyping biological samples. These genotyping assays function optimally with a relatively narrow range of template DNA, necessitating accurate and consistent DNA quantitation. However, the quantitation and amplification process may be complicated by the presence of DNA from other species. Quantitation of human DNA in a sample can be distinguished from other species DNA through the use of human-specific DNA probes. Use of such probes often requires immobilization of the target DNA on a solid support, and hybridization of the probe to the DNA followed by a series of washing steps at various stringencies to remove unbound probe. This process can be time consuming, produces variable results and has a limited quantitation range.

The AluQuant™ Human DNA Quantitation System[a] uses specific DNA probes and does not require target immobilization or washing steps. This system uses a probe that is specific to repetitive human elements, allowing quantitation without PCR amplification. Additional specificity is provided by the fidelity of DNA polymerase in recognition of perfect hybrids. Consequently, the AluQuant™ Human DNA Quantitation System has been shown to be unaffected by the presence of DNA from other species even when the DNA is in 10-fold excess of the human DNA. The optimal quantitation range of this system with the suggested protocol is 0.02–4ng/µl (with a maximum of 10µl of sample/reaction). The signal-to-DNA quantity relationship remains proportional above this range but accuracy decreases.

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Quantitation of human DNA by the AluQuant™ Human DNA Quantitation System is provided by a series of reactions (Figure 1). Following an initial denaturation, DNA samples are incubated with the AluQuant™ Enzyme Solution and the human-specific AluQuant™ Probe. The AluQuant™ Enzyme Solution contains READase™ Polymerase and READase™ Kinase. This coupled enzymatic reaction produces ATP relative to the amount of human DNA present. In a second incubation, ATP produced in the first reaction is used by luciferase to produce a proportional and measurable amount of light. Background noise is determined by concurrent analysis of the sample without the human-specific probe. The quantity of DNA can then be calculated through comparison of the signal to standards of known DNA quantity. Refer to Section VII for more information on the theory of the AluQuant™ Human DNA Quantitation System.

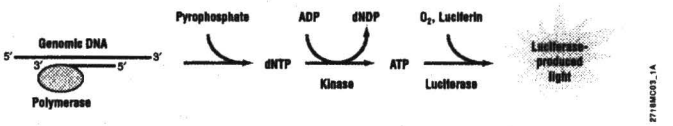

Figure 1. Detection of human DNA using the AluQuant™ Human DNA Quantitation System.

The general protocol for the AluQuant™ Human DNA Quantitation System consists of the following steps:

1. Prepare DNA dilutions to be used as standards.
2. Prepare the Luciferase/Luciferin (L/L) Reagent.
3. Prepare two Master Mixes (one with and one without the AluQuant™ Probe).
4. Denature samples and DNA standards.
5. Neutralize, then incubate samples and DNA standards with the Master Mixes.
6. Transfer the sample or standard reaction into L/L Reagent.
7. Measure light output in a luminometer.
8. Calculate DNA concentration using the AluQuant™ Calculator.

II. Product Components

Product	Size	Cat.#
AluQuant™ Human DNA Quantitation System	400 determinations	DC1011
	80 determinations	DC1010

Not For Medical Diagnostic Use. Cat.# DC1010 contains sufficient reagents for 80 determinations. Cat.# DC1011 includes:

- 10 × 1.25ml AluQuant™ Enzyme Solution
- 12ml Denaturation Solution
- 6ml AluQuant™ Neutralization Solution
- 3ml AluQuant™ Probe Mix
- 25ml Nuclease-Free Water
- 12ml Hydrochloric Acid
- 10µg Human Genomic DNA Standard (20ng/µl)
- 5 vials ENLITEN® Luciferase/Luciferin (L/L) Reagent
- 5 × 12ml ENLITEN® Luciferase/Luciferin (L/L) Reconstitution Buffer
- 1 Protocol

Storage Conditions: Store all components at –20°C or as listed on labels. Freeze remaining reconstituted L/L Reagent in aliquots, at –20°C protected from light (up to two weeks) or –70°C.

Avoid multiple freeze-thaw cycles of the reconstituted L/L Reagent. Slight decreases in the light signal may be seen due to a gradual decline in luciferase activity.

III. Protocol for the AluQuant™ Human DNA Quantitation System

Note: An *Experienced User's Protocol* can be found at the end of this Technical Bulletin.

Materials to Be Supplied by the User

- luminometer (single tube or 96 well plate reader)
- luminometer tubes or plates
- 1.5ml microcentrifuge tubes and 0.2–0.5ml microcentrifuge/amplification tubes
- 55°C heating block, water bath or thermal cycler
- micropipettes
- vinyl or latex gloves
- TE (10mM Tris-HCL, 1mM EDTA [pH 7.4]), 0.1X TE or TE^{-4} (10mM Tris-HCl, 0.1mM EDTA [pH 7.4])
- **recommended:** ART® Aerosol Resistant Tips

Before You Begin

Gloves should be worn during the reconstitution of L/L Reagent and while performing the assay to avoid contamination with ATP.

Thaw the desired amount of system components at room temperature before starting the assay. Store the AluQuant™ Enzyme Solution on ice once thawed.

Label tubes for reactions (see Section III.D or Section III.E).

A. Human Genomic DNA Standard Preparation

1. Prepare **fresh** dilutions of the Human Genomic DNA Standard to obtain desired experimental concentrations. Use TE, 0.1X TE or TE^{-4} to make dilutions and vortex well between each dilution. Figure 2 provides an example scheme for generating dilutions.
2. Always include a negative control without genomic DNA.

Note: When diluting the Human Genomic DNA Standard, choose the buffer closest in composition to that used for your sample DNA.

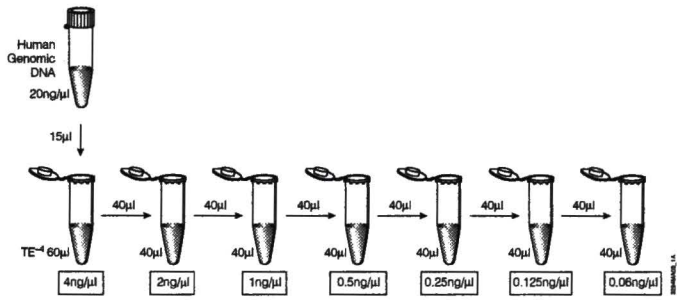

Figure 2. Recommended dilution scheme for generating the Human Genomic DNA Standard. When making dilutions, be sure to vortex between each dilution.

Note: If desired, an independent dilution of the Human Genomic DNA Standard can be prepared for use as a control. For example, to create a 1ng/µl control, mix 5µl of the Human Genomic DNA Standard and 95µl buffer.

Thaw Reconstitution Buffer at room temperature.

B. Luciferase/Luciferin (L/L) Reagent Preparation

1. Gently tap the vial of ENLITEN® L/L Reagent before opening to collect all of the dried material to the bottom of the vial.
2. Transfer 12ml of the ENLITEN® L/L Reconstitution Buffer into one vial of ENLITEN® L/L Reagent.
3. Replace the stopper carefully, and gently invert the vial several times to dissolve the contents.
4. Equilibrate the reconstituted L/L Reagent at room temperature for at least 60 minutes prior to use. Proceed to Section III.C while equilibration is taking place.

   Note: Luciferase activity is temperature-dependent; therefore, ensure that the temperature of the reagent does not fluctuate during a set of readings. After the reconstitution step is completed, the prepared L/L Reagent may be kept at room temperature during the course of the experiment (up to a total of 8 hours). Unused reconstituted reagent should be dispensed into aliquots in sterile microcentrifuge tubes and frozen at –20°C protected from light. Reconstituted L/L Reagent loses activity after being stored at room temperature for more than a total of 8 hours, including the time for each freeze-thaw.

C. Master Mix Preparation

Note: Prepare the Master Mix solutions just prior to use.

1. Determine the number of reactions needed for the samples and a standard curve. There should be two reactions for each sample and standard, one reaction with probe and one control reaction without probe.
2. Prepare both Master Mix solutions using Table 1 as a guide. Mix each of the reagents well by vortexing or inversion before use.

Do not store Master Mix solutions for more than one hour.

3. Vortex the Master Mix solutions and keep **on ice** until ready to use. Once mixed, do not store solutions for more than 1 hour. Vortex the Master Mix solutions before dispensing.

Table 1. Volume of Master Mix Components Required by Number of Reactions (n)*.

Component	Master Mix (with probe)	Master Mix Control (without probe)
AluQuant™ Neutralization Solution	n × 5µl =	n × 5µl =
AluQuant™ Probe Mix	n × 5µl =	
Nuclease-Free Water		n × 5µl =
AluQuant™ Enzyme Solution	n × 10µl =	n × 10µl =
Total Volume	n × 20µl =	n × 20µl =

*Make extra Master Mix to allow for losses during pipetting.

D. AluQuant™ Human DNA Quantitation System Assay—Single-Tube Luminometer (Figure 3)

For instructions on the use of 96 well luminometer plates refer to Section III.E.

1. Label three tubes (0.2 or 0.5ml tubes) per sample or standard: One for the denaturation step, the second for the sample to be treated with the Master Mix (with probe), and the third for the Master Mix Control sample (without probe).

   Note: Incubate all samples, including the DNA standard reactions, for the same period of time.

2. Pipet 5µl of the Denaturation Solution (NaOH) into each of the labeled denaturation microcentrifuge tubes.
3. To each denaturation tube, add 10µl of the appropriate sample or DNA standard. A smaller amount of sample diluted to a volume of 10µl can be used.
4. Vortex gently and incubate the tubes at room temperature for 10 minutes.
5. While samples are incubating, pipet 20µl of the Master Mix (with probe) to the appropriately labeled reaction tubes and 20µl of the Master Mix Control (without probe) to the control labeled reaction tubes.
6. Add 10µl of Hydrochloric Acid to each of the denaturation tubes (from Step 4) and vortex briefly or pipet to mix. **Do not** add Hydrochloric Acid directly to the Master Mix.
7. Transfer 10µl from the denaturation tubes to each of the Master Mix reaction tubes.
8. Vortex briefly to mix, and centrifuge briefly to spin down liquids.
9. Tightly cap the tubes and incubate at 55°C for 60 minutes in a heat block, water bath or thermal cycler. It is important to ensure that efficient thermal transfer occurs. If using a heat block, make sure the tubes fit well and have good contact with the block.
10. Set up the same number of luminometer tubes as reaction tubes. Place 50µl of reconstituted L/L Reagent into each luminometer tube. This may be set up during the last 15 minutes of the incubation step.
11. After incubation, move the reaction tubes to room temperature to cool briefly then spin each tube to collect condensate.
12. Transfer 25µl of the reaction to the luminometer tube. Vortex briefly, then tap the tube to collect all liquid to the bottom.
13. Place the tube in the luminometer and initiate reading. The number given by the luminometer is in Relative Light Units (RLU).
14. Repeat Steps 12–13 for each tube. Read the results **immediately** upon mixing.
15. Proceed to Section IV for calculating results.

    Note: The maximum concentration calculated for a sample by the AluQuant™ Calculator is determined by the highest DNA Standard concentration (i.e., 4ng/µl). For samples of higher concentration the result will read ">4ng/µl".

Important: The Denaturation Solution bottle needs to be tightly sealed when not in use.

Do not exceed 20 minutes for denaturation.

Make sure that the thawed reconstituted L/L is at room temperature before use.

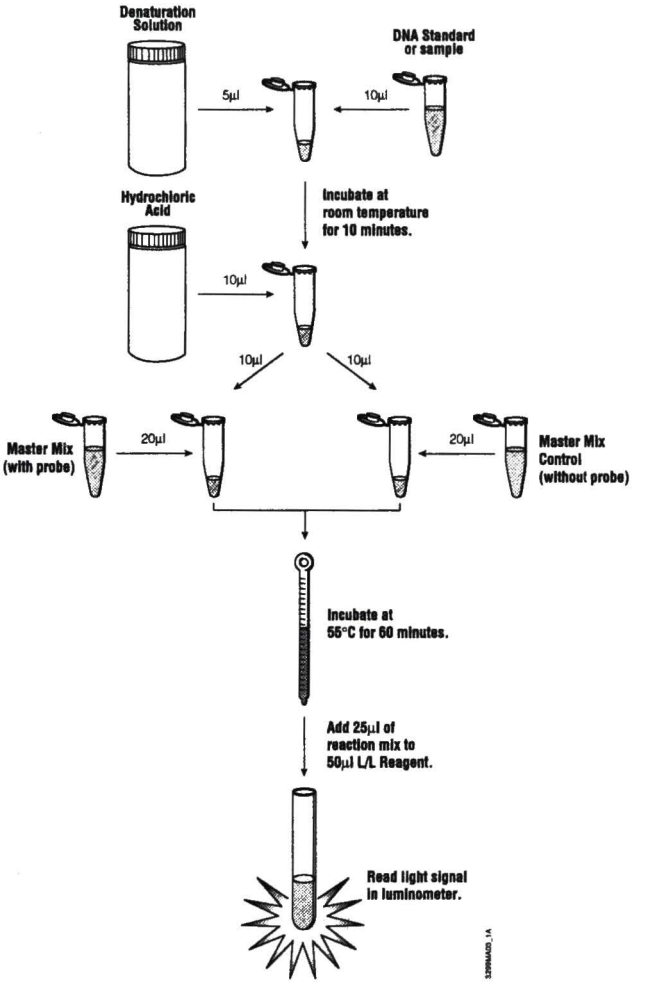

Figure 3. Schematic diagram of the AluQuant™ Human DNA Quantitation System.

E. AluQuant™ Human DNA Quantitation System Assay—Turner Reporter™ Microplate Luminometer (Cat.# E5030)

The following protocol describes the use of the AluQuant™ Human DNA Quantitation System using 96 well luminometer plates and the Turner Reporter™ Microplate Luminometer. It is possible to perform the assay in single tubes as described in Section III.D or thermal cycler strip tubes, then transfer reactions to a 96 well plate for reading in the Reporter™ luminometer as described below.

1. Label one well per sample or DNA standard on a plate designated for the denaturation step. Label two wells per sample or DNA standard on a second plate (Robbins Scientific CyclePlate® or equivalent) designated for the Master Mix and Control reactions.

   Note: For ease of data transfer to the AluQuant™ Calculator, arrange the Master Mix reactions in the odd-numbered columns and the Master Mix Control reactions in the even-numbered columns. Place the DNA standards in columns 11 and 12 with the lowest concentration in row A and highest in row H.

2. Pipet 5µl of the Denaturation Solution (NaOH) into each of the labeled denaturation wells.

3. To each denaturation well, add 10µl of the appropriate sample or DNA standard. A smaller amount of sample diluted to a volume of 10µl can be used. Mix each sample while adding by pipetting.

4. Incubate the tubes at room temperature for 10 minutes.

5. In the second plate, pipet 20µl of the Master Mix (with probe) to the appropriately labeled reaction wells and 20µl of the Master Mix Control (without probe) to the control labeled reaction wells.

6. Add 10µl of Hydrochloric Acid to each of the denaturation wells (from Step 4) and pipet to mix.

7. Transfer 10µl from the denaturation wells to each of the Master Mix and Master Mix Control wells (Figure 4) and seal the plate (Robbins Scientific CycleFoil® Plate Sealer).

8. Incubate the tubes at 55°C for 60 minutes in a water bath or thermal cycler. Use tubes or plates that ensure even heat transfer during this step.

9. Remove the tubes to room temperature. Gently tap the tubes to drop any condensed liquid to the bottom.

10. Transfer 50µl of reconstituted L/L Reagent (from Section III.B) to a 96 well luminometer plate. The number of wells with L/L Reagent should be the same as the number of samples. (If a luminometer that automatically injects L/L Reagent is used, transfer the reactions to the luminometer plate and follow the luminometer instruction manual for L/L Reagent addition.)

11. Mix the contents of the Master Mix sample wells and transfer 25µl to the corresponding wells in the luminometer plate. If a multichannel pipettor is used, check that all tips are pipetting the correct volume. Mix the contents by pipetting in the wells avoiding bubbles.

    Note: To maintain relative strength of light signal across the plate, all samples should be transferred to the luminometer plate within 5 minutes from start to finish.

Important: The Denaturation Solution bottle needs to be tightly sealed when not in use.

Do not exceed 20 minutes for denaturation.

Make sure that the thawed reconstituted L/L is at room temperature before use.

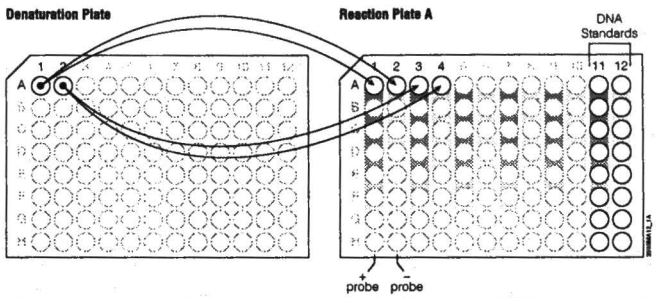

Figure 4. Schematic diagram illustrating transfer of denatured DNA to reaction plate containing master mix.

12. Immediately place the plate in the Reporter™ Microplate luminometer and initiate read.

    Note: The luminometer can be set to read only preselected wells.

13. Save the results and proceed to Section IV.

IV. Calculation of DNA Concentration

Note: Other DNA quantitation methods may produce different results when compared to the AluQuant™ System due to variations in the quantitation standards and chemistry used. First time users of the AluQuant™ System may need to optimize the calculated quantity of template DNA needed to maintain the desired STR signal intensity.

The first time the AluQuant™ Calculator (Cat.# DG2940 [PC Version*]) is used: Transfer the file "MezMATH32.dll" to the C:\windows\system32 subdirectory. Depending on the operating system, it may be called windows or winnt, etc. If there is no "system32" folder, then transfer the file to the "system" folder. The "AluQuant.xls" file can be transferred to the same or a different folder on the computer.

1. Open the AluQuant™ Calculator by double-clicking on "AluQuant.xls", then select "enable macros".

   Note: Macros must be enabled for the AluQuant™ Calculator to function. Low and high Excel security settings may not prompt the "enable macros" option.

2. Enter the case, analyst and date information as desired on the top of the worksheet.

3. Enter the DNA standard concentrations (ng/µl) in the Standard Curve section (or use the default concentrations). List in order from **lowest to highest** concentration. Enter the data from the Master Mix reactions into the column labeled "+probe" and the Master Mix Control reactions into the "–probe" column. There must be **at least** 5 different concentrations of DNA including the negative control (0ng/µl). Do not skip any cells when entering data.

   *Macintosh® version available on request. Please contact Promega Technical Services (techserv@promega.com).

4. In the Unknown section, enter the sample names and data from the quantitated samples.

   Note: Data can be typed manually, copied and pasted, or imported directly into the worksheet:

   - **To copy data from an 8 × 12 displayed array:** Copy and paste data into cells A1–H12 of the Raw Data worksheet. Select "Transfer to Template" to transfer data to the AluQuant™ page. To label samples individually, choose the "No Sample Names" option from the pop-up selections. If a 96 well plate was used and the assay was performed in two reaction plates, choose the "Reaction Plate A" or "Reaction Plate B" option to insert the sample well names.
   - **To import data:** The "Import" function operates when using data saved in Excel format from a Labsystems Luminoskan® Luminometer. To use this function, select "Import" from the AluQuant™ page. Choose the Sample Name label option as described above then click on the Excel data file to automatically enter data into the Raw Data worksheet and the AluQuant™ page.

5. After all of the data is entered, select the "Calculate" button at the top of the page, and it will calculate the net RLU and the DNA concentration (ng/µl).

   Notes:

   a. The generated graph displays two curves. The blue curve connects the data points and the pink one is the calculated trendline.

   b. The DNA concentration of the unknown sample is determined by two trendline curves. The Lower Curve (not displayed on the graph) includes the first 5 DNA standard concentrations (e.g. 0–0.5ng/µl). The Full Curve (pink curve displayed on graph) includes all DNA standard points.

6. Use "Save As" with a descriptive name to save the data.

   Note: Do not use any letters or spaces with the RLU numbers or DNA standard concentrations. For example, do not use "2ng/µl" or " 2", simply use "2". The AluQuant™ Calculator will not recognize any numbers with a space or letter in the cell when determining the standard curve or the concentration of the unknown samples. The Sample Name column can contain letters, numbers and spaces.

   Additional Calculator Features:

   - To determine the volume of quantitated sample required for subsequent amplification reactions, enter the amount of template DNA (ng) required per PCR tube and the maximum volume of template (µl) to be added in the upper right hand corner of the worksheet.
   - To determine the total yield of DNA, enter the elution volume (if it is the same for all quantitated samples) in the upper right hand corner of the worksheet. If quantitated samples were diluted, the dilution factor will need to be taken into consideration.

*RLU = Relative Light Units, which is the number generated by the luminometer.

Important: To use the Raw Data worksheet, the Master Mix results must be in the odd numbered columns, the Control result in the even numbered columns and the DNA standards in columns 11–12 in ascending order of concentration.

Note: When the DNA concentration is low or zero, the maximum template volume will be listed. Always check the DNA concentration so that you are aware when the listed volume may contain less DNA than desired.

Promega

For questions not addressed here, please contact your local Promega Branch Office or distributor. Contact information available at: **www.promega.com**.

E-mail: **techserv@promega.com**

V. Troubleshooting

Symptoms	Possible Causes	Comments
High background in sample but not in DNA standard reaction	Nucleotide contaminants in the sample	Clean up samples (e.g., ethanol precipitation).
High background in both the sample and the DNA standard reactions	Contamination of the reaction or the L/L Reagent	Read the L/L Reagent alone in the luminometer to insure that it is not contaminated (there should be little or no signal).
	Cross-contamination	Avoid splashing of samples; use a fresh tip each time when pipetting into tubes already containing reagents or DNA.
	Double-stranded DNA in the reaction	Make sure the Denaturation Solution was added and incubated for a minimum of 10 minutes. Try using a fresh bottle of Denaturation Solution.
	Reactions sitting at room temperature for long periods of time prior to incubation at 55°C	Incubate reactions in the heat bath/block as soon as possible.
Low signal	Loss of activity of the L/L Reagent	Read 10µl of 10^{-7}M ATP in 100µl of L/L Reagent. Do not use the reagent if the signal has dropped more than 25% relative to a freshly reconstituted one. Do not use reconstituted L/L Reagent if it has been at room temperature for a total of more than 8 hours.
	L/L Reagent not equilibrated to room temperature	Make sure that the thawed reconstituted L/L is at room temperature before use.
	AluQuant™ Probe Mix not added to Master Mix	Add the AluQuant™ Probe Mix to the "with probe" Master Mix. Do not add probe to the "without probe" Master Mix Control.
	Storage of prepared Master Mix at room temperature for >30 minutes	Use Master Mix promptly after preparation.
	Inhibitors present in the the DNA reaction	DNA purified using unwashed Chelex® resin or phenol/chloroform might have residuals that can inhibit the reaction. Add the unknown sample to a 1ng/µl DNA standard sample and repeat the experiment. If the value for the suspect sample is less than the 1ng/µl DNA standard inhibitors are present. Clean up the samples (e.g., ethanol precipitation).
	Wrong pH	Make sure that correct volumes of Denaturation Solution and hydrochloric acid were added.

V. Troubleshooting (continued)

Symptoms	Possible Causes	Comments
No signal differences between the Master Mixes (with and without probe)	AluQuant™ Probe Mix not added to Master Mix	Add the AluQuant™ Probe Mix to the "with probe" Master Mix. Do not add probe to the "without probe" Master Mix Control..
Insufficient volume in the reaction after incubation	Tubes were left open too long, allowing too much liquid to evaporate	Close reaction tubes to prevent evaporation if there is a long time delay before adding DNA to the Denaturation Solution.
	Pipetting error	Check calibration/accuracy of pipettes. Make sure tips are firmly secured to the pipette when using.
	Not all reaction components were added	Make sure DNA, Denaturation Solution, hydrochloric acid and Master Mix were added to the reaction mixture.
Wide variation between data points on the standard curve	Pipetting error	Check calibration/accuracy of pipettes. Make sure tips are firmly secured to the pipette when using.
	Inadequate mixing of DNA Standard	Vortex DNA Standard before removing the aliquots.
		Let DNA standard dilution sit for 5 minutes, revortex and remove an aliquot for the next dilution.
	Heat source variability	Make sure that all tubes have good contact with the heat source during incubation.
	Luminometer cuvette is at a variable height in the luminometer	Gently push the cuvette completely into the adapter each time.
Inconsistent concentration values	Insufficient mixing of high molecular weight DNA	To ensure homogenous solutions allow genomic DNA to thaw overnight at 4°C.
Error message: "the macros in this project are disabled"	Microsoft® Excel security settings is set on high	Change Excel security settings to medium. Security settings can be accessed under Tools/Macros/ Security.
Error message: "an error has occurred fitting this curve"	The "MezMATH32.dll" file is not installed	Transfer the file "MezMATH32.dll" to the C:\windows\system32 subdirectory.
Error message: "You entered standard	Standard entered in the wrong order	Re-enter standard in order from lowest to highest concentration.
concentrations in decreasing order, which can lead to invalid results. Please reorder increasing and restart the program"	A line was skipped when entering data	Re-enter data without skipping cells.

VI. Related Products

Product	Size	Cat.#
ENLITEN® rLuciferase/Luciferin Reagent	100 assays	FF2021

Promega

VII. Appendix

A. Overview of AluQuant™ Technology

The AluQuant™ Human DNA Quantitation System uses two incubation steps to measure the amount of human DNA (1). In the first incubation, a coupled enzymatic reaction takes place. The first reaction is known as a depolymerization reaction. This reaction is the reversal of the DNA polymerization reaction, where a DNA polymerase, known as the READase™ Polymerase, catalyzes the addition of a pyrophosphate across the 3′-terminal bond of double-stranded DNA. This addition results in the release of the 3′-terminal base from the DNA strand as a deoxyribonucleoside triphosphate (dNTP). The terminal phosphate of the released dNTP is then transferred to ADP to form ATP using the second enzyme in the reaction mixture, the READase™ Kinase. In the second incubation, the amount of ATP produced in the first reaction is quantitated by measuring the amount of light produced from the luciferase activity in the reaction (Figure 1).

Specificity

These reactions will take place with double-stranded DNA templates that are either blunt-ended or have a 5′-overhang end. To specifically measure the amount of human DNA in a sample, the target DNA is denatured in solution to form single-stranded target DNA. The reaction solution is prepared using human-specific probes (AluQuant™ Probe Mix), AluQuant™ Neutralization Solution and the AluQuant™ Enzyme Solution containing the READase™ Polymerase / Kinase enzyme mixture. In cases where the probe hybridizes to the target DNA with no mismatches, the READase™ Polymerase recognizes the hybrid as a substrate for depolymerization, and dNTPs are produced. However, if there are mismatched bases near the 3′-end of the probe-target DNA hybrid, the complex is not an effective substrate for the polymerase, and very few dNTPs are generated.

Background

Any double-stranded DNA template without mismatched bases near the 3′-end and either a blunt end or a 5′-overhang can be a substrate for the polymerase. If sufficient DNA is present in the denaturation reaction, some fraction of the DNA duplexes can reform and can give a signal in the absence of probe. Thus, measurement of the amount of human DNA in a sample uses two reactions: One reaction in the absence of added probe to determine the signal that is generated by reannealed double-stranded DNA (not probe specific) and a second reaction in the presence of probe to measure the combined signal from both the probe-target hybrids and the reannealed double-stranded DNA formed during rehybridization. The net signal of the probe/template for each sample or DNA standard is determined by subtracting the signal of the control reaction from the signal measured in the presence of probe.

Standard Curve

An example of a standard curve of the net signal produced from human DNA using the system is given in Figure 5. As illustrated, a linear relationship exists between the amount of human DNA present in the sample and the amount of net signal (net light units) measured using the system over a wide range of DNA concentrations. In general, 0–4ng/µl of human DNA will show a linear relationship. The relationship between net signal and input DNA remains proportional but is no longer linear above 4ng/µl of human DNA.

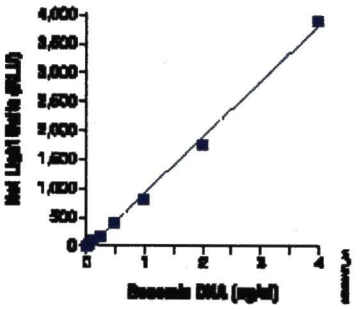

Figure 5. Example standard curve. Net signal produced using the AluQuant™ Human DNA Quantitation System.

B. Reference

1. Mandrekar, M.N. *et al.* (2001) Development of a human DNA quantitation system. *Croat. Med. J.* 42, 336–9.

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AluQuant™ Human DNA Quantitation System: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Section III) the first time you use this system.

DNA Standard, L/L Reagent and Master Mix Preparations (Sections III.A–III.C)

1. Thaw all reagents.
2. Prepare serial dilutions of the Human Genomic DNA Standard in TE, 0.1X TE or TE^{-4} (4, 2, 1, 0.5, 0.25, 0.12, 0.06ng/µl). Include a negative control (0ng/µl).
3. Prepare L/L by transferring 12ml of the ENLITEN® L/L Reconstitution Buffer to the vial of ENLITEN® L/L Reagent. Invert to mix and equilibrate for 60 minutes at room temperature.
4. Prepare Master Mix and Master Mix Control to analyze samples and standards using the following table:

Component	Master Mix (with probe) n reactions*	Master Mix Control (without probe) n reactions*
AluQuant™ Neutralization Solution	n × 5µl =	n × 5µl =
AluQuant™ Probe Mix	n × 5µl =	
Nuclease-Free Water		n × 5µl =
AluQuant™ Enzyme Solution	n × 10µl =	n × 10µl =
Total Volume	n × 20µl =	n × 20µl =

*Make extra Master Mix to allow for losses during pipetting.

5. Vortex Master Mixes and place on ice.

AluQuant™ Human DNA Quantitation System Assay (Section III.D)

1. Label tubes for analysis of samples and standards (three tubes for each analysis).
2. Pipet 5µl of Denaturation Solution to each denaturation tube.
3. Add 10µl of sample or standard to the correspondingly labeled tubes.
4. Vortex and incubate samples at room temperature for 10 minutes.
5. During incubation, pipet 20µl of Master Mix (with probe) or Master Mix Control (without probe) to appropriately labeled tubes.
6. Add 10µl of Hydrochloric Acid to each denaturation tube.
7. Vortex and centrifuge tubes briefly.
8. Transfer 10µl of denatured sample to each of the Master Mix tubes.
9. Incubate tubes at 55°C for 60 minutes. Move to room temperature when complete.
10. Place 50µl of prepared L/L in luminometer tubes (set up one tube for each reaction to be analyzed from Step 9).
11. Transfer 25µl of reaction mix to a luminometer tube, vortex briefly and read light output in luminometer. Repeat this step for each reaction.
12. Enter RLU values into AluQuant™ Calculator and calculate DNA concentration of unknowns. Refer to Section IV for calculating DNA concentrations.

APPENDIX B

LABORATORY NOTEBOOK / DAILY LOG

The purpose of this internship is to examine and evaluate the AluQuant™ Human DNA Quantitation System for possible implementation by Orchid Cellmark Dallas. Specifically, the project will focus on using the 96-well plate format. The evaluation will include examining the variables in the protocol for optimal performance by the quantitation system. Variables to concentrate on include: reproducibility and sensitivity of the Reporter™ Microplate Luminometer, reagent preparation, importance of constant 55°C temperature during the hour incubation, reproducibility of the standard curve, and the characteristics of an acceptable curve.

See notebooks for all Worksheets, Excel® Raw Data Spreadsheets, Calculator™ Excel Spreadsheets, and electropherograms.

3-7-02

- Meet with Judy Floyd, Technical Manager, to begin the evaluation of the kit. She gave me an initial orientation with my work area and the storeroom where supplies are located. The luminometer, computer, and water bath were set up and additional supplies (i.e. pipettes, tips, etc.) were gathered.
- Upon instruction by Judy [per request by Lisa Lane, Regional Technical Representative (405-364-6214)], I called Promega (1-800-356-9526) to speak with Kimberly Houston at ext. 1389, a Technical Representative, to try to determine what has been done and what needs to be done to streamline the process. Kimberly did not know what to tell me, so I asked her for any

information that would help someone that has never used the assay. She told me the following: (1) denaturation plates->any; 55°C incubation plates->Robbins Scientific CyclePlate® with cover; luminometer plates->white (opaque) not clear (2) 55°C exactly no variation not even ½°C (3) add L/L reagent to luminometer plate then add reaction mix not the other way around (4) L/L reagent and samples must be at room temperature (5) luciferase has a 15-minute half-life.

- I called Robbins Scientific (1-800-752-8585, Sunnyvale, CA) and spoke with Michael at ext. 183 in order processing. I asked him to send sample plates for me to try before buying; he will send me three purple CyclePlates® with CycleFoil® (aluminum sits on top-> adhesive hold) and CycleSeal® (plastic sheet-> heat seals). Michael stated a five-day delivery.
- I called Corning CoStar (978-635-2200, Corning, NY) and spoke with Sandy in order processing. I asked her to send me sample plates for me to try before buying; she will send me five white high throughput plates that fit the Turner Reporter™. Sandy stated a five-day delivery.

3-8-02

- Judy called me at home to discuss the procedure and told me to run standards next week if plates arrive. I asked her the following questions: (1) how to set trays in water bath? she will check on it (2) overall steps/goals? to come (3) timer, ice, and container? ask paternity (4) where to save data? hard drive (5) how to aliquot L/L? freezer storage box with foil around it, try using 2 ml tubes with 1.7 ml in

them (6) how many times do I do standards? twice (7) how many scans? three (8) any delay? zero (9) extra sensitivity? yes

3-14-02

- No plates have arrived. I checked with both Corning CoStar and Robbins; both say the plates are on the way.
- I worked on developing a worksheet in Excel® for use with the assay.
- The "Reporter™" software on the computer does not work. The "Calculator™" software seems to be fine.

3-21-02

- Plates arrived and the "Reporter™" software is loaded properly now.
- Experiment 1: "Standards 32102"

Purpose: To become familiar with the procedure. Two sets of standards were run to evaluate the reproducibility of the procedure. Three scans were performed to evaluate the reproducibility of the luminometer. The extra sensitivity option was chosen to determine the sensitivity of the luminometer.

Materials & Methods: ran 2 sets of the standards with 3 scans, zero delay, and the extra sensitivity option chosen; standards diluted with TE^{-4}; 3 different tray types recommended were used with the CycleSeal® cover; water bath used is a GeneMate model # 180051SC made by Lab-Line Industries (Melrose Park, IL)

****The same kit was used throughout the entire evaluation, so Lot #s etc. will not be listed each time!****

Assay-> Kit # DC1011 & Lot # 146465 with the following components:

Human Genomic DNA Standard	Lot #: 13404002
ENLITEN® L/L Reagent	Lot #: 13074901
ENLITEN® L/L Reconstitution Buffer	Lot #: 12867001
Neutralization Solution	Lot #: 13336502
Probe Mix	Lot #: 13329402
Nuclease Free Water	Lot #: 14255601
Enzyme Solution	Lot #: 13359202
Denaturation Solution (NaOH)	Lot #: 14433401
Hydrochloric Acid (HCl)	Lot #: 14433301
Not in the kit:	
TE^{-4} buffer pH 8.0	Lot #: 610502

Observations: need a multiple channel pipette for sure; the reagents take a long time to thaw; water bath varies from 55°C to 56°C to 57°C; timing is very tight and unforgiving throughout the procedure.

Results:

- the two standard curves were not precisely reproduced
- the luminometer read each set of standards with good reproducibility from what I understand especially considering the L/L reagent was certainly reaching its 15-minute half-life by the time the third set was finished being scanned
- the extra sensitivity option causes the read time to increase which would not be desirable with an already time sensitive assay -> the RLUs (relative light units) are at 300,000 to 400,000 -> so I am not sure what to conclude about the extra sensitivity option or its effects
- the first curve on all three sets has fairly good curve fitting statistics, all greater than 0.98

- if the 1 ng/μl point is taken out, an even better curve is obtained for all of the first curves
- the second curve on all 3 sets had a pipetting error at 2 ng/μl
- if 2 ng/μl point is taken out, a more acceptable curve is obtained on all of the second curves
- the calculated trend line is decent on curve one but obviously off on the second curve

3-22-02

- Joe Warren is to order more Robbins CyclePlates® and CycleSeal® for me.
- I aliquotted reagents.
- Experiment 2: "Standards 32202"

Purpose: To become more familiar with the procedure, improve pipetting, and obtain two acceptable curves. The standards were run in duplicate again to evaluate the reproducibility of the procedure. Three scans were performed to evaluate the reproducibility of the luminometer. The extra sensitivity was again selected to try to determine how sensitive the luminometer can detect.

Materials & Methods: ran 2 sets of the standards with 3 scans, zero delay, and the extra sensitivity option chosen; standards diluted with TE^{-4}; 3 different tray types recommended were used with a CycleSeal® cover; water bath used again; single pipette used again because the multi-channel given to me cannot pipette small enough amounts

Observations: a multi-channel pipette would still be very beneficial even though I obtained a straight line with a single pipette; I can save time by aliquotting reagents; the water bath still varies from 55°C to 56°C to 57°C on its own; timing is too critical rendering the assay not robust

Results:

- the two standard curves were more similar to each other than those in Experiment 1
- the luminometer scanned the standards three times with less reproducibility than in Experiment 1 -> keep in mind that the L/L reagent was certainly reaching its 15-minute half-life by the time Set 3 was finished being scanned
- the RLUs were from 250,000 to 400,000 this time -> until I do a run without the extra sensitivity option, I cannot make any conclusions about its effect
- the first curve on all three sets was acceptable (possibly) -> I would like to see the calculated trend line closer to the actual data points; *R*-values were greater than or equal to 0.98
- the second curve on set one is certainly the best I have seen with *R*-values = 1.0 -> with each successive set scan the curve fitting statistics decrease slightly but never below 0.9998; the second curve has a great trend line because I cannot tell the difference between the curve and the trend line

- I discovered I must click on the "Calculate" button on the screen each time new data is imported even though one already appears (it modifies the previous one but does not recalculate)

3-28-02

- Before proceeding onward, I spoke with Kimberly at Promega about the curves from 3-22-02. Kim said do not use the extra sensitivity option and only scan once (that is what Promega does). She also said that the RLU's between the first well read and the last well read will decrease especially with the extra sensitivity option selected because that option increases the read time per well.
- I clarified with Promega that the kit number on the Calculator™ program refers to the catalog number on the kit and that the lot number on the kit corresponds to all lot numbers of all reagents.
- I received the 55°C plates today.

3-29-02

- I aliquotted reagents.
- Experiment 3: "Standards 32902"

Purpose: To gain more experience with the assay as well as the multiple channel pipette. To obtain two curves which are acceptable.

Materials & Methods: ran standards in duplicate with 1 scan, zero delay, and the extra sensitivity option off; standards diluted with TE^{-4}; 3 different tray types recommended were used with the CycleSeal® cover; water bath used; a multi-channel pipette used for the first time

Results:

- both curves are decent
- the first curve is almost straight with the calculated trend line on top of the plotted data points and *R*-values greater than 0.99
- the bottom part of the second curve is not as good with the trend line off somewhat -> the full curve statistics are greater than 0.99

- Experiment 4: "Test 32902"

Purpose: To evaluate the assay's performance with case samples.

Materials & Methods: 1 standard curve was run with 16 samples (first samples to be tested->samples were previously quantitated using Quantiblot® and were extracted with phenol/chloroform and precipitated with ethanol); 1 scan was performed with no extra sensitivity; the standards were diluted with TE^{-4}; 3 different tray types recommended were used with CycleSeal® cover; used the water bath and a multi-channel pipette; 10 µl of sample was used to quantitate

Observations: I must have reversed or switched the Master Mix and Master Mix Control columns on the standard curve because, as is, no curve is plotted; if I flip flop results of the "+ probe" and the "- probe", then a curve is produced; it makes sense just looking at the raw data that the "+ probe" values should be greater than the "- probe" values; due to the failure of the standard curve, I should have run two curves; even though I used previous acceptable curves to analyze the samples due to the unacceptable nature of the curve ran with the samples, this could not be standard

practice because the curve serves as a control; this was done only to get an idea of how the AluQuant™ and Quantiblot® values compare.

Results:

- a true comparison between AluQuant™ and Quantiblot® values should not be made due to the unacceptable nature of the curve ran with the samples
- I believe something happened at both 2 ng/µl and 4 ng/µl....they both have extremely high background or "- probe" values relative to their "+ probe" values
- as stated in the Observations section, it would not be acceptable to routinely use previous curves to analyze samples -> I believe the results obtained might be revealing...they are significantly lower than those obtained with Quantiblot®

4-4-02

- I spoke with Judy about results from 3-29-02, and together we came up with questions for me to ask Kim at Promega: (1) how do I know if I have high background in a sample or standard and what are reasonable "- probe" values? "+ probe" = 10,000 RLUs and "- probe" = 9,000 RLUs this would be considered high but if the RLUs were 10,000 and 100 this would be considered not high (2) RLU so high still with no extra sensitivity and 1 scan? "luminometers vary" (3) denature more than 10 minutes? no (4) Quantiblot® values at 10 ng/µl and

AluQuant™ around 1 ng/μl? "quantitation methods are variable" (5) if override the system with too much DNA what is the effect? "curve tapers off at end"

- Kim at Promega also suggested going ahead and running samples through PCR and STR analysis with values given by AluQuant™ to see the difference in peak heights.
- I faxed to Kim samples from Exp.4: "Test 32902" with curve from 3/22/02.

4-5-02

- Kim from Promega called to recommend the following based on the data I faxed to her on 4-4-02: (1) run standard curve at the beginning and end of the plate to look for consistency throughout procedure (2) redo a few of the samples with high "- probe" values resulting in 0 ng readings or values (3) to those I redo add both sample and standard...5 μl of 2 ng/μl standard + 1 μl sample + 4 μl water for a total of 10 μl volume (4) dilute standards with water not TE^{-4} to check purity. Kim felt as though these suggestions would help pinpoint technique or sample related issues. She does not feel as though the high "- probe" values will repeat.
- Joe will order luminometer plates and the correct cover, CycleSeal®.
- I aliquotted reagents.
- Experiment 5: "Test 4/5/02"

Purpose: To follow Kim's suggestions listed above to see any effects on results as well as to identify and correct technique issues related to user error. Also, to check the purity of the TE^{-4} and to determine if consistency issues exist. To determine if the high "- probe" values are reproducible.

Materials & Methods: ran two sets of standards with 1 scan and no extra sensitivity; standards diluted with water; 3 different tray types recommended were used; used the CycleSeal® cover; used water bath; used multi-channel pipette; ran 8 samples as repeats -> first three of which were treated as follows: 5 µl of 2 ng/µl standard + 1 µl sample + 4 µl water for a total of 10 µl volume, therefore, at least 1 ng of DNA should be recovered on those samples; other five samples repeated were treated as follows: 1 µl sample + 9 µl water for a total of 10 µl volume

Observations: due to the unacceptable nature of both standard curves, the samples were analyzed using a previous acceptable curve from Exp.2: "Standards 32202"; again, I know this would not be normal protocol -> in order to evaluate the "- probe" values of the samples this was the only way to see if they repeated as high as they were previously

Results:

- obviously, sample values obtained from AluQuant™ and Quantiblot® cannot be compared due to the unacceptable nature of the curves they were run with
- both curves have problems at 0.063 ng/µl, 0.25 ng/µl, and 4 ng/µl
- the two standard curves ran to check consistency throughout the procedure only showed consistently inconsistent results
- water dilution of standards seemed to make no difference as compared to TE^{-4} dilutions

- the "- probe" values with the curve from Exp.2 on the first three samples that had previously had extremely high values was not repeated; it appears to me that the 1 ng/µl of DNA from the standard added to the samples was all that was recovered; they are all three approximately 1 ng/µl, Where is the sample?-> have to remember to correct for the dilution factor so really more than 1 ng/µl was recovered in fact on one sample 14 ng/µl was recovered where only 1 ng/µl of it is standard and it was previously quantitated with Quantiblot® to be 2 ng/µl ....??? I don't have an explanation
- I think these results show assay related issues not sample and not entirely technique
- examples of those samples where only 1 µl was quantitated without any standard added: 4.3 ng/µl and 5.9 ng/µl for 10 ng/µl samples quantitated with Quantiblot® and .1 ng/µl for 0 ng/µl sample per Quantiblot®

- did as Promega Representative Kimberly suggested in Experiment 5 -> still has not solved standard curve issues so I called Promega back and spoke with a different Representative (Abigail) who said the following (1) [I had aliquotted the standard DNA into tubes with just over what would be needed per curve -> approx. 16 µl] she said do not aliquot DNA but I thought repeated freeze/thaw of DNA was not good...she agreed finally and said to aliquot in larger volumes

(maybe 50 µl) but to first vortex for much longer (1- 2 minutes) than I had been doing (30 sec- 40 sec)...she will send me new DNA (2) are pipettes calibrated? (3) test TE^{-4} and water in luminometer by themselves to check for contamination (4) suggested luminometer problem if none of this helps (5) use another standard of DNA

4-11-02

- Spoke with Judy-> wants me to use the new DNA shipped from Promega with 4 samples from before that had been precipitated with ethanol and get 4 new samples that were cleaned using Microcon™-> said pipettes are calibrated-> I will test the TE^{-4} and water-> doubts the luminometer is the problem-> standards on curve serve as standards...if the curve does not work or is unacceptable no other standards tested would give any additional information at this point-> I mentioned water bath variability again
- I first tested stock solutions of TE^{-4} and water in the luminometer for contamination...the results were 000.00 and can be found on "AluQuant Quantitation Sheet" from 4-5-02...unfortunately due to the readings the computer program would not allow me to print the results or save them to an Excel® file
- Experiment 6: "Test 4/11/02"

Purpose: To follow Promega's and Judy's suggestions...test new DNA after more vortexing as standard dilutions are made. To test two different clean up extraction

procedures (ethanol vs. Microcon™) to see if either gives better results with AluQuant™. To obtain two consistent, reproducible, acceptable curves on the same plate.

Materials & Methods: ran 2 sets of standards with 1 scan, no extra sensitivity, using water bath, 3 tray types recommended, CycleSeal® cover, standards diluted in water, multiple channel pipette used, new DNA used, 8 samples were also run (4 ppt with ethanol and 4 cleaned up with Microcon™), samples prepared as follows: 1 µl of 1 ng/µl standard + 1 µl sample + 8 µl water = total 10 µl so should recover at least 0.1 ng/µl, vortexed new DNA 2 minutes to begin with followed by 30 sec vortex, 5 min sit, 30 sec vortex in between each dilution

Results:

- still have not obtained 2 identical curves on same plate…not reproducible or robust
- first curve appears acceptable to me…trend line is good…first believed only straight line is acceptable however I've only gotten 1 of those-> what is acceptable?-> "Certificate of Analysis" claims $R^2 >$ 0.98…how does this fit in? does that define acceptable?
- even though the first curve is acceptable (I think), the samples were analyzed with a previous standard curve from 3/22/02 (Experiment 2: Standards 32202) for the sake of comparison
- second curve does not have as good of a trend line but $R^2 > 0.98$ so is it acceptable or not?

- if the first curve is acceptable, one can compare the Quantiblot® to AluQuant™ values…overall they are different but some values are close to each other…Quantiblot® gave 10 ng/µl and AluQuant™ gave 5.4 ng/µl of which 0.1 ng/µl is standard however another example Quantiblot® gave 2.5 ng/µl and AluQuant™ gave 2.2 ng/µl …remember to correct for dilution factor
- new DNA showed no significant difference from original DNA
- no difference seen between ethanol ppt. samples and those cleaned with Microcon™

4-12-02

- Called Promega to ask the following questions: (spoke with another Technical Representative, Bob McLaren) (1) water bath variable temperature? "critical 55° only" (2) bubbles in L/L? "set out at beginning of experiment and do not vortex" "22°-24° C for 1-2 hours is best" (3) condensation on cover? "no problem, we tested for that" (4) ask about other labs consensus and can I contact one of them? "can't say" "can't tell me" "private information" (5) high RLUs even without extra sensitivity on? "protocol used a different luminometer so can't compare"….Bob asked me to fax him the runs from yesterday 4-11-02…he suggested doing a dilution series of ATP with L/L to rule out the first part of the reaction and/or rule in the L/L reagent and luminometer
- Judy will look for a heat block and ATP…she does not want to take samples through to obtain a profile based on AluQuant™ values as had been suggested by

Promega to compare RFUs, etc. to those obtained using Quantiblot® values because her thought is that if a DNA sample truly had approx. 1 ng/μl (as AluQuant™ says) but Quantiblot® says 10 ng/μl then profiles would not have been obtained because too little DNA would have been added to the PCR reaction...or if the true value was 10 ng/μl (like Quantiblot® says) and we thought we had 1 ng/μl (like AluQuant™ says) then the electropherograms would have been overblown because too much DNA would have been added to the PCR reaction

- Waiting on Joe to order luminometer plates
- Abigail, another Technical Rep. at Promega, called me back after she and Bob had reviewed the data I faxed them-> she said first curve is acceptable, stressed water bath temperature at 55°C with no variation and L/L sit at room temperature for 2 hours, admitted should not be seeing such differences between Quantiblot® and AluQuant™ values

4-16-02

- Spoke with Judy-> only 96-well heat block is in use so I told her I would try Promega's suggestion about the L/L reagent first to see what difference it makes; we are both uncertain of the water bath being the issue because I have obtained both acceptable and unacceptable curves on the same plate with the water bath

4-18-02

- Still have not received luminometer plates; I did receive the CycleSeal® sheets

4-19-02

- Received luminometer plates
- Experiment 7: "Test 4/19/02"

Purpose: To try Promega's suggestion...leave L/L reagent at room temperature 2 hours before using (I had been only leaving the L/L out for 30-45 minutes before use in order to save time on the 8 hour life of the reagent.) Still trying to obtain 2 acceptable curves on the same plate.

Materials & Methods: ran 2 sets of standards with 1 scan, no extra sensitivity, using the water bath, 3 tray types, CycleSeal®, standards diluted in water, multiple channel pipette used, 8 samples were also run (all ppt. with ethanol), samples prepared as follows-> 1 µl sample + 9 µl water = 10 µl total, new DNA used, again DNA standards were prepared by first vortexing for 2 min followed by 30 sec vortex, 5 min sit, 30 sec vortex in between each dilution made, L/L left out at R.T. for 2 hours

Results:

- still did not obtain 2 acceptable curves on the same plate
- due to the unacceptable nature of both curves, the samples were analyzed with 2 previously acceptable curves for comparison purposes; first curve used was from Experiment 2: Standards 32202 and the second from Experiment 6: Test 41102; again I know this would not be standard protocol
- don't see that L/L at room temperature for 2 hours made any difference; standard curves are still non-reproducible and sporadic

- first curve has good lower curve *R* stats, but the 2 ng/μl point throws the upper curve off-> can that point be taken out?
- second curve *R* stats are all above 0.98 so why does the curve look so funny? this curve proves that one can not rely on *R* stats alone to define an acceptable curve, the 1 ng/μl point is off and if taken out could possibly make the overall curve more accurate
- both curves as they are gave DNA quantities way off from Quantiblot® values…this is not new! (even with better curves) even taking into account the dilution factor
- using previous curves with samples from this experiment also give DNA concentrations very different from Quantiblot® values even after the dilution factor is corrected for…that is to be expected because the chemistry of the assay seems to vary from experiment to experiment

4-23-02

- left message with Lisa Lane (Regional Technical Representative) to call me…Judy wanted me to talk to her about the feeling of other labs trying the 96-well assay…she never returned my call-> also contacted Christy at UNTHSC who has done some testing with the single tube assay…Christy said the assay was "not robust or reproducible" "I do not like it!"

4-24-02

- Dr. Eisenberg graciously loaned me a 96-well heat block to try

4-26-02

- Experiment 8: "Standards 4/26/02"

Purpose: To try a 96-well heat block which should provide a more constant 55°C temperature than the water bath which varies from 55°-57°C. To obtain 2 acceptable curves on the same plate.

Materials & Methods: ran 2 sets of standards with 1 scan, no extra sensitivity, using a Boekel Digital Dry Bath Incubator 96-well heat block(Dry Bath model # 113002 and 96-well block model # 110096, Boekel Scientific, Feasterville, PA), 3 tray types, CycleSeal®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 2 min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use

Observations: I stepped away from the heat block for 20 minutes during the one-hour incubation and when I returned, the edges of the CycleSeal® (plastic cover) had rolled up on the ends; therefore, I expected evaporation at least on the wells on the ends i.e. 0 ng/µl and 4 ng/µl; actually when I performed the last pipetting steps, all samples had evaporated some

Results:

- due to the evaporation, I am unable to make a statement regarding the heat block's effects
- the first cure is certainly unacceptable due to evaporation I believe at 4 ng/µl for sure-> possible evaporation at other points also

- second curve is almost straight with *R* stats (that it would calculate) above 0.98 however I got an error message I have never seen before, "Marquardt's Compromise Algorithm cannot fit this data...re-assay curve,...fresh NaOH,...fit with linear regression..."
- NaOH was the problem in the alpha kit and "fit with linear regression"?-> that is what Promega claims in the "Certificate of Analysis" occurs all the time
- out of curiosity, I used standard points from the first bad curve as unknowns on the second curve with the funny error message to see what values it would calculate...fairly good values for the first 4 points of the curve but not the upper 4 points which corresponds to the lack of "full curve statistics" which would incorporate the top part of the curve

- I called Promega and spoke with Alyssa, Technical Representative; I faxed her today's results and asked about the error message...I am to call her Monday at ext. 1388 to get her suggestions-> she claims she knows nothing about the error message (I saw a similar message about the NaOH in Christy's (UTHSC) alpha test results)

4-29-02

- I called Alyssa at Promega-> (1) has to look into (talk with a programmer) error message...does not know why I got message because she says the curve looks good...I will call her tomorrow about this (2) I asked about my software-> she

thinks I have the correct software and that it is ok (3) I asked if points can be taken out of the standard curve-> she does not know and will ask a programmer (4) only suggestion she had was to repeat experiment with foil cover which I had already told her about the evaporation occurring (5) she said a water bath with circulating water is best...heat blocks can have problems (6) she said variable temperature is ok....*** comments 5 and 6 are in direct contradiction to 3 other Tech. Reps. I have spoken with at Promega!!!

4-30-02

- I called Alyssa at Promega about programmer's response to error message-> she said programmer has not emailed her back yet and she is waiting to hear advice on water bath from someone and she will not answer the question about leaving points out of the standard curve
- Experiment 9: "Standards 4/30/02"

Purpose: To prevent evaporation by using the CycleFoil® cover on the 96-well plate. Heat block used to provide constant 55°C temperature. Still striving for 2 acceptable curves on the same plate.

Materials & Methods: ran 2 sets of standards with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 2 min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use

Results:

- I do not believe evaporation occurred this time because the CycleFoil® completely covers the top of the 96-well plate as well as the sides of the wells
- still can not draw a conclusion about the water bath vs. heat block
- first curve is way off-> I do not know what happened, problems occurred at 0.5, 1, and 2 ng/µl in the "- probe" column only-> I have never seen this type of curve before
- second curve is fairly good though, *R* stats are all above 0.98 and the trend line is almost entirely superimposed on the raw data curve, in fact this curve is very similar to the second curve on 4-26-02 that gave the weird error message
- Judy, Dr. Joe, and Dr. Eisenberg all tell me to call Paul Newman at Promega for help/input

5-1-02

- Contacted Paul Newman at Promega (1-800-356-9526 ext. 2646 or direct 608-277-2646), I asked him the following: (1) water bath vs. heat block temp. variation? (2) error message? (3) leave points out? (4) scheme for vortexing DNA? (5) Quantiblot® variation? (6) upper curve problems 0.5-4 ng/µl range? (7) luminometer RLUs 4,000 in bulletin vs. 300,000 with the Reporter™ (8) condensation? (9) bubbles?→ all he told me as of yet is that he thinks a

thermocycler is best, he has only used the one tube luminometer, he leaves points out, he will get back to me on rest of questions

5-2-02

- Spoke with Alyssa (Tech. Rep.) at Promega to ask the following: (1) programmer response on error message? "according to programmer need "S" shape curve-> if you get a straight line the computer will ask you if you want to fit the curve with linear regression because it can not fit the curve with the Marquardt's Compromise Algorithm which is a non-linear fitting algorithm"→ this makes no sense to me because it is the only time I've gotten the message but have had plenty of straighter lines and have never gotten the message before, also they claim it is linear regression but now they say it is not??? (2) water bath thoughts? she still thinks I should use a water bath even though temperature varies (3) leave out points and its effect on program? does not know and says never heard of anyone else doing it, validate for your lab and it is ok (4) ATP? will send me 100mM ATP, "dilute way down with water" (5) stats on curves or what is acceptable vs. unacceptable? would not tell me, "it is up to individual labs to determine", "they do not want to set standards for all labs because depends on needs of the individual lab" (6) fax me example of acceptable and unacceptable curves? see #5
- Experiment 10: "Test 96-Well Heat Block"

Purpose: To test all 96-wells of the heat block with a Temperature Verification System to ensure each well is heating to 55°C. (the first curve or the one on the

outside of the plate in the only 2 experiments with the heat block have been unacceptable so are those wells at correct temperature?)

Materials & Methods: I used AB Gene Amp PCR System Temperature Verification System to test all 96-wells.

Results:

- the temperature of all 96-wells fell between 54.7°C and 55.0°C
- failure of previous curves with heat block was not due to temperature

5-3-02

- Spoke with Paul Newman at Promega-> emailed him some of my raw data both good and bad, water bath and heat block
- Received ATP from Promega but Paul wants me to wait before I do anything else so he can review the data
- Paul said he spoke with the developer of the READIT® Technology who said that he uses a heat block and that condensation is not a problem but Paul agrees with me that condensation could certainly be a problem
- Paul said that there are 2 parts or algorithms to the standard curve-> bottom part of curve uses parabolic algorithm and top part uses Marquardt's non-linear algorithm
- Questions to Paul: (1) how much temperature matters? referred to the developer (2) error message? bottom parabolic and top Marquardt's (3) leave out points? he is talking with programmers etc. to see if the program itself would take out bad

points for the forensic analyst (4) acceptable curve or not? stats? did not answer me, said yes need to define but did not answer what the definition should be

5-6-02

- Emailed Paul "Calculator" data that he gave to the developer of the kit...waiting for response
- Met with Judy to update her, next step to do the following: (1) go back through old curves and take out bad points to see effects (2) ATP dilutions 100x dilution serially out 10 times (3) received permission to spin plate in post PCR paternity lab as long as I decontaminate well (10% bleach followed by ethanol, clean before and after use, place plate on 10% bleach paper towel, bleach counter well, bleach pens, pipette handles, etc.)

5-7-02

- Experiment 11: "ATP dilutions"

Purpose: To rule out the L/L reagent and luminometer as contributing to unacceptable, non-reproducible standard curves. If the L/L reagent and the luminometer both rule out due to acceptable results with the dilutions, then it will be proven that the real problem is "upstream" from there in the procedure. The problem will be in enzymatic reactions that are suppose to create the ATP. Adding ATP directly into the reaction with the L/L bypasses the enzymatic reactions. The dilution series results will also give an idea of the sensitivity of the Reporter™ to the luciferase reagent.

Materials & Methods: rATP (100mM) lot # 13168611 from Promega, performed 2 different dilution schemes with ATP and water-> (1) 1:1, 1:2, 1:10-> out 7 tubes to 1:640, 1:100-> out 10 tubes to 1:51,200 (2) took 1:51,200 diluted both 1:100 so 1:5,120,000-> out 5 tubes to 1:81,920,000 and 1:1000 so 1:51,200,000-> out 3 tubes to 1:204,800,000; placed 25 µl of dilution into 50 µl of L/L that had set at R.T. for at least 2 hours read in luminometer 1 scan and no extra sensitivity

Results

- the second dilution series had to be preformed because the first series was all saturated-> no values were obtained except for the blanks that consisted of water and L/L only no ATP …so is my water or L/L contaminated with ATP or perhaps ATP was in those wells already?…keep in mind stock water was tested previously and there were no values obtained all read "0"…also L/L might produce light by itself, a little maybe but I don't know that it would give approx. 3,000-88,000 RLUs
- the second set of dilutions looked good-> each was approximately half of the previous and the blank (water and L/L) was really blank "0"
- what do the previous blank readings mean?
- the Reporter™ saturates somewhere between 1:51,200 and 1:5,120,000 dilution of ATP (moles ATP)
- also the L/L reagent is sensitive to at least 1:204,800,000 or 4.88×10^{-9} moles of ATP

- overall I think the L/L is fine as is the luminometer? maybe problem with the Reporter™ but also problem with the chemistry upstream

5-8-02

- answered Paul's email about brand and kind of 96-well plates I use-> Robbins Scientific CyclePlate® polypropylene for the 55°C incubation; also called Paul to ask for the developer's thoughts about my data
- Experiment 12: "Standards 5/8/02"

Purpose: To determine the effect of "flash spinning" the 96-well plate both before and after the 55°C incubation (to bring all condensation down off the foil and to force all liquid on sides of wells down to the bottom). This is all in an attempt to obtain 2 acceptable curves on the same plate.

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 2 min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, first time to flash spin plate both before and after the one-hour incubation, centrifuge used was a Labnet International, Model # Hermle Z300, Edison, NJ

Results:

- I am not ready to make a statement about the "flash spin" effect because I think maybe the plate set too long before incubation due to decontamination steps I had to take

- obviously, curve 1 is unacceptable mainly due to the 4 ng/μl point or lack of -> had that point worked properly I think the curve may have been good-> the lower curve stats are good with *R* values >0.99
- the second curve is acceptable I think: looks good, *R* stats are all >0.99 and standard points as unknowns gives fairly accurate values furthest off is the 2 ng/μl point (.18 off) and 1 ng/μl point is second furthest off (.15 off) but are these values that bad considering the range? I don't think so, but this does pose the question on acceptability of curve on standard points as unknowns... how off is ok?

5-9-02

- Phone tag with Paul Newman at Promega
- First I looked back at the 3 best curves thus far including the curve deemed acceptable by Promega and put the standard points in as unknowns to see what DNA concentrations it calculated; I did this for several reasons (1) I am looking at the accuracy of the system (2) I wanted to see how close the calculated values were to the known values to give me an idea of how off or the % different they can be so that I can try to establish guidelines for an acceptable curve (3) to see if variation exists in accuracy between different acceptable curves using standard points as unknowns from other acceptable curves and if it exists how much exists
- Second I looked back over data collected thus far focusing on those curves that could possibly be acceptable if just one point on the curve were thrown out-> I

took points out and made standard points as unknowns to see how accurate the calculated concentrations would be

- Results from 3 best curves thus far...(1) assay seems most accurate with the curve the points were run with (2) assay becomes inaccurate when use standard points from one acceptable curve with another acceptable curve and when *R* stats are less than 0.99 (each lab could have different acceptable stats) (3) how "off" or better termed % difference I think as of now should be 10% or less (again each lab could define their own) (4) basically, as of now, I am thinking that an acceptable curve would be defined by: looks which are reflected in the *R* stats as well as the standard points being analyzed as unknowns with only 10% maximum % difference, also trend line very close to being superimposed on the raw data curve which seems to occur when previously mentioned criteria are met, and would probably be best to not leave points out because the assay should work without having to do that but Promega (Paul) is still checking into possibly having the program do that for the analyst so still unsure about leaving points out (5) curve deemed acceptable by Promega (Exp. 6 on 4/11/02) actually has a large % difference (83%) at 0.06 ng/µl and 12% difference at 0.125 ng/µl so I actually would not call this curve acceptable, in addition the lower curve R^2 stat is 0.9886 with the rest at 0.99 (6) can not go by *R* stats alone and the further the *R* stats are from 1.0 the less accurate the curve becomes
- Results from curves with points taken out...(1) taking a point out will improve the *R* stats but no the % difference of each point necessarily (2) can have *R* stats

>0.99 and not be straight line-> have a crook in it (3) can have *R* stats >0.99 and have well over 10% difference (4) can have *R* stats >0.99 and have % difference of 11% or 12% (5) last point on curve whether it is 1 or 4 ng/µl will give >X (that number) on the concentration readings (6) can have *R* stats >0.99, "S" shape curve, and large % difference especially on lower end (7) can have *R* stats >0.99, "handgun" shape curve, trend line off, and % difference >10% (8) can have *R* stats >0.99, crook in curve, and >10% difference on smaller points, taking out a point to improve curve does change any samples run slightly-> some more accurate and some less possibly

5-10-02

- No word from Paul Newman (Promega)
- Experiment 13: "Standards 5-10-02"

Purpose: To determine the effect of flash spinning the 96-well plate after the 55°C incubation. To determine if non-ART® tips help last stages occur quicker (to see if decreasing the time between post incubation to the reading of the plate helps obtain 2 acceptable curves).

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, first time to flash spin plate only after the one-hour incubation and to use non-ART® tips to do last 50 µl

L/L and 25 µl sample pipetting (due to the available pipette and tips, using ART® tips requires multiple pipetting steps)

Results:

- first time to ever get 2 curves this good on same plate, 2 of best curves ever, YEAH!!!
- upon further examination of the data both curves have *R* stats >0.99, trend lines look good, overall appearance of curve looks really good however when standard points are put in as unknowns the 0.063 ng/µl point has a % difference of 43% on both curves and 0.125 ng/µl point has a % difference of 20% on one curve-> rest % differences are <10%-> so what does this mean?... perhaps I should not make % difference a requirement to be an acceptable curve or maybe the lower part of the curve should have a larger % difference allowed (50%) or maybe I should just use +/- amount allowed ...I do not know!
- just when I finally achieved what I had been aiming for (2 good curves on the same plate) and had decided upon what I would characterize an acceptable curve as-> the 2 don't fit together however I do think that the flash spinning after incubation made a positive impact as well as using non-ART® tips on the last step to save time (time is so critical at so many places in this assay...not good)

5-13-02

- Experiment 14: "Standards 5-13-02"

Purpose: To try to reproduce Experiment 13's results.

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after the one-hour incubation, used non-ART® tips to perform last pipetting steps

Results:

  - certainly the most reproducible results from assay to assay ever seen-> curves are very similar to those in Exp.13
  - first curve is better than the second however both have *R* stats >0.99
  - second curve's trend line is off a bit so I took out the 2 ng/µl point to see its effects-> improved overall *R* stats and decrease % difference for 1 ng/µl from 23% to 3% and improved trend line
  - even though I am still undecided about using % difference I believe both curves are acceptable

- Paul with Promega called-> he said they are stumped and that a group of them have been putting their heads together to try to determine what may be happening and made these comments-> as far as the curve is concerned (1) he said incomplete denaturation (2) incomplete mixing with HCl (3) use of any

polystyrene products...so I told him I mix 5 times at every mixing step and he said that was plenty and as far a polystyrene goes nothing I use is that except the white luminometer plates which they told me to buy in the beginning (the concern is that the DNA sticks to it so maybe it is not all transferred from tray to tray however use of it in the last step is not important I would not think because in the luminometer plate the reaction occurring is between L/L and ATP not DNA)-> as far a high background or (- probe) values is concerned (1) the one tube luminometer does not have high background like my results (2) luminometer contaminated (3) general contamination (4) cross-talk with luminometer...so I said "what do others using 96-well luminometer get?"-> no answer because they have not done those experiments, I do not know about the luminometer or general contamination (if these were true why would have I gotten the last 2 experiments to finally produce such good curves?), I do not touch trays with ungloved hands nor do I take the trays out of their shipping container until I am ready to use them...so he wants to look into the luminometer and cross-talk some more and he suggested I perform an experiment with ATP saturated and unsaturated concentrations indifferent patterns to see if cross- talk occurs into adjacent wells as it did in my ATP dilution Exp.11

- Shared my results and Paul's comments with Judy-> "assay not work for their use but perhaps I can prove cross-talk to add to other reasons why kit not good for high throughput" and suggested I do standards and skip columns to try to prove cross-talk and speak with Turner about cross-talk

5-14-02

- Aliquotted reagents.
- Paul called and said developer of assay said my "- probe" values are good...they should increase as the "+ probe" increases and gave explanation of what is occurring or why there is background (see Technical Bulletin)...he confused me because I had always gotten "- probe" values that increased and I thought I had understood...also now says luminometer is ok...very confused but he is trying to help
- Called Turner Designs (Sunnyvale, CA) about the Reporter™ (877-316-8049) original one Model # 9600-002 and Serial # 960011...spoke with Product Manager Brian Quast (direct 408-212-4002 or ext.102) about the cross-talk...he said most cross-talk comes from light coming out of wells in all directions not from failure of the detector, the Reporter™ has 1 photomultiplier tube (PMT) that reads all 96-wells from above, example of cross-talk values-> if well reads 100,000 RLUs then 100,000 x 10^{-5} or approximately 1 RLU will be due to cross-talk...manual says $5x10^{-5}$ but he said 10^{-5} ...Reporter™ for AluQuant™ saturates at 1.5 million RLUs which would cause 15 or greater RLUs from cross-talk...20-30 fold decrease in RLU with black plates...told me to run an empty white plate...should get approximately 300 RLU (+/- 20) in every well and if I don't let the luminometer run multiple times to try dry out any "moisture on the internal circuitry"

- Experiment 15: "Luminometer"

Purpose: To run empty white plate to see what the luminometer is reading at each well. (According to Brian Quast, Product Manager for Turner, each well should read approximately 300 RLUs (+/- 20).)

Results:

  - the wells did not read 300 RLUs; they varied from zero to 217 RLUs

- Experiment 16: "Luminometer 5"

Purpose: To let the luminometer run multiple times to dry out the internal circuitry. (First, I set the luminometer for 75 runs; as it ran the RLUs approached zero, so I set up 5 more runs. To begin with the RLUs were larger overall, but they still were not reading 300 RLUs. So, I set the luminometer to run its maximum, 255, runs for overnight.)

5-15-02

Results:

  - after a total of 335 runs, the values were still not 300 RLUs, the values were larger overall, and some were approaching 300 RLUs; however, some were virtually unchanged

- Spoke with Brian at Turner-> wanted me to email him this data along with dark current measurements (very low 101.98, low 150.088, medium 174.924)…I asked what water should read and he answered "like an empty tray"…I also asked what should L/L read and he answered "give off some light"-> he called me back after looking over the data and told me that he spoke with an engineer who said all

wells should read zero not 300 and should not vary from 0-250 so I have 2 choices (1) send it to them to fix but it can take at least 2 weeks or (2) let run over and over or leave open or put in oven without humidity control at 42°C

- I chose to look for an oven because I do not have 2 weeks to lose, no oven so I let it air dry overnight with the drawer open…I had also asked Brian if the luminometer should have 4 gains like their web site or 3 gains like it has…he said it should only have 3 gains per Promega for AluQuant™ assay to cut time down and that it is part of the firmware…he also explained dark current "is when PMT sees no light" and for very low gain it should be approximately 30 RLU

5-16-02

- Experiment 17: "Luminometer 9"

Purpose: To determine if the overnight air dry with drawer open worked or not. (Values should read zero or very close to it.)

Results:

  - values (RLUs) are moving closer to zero; however, they still range form zero to 206. Why are they varying so much from one run to the next with the same tray? Why did they read in the 200 RLUs and are now back down? Is the interior drying out?

- Experiment 18: "Luminometer 11"

Purpose: To determine if blow-drying on low setting will help dry out the luminometer so that RLUs read closer to zero. (trying this because did not have an oven)

Results:

- the RLU values actually increased on many wells, range of RLUs is from zero to 192, the values seem to be sporadic/jumping around

- Called Brian back with results…he wants the luminometer back, he says there is something else wrong with it possibly contamination on the PMT or something worse, the one and only one they have to send me will take several days to get it ready, he says it will be next Tuesday or Wednesday and suggests me calling Promega to see if they can get me one faster
- Very long story short, I spoke with 3 different Tech. Reps. (Abigail, Bob, Joe)…Abigail wanted me to speak with Paul who I could not get in touch with…Bob called Cherrie and Lisa Lane for me and I also left a message with Lisa myself…Joe said final word was no one had one to send to me, Joe also told me that they had just received an email about plates and the 96-well format…do not use polystyrene which is what they told me to use from Corning how frustrating!! they had just finished experiments about it he said, again I say what difference does it make if they are used in the last step where ATP and L/L react because we are not concerned about DNA at that point
- I then notified Brian that I would need the Reporter™ he has, he gave me information to return the one I have-> the RMA # 3686 and shipping address (Turner BioSystems 845 W. Maude Sunnyvale, CA 94085), wrap in bubble wrap, they do not pay for returns, send it next day air

- Paul heard what was going on by this time and called me, he is skeptical if there is really something wrong with the luminometer, "what is 200 RLUs when you get such large RLUs to begin with?", I agree to a certain extent, I do not believe the luminometer is all of the problem, I believe there are several problems, however, if there really is something wrong with it I do not need that added problem compounding my evaluation, Paul agreed to pay for the return of the Reporter™ even though he was not happy about it, he agreed to insure the Reporter™ also

5-17-02

- I packaged the luminometer up in bubble wrap and prepared it for shipment, I called for pickup, I emailed Paul the air bill # for tracking, weighed 15.11 lbs, air bill # 6660409324, should arrive at Turner Monday by 12:00, only could insure it for $4,999 because Airborne will not do any more than that for anything being sent for repair

5-20-02

- Verified package arrived at Turner shipping dock at 11:07 a.m. May 20th signed for by E. DeCastro, Lisa Lane at Promega called me at Orchid

5-22-02

- Spoke with Lisa Lane, she will be at Orchid next Tues, according to Lisa it is not the first time conflicting information has been given out by Customer Support, I asked her for others who have worked with 96-well format…she said she would bring the information next week…she could not remember exactly but mentioned DPS in Austin and LASD in CA

- Not received luminometer yet so called Angela Tran in customer support at Turner…she said it will be here next Tues. May 28th, I told her that was unacceptable nor was it what Brian had promised (he promised it would be here yesterday), later Brian called to tell me the one and only one they had to send me failed QC that morning and he does not know what is wrong with it but he will email me as soon as he is told (2 hrs) to give me projected time line, notified both Paul and Lisa with Promega

5-23-02

- Paul called me very upset with Turner, I spent all day on the phone and emailing Turner (Angela and Brian) and Promega (Paul and Lisa) trying to get answers, poor customer support all the way around except for Paul who seemed to care and was trying; however, in the end I was promised a luminometer next Monday or Tuesday that I can have for 2 weeks, Brian apologized for bad customer support and told me to please contact him with results of the new Reporter™ if need be and he will not be calling in 2 weeks wanting the luminometer back…"finish my evaluation", Paul spoke to Lisa who then spoke to Brian…Paul said the customer, me, should not have to be dealing with all of this, due to the short time I have left both Paul and Lisa want to keep in close contact, Brian said new luminometer should read all zeros with empty white tray (+/- 10%)

5-28-02

- Still no luminometer, false promises real old, UPS says tomorrow, Joe at Promega said "elution volume" is amount extracted, met with Lisa and Cherrie briefly-> no answers

5-29-02

- Gathered 13 samples that I plan to take all the way through profiling as well as information needed to do that (Quantiblot® values, electropherograms, PCR information, 3100® information, etc.), new luminometer finally arrived at 3:30, Serial # 960031 and Model # 9600-002, empty white plate all read 000.00 with Reporter™ software version 2.2 currently loaded and with version 2.3 sent with the new Reporter™, dark gain settings: very low 176, low 213, and medium 235

5-30-02

- Experiment 19: "New Reporter™ 53002"

Purpose: To examine the reproducibility of the curves by running them in duplicate. To examine the acceptability of the curves...are these any different than before? To test the sensitivity of the assay by examining dilutions made around the lower end of the range (0.02 ng/μl) claimed by Promega. To test the assay's accuracy by using 4 standards of different concentrations taken straight from manufacturer's tubes of DNA. Overall, see how this Reporter™ performs as compared to the previous one.

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared

by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after the one-hour incubation, used non-ART® tips to do last pipette step, first experiment with new luminometer other than empty white plate tests, 4 standards ran in duplicate using 1 µl, 4 additional dilutions ran in duplicate using 1 µl, standards used include: K562 10 ng/µl (Applied Biosystems, Foster City, CA), 9947A 10 ng/µl and 9947A 0.1 ng/µl (Promega Corporation), ATCC 45514 2.5 ng/µl (American Type Culture Collection, Manassas, VA), dilutions used include: 0.03125 ng/µl, 0.015625 ng/µl, 0.0078125 ng/µl, 0.00390625 ng/µl.

Results:

- overall, I do not think this Reporter™ made a significant difference in my results because they are similar to results from before, as I have thought all along, the true problems are with the assay itself, I will not dispute the fact that not having sporadic background helps, but it has not "solved" all problems, keep in mind I did get the best curve ever with the first Reporter™ as well as several other "acceptable" curves
- the curves are more reproducible now than when I began, however to use in a high throughput lab you would only want to run one curve and be able to rely on it to give reproducible and acceptable curves, I just do not think this assay in this format can do that, I would think the 2 curves should be more alike both with *R* stats >0.99 every time but that does not seem to be the norm

- first curve is certainly acceptable-> R >.99 with a good trend line and standards as unknowns are less than 10% except for 0.063 ng/µl with 27% difference…so in light of this curve and my previous analysis on "% difference" I do not believe that it should be used as a parameter to judge acceptability of curves
- second curve has lower R stats of >0.98 and it would not calculate the full curve stats, it has an off trend line, standards as unknowns have % differences from the 0.5 ng/µl point and below of > 10% so I took out the 0.25 ng/µl point which gave R >0.99, better trend line, and only point 0.063 ng/µl is > 10% different…so in light of this curve as well as my previous analysis of "points taken out" I believe that it should be used if needed as a parameter to obtain an acceptable curve
- sensitivity of the assay-> Promega claims 0.02-4 ng/µl-> below 0.02 ng/µl the assay does give zero readings however it gave 0.02 ng/µl readings not on the correct samples meaning dilutions made 0.015625 ng/µl or approx. 0.02 ng/µl gave readings of 0.4 to 0.9 ng/µl (with correction factor for only 1 µl quantitated), perhaps the dilution did not contain the amount I thought because when I used a manufacturer standard 0.1 ng/µl it read 0.2 and 0.3 which is very close, it only reports values to 2 decimal points so if I quantitated 1 µl of a 0.02 ng/µl then it would have to be able to read 0.002 ng/µl and it

can't, I do think the assay is sensitive with lower values not necessarily high ones because a 10 ng/µl standard read from 4.5 to 11.5 ng/µl

- accuracy of the assay-> Promega claims 2 fold accuracy-> some values I obtained fell within that range and others did not, 2 fold is very broad for example, a 10 ng/µl standard could read from 5 ng/µl to 20 ng/µl...isn't that a broad range when my curve goes from 0-4 ng/µl and the "optimal range is 0.02-4 ng/µl", using 1 µl of extract allows larger DNA concentrations to be quantitated without repeat but using 1 µl of lower concentrations can cause samples to quantitate as zero
- basically, I think the assay is sensitive so that it can give low readings but not necessarily accurate

- Experiment 20: "Test 53002"

Purpose: To quantitate 13 samples, to use those values to set up PCR reactions, and then to analyze the PCR product on the ABI Prism® 3100 Genetic Analyzer for comparison to genetic profiles obtained with Quantiblot® values. To examine the reproducibility and acceptability of the standard curves.

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after

the one-hour incubation, used non-ART® tips to do last pipette step, new luminometer, quantitate 13 samples (NY victim) previously quantitated with Quantiblot® and ran on the ABI Prism® 3100-> 1 µl of samples quantitated, 1 µl of 3 DNA standards also ran (K562 10 ng/µl, 9947A 0.1 ng/µl, ATCC 45514 2.5 ng/µl)

Results of Quantitation:

- DNA concentrations can be seen in lab notebook with corrected values for last 3 columns due to dilution factor, overall AluQuant™ values were very similar to Quantiblot® -> they will be used in PCR setup
- the curves were neither acceptable nor reproducible...not even bad at same points...typical results though
- I took the 2 ng/µl point out of the first curve to use for PCR because it has the better of the 2 "lower curve" *R* stats-> this made the *R* stats >0.99, improved the trend line dramatically, improved the standards as unknowns values, fairly straight, and the 3 standards ran as unknowns improved
- other standards ran were as accurate as before if not more (0.1 ng/µl = 0.1 ng/µl, 2.5 ng/µl = 1.3 ng/µl, and 10.0 ng/µl = 9.0 ng/µl) and the curve this time was not as good as the one before (Exp. 19)

5-31-02

- I called Promega-> Alyssa (Tech. Rep.) agreed dilutions must be corrected for

Materials & Methods continued (amp. and analyze): 13 samples plus positive and negative controls were amplified with Profiler Plus™ using PE Gene Amp PCR

System 2400 (both are ABI, Foster City, CA), 200 µl amp. tubes used, $V_1C_1=V_2C_2$ used to calculate dilution down to 0.125 ng/µl so that when 10 µl of extract is added 1.25 ng of DNA is added to the PCR reaction, extraction volume = 100µl, (1 µl quantitated with Quantiblot®), 25 µl PCR reaction volume, (PCR Master Mix = 10.5 µl reaction mix, .5 µl *Taq* Gold, 5.5 µl primers), 15 µl MM + 10 µl template DNA = 25 µl total, pos control (9947A 0.1 ng/µl) 10 µl + 15 µl MM, neg. control 10 µl water + 15 µl MM, amp. program used: 95°C for 11 min, 28 cycles of {94°C for 1 min, 59°C for 1 min, and 72°C for 1 min}, 60°C for 60 min, 4°C hold→ to load on ABI Prism® 3100 first make dilution tray 1:25 (2 µl product + 48 µl water), dilute ladder (8.5 µl water + 1.5 µl ladder), vortex, spin down, add 1µl of dilutions to 9 µl ROX500 + formamide in loading tray, vortex, spin down, 5 min at 95°C to denature, 5 min on ice, check for bubbles in tray wells, so actually only load 1:250 dilution of PCR product

Results of amplification and analyze:

- used GeneScan® Analysis and Genotyper® software-> positive control did not work however since we already know results the run was not repeated (in case work or a validation study it would have to be repeated), neg. control worked, of the 13 samples 2 did not work at all and 2 had at least one locus drop out, I believe the 0 ng/µl sample did not work because I diluted it as I did the others, overall my samples were cleaner (they had matrix problems), I had some alleles

called that they did not due to threshold and visa versa, overall RFUs very comparable in fact mine were a little higher with better peak height balance, ladder and ROX 500 both look good

- the curve used (characteristics previously mentioned) resulting in comparable results between the 2 systems confirms the characteristics I have defined as an acceptable curve but the catch is in obtaining acceptable curves or ones that could be altered to be acceptable on a repeated consistent basis

6-3-02

- Experiment 21: "Cross-talk 60302"

Purpose: To examine any cross-talk that occurs between wells containing Master Mix with probe to those with Master Mix Control without probe. To examine the reproducibility of the new Reporter™ by having it scan the plate 3 times with zero delay. As usual, to examine the reproducibility and acceptability of the standard curves.

Materials & Methods: performed standards in duplicate (one separated by a column of wells and one side by side) with 3 scans, no extra sensitivity, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after the one-hour incubation, used non-ART® tips to do last pipette step, new Reporter™

Results:

- it does not appear to me that cross-talk between wells is affecting the "- probe" readings, in fact as a whole the "- probe" values of the curve that was set up side by side are smaller that of the "- probe" that was set up by leaving a blank column in between
- in examining the reproducibility of the new Reporter™ by scanning in triplicate, I found that the luminometer is reproducible in a somewhat narrow range, it is similar to the first Reporter™ in Experiments 1 and 2 where plates were scanned in triplicate, keep in mind the L/L half-life is near by the time the plate is scanned for the third time, reproducibility can be seen by looking at both the raw data values and the *R* stats for each curve because they are reflections of the raw data
- I would say this is some of the best reproducibility of the curve seen, the 2 curves are very similar, the second curve is acceptable by my definition and the first curve would be if the 0.5 ng/μl point were taken out, the first curve is not too far from acceptable as is

- Experiment 22: "ATP Cross-talk"

Purpose: To examine any cross-talk that occurs between wells when wells are saturated and unsaturated using dilutions of ATP, water, and L/L in different patterns on a plate. [I found in a catalog that the L/L used in this assay, ENLITEN®, is designed to measure 10^{-11} to 10^{-16} moles of ATP; I also found out from Brian Quast, Turner Product Manager, that the AluQuant™ Reporter's™ sensitivity is "not more

than 10x" different from the regular Reporter™ that can detect as little as 10^{-19} moles of Bright Glo™ Luciferase, (Bright Glo™ is different from ENLITEN® according to Paul at Promega)]

Materials & Methods: 1 scan with no extra sensitivity, Corning® white microplate, L/L left out > 2 hours, new Reporter™, ATP dilutions used both "saturated" and "unsaturated"→ these were determined from previous Exp. 11 "ATP dilutions"

Results:

- cross-talk occurred in all 4 adjacent wells to those with a saturated dilution of ATP (a dilution of ATP that I knew would saturate the PMT in the luminometer), it did not seem to matter whether the wells had water, L/L, or a combination (saturation is expected at 1.5 million RLU and can cause cross-talk of 15 RLU or more according to Brian Quast, Turner), cross-talk values of 350-750 RLUs
- the wells containing a dilution of ATP that would not saturate the PMT as determined in Exp. 11 did not cause cross-talk when surrounded by water or L/L however when surrounded by water + L/L each well to the right and left of the unsaturated well gave readings…I believe this is due to natural light given off by L/L because the unsaturated value was similar to the other two that did not have cross-talk…however it was the largest reading so maybe it is cross-talk, if it is the L/L giving off light why only those 2 wells?

- why is well D7 giving a reading? is it the L/L reagent alone or could it be cross-talk from the well at a slant? is it possible that L/L can give off light from 60-100 RLU's by itself? and at other times give off no light? (according to Brian Quast, L/L can give off light by itself)

6-4-02

- finally heard back from Paul Newman and Lisa Lane at Promega: Paul-> (1) 2 fold accuracy means if should get 1 ng/µl then the range can be from 0.5 to 2.0 ng/µl (2) luciferase is firefly and not Bright Glo™ (3) bottom curve is actually a straight line (first 5 points) above this uses Marquardt's non-parametric algorithm...*R* values will give an indication of how close the values are to the expected-> this contradicts what he told me before! (4) the range test on the "Certificate of Analysis" simply states that the system will show linearity to 1 ng/µl and the proportionality test is simply to show that the signal will continue to increase the more DNA is added...the distinction is between the amount of DNA in the actual reactions vs. the concentration of input DNA-> reaction set up by using 10 µl of DNA sample + 5 µl NaOH + 10 µl HCl Solution = 25 µl which is split into "+" and "-" probe reactions using 10 µl so in 1 ng/µl tube actually have 4 ng total DNA (5) algorithm plots trend lines based on net RLUs and then from the trend lines the unknown samples are calculated...the lower trend line is not displayed but is still used to calculate; Lisa Lane-> contacts "2 experienced AluQuant™ users" (1) Jenny LaCoss, Houston PD, uses 96-well format but reads with TD-20/20, 1-713-308-2600 (2) Steve LaBonne, Lake Co. Reg. Crime Lab,

uses TD-20/20, 1-440-350-2129 (3) only Reporter™ users were LASO over a year ago and GeneScreen!!!→ isn't that interesting...could explain quite a bit

- Experiment 23: "wbs 60402"

Purpose: To determine if the temperature variation (55°-57°C) in the water bath is as critical as some at Promega claim. To again examine the reproducibility and acceptability of the standard curves.

Materials & Methods: performed standards in duplicate with 1 scan, no extra sensitivity, using water bath, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after the one-hour incubation, used non-ART® tips to do last pipette step, new luminometer

Results:

- first curve is acceptable-> R .0.99, good trend line, do not need to leave a point out
- second curve is unacceptable due to 0.125 ng/µl point so took that point out then $R > 0.99$, trend line ok
- both curves standards as unknowns-> give within 2 fold accuracy
- typical curves from my experience...reproducibility and acceptability of curves no different than when heat block is used
- I conclude the 55°-57°C variation is not as critical as Promega would have me believe

- Experiment 24: "snr 60502"

Purpose: To examine the sensitivity of the new Reporter™ by selecting the extra sensitivity option. To examine the reproducibility of the new Reporter™ when the extra sensitivity option is selected. To examine the reproducibility and acceptability of the standard curves.

Materials & Methods: performed standards in duplicate with 3 scans and zero delay, with the extra sensitivity option selected, using 96-well heat block, 3 tray types, CycleFoil®, standards diluted in water, multiple channel pipette used, new DNA used, DNA standards were prepared by first vortexing for 1½ min followed by 30 sec vortex, 4 min sit, 30 sec vortex in between each dilution, L/L left out at R.T. 2 hours before use, flash spin plate after the one-hour incubation, used non-ART® tips to do last pipette step, new luminometer

Results:

- as before with the first Reporter™, selecting the extra sensitivity option does not seem to make any difference…the RLUs are still the same with and without the option selected (approx. 250,000 – 400,000)
- the reproducibility of the Reporter™ is as good with and without the sensitivity option selected, keep in mind the L/L is reaching its half-life by the time the Reporter™ scans 3 times with the option selected

- set 1 curve 1 has $R^2 = 0.98$ on lower curve so I took 0.5 ng/µl out to obtain an acceptable curve, curve 2 has a strange trend line I am not familiar with or understand why but if I take out the 1 ng/µl point the curve becomes acceptable
- set 2 curve 1 is similar to set 1 and if 0.5 ng/µl were taken out it would become acceptable, curve 2 does not have a weird trend line and would be acceptable as is…I have no explanations for this…it has to be the Marquardt's algorithm I guess
- set 3 curve 1 is acceptable as is as far as *R* stats and trend line however I think better values would be obtained at 0.25 ng/µl if 0.5 ng/µl were removed, curve 2 has strange trend line again like in set 1 curve 2 so if 1 ng/µl were taken out the curve would become acceptable
- the curves were fairly reproducible between scans and when comparing curve1 and 2

- I contacted those Lisa Lane had given me information on to ask the following questions: (1) what do you think of AluQuant™? (2) do you obtain acceptable curves? (3) are your curves reproducible? (4) water bath, heat block, thermocycler? (5) how do you make the standard dilution series (6) how previously quantitate?
- Steve LaBonne, Lake County Regional Crime Lab (1-440-350-2129) outside Cleveland, Ohio…uses one tube system, adopted alpha kit (goes up to 10 ng/µl), runs on ABI Prism® 310, uses incubator, not as robust as he would like, can see

high throughput as a problem, very sensitive to least little mistake/incomplete mixing and vortexing, he does have problems with acceptable curves and it does concern him but he said he is not trying to get absolute accurate results just wants to be close, says used Quantiblot® before and he feels AluQuant™ is better than Quantiblot® so he is satisfied, he does not have the workload of Orchid Dallas

- Jenny LaCoss, Houston PD (1-713-308-2600), is using single tube, 9700 thermocycler, says AluQuant™ is very sensitive, uses Quantiblot® still because are in process of validating AluQuant™ (they are also doing validation studies for Promega), however for themselves they are suppose to use the Biomek® automated handling system in the near future as well as DNAIQ™ (Promega's extraction kit), she "adjusts values" to get a straight line…is that better than removing a point (I don' think so!), she has trouble with the lower part of the curve forming a hump…I have seen that too!…she said she would not recommend the one tube to anyone, she will be leaving in a month and a Joe Chu will be setting up the Biomek®
- I contacted the Los Angeles Sheriff's Office because they are the only other lab to have used the 96-well format (according to Lisa) and the Reporter™ but Lisa did not give me the contact information but Dr. Joe did…Steve Renteria (1-213-989-2160)…Steve had me speak with Learden Matthew who actually worked with the kit…she did not like the kit, she had curve problems, had to use multi-channel pipette, "never successfully got standard curves to work to even work with samples", bubbles a problem…(I asked Promega about bubbles because I have

them too, the L/L is frothy, and they said it should not be a problem...sounds familiar)...she said "wait for better technology", "no good-scrap it", uses chemiluminescent Quantiblot®

- Cost Analysis-> gathered the following information over several days by the web or calling companies...all prices are list and are the prices for equipment used in this evaluation:
    - AluQuant™-> $595 for 400 determinations, $10,000 for Reporter™, Boekel dry bath $586 + $175 for 96-well insert, centrifuge HermleZ300 $2,495, water bath $811...so that is $1.49 per determination and 40 samples + 1 curve can fit per 96-well plate (if only have to run one), given enough L/L for 1200 wells @ 50 µl per well (60ml) so that is 12.5 96-well plates so if only one curve is run per plate can get 496 samples per 12.5 plates (992 wells) and 13 curves per 12.5 plates (208 wells), takes approx. 3 hours to perform but I think that could be shortened
    - Quantiblot®-> $200 for 480 tests so $0.42 per test, Orchid already has all equipment necessary (slot blot apparatus, x-ray film, machine to develop it, etc.), 10 hybrid reactions per kit which is 10 control samples (7 DNA standards, 2 calibrators, 1 blank) plus 38 samples so 48 total tests per hybrid reaction, takes approximately 3½ hours to perform

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