

The following report is a summary of the conclusions from the Quantifiler® Duo Internal Validation conducted by the Forensic Biology Unit of the Department of Forensic Sciences Forensic Science Laboratory. This validation was completed while the Forensic Biology Unit was a part of the Metropolitan Police Department.

Quantifiler® Duo DNA Quantification Kit Using the AB 7500 Real-Time PCR Instrument Validation Report Summary

The internal validation of the Quantifiler® Duo DNA Quantification Kit using the AB 7500 Real-Time PCR instrument was performed by the MPD Crime Laboratory following the guidelines prepared and approved by the Scientific Working Group on DNA Analysis Methods (SWGDM).

1. Introduction

This report describes the internal validation of the Applied Biosystems® Quantifiler® Duo DNA Quantification Kit performed according to the Quality Assurance Standards for Forensic DNA Testing Laboratories (2004) issued by the Director of the Federal Bureau of Investigation (FBI) and the revised validation guidelines issued by the Scientific Working Group on DNA Analysis Methods (SWGDM, 2004).

SWGDM guidelines provide the following definitions regarding the purpose of validation:

Validation is the process by which the scientific community acquires the necessary information to:

- (a) Assess the ability of a procedure to obtain reliable results.
- (b) Determine the conditions under which such results can be obtained.
- (c) Define the limitations of a procedure.

The validation process identifies aspects of a procedure that are critical and must be carefully controlled and monitored.

Internal validation is conducted by each forensic DNA testing laboratory and is the in-house demonstration of reliability and limitations of the procedure. Prior to using a procedure for forensic applications, a laboratory must conduct internal validation studies. These studies must be sufficiently documented and summarized, lead to the establishment of documented quality assurance parameters and interpretation guidelines.

To verify the performance of the Quantifiler® Duo DNA Quantification Kit the following studies were performed as part of the internal validation: Precision, Reproducibility, Accuracy, Sensitivity, Contamination, Standard Curve, Mixture, and NIST Study.

2. Precision Study

To demonstrate precision, two full plates consisting of an individually prepared standard dilution series of the Quantifiler® Duo Standard were run by the same analyst. The averages and standard deviations of the C_T values for each detector (Human, Male, and IPC) of each standard were evaluated to determine the range of variation within a plate and between plates.

According to this study, standards 1 through 5 gave consistent C_T values with a standard deviation of 0.2 and below. Standards 6, 7, and 8 showed more variability in C_T value with a maximum of 0.98. Using the following equation,

$$\text{Concentration difference} = 2^{\Delta C_T},$$

a standard deviation of 0.2 in C_T value could result in a possible 1.15-fold difference in concentration. A standard deviation of 0.98 could result in a possible 1.97 fold difference in concentration. Therefore, a 1.0ng sample could quantify as high as 1.15ng or as low as 0.87ng. A 0.1ng sample could quantify as high as 0.197ng or as low as 0.051ng. The pattern of higher standard deviations in the lower concentration standards is present in both the Human C_T and Male C_T standard values. According to the Quantifiler® Duo manual, it is expected to see a higher amount of variation in the lower concentration standards, especially the 23pg standard, due to the stochastic effects in low concentrations of DNA during PCR.

Also demonstrated in this study was the reliability of the IPC C_T value despite the concentration of DNA in the sample. The IPC C_T value from a sample can be used to indicate the possible presence of inhibitors in the DNA extract. A high IPC value can be used to determine if a sample may require additional cleanup, dilution or re-extraction. The average IPC C_T was 28.94 with a standard deviation of 0.144. Using three standard deviations, the normal range of IPC C_T values should be between 28.508 and 29.372. This data is within the limitations listed by Applied Biosystems in the Quantifiler® Duo User's Manual which states the following:

“...the reactions should show normal IPC amplification across a broad range of input DNA; that is, a NED™ C_T which falls between 28 and 31 with a variation of approximately 1 C_T across the standard curve.”

The range of IPC C_T values will continue to be evaluated in the subsequent plates of this validation to determine how the range may change with different samples.

The information obtained in this study demonstrates that the precision of the quantification is sufficient for purposes of accurately and reliably adjusting concentrations of DNA in extracts for amplification.

3. Reproducibility

To demonstrate reproducibility, two full plates consisting of an individually prepared standard dilution series of the Quantifiler® Duo DNA Standard were run by the same analyst. The quantities of each sample were evaluated by using two columns of samples as standards and the other columns of samples as unknown quantities. All possible combinations of columns were used to create the standard curve with which to determine each sample's quantities. The averages and standard deviations of the quantities for each detector (Human, Male, and IPC) of each sample were evaluated to determine the range of variation within a plate and between plates when the assigned standard columns are in different locations.

According to this study, up to an approximate 20% difference may be observed from an expected human quantity especially samples with DNA concentrations above 50ng or below 0.068ng. Up to an approximate 30% difference may be observed from an expected male quantity especially in samples above 50ng or below 0.068ng. An average percent difference in expected quantity is 4% in the human detector and 6% in the male detector. As shown by the bar graphs, the data also indicates that the location of the columns of standards on the plate will not affect the standard curve generated.

This study used the Quantifiler® Duo DNA Standard to assess reproducibility. This standard is generated from pooled human male genomic DNA. The reproducibility of the Quantifiler® Duo DNA Quantification Kit on the 7500 Real-Time PCR instrument will be further evaluated with mock case samples and various dilutions of single source samples in the accuracy and sensitivity studies.

4. Accuracy Study

To demonstrate accuracy, two plates consisting of two columns of a serial dilution of the Quantifiler® Duo DNA Standard which comes with the kit, NIST's 2372 Quantitation Standard, Promega's Human Genomic Male Standard and Promega's Human Genomic Female Standard. The average concentrations and standard deviations obtained were compared to their expected values to determine the accuracy of the Quantifiler® Duo DNA Quantitation Kit and 7500 Real-Time PCR instrument.

The smallest differences in observed to expected quantities were in concentrations in the middle of the dilution set while the larger differences were observed in the higher and lower concentrations. This data is consistent with the results obtained from the precision and reproducibility studies. Samples with concentrations in the higher and lower areas of the standard curve show more variability and may therefore exhibit more variability in observed concentrations than expected concentrations of DNA.

According to this study, an average 1.14 difference in human DNA sample concentration and 0.956 difference in male DNA sample concentration were observed. Although these averages indicate a slight overestimation in human DNA and a slight underestimation in male DNA, they are within the calculated possible differences between plates which may be observed (see precision study conclusions). Therefore, accurate values are being obtained from the Quantifiler[®] Duo DNA Quantification kit on the 7500 Real-Time PCR instrument.

5. Sensitivity and Reproducibility Study

Sensitivity

For the sensitivity study, serial dilutions of two DNA samples (ranging from 1.0ng to 0.007ng) were prepared and used to assess the ability of the Quant Duo system to accurately quantify samples, with particular consideration to low level DNA samples.

Genotyped data generated in this study was used to evaluate allele and locus dropout, peak heights, and artifacts, and correlated back to the quantitation results.

In this study, the ability of Quant Duo to detect and accurately assess the quantity of DNA in a sample, with particular attention paid to low level DNA samples, was evaluated. The greatest variability in Ct values between replicates was seen at the lower concentration amounts. The standard deviation was noted to be inversely proportional to the concentration. Loss of detection of the sample by Quant Duo was observed at both the 0.015ng and 0.007ng levels. It is important to note that both of these quantities fall outside of the Quant Duo standard curve range. The samples do follow the expected results pattern, with an increase in Ct value as the sample concentration decreases. The detection of male DNA is another feature of the Quant Duo chemistry that was also evaluated. The standard deviation for the male DNA samples was seen to be more random in its pattern as the sample concentration was decreased. This may be due to the fact that there is only one copy of male DNA to begin, whereas there are two copies of human DNA. The decreased amount of copies of male DNA may lead to more variability in the results.

Additionally, the study also correlated the amplified Identifiler data (DNA profiles) back to the results obtained from Quant Duo. One replicate of NMY08-TR1-002.1-0.5ng experienced injection failure and the data was not used in evaluation. All samples with a starting dilution concentrations of 0.06ng and greater gave full profiles (total amplification input was 0.36 to 1.5ng). Using a Peak Height Ratio (PHR) of 55%, imbalance in heterozygotic loci was not seen in NMY08-TR1-002.1 samples of 0.06ng or greater, or in BK-1 samples of 0.125ng or greater. Conversely, Off-Ladder (OL) alleles were seen starting with dilution samples of 0.125ng for both NMY08-TR1-002.1 and BK-1. Of the OL alleles, 107 of 111 were observed to be a result of pull-up of a larger peak and could be explained. Additionally, if the Peak Amplitude Threshold (PAT) was raised to a value between 70-80 RFU, 96 of those 102 peaks would be removed by GeneMapper in analysis.

Based on this study, it is seen that Quant Duo is able to detect low level DNA samples. However, the results are more variable than samples of higher concentration. With this in mind, results from low level DNA samples, particularly those that fall outside of the standard curve values (<0.023ng) should be interpreted with caution. With the data obtained from the Identifiler analysis, it can be concluded that although low levels of DNA may be quantitated by Quant Duo, they will not yield usable DNA profiles. One step that may be considered in the case of a low level DNA sample is to concentrate the sample prior to amplification. These findings should be taken into account when analyzing casework quantitation and its subsequent amplification data.

Reproducibility

For Reproducibility, serial dilutions of two DNA samples (ranging from 1.0ng to 0.007ng) and six simulated case samples were prepared and used to assess the ability of the Quant Duo system to reliably produce reproducible quantitation values for both Human and Male over a range of DNA concentrations.

Genotyped data generated in this study was used to evaluate allele and locus dropout, peak heights, and artifacts, and correlated back to the quantitation results.

In this study, the ability of Quant Duo to yield reproducible values across multiple replicates and multiple plates was evaluated. The least variability in results was seen with the simulated case samples. These were samples ranging from 2.0 to 10.0ng, indicating that the system works best with samples that have a higher DNA concentration. When evaluating the dilution series, the least amount of variability is seen in the 0.5ng dilution sample for both the NMY08-TR1-002.1 and BK-1 samples. The greatest variability is seen at the lower level concentrations. This variability is also seen when evaluating the male DNA aspect of the BK-1 samples.

Based on this study, Quant Duo is capable of yielding reproducible results both within and between plates. However, as the sample concentration decreases the variability of the quantitation results increases, which leads to more unreliable information from the quantitation procedure. Although quantitation is in essence an estimate of the amount of DNA present in a sample, it is probable that with a low level DNA sample the actual amount of DNA present may be significantly different than the result provided by Quant Duo. As seen with the sensitivity data, any data obtained from low level DNA samples must be viewed and interpreted with caution.

6. Contamination

Demonstrated in this study are the laboratory's procedures to minimize contamination that would compromise the integrity of results. Not only were all instrument calibrations run prior to validation, but procedures have been put in place to maintain the instrument on a monthly, bi-monthly, quarterly, and yearly basis as recommended by the manufacturer. Each plate also contains a Non-Template Control. The quantities and C_T values of this control in all plates run for this validation will be evaluated in this study. This will determine appropriate values with which to evaluate each plate to confirm contamination does not exist and therefore has not affected results.

The human and male C_T values of the Non-Template Controls for the plates run in this validation project showed no evidence of amplification, indicating an undetectable amount of DNA present. The IPC C_T values were consistent with the range of IPC C_T values established by the precision study. This indicates that no inhibition was present.

According to this study, regular instrument calibrations and checks along with a Non-Template Control per plate can be used to ensure the detection of contamination which may influence quantification results. The monthly, bi-monthly, quarterly and annual calibrations and checks of the 7500 Real-Time PCR instrument suggested by Applied Biosystems ensures proper maintenance and control of the instrument. A Non-Template Control (NTC) has been demonstrated to ensure the detection of possible contamination in the quantification process. However, it should be noted that a detectable quantity of DNA in an NTC may not be definitive evidence of a contamination event and should be interpreted with caution.

7. Standard Curve

The objective of this study was to evaluate the range of values for an acceptable standard curve, averages and standard deviations were calculated for the slopes, y-intercepts and R^2 values of each plate run for the Quantifiler[®] Duo validation and Identifiler3 validation. These values were used to establish an acceptable range of values which should be expected within a lot or shipment of Quantifiler[®] Duo DNA Quantification Kits on the 7500 Real-Time PCR instrument.

Results:

Human Standard Curve

Plate Name	Slope	Y-Intercept	R ²
010709js	-3.496499	29.424158	0.991789
010709js1	-3.361966	29.50835	0.993006
011609JS	-3.37952	29.497553	0.99629
011609JS1	-3.438747	29.592628	0.996449
011509JLZ1	-3.269321	29.254559	0.994476
011509JLZ2	-3.209854	29.22134	0.990072
012309LAM	-3.596447	29.6103	0.996645
ID3_SENS_5 (011509NY)	-3.415382	29.469028	0.99513
ID3_Acc_Rep_1 (012209JS)	-3.451871	29.402576	0.996504
ID3_Mix_1(012609NMY)	-3.547779	29.775286	0.990162
average	-3.4167386	29.4755778	0.9940523
std dev	0.118540833	0.164902355	0.002625089
average + 3 std dev	-3.061116102	29.97028486	1.001927568
average - 3 std dev	-3.772361098	28.98087074	0.986177032

Male Standard Curve

Plate Name	Slope	Y-Intercept	R ²
010709js	-3.469872	30.244131	0.988429
010709js1	-3.494422	30.432837	0.985342
011609JS	-3.716688	30.474197	0.988865
011609JS1	-3.363768	30.307486	0.992438
011509JLZ1	-3.718771	30.369982	0.993925
011509JLZ2	-3.466251	30.130386	0.991211
012309LAM	-3.6766	30.393	0.9941
ID3_SENS_5 (011509NY)	-3.360514	30.204933	0.985885
ID3_Acc_Rep_1 (012209JS)	-3.71783	30.387617	0.983405
ID3_Mix_1(012609NMY)	-3.771572	30.746088	0.984325
average	-3.5756288	30.3690657	0.9887925
std dev	0.159895596	0.170454069	0.00398792
average + 3 std dev	-3.095942013	30.88042791	1.000756259
average - 3 std dev	-4.055315587	29.85770349	0.976828741

The ranges listed above demonstrate the amount of variation which may be observed in the values associated with the standard curve (slope, y-intercept, and R²). These ranges should be used as guidelines when evaluating the standard curve obtained from each plate and the consistency from lot to lot of kits received. If values outside of these ranges are obtained, the possibility of omitting a point (especially in standards 7 and 8) or re-performing the quantification should be considered. The ranges obtained from this study are slightly larger than the ranges suggested in the Quantifiler[®] Duo DNA Quantification User's Manual. This manual suggests a slope range of -3.0 to -3.6 and an R² greater than or equal to 0.99. No range is listed for the y-intercept value.

Overall, the male standard curve produced higher standard deviations in slope, y-intercept, and R² values than the human standard curve. This indicates more variability in the male values obtained from the standards.

It should be noted in this study's conclusions that previous plates not listed above produced highly inconsistent values due to the freezing of the serial dilution set of standards. These plates were omitted from the validation results. Storage of the standards at 2 to 8°C for up to two weeks produced the results used in this validation.

8. Mixture Study

In order to demonstrate the ability of Quantifiler® Duo DNA Quantification kit with the 7500 Real-Time PCR instrument to estimate the ratio of human to male DNA present in a mixed sample, three mixture sets were prepared, quantified, amplified and run in triplicate. The ratio of male to female DNA was calculated from the data obtained from the quantification step and then visually evaluated from the data obtained from the 3130xl. These ratios were then compared to determine the maximum ratio at which a minor contributor can be detected in the quantification step. The results for this study will be analyzed and reported; however, conclusions will not be drawn until data from more mixture sets can be obtained.

As noted above, no conclusions will be drawn at this time.

9. NIST Standards

The NIST standards will be quantified with Quantifiler® Duo on the 7500, amplified at a 1.5ng target with Identifiler and run on the 3130xl. The results will be technically and administratively reviewed.

All results were obtained and confirmed as correct by technical and administrative review.

10. Final Conclusions

Based on the studies and tests performed in this internal validation, the following conclusions regarding the use of the Quantifiler® Duo DNA Quantification kit and 7500 instrument on casework can be made:

- The precision of the instrument is sufficient for the purposes of accurately and reliably adjusting concentrations of DNA in extracts for amplification.
- The values of the IPC can be used to detect possible inhibitors in an extract.
- The location of the standards on the plate does not influence the results of the samples being quantified.
- More variability was observed in concentrations above and below the standard curve range than within the standard curve range. The most variability was observed in concentrations below the standard curve range.
- Using an NTC (Non-Template Control) sample is a reliable way to detect contamination in the quantification step.
- The reliability of the standard curve to accurately quantify samples should be evaluated using the following ranges:

	Human	Male
Slope	-3.77 to -3.06	-4.06 to -3.10
Y-intercept	28.98 to 29.97	29.86 to 30.88
R ²	≥0.986	≥0.977

- Prepared standards should be stored 2°C to 8°C and should expire after no more than two weeks. The best results were observed in standards used for only one week.
- More mixture samples will need to be run and evaluated to draw conclusions regarding the ability of the Quantifiler® Duo DNA Quantification kit and the 7500 instrument to predict mixture ratios.