An Internal Validation of the Qiagen® QIAgility™ Liquid Handling Robot

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Abstract

As advances in robotics are made, DNA analysis methods have begun to shift toward automation. While instruments with a single function are relatively easy to integrate into the workflow of a laboratory, robots that involve multiple steps in the workflow, such as liquid handlers, have proven to be more difficult. The Qiagen® QIAgility™ is an automated liquid handler designed for real-time PCR setup and can be adapted for any step that requires liquid to be transferred. The robot is intended to provide a means to increase casework efficiency, reduce pipetting errors, and improve the quality of DNA standards used in quantitation. An internal validation was performed at the San Mateo County Sheriff's Office Forensic Laboratory (SMCSOFL) according to the Scientific Working Group on DNA Analysis Methods (SWGDAM) validation recommendations to ensure the instrument's reliability, precision, and accuracy prior to being implemented for use with casework.

Automated protocols were designed on the QIAgility™ software for DNA quantitation setup using the Applied Biosystems® Quantifiler® Human and Quantifiler® Duo DNA Quantification kits. A protocol was created to normalize each of the samples to 0.1 ng/µL based on the quantitation results. Amplification and capillary electrophoresis setup using the AmpFℓSTR® Identifiler® kit could also be accomplished using the corresponding designed protocols of the QIAgility™ software. Each of the protocols was designed to allow flexibility and can be adjusted based on the number of samples that need to be analyzed.

The results of the reproducibility study demonstrated that the instrument is capable of accurately making DNA standards for quantitation. The outcome of the contamination

study indicated that no cross-contamination occurred during the QIAgility™ quantitation, normalization, amplification, and capillary electrophoresis setup. To ensure that the automated method consistently produced accurate and reliable DNA profiles, known samples were used to verify the process. Complete DNA profiles of each of the known samples were obtained after capillary electrophoresis. Finally, a comparison study was also completed to ensure that the QIAgility™ would perform equally as well as the manual methods currently employed by the laboratory. Results of this study indicated that the QIAgility™ produced a quantitation value that was more accurate than the manually prepared quantitation. The analysis of the STR profiles of the comparison study samples showed that the instrument produced comparable results to the manual method. As a result of the validation studies, the QIAgility™ will be adapted into the DNA analysis workflow of the laboratory in order to decrease the amount of human error and increase the efficiency of the laboratory.

Introduction

In today's forensic laboratory, automation of several different stages in the DNA analysis process is allowing analysts the opportunity to reduce backlogs and keep up with an increasing number of samples that are being submitted. The increase in the workload has caused the shift away from manual processes and toward automation (Stangegaard 378). Instruments that have a single function, such as the Qiagen® EZ1 Instrument (Qiagen®, Hilden, Germany), are the simplest way to integrate automation into the laboratory workflow. They require less training for the analysts, as well as fewer changes to the existing workflow. Liquid handling robots, such as the Qiagen® QIAgility™ (Qiagen®, Hilden, Germany), require a greater degree of training and adjustments in order to fully

integrate the instrument into the workflow of the laboratory because multiple different steps of the process are affected. By using these automated instruments, there is the potential to decrease the number of human errors that occur during repetitive tasks throughout the DNA analysis process, which is made up of multiple steps that require a liquid to be transferred from one location to another. Examples of human errors can include analysts pipetting a sample into the incorrect well or when the wrong volume of a reagent or sample gets distributed. A liquid handling instrument reduces these types of errors because it performs the same repeatable motions exactly as it is programed. Any of the steps that require liquid to be transferred during setup, including quantitation, normalization, amplification, and capillary electrophoresis, can be modified to include the use of a liquid handling robot (Myers 1570).

The Qiagen® QIAgility™ is an automated liquid handling robotic system primarily designed for real-time PCR setup. The instrument has the ability to set up reactions for the remaining steps of the forensic workflow after extraction of the samples has been performed. This includes quantitation, normalization, amplification, and capillary electrophoresis. The QIAgility™ uses a single-channel pipette to set up as many as 96 reactions in approximately 30 minutes (Qiagen 25).

Prior to using a new instrument for casework, it must be verified through the validation process. “Validation refers to the process of demonstrating that a laboratory procedure is robust, reliable, and reproducible in the hands of the personnel performing the test in that laboratory” (Butler 300). There are two different types of validation. The manufacturer of an instrument typically performs the developmental validation. However, an internal validation is performed to verify that the established procedures will work in

the individual laboratory implementing the new instrument (Butler 300). The Scientific Working Group on DNA Analysis Methods (SWGDAM) provides guidelines to assist laboratories during the validation process. Each laboratory is responsible for determining which validation studies are relevant to the methodology and the number of samples needed to satisfy each study (SWGDAM 2).

An internal validation was performed at the San Mateo County Sheriff's Office Forensic Laboratory to ensure the QIAgility's™ reliability, precision, and accuracy. The laboratory purchased two QIAgility™ instruments for use, one for pre-amplification procedures and the other for post-amplification procedures. The studies for the validation included reproducibility, contamination, and a mixture study. A comparison study was also done to ensure that the performance of the instrument matched the manual methods currently being used by the laboratory. A set of samples with known profiles, as well as a serial dilution of two of these samples, was also used to confirm that full profiles could be obtained when using the QIAgility™.

During quantitation, DNA standards of known concentrations are used to produce a standard curve. Typically, an analyst will make these standards by performing a serial dilution of the standard DNA provided in the quantitation kit. If these standards are not accurately made, the concentration results of the samples can be unreliable. Amplification and capillary electrophoresis can be negatively impacted if these concentrations are inaccurate. Reproducibility of real-time PCR results is dependent on the production of a consistent standard curve based on accurate volume transfers along with instrument performance (Grgicak 1331). The QIAgility™ can be used to produce the DNA standards

used in quantitation. The reproducibility study was designed to confirm that the robot could consistently generate accurate DNA standards and serial dilutions.

A contamination study was performed in order to determine if the automated method introduced contamination while the pipette of the instrument was in motion. This contamination could be introduced into the source well, which contains the DNA, or the destination well, which is empty and the location of the reaction, by a contaminated pipette tip. The design of the QIAgility™ allows for all of the reagents and samples to be placed on the worktable of the instrument simultaneously. While a protocol is being run, all of the samples and reagents must remain open for the entirety of the run. Because of this design, it is essential to ensure that cross-contamination of the reagents and/or samples is not taking place while the QIAgility™ is in use.

To ensure that the automated method consistently produced accurate and reliable DNA profiles, samples with known profiles were used to verify the process. After the samples were extracted, the quantitation plate for these samples was set up using the QIAgility™. The instrument was also used to normalize these samples and make amplification and capillary electrophoresis plates containing the samples with the known profiles. Several different sample types were used to ensure that the type of substrate containing the DNA did not affect the results.

It is important when working with forensic samples to be able to consistently obtain reliable results from mixed-source samples. Determination of the number of contributors and contributor ratios of the mixture is of primary concern when analyzing a sample with multiple donors. A mixture study was designed using three known female donors with a

high degree of variability at each of the tested loci. Several mixture ratios were chosen to demonstrate different contributor ratios that may be found in forensic samples.

Finally, a set of samples with known profiles was compared using automated and manual methods. For the automated method, the QIAgility™ was used for quantitation setup, normalization, amplification setup, and capillary electrophoresis setup. An analyst performed the same steps using the manual methods currently employed by the laboratory. The comparison study was used to determine if the reliability of the QIAgility™ equaled that of the manual methods.

Materials and Methods

General

The Qiagen® QIAgility™ instruments were installed and calibrated (volume, position, height, tip-offsets) according to the manufacturer's instructions before use (Qiagen® 41). A manual calibration was performed for the plate height of each of the blocks of the QIAgility™ worktable. This was done for each of the protocols to help reduce the number of insufficient liquid volume errors that may result if the recommended extra volume is not present.

DNA Extraction

Each of the samples chosen for this validation was from a known source with the DNA profile having previously been analyzed by the laboratory. A variety of different sample types were used, including buccal swabs, FTA cards, and liquid blood. All samples were extracted using the Qiagen® EZ1 Advanced or EZ1 Advanced XL and eluted into TE^{-4}.

DNA Quantification and Analysis

The Applied Biosystems® Quantifiler® Human and Quantifiler® Duo DNA Quantification kits (Life Technologies™, Foster City, CA) were used in all of the studies performed for this validation. The master mix was made by the analyst, according to the manufacturer's recommendations, and then placed on the worktable of the robot prior to beginning quantitation plate setup (Applied Biosystems® 3-5). Specific QIAgility™ protocols were designed for Quantifiler® Human and Quantifiler® Duo kits to prepare the DNA standards and allocate the master mix, standards, and samples into a 96-well plate (See Figure 1). The first standard of the dilution series must be made by the analyst and placed in position I of the Reagent Plate on the robot worktable. Empty 1.7 mL tubes need to be placed in positions J-P of the Reagent Plate, which will be used by the instrument for standards 2-8. Quantifiler® Duo DNA standards are required to be made with Duo Buffer, and Quantifiler® Human DNA standards are made using TE^{-4} prepared by the laboratory. The QIAgility™ was used to pipette 23 µL of the master mix into each well of the 96-well quantitation plate and 2 µL from each sample or DNA standard. The prepared plates were sealed with a clear optical seal by the analyst and amplified using the Applied Biosystems® 7500 real-time PCR instrument (Life Technologies™, Foster City, CA). The Sequence Detection Software (SDS) was used to process the information and analyze the quantitation data.

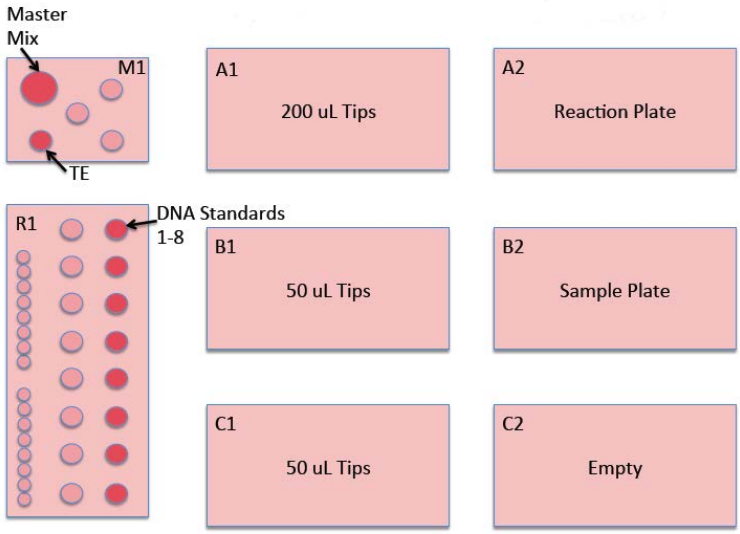

Figure 1: QIAgility™ Workbench Setup for the Quantifiler® Human Kit

DNA Amplification and Profiling

All samples that required DNA typing were amplified using the Applied Biosystems® 9700 thermal cycler and the Applied Biosystems® Identifiler® kit (Life Technologies™, Foster City, CA) per the manufacturer's 28-cycle protocol. The target amount of template DNA for amplification was 1.0 ng. This was accomplished by normalizing each sample to 0.1 ng/µL on the QIAgility™ and adding 10 µL of the normalized sample to the amplification plate. The amplified samples were injected on an Applied Biosystems® 3130 Genetic Analyzer (Life Technologies™, Foster City, CA) and analyzed using Applied Biosystems® GeneMapper® ID software v3.2.1.

Reproducibility Study

A 96-well quantitation plate was made on the QIAgility™ using the Quantifiler® Duo reagents and protocol. For this study, replicates of the DNA standards were aliquoted into columns 1-6 of the Reaction Plate. Table 1 lists the expected concentrations for the Quantifiler® Duo DNA standards. Columns 1 and 2 were used as the standards for

quantitation to produce the standard curve. The concentrations of the remaining four columns were analyzed to determine the consistency of the robot. To continue the analysis of the standards produced by the instrument, columns 3 and 4 were then used for the standard curve, and the concentrations of columns 1, 2, 5, and 6 were analyzed based on the adjusted standard curve. Finally, columns 5 and 6 were used for the standard curve, and the concentrations of columns 1-4 were analyzed. The three sets of data were compared to determine any inconsistencies with the standards that were made for the study.

Table 1: Expected Concentrations of the Serial Dilutions of the Quantifiler® Duo DNA Standard

Dilution	Concentration (ng/µL)
1	50
2	16.67
3	5.56
4	1.85
5	0.617
6	0.206
7	0.0686
8	0.0229

A serial dilution of Promega® Human Genomic DNA Male standard G147A (Lot # 27740001) was made using a second QIAgility™ protocol, and the eight dilutions were aliquoted into columns 7-9 of the same plate containing the replicates of the DNA standards. The initial concentration of the Promega® standard was 254 ng/µL. A manual dilution was made to adjust the concentration of the first standard to 50 ng/µL. To achieve this concentration, 9.6 µL of the standard DNA was added to 39.4 µL of TE^{-4} to produce 49 µL of the Promega® Male standard with the new concentration. This initial dilution was placed in the Reagent Plate at position I. Empty 1.7 mL tubes were placed in positions J-P of

the Reagent Plate, and the instrument made dilutions 2-8 during the protocol. The expected concentrations of the serial dilutions are listed in Table 2.

Table 2: Expected Concentrations of the Promega® Male Standard Serial Dilution

Dilution	Concentration (ng/µL)
1	50
2	16.67
3	5.56
4	1.85
5	0.617
6	0.206
7	0.0686
8	0.0229

Using a third protocol, a serial dilution of an extracted quality control sample, QC93, was made. The initial concentration was determined to be approximately 40 ng/uL based on a quantitation that was completed prior to the beginning of the study. Table 3 lists the expected concentrations of each of the serial dilutions for the extracted sample. The sample was not diluted prior to beginning the serial dilution setup, and the extract was placed in position I on the Reagent Plate. Empty 1.7 mL tubes were placed in positions J-P of the Reagent Plate, and dilutions 2-8 were made by the instrument during the protocol. The dilutions were distributed into columns 10-12 of the Reaction Plate. Figure 2 shows the 96-well Quantifiler® Duo plate and the location of each of the serial dilutions for the reproducibility study.

Table 3: Expected Concentrations of the QC93 Serial Dilution

Dilution	Concentration (ng/µL)
1	40
2	13.3
3	4.44
4	1.48
5	0.494
6	0.165
7	0.0549
8	0.0183

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 50 ng/uL	Std 1 50 ng/uL	Std 1 50 ng/uL	Std 1 50 ng/uL	Std 1 50 ng/uL	Std 1 50 ng/uL	Promega Male 50 ng/uL	Promega Male 50 ng/uL	Promega Male 50 ng/uL	QC93 40 ng/uL	QC93 40 ng/uL	QC93 40 ng/uL
B	Std 2 16.7 ng/uL	Std 2 16.7 ng/uL	Std 2 16.7 ng/uL	Std 2 16.7 ng/uL	Std 2 16.7 ng/uL	Std 2 16.7 ng/uL	Promega Male 16.7 ng/uL	Promega Male 16.7 ng/uL	Promega Male 16.7 ng/uL	QC93 13.3 ng/uL	QC93 13.3 ng/uL	QC93 13.3 ng/uL
C	Std 3 5.56 ng/uL	Std 3 5.56 ng/uL	Std 3 5.56 ng/uL	Std 3 5.56 ng/uL	Std 3 5.56 ng/uL	Std 3 5.56 ng/uL	Promega Male 5.56 ng/uL	Promega Male 5.56 ng/uL	Promega Male 5.56 ng/uL	QC93 4.44 ng/uL	QC93 4.44 ng/uL	QC93 4.44 ng/uL
D	Std 4 1.85 ng/uL	Std 4 1.85 ng/uL	Std 4 1.85 ng/uL	Std 4 1.85 ng/uL	Std 4 1.85 ng/uL	Std 4 1.85 ng/uL	Promega Male 1.85 ng/uL	Promega Male 1.85 ng/uL	Promega Male 1.85 ng/uL	QC93 1.48 ng/uL	QC93 1.48 ng/uL	QC93 1.48 ng/uL
E	Std 5 0.617 ng/uL	Std 5 0.617 ng/uL	Std 5 0.617 ng/uL	Std 5 0.617 ng/uL	Std 5 0.617 ng/uL	Std 5 0.617 ng/uL	Promega Male 0.617 ng/uL	Promega Male 0.617 ng/uL	Promega Male 0.617 ng/uL	QC93 0.494 ng/uL	QC93 0.494 ng/uL	QC93 0.494 ng/uL
F	Std 6 0.206 ng/uL	Std 6 0.206 ng/uL	Std 6 0.206 ng/uL	Std 6 0.206 ng/uL	Std 6 0.206 ng/uL	Std 6 0.206 ng/uL	Promega Male 0.206 ng/uL	Promega Male 0.206 ng/uL	Promega Male 0.206 ng/uL	QC93 0.165 ng/uL	QC93 0.165 ng/uL	QC93 0.165 ng/uL
G	Std 7 0.0686 ng/uL	Std 7 0.0686 ng/uL	Std 7 0.0686 ng/uL	Std 7 0.0686 ng/uL	Std 7 0.0686 ng/uL	Std 7 0.0686 ng/uL	Promega Male 0.0686 ng/uL	Promega Male 0.0686 ng/uL	Promega Male 0.0686 ng/uL	QC93 0.0549 ng/uL	QC93 0.0549 ng/uL	QC93 0.0549 ng/uL
H	Std 8 0.0229 ng/uL	Std 8 0.0229 ng/uL	Std 8 0.0229 ng/uL	Std 8 0.0229 ng/uL	Std 8 0.0229 ng/uL	Std 8 0.0229 ng/uL	Promega Male 0.0229 ng/uL	Promega Male 0.0229 ng/uL	Promega Male 0.0229 ng/uL	QC93 0.0183 ng/uL	QC93 0.0183 ng/uL	QC93 0.0183 ng/uL

Figure 2: Reproducibility Study Plate Setup

Upon completion of the three QIAgility™ protocols that were required for the plate setup, the quantitation plate was sealed with an optical adhesive seal and placed on the 7500 to proceed with real-time PCR. The Promega® Male and QC93 dilutions were selected for amplification on the 9700 and capillary electrophoresis with the 3130 Genetic Analyzer. The data from the 3130 Genetic Analyzer was analyzed using GeneMapper® ID.

Contamination Study

A 96-well quantitation plate was set up using the Applied Biosystems® Quantifiler® Duo reagents and protocol. In preparation for the quantitation, a plate of extracted samples was made which alternated the samples with blank wells of TE^{-4} in a checkerboard pattern (see Figure 3). Extracted samples were added to each of the sample well positions by manually pipetting 25 µL into the designated wells. The other positions contained 25 µL of TE^{-4} added through manual pipetting by the analyst. A total of 38 TE^{-4} blanks were used for this study. The checkerboard extract plate was placed in the Sample Plate position of the QIAgility™ worktable.

The analyst made the master mix according to the Quantifiler® Duo protocol, and it was placed on the worktable in position A of the Mix Plate. Standard 1 was made by adding 11.5 µL of the Quantifiler® Duo DNA standard to 34.5 µL of Duo Buffer to produce 46 µL of the first standard with a concentration of 50 ng/µL. The prepared first standard was placed, along with seven empty 1.7 mL tubes, in the Reagent Plate, and standards 2-8 were made by the instrument. These DNA standards were aliquoted in duplicate into columns 1 and 2 of the Reaction Plate. The quantitation negative control was made by adding 2 µL of TE^{-4} located in position B on the Mix Plate to 23 µL of master mix in well position A3 of the quantitation plate. The quantitation plate was placed on the 7500 to proceed with real-time PCR.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 50 ng/µL	Std 1 50 ng/µL	Blank	Sample 5	Blank 8	Sample 13	Blank 16	Sample 21	Blank 24	Sample 29	Blank 32	Sample 37
B	Std 2 16.7 ng/µL	Std 2 16.7 ng/µL	Sample 1	Blank 4	Sample 9	Blank 12	Sample 17	Blank 20	Sample 25	Blank 28	Sample 33	Blank 36
C	Std 3 5.56 ng/µL	Std 3 5.56 ng/µL	Blank 1	Sample 6	Blank 9	Sample 14	Blank 17	Sample 22	Blank 25	Sample 30	Blank 33	Sample 38
D	Std 4 1.85 ng/µL	Std 4 1.85 ng/µL	Sample 2	Blank 5	Sample 10	Blank 13	Sample 18	Blank 21	Sample 26	Blank 29	Sample 34	Blank 37
E	Std 5 0.617 ng/µL	Std 5 0.617 ng/µL	Blank 2	Sample 7	Blank 10	Sample 15	Blank 18	Sample 23	Blank 26	Sample 31	Blank 34	Sample 39
F	Std 6 0.206 ng/µL	Std 6 0.206 ng/µL	Sample 3	Blank 6	Sample 11	Blank 14	Sample 19	Blank 22	Sample 27	Blank 30	Sample 35	Blank 38
G	Std 7 0.0686 ng/µL	Std 7 0.0686 ng/µL	Blank 3	Sample 8	Blank 11	Sample 16	Blank 19	Sample 24	Blank 27	Sample 32	Blank 35	Sample 40
H	Std 8 0.0229 ng/µL	Std 8 0.0229 ng/µL	Sample 4	Blank 7	Sample 12	Blank 15	Sample 20	Blank 23	Sample 28	Blank 31	Sample 36	Promega® Male 10ng/µL

Figure 3: Quantifiler® Duo 96-well Contamination Study Plate

To ensure that no amplifiable DNA was present in the wells of TE^{-4}, the blank wells were amplified using the AmpF*ℓ*STR® Identifiler® kit on the thermal cycler. The QIAgility™ was used to add 10 µL of each of the blank samples to the amplification plate. The amplified samples were run on the 3130 Genetic Analyzer. Analysis of the data was performed using GeneMapper® ID.

Known Profile Study

A selection of seventeen samples with known profiles was extracted using the Qiagen® EZ1 Advanced and EZ1 Advanced XL BioRobots. A variety of sample types were used including buccal swabs, liquid blood, and FTA cards. A 96-well quantitation plate was made by the QIAgility™ using the Quantifiler® Duo reagents and protocol.

Based on the results of the initial quantitation, a second 96-well quantitation plate was made with serial dilutions of the "WL liquid blood" sample extract and the "QC93 buccal swab" sample extract. A 1:3 serial dilution was performed seven times to produce eight different concentrations for the two samples. The QIAgility™ was used to make the

serial dilutions in 1.7 mL tubes placed on the Reagent Plate. Each dilution was added to wells of the second quantitation plate and analyzed using the Applied Biosystems® 7500.

After quantitation, the seventeen known samples were normalized to 0.1 ng/μL using the QIAgility™ normalization protocol. The Identifiler® protocol of the QIAgility™ was used to add the normalized samples to the amplification plate. The serial dilutions were not normalized, and 1 μL of each dilution and 9 μL of TE^{-4} were added to the Identifiler® master mix on the same amplification plate as the normalized samples. The samples were amplified in the 96-well plate on the 9700 thermal cycler, and 1 μL of the amplification product was used for capillary electrophoresis on the 3130 Genetic Analyzer. The STR analysis was performed using GeneMapper® ID v3.2.1.

Comparison Study

Buccal swabs from five subjects were used for the manual comparison. Quantitation, normalization, amplification, and capillary electrophoresis plates were prepared using the QIAgility™ protocols for each process. The same sample extracts were used by an analyst to set up a quantitation using the current validated protocol in place at the laboratory. For the manual process, an aliquot of 2.5 μL was removed from each of the extracts and placed in a 0.6 mL tube. This aliquot was used for quantitation plate setup. The analyst pipetted 2 μL of the aliquot into 23 μL of Quantifiler® Human master mix in the 96-well plate, and the plate was run on the 7500. Based on the quantitation results, necessary dilutions of the samples were made to normalize each of the samples to 0.1 ng/μL. Rather than using an 96-well plate, each individual Identifiler® amplification reaction was added to a 0.6 mL amplification tube and placed on the 9700 thermal cycler. The capillary electrophoresis 96-

well plate was made from the amplified product, and the results were analyzed by GeneMapper® ID.

Mixture Study

Three different female quality control samples (QC19, QC62, and QC63) were used to make the mixtures for this study. Prior to beginning work on this study, the profiles of each of the three contributors were compared for loci with a high degree of heterozygosity. Multiple different ratios were chosen for the mixture series in order to simulate forensic samples with different contributor ratios. Buccal swabs were extracted on the EZ1 Advanced into 100 µL of TE^{-4}, and quantitation was performed on each of the three samples using the QIAgility™ and Quantifiler® Duo reagents to make the quantitation plate.

Based on the quantitation results, each sample was normalized to 1 ng/µL, and 10 different mixtures (Table 4) were made. Each mixture was then normalized to 0.5 ng/µL by adding 25 µL of the 1ng/µL mixture sample to 25 µL of TE^{-4} in a new 1.7 mL tube. All of the normalized 0.5 ng/µL mixture samples were quantitated to ensure an accurate starting concentration. The QIAgility™ made serial dilutions of each mixture to the following concentrations: 0.5 ng/µL, 0.25 ng/µL, 0.125 ng/µL, 0.0625 ng/µL, and 0.03125 ng/µL. Amplification of each of the serial dilutions was accomplished by adding 1 µL of the normalized product and 9 µL of TE^{-4} to the 96-well plate, along with the Identifiler® master mix. After amplification, the samples were run on the Applied Biosystems® 3130 Genetic Analyzer.

Table 4: Mixture Ratios and Components

Sample Name	Ratio	Mixture Components
Mx1	1:1	QC62:QC63
Mx2	3:1	QC62:QC63
Mx3	5:1	QC62:QC63
Mx4	1:1:1	QC19:QC63:QC62
Mx5	1:5:1	QC19:QC63:QC62
Mx6	5:1:1	QC19:QC63:QC62
Mx7	5:5:1	QC19:QC63:QC62
Mx8	1:3:1	QC19:QC63:QC62
Mx9	3:1:1	QC19:QC63:QC62
Mx10	3:3:1	QC19:QC63:QC62

Results

Reproducibility Study

Using the first 2 columns of the Quantifiler® Duo standards for the human standard curve resulted in a slope of -3.276, y-intercept of 27.74, and an R^2 value of 0.996. The male standard curve had a slope of -3.304, a y-intercept of 28.37, and an R^2 value of 0.996. Table 5 lists the quantitation values for the remaining columns along with the mean and standard deviation values. The percentage difference between the largest and smallest concentration was also determined.

Table 5: Quantifiler® Duo Standard Quantitation Results with Columns 1 and 2 Used as Standards

	Column 3 (ng/µL)	Column 4 (ng/µL)	Column 5 (ng/µL)	Column 6 (ng/µL)	Mean	Standard Deviation	% Difference Between the Largest and Smallest Value
A	53.25	56.37	57.8	51.96	54.84	2.703	10.6%
B	14.54	14.02	14.39	15.29	14.56	0.5335	8.67%
C	5.58	5.64	5.65	5.73	5.65	0.06164	2.65%
D	1.85	1.67	2.03	1.96	1.877	0.1569	19.5%
E	0.615	0.6	0.693	0.508	0.604	0.07588	30.8%
F	0.187	0.178	0.296	0.208	0.2172	0.05398	49.8%
G	0.0675	0.0666	0.0588	0.0541	0.06175	0.006424	22.0%
H	0.0173	0.0167	0.0239	0.0327	0.02265	0.007452	64.8%

Using columns 3 and 4 as the standards, the quantitation values differed slightly. The human standard curve had a slope of -3.36, a y-intercept of 27.85, and an R^2 value of 0.998. The male standard curve had a slope of -3.42, a y-intercept of 28.52, and an R^2 value of 0.998. Table 6 lists the quantitation values based on the second standard curve. The mean and standard deviation of each of the standards was also calculated along with the percent difference between the largest and smallest concentration.

Table 6: Quantifiler® Duo Standard Quantitation Results with Columns 3 and 4 Used as Standards

	Column 1 (ng/µL)	Column 2 (ng/µL)	Column 5 (ng/µL)	Column 6 (ng/µL)	Mean	Standard Deviation	% Difference Between the Largest and Smallest Value
A	48.23	52.21	55.97	50.45	51.72	3.271	14.9%
B	16.23	13.57	14.45	15.33	14.90	1.144	17.9%
C	5.35	5.97	5.81	5.89	5.755	0.2777	11.0%
D	2.20	2.05	2.14	2.07	2.115	0.06856	7.06%
E	0.593	0.641	0.752	0.556	0.6355	0.08510	29.9%
F	0.259	0.314	0.328	0.232	0.2832	0.04532	34.3%
G	0.0793	0.0937	0.0680	0.0627	0.07592	0.01372	39.6%
H	0.0186	0.0277	0.0283	0.0384	0.02825	0.008092	69.5%

Using columns 5 and 6 as the standards, the quantitation values of the four other columns of the Quantifiler® Duo standard DNA dilutions change. The human standard curve had a slope of -3.25, a y-intercept of 27.70, and an R^2 value of 0.996. The male standard curve had a slope of -3.38, a y-intercept of 28.44, and an R^2 value of 0.993. The resulting quantitation values, means, standard deviations, and percent difference have been listed in Table 7.

Table 7: Quantifiler® Duo Standard Quantitation Results with Columns 5 and 6 Used as Standards

	Column 1 (ng/µL)	Column 2 (ng/µL)	Column 3 (ng/µL)	Column 4 (ng/µL)	Mean	Standard Deviation	% Difference Between the Largest and Smallest Value
A	49.77	54.02	53.46	56.61	53.47	2.819	12.9%
B	16.14	13.41	14.46	13.94	14.49	1.182	18.5%
C	5.12	5.74	5.51	5.56	5.483	0.2611	11.4%
D	2.05	1.90	1.82	1.64	1.852	0.1708	22.2%
E	0.527	0.571	0.598	0.583	0.5698	0.03057	12.6%
F	0.224	0.273	0.180	0.171	0.212	0.04680	45.9%
G	0.0657	0.0782	0.0645	0.0636	0.068	0.006854	20.6%
H	0.0147	0.0222	0.0164	0.0158	0.01728	0.003358	40.7%

Tables 8 and 9 list the resulting quantitation values for each of the replicated serial dilutions based on columns 1 and 2 being used as the DNA standards for the standard curve. The means and standard deviations, along with the percent difference between the largest and smallest concentrations were calculated.

Table 8: Promega® Male Quantitation Results

	Column 7 (ng/µL)	Column 8 (ng/µL)	Column 9 (ng/µL)	Mean	Standard Deviation	% Difference Between the Largest and Smallest Value
A	26.99	27.61	30.91	28.50	2.107	13.5%
B	9.59	9.71	9.58	9.626	0.07234	1.35%
C	3.1	2.95	3.28	3.11	0.1652	10.6%
D	1.08	1.1	1.13	1.103	0.02516	4.52%
E	0.313	0.301	0.325	0.313	0.012	7.67%
F	0.118	0.095	0.106	0.1063	0.01150	21.6%
G	0.031	0.0261	0.0398	0.0323	0.006942	41.6%
H	0.0244	0.0143	0.0158	0.01816	0.005450	52.2%

Table 9: QC93 Quantitation Results

	Column 10 (ng/µL)	Column 11 (ng/µL)	Column 12 (ng/µL)	Mean	Standard Deviation	% Difference Between the Largest and Smallest Value
A	16.54	16.24	14.08	15.62	1.342	16.1%
B	5.23	4.95	5.46	5.213	0.2554	9.80%
C	1.78	1.59	1.68	1.683	0.09504	11.3%
D	0.622	0.519	0.511	0.5506	0.06191	19.6%
E	0.182	0.174	0.178	0.178	0.004	4.49%
F	0.0736	0.0579	0.0705	0.06733	0.008315	23.9%
G	0.0173	0.0277	0.0166	0.02053	0.006216	50.1%
H	0.00573	0.00667	0.00587	0.00609	0.0005071	15.2%

Contamination Study

The results of the checkerboard-patterned quantitation plate which was evaluating the potential for contamination showed that 38 out of the 38 blank wells yielded a quantitation value of 0.00 ng/µL for both total DNA and male DNA. The human standard curve for this quantitation had a slope of -3.396, a y-intercept of 28.56, and an R^2 value of 0.9966. The male standard curve had a slope of -3.396, a y-intercept of 29.32, and an R^2 value of 0.9948. All of these values were within the acceptable range for the standard curve of the Quantifiler® Duo kit.

Amplification of the blank samples was performed, and STR analysis was conducted on these samples, as well as on the positive amplification controls, negative amplification control, and allelic ladders. This was done in order to verify that there was no amplifiable DNA present in any of the blank samples. The positive amplification control located at well F6 produced a complete profile. However, the positive amplification control located at well F1 exhibited dropout at the D2S1338 locus and contained low peaks with an RFU between 50-199 at the D3S1358, D13S317, and D16S539 loci. The negative amplification control produced no profile as expected.

Known Profiles Study

Table 10 lists the concentration results for each of the seventeen samples with known profiles after quantitation. The concentrations of the two serial dilutions can be seen in Table 11.

Table 10: Sample Quantitation Results

Sample Name	Concentration (ng/µL)
EZ130620B- PEC	0.507
EZ130620B- NEC	0
QC63- Lot 051711	0.875
EZ130620C- NEC	0
EZ130620C- LB 1	6.11
EZ130620C- LB 2	3.27
EZ130620D- PEC- QC93	7.73
EZ130620D- NEC	0
Alex- buccal	4.05
Scott- buccal	3.31
WL- liquid blood	4.89
Mona- liquid blood	4.11
Alice- liquid blood	1.36
Jim- liquid blood	2.76
QC1- stain	0.0823
QC3- stain	0.471
QC4- stain	0.685
QC5- stain	0.476
QC6- stain	0.417
QC7- stain	0.401

Table 11: Serial Dilution Quantitation Results

Sample Name	Predicted Concentration (ng/µL)	Concentration (ng/µL)
WL- liquid blood	4.89	4.78
WL 2	1.63	1.76
WL 3	0.543	0.521
WL 4	0.181	0.19
WL 5	0.0604	0.0783
WL 6	0.0201	0.0247
WL 7	0.0067	0.0138
WL 8	0.00224	0.0033
EZ130620D- PEC- QC93	7.73	7
QC93 2	2.58	1.93
QC93 3	0.859	0.737
QC93 4	0.286	0.215
QC93 5	0.0954	0.102
QC93 6	0.0318	0.0304
QC93 7	0.0106	0.0103
QC93 8	0.00353	0.00207

Comparison Study

Table 12 compares the quantitation results of the five samples used for the comparison study.

Table 12: Comparison Study Quantitation Results

Sample Name	QIAgility™ Concentration (ng/µL)	Manual Concentration (ng/µL)
QC134 AB	1.97	1.06
QC135 SG	3.17	2.51
QC136 KM	3.13	1.61
QC137 HV	0.789	0.398
QC138 SW	0.363	0.219

Mixture Study

The target concentration for each of the mixtures was approximately 0.5 ng/µL. Based on the quantitation results (Table 13), it was determined that additional adjustments were not necessary before making the serial dilutions of each sample.

Table 13: Mixture Sample Quantitation Results

Sample Name	Concentration (ng/μL)
Mx1	0.423
Mx2	0.473
Mx3	0.423
Mx4	0.427
Mx5	0.429
Mx6	0.495
Mx7	0.468
Mx8	0.45
Mx9	0.451
Mx10	0.569

Discussion and Conclusions

Reproducibility Study

The first and second dilutions of the Quantifiler® Duo DNA standard dilution series exhibited the greatest amount of variation from the expected values of 50 ng/μL and 16.67 ng/μL respectively. The standard deviation that was calculated for each dilution was within an acceptable range. The first dilution of each of the 3 series of samples showed the greatest amount of variability between each replicate. The percent differences between the highest and lowest concentrations that were calculated indicated that dilutions 5-8 of each dilution series typically had the highest percent differences. This is due to the low concentration amounts of DNA in these dilutions.

Based on the quantitation results of Table 8, it was determined that the Promega® Male sample has degraded over time. This conclusion was further supported by the determination that the expiration date was March 9, 2013, three months prior to the start of the study. This would account for the quantitation results being lower than expected for the serial dilution series. It was concluded that based on the STR analysis results, the Promega® Male standard is made up of multiple donors, which explains the 3-person

mixture that was present in each of the serial dilutions. The insert including with the packaging of the sample confirmed that it was made up of pooled DNA. Because of this, further analysis of these samples was not performed.

The starting concentration of the QC93 sample was calculated at 40 ng/µL based on the results of a previously performed quantitation for that sample using the manual method. However, based on the serial dilution quantitation results, this value was incorrect. The starting concentration of the sample was likely around 16.5 ng/µL based on the results of the quantitation of the serial dilutions. The concentrations of the first dilution of the three replicates were 16.54 ng/µL, 16.24 ng/µL, and 14.08 ng/µL respectively. The standard curve of the manual quantitation may have led to the inaccurate initial concentration results. Although the starting concentration was inaccurate, the sample was diluted correctly in a 1:3 manner for each of the seven dilutions. The QC93 samples produced a single source profile when analyzed with GeneMapper® ID. No contamination was observed in any of the samples, and each dilution produced the expected profile. The peak levels of each sample appeared to be consistent with the dilution that was made. Based on the results of the reproducibility study, it can be concluded that the QIAgility™ instrument is capable of making accurate and reproducible DNA standards to be used for quantitation.

Contamination Study

The contamination study indicated that the robot caused no cross-contamination between the samples or within the reagents that were used to prepare the quantitation plate. This was confirmed by the results of the quantitation and the STR analysis of the blanks wells that were positioned next to wells containing varying amounts of DNA.

Dropout that was seen in the positive amplification control was likely caused by a malfunction of the thermal cycler at position F1 during amplification. No alleles were detected in any of the blank samples when the capillary electrophoresis results were analyzed. Blank 34 had peaks present below the 50 RFU threshold at the D8S1179 and D13S317 loci which may be an indication of some low level contamination for that sample. One possible explanation for these peaks may be carry over from a previous injection on the 3130 Genetic Analyzer, which has been an issue in the laboratory. The QIAgility™ may be another option to consider as a possible source of the contamination. The level of contamination, however, was minimal and would not affect casework. All of the other blank samples had no detectable peaks below the 50 RFU threshold.

Known Profile Study

Different types of samples were used for this study. Full profiles of each of the known samples were obtained after capillary electrophoresis and analysis by GeneMapper® ID regardless of the sample type. The serial dilutions of samples "WL" and "QC93" also resulted in full profiles. This indicated that the QIAgility™ could be used with samples having a wide range of concentrations, and a full profile will still be produced.

Comparison Study

Based on the quantitation results, it is evident that the QIAgility™ resulted in higher concentration values for each of the five samples used in the comparison study. After the amplification and analysis of the data from the 3130 Genetic Analyzer with GeneMapper® ID, it was determined that the manual quantitation underestimated the amount of DNA in each sample which led to overamplification. Capillary electrophoresis had to be performed on the manual samples again once the samples were diluted, and full profiles were

produced. The comparison study proved that the QIAgility™ is capable of setting up quantitation, normalization, amplification, and capillary electrophoresis plates comparable to or better than the manual processes currently being used by the laboratory.

Mixture Study

The targeted starting concentration of the initial sample of each mixture was 0.5 ng/µL. Based on the results of the quantitation, it was determined that the concentrations of each mixture was acceptable and further normalization was not necessary. When the mixture samples were analyzed in GeneMapper® ID, it was evident that the amplification had been unsuccessful due to the high degree of drop out for many of the samples. Based on the inconsistent results of previous amplifications, it was determined that the 9700 thermal cycler was malfunctioning. The results of the mixture study are inconclusive and will need to be continued after a replacement thermal cycler is received by the laboratory. In the future, the laboratory will reanalyze the mixtures that were made for this study.

As the number of forensic samples continues to increase, the risk of errors also increases for manually performed processes. When more than one sample is manually processed, sample misplacement is a possibility as well as pipette variation (Stangegaard 381). This validation demonstrated that the QIAgility™ produces accurate and reliable results comparable to the manual processes in place at the San Mateo County Sheriff's Office Forensic Laboratory. The Qiagen® QIAgility™ has been validated for use in quantitation setup using Quantifiler® Human and Quantifiler® Duo kits, normalization, Identifiler® amplification setup, and capillary electrophoresis setup.

Currently, when a new lot number of quantitation and amplification kits arrive at the SMCSOFL, an analyst validates the kits. In the future, integrating the QIAgility™ into this

process may allow for more accurate results. The difficulty will be in factoring in the excess volume required by the instrument for operation. The NIST standards that are used for the validation are not purchased in large quantities, so insufficient liquid errors may result on the QIAgility™. As new quantitation and amplification kits are implemented in the SMCSOFL, the kits will also need to be validated for use on the QIAgility™. New protocols will need to be made within the QIAgility™ software to factor in the requirements of the new kits.

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