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Validation of two QuantStudio™ 5 Real-Time PCR Systems

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Document Details

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Contents

Abstract

The purpose of this project was to validate both QuantStudio™ 5 (QS5) instruments for the analysis of Quantifiler® Trio (Quant Trio) DNA quantification reactions. Both QS5-A and QS5-B were validated separately using the experiments outlined below.

The following experiments were performed on both QS5-A and QS5-B:

- Sensitivity and Limit of Detection
- Comparison of QS5 and 7500
- Repeatability and Reproducibility
- Y Intercept Thresholds

The results of this verification found that both QS5-A and QS5-B instruments are suitable to perform DNA quantification using the Quantifiler® Trio quantification kit, and can replace the two 7500 instruments that are currently in use.

Introduction

Forensic DNA Analysis has two 7500 Real-Time PCR instruments (7500s) which are used to analyse Quantifiler® Trio DNA quantification reactions. Both 7500s are at the end of life and are being replaced under the Health Technology Equipment Replacement Program (HTER). The HTER process identified the QuantStudio™ 5 Real-Time PCR System (QS5) as the most suitable replacement for the 7500s. Two QS5s were purchased.

Both QS5s were validated for the analysis of Quantifiler® Trio DNA quantification reactions by the manufacturer. The QS5s were delivered with pre-installed protocols for the Quantifiler® Trio kit.

Validation of the two QS5s were performed separately, QS5-A followed by QS5-B. Both QS5s will be implemented concurrently and replace the two 7500s. The validation experiments for both QS5s were the same.

Resources

All reagents, materials and equipment used in this project were as specified in the approved in-house document Project Proposal #185 – Validation of QuantStudio™ Real-Time PCR Systems (June 2017) [4]. This document will be referred to as the Experimental Design. The following QIS documents are referenced throughout this report:

- Operation and Maintenance of the Microlab STARlet and LabElite Integrated I.D.Capper. QIS 34050. [5]
- Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit. QIS 33407. [6]

Methods

The methods for each experiment in this verification were as per the Experimental Design unless otherwise specified.

Sample Selection

NIST standards were used for this validation. NIST Standard sets A, B and C were used to create serial dilutions using TE-4 buffer with final concentrations as per the Experimental Design. NIST Standards A, B, and C, are derived from a single male donor, multiple female donors, and multiple male and female donors, respectively [3].

Experiments and Results

Experiment 1: Sensitivity, Limit of Detection and Inaccuracy

Purpose

Quantifiler® Trio has been shown to have a single source sensitivity down to concentrations of 0.005 ng/µL[1]. The validation of Quantifiler® Trio on the 7500s determined the Limit of Detection (LOD) to be 0.001 ng/µL[2]. Serial dilutions of NIST standards were used to determine the LOD for Quantifiler® Trio on the QS5 instruments. Percent change (inaccuracy) was calculated from the expected and observed result. This was performed for each of the quantification targets: SAT, LAT and Y-Target for both QS5 instruments and 7500-A.

Results

Two plates of NIST standards A, B, and C serial dilution duplicates were prepared each for the 7500-A and both QS5s as outlined in Tables 1 and 2 below. Dilutions ranged from 5 – 0.0001 ng/µL.

Table 1: NIST Standards Serial Dilutions – Platemap 1 of 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 5 0.005 ng/µL	NIST C 5.0 ng/µL	NIST B 0.5 ng/µL	NIST A 0.1 ng/µL	NIST C 0.09 ng/µL	NIST B 0.05 ng/µL	NIST A 0.03 ng/µL	NIST C 0.01 ng/µL	NIST B 0.008 ng/µL	NIST A 0.007 ng/µL	NIST C 0.006 ng/µL
B	STD 1 50 ng/µL	STD 5 0.005 ng/µL	NIST A 1.0 ng/µL	NIST C 0.5 ng/µL	NIST B 0.1 ng/µL	NIST A 0.07 ng/µL	NIST C 0.05 ng/µL	NIST B 0.03 ng/µL	NIST A 0.009 ng/µL	NIST C 0.008 ng/µL	NIST B 0.007 ng/µL	NIST A 0.005 ng/µL
C	STD 2 5.000 ng/µL	Reagent Blank	NIST B 1.0 ng/µL	NIST A 0.5 ng/µL	NIST C 0.1 ng/µL	NIST B 0.07 ng/µL	NIST A 0.05 ng/µL	NIST C 0.03 ng/µL	NIST B 0.009 ng/µL	NIST A 0.008 ng/µL	NIST C 0.007 ng/µL	NIST B 0.005 ng/µL
D	STD 2 5.000 ng/µL	NIST A 5.0 ng/µL	NIST C 1.0 ng/µL	NIST B 0.5 ng/µL	NIST A 0.09 ng/µL	NIST C 0.07 ng/µL	NIST B 0.05 ng/µL	NIST A 0.01 ng/µL	NIST C 0.009 ng/µL	NIST B 0.008 ng/µL	NIST A 0.006 ng/µL	NIST C 0.005 ng/µL
E	STD 3 0.500 ng/µL	NIST B 5.0 ng/µL	NIST A 1.0 ng/µL	NIST C 0.5 ng/µL	NIST B 0.09 ng/µL	NIST A 0.07 ng/µL	NIST C 0.05 ng/µL	NIST B 0.01 ng/µL	NIST A 0.009 ng/µL	NIST C 0.008 ng/µL	NIST B 0.006 ng/µL	NIST A 0.005 ng/µL
F	STD 3 0.500 ng/µL	NIST C 5.0 ng/µL	NIST B 1.0 ng/µL	NIST A 0.1 ng/µL	NIST C 0.09 ng/µL	NIST B 0.07 ng/µL	NIST A 0.03 ng/µL	NIST C 0.01 ng/µL	NIST B 0.009 ng/µL	NIST A 0.007 ng/µL	NIST C 0.006 ng/µL	NIST B 0.005 ng/µL
G	STD 4 0.050 ng/µL	NIST A 5.0 ng/µL	NIST C 1.0 ng/µL	NIST B 0.1 ng/µL	NIST A 0.09 ng/µL	NIST C 0.07 ng/µL	NIST B 0.03 ng/µL	NIST A 0.01 ng/µL	NIST C 0.009 ng/µL	NIST B 0.007 ng/µL	NIST A 0.006 ng/µL	NIST C 0.005 ng/µL
H	STD 4 0.050 ng/µL	NIST B 5.0 ng/µL	NIST A 0.5 ng/µL	NIST C 0.1 ng/µL	NIST B 0.09 ng/µL	NIST A 0.05 ng/µL	NIST C 0.03 ng/µL	NIST B 0.01 ng/µL	NIST A 0.008 ng/µL	NIST C 0.007 ng/µL	NIST B 0.006 ng/µL	Reagent Blank

Plates were prepared as per Operation and Maintenance of the Microlab STARlet and LabElite Integrated I.D.Capper (QIS 34050) [5] and Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit (QIS 33407) [6] for 7500-A and both QS5s.

Combined results for NIST A, B and C were used to determine the LOD for the SAT and LAT. Results from only NIST A were used to determine the LOD for the Y-Target.

Table 2: NIST Standards Serial Dilutions – Platemap 2 of 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD 1 50 ng/µL	STD 5 0.005 ng/µL	NIST C 0.004 ng/µL	NIST B 0.002 ng/µL	NIST A 0.001 ng/µL	NIST C 0.0001 ng/µL						
B	STD 1 50 ng/µL	STD 5 0.005 ng/µL	NIST A 0.003 ng/µL	NIST C 0.002 ng/µL	NIST B 0.001 ng/µL							
C	STD 2 5.000 ng/µL	Reagent Blank	NIST B 0.003 ng/µL	NIST A 0.002 ng/µL	NIST C 0.001 ng/µL							
D	STD 2 5.000 ng/µL	NIST A 0.004 ng/µL	NIST C 0.003 ng/µL	NIST B 0.002 ng/µL	NIST A 0.0001 ng/µL							
E	STD 3 0.500 ng/µL	NIST B 0.004 ng/µL	NIST A 0.003 ng/µL	NIST C 0.002 ng/µL	NIST B 0.0001 ng/µL							
F	STD 3 0.500 ng/µL	NIST C 0.004 ng/µL	NIST B 0.003 ng/µL	NIST A 0.001 ng/µL	NIST C 0.0001 ng/µL							
G	STD 4 0.050 ng/µL	NIST A 0.004 ng/µL	NIST C 0.003 ng/µL	NIST B 0.001 ng/µL	NIST A 0.0001 ng/µL							
H	STD 4 0.050 ng/µL	NIST B 0.004 ng/µL	NIST A 0.002 ng/µL	NIST C 0.001 ng/µL	NIST B 0.0001 ng/µL							

Table 3 outlines the expected and the average quantification values and % inaccuracy for each serial dilution obtained from the 7500-A and QS5 instruments. The SAT, LAT and Y-Target results for both instrument types all gave quantification results down to 0.0001 ng/µL.

The % inaccuracy for SAT and LAT for the 7500-A was markedly higher (>180%) at 0.0001 ng/µL than for QS5-A (<70%) and QS5-B (<117%), which supports the recommendation of previous studies [2] that the LOD for Quant Trio on the 7500s should be set at 0.001 ng/µL. The data indicates that both QS5s are more accurate than 7500-A at the lowest dilution concentration tested (0.0001 ng/µL) for SAT and LAT, although it should be noted that the inaccuracy % for all instruments fluctuates across the range of dilutions tested (Figures 1-3).

Y-Target % inaccuracy appeared to increase with decreasing concentration for all instruments with QS5-B registering the greatest inaccuracy reading for the data set at 0.0001 ng/µL which was produced by a single outlying quantification value (0.00056 ng/µL) as the replicate failed to produce a value from which an average could be calculated.

Table 3: Average quantification results and % inaccuracy

	7500-A						QS5-A						QS5-B					
Concentration (ng/µL)	SAT Average (ng/µL)	SAT % Inacc.	LAT Average (ng/µL)	LAT % Inacc.	Y-Target Average (ng/µL)	Y-Target % Inacc.	SAT Average (ng/µL)	SAT % Inacc.	LAT Average (ng/µL)	LAT % Inacc.	Y-Target Average (ng/µL)	Y-Target % Inacc.	SAT Average (ng/µL)	SAT % Inacc.	LAT Average (ng/µL)	LAT % Inacc.	Y-Target Average (ng/µL)	Y-Target % Inacc.
5	5 23438	4.7	5.65350	13.1	7.69158	53.8	5.93264	18.7	6.55684	31.1	7.69477	53.9	5.64724	12.9	6.12456	22.5	7.92748	58.5
1	0 83839	-16.2	1.00262	0.3	1 29179	29.2	0.92602	-7.4	1.15516	15.5	1.29869	29.9	0.68532	-31.5	0.81726	-18.3	0.84837	-15.2
0.5	0.40486	-19.0	0.47043	-5.9	0 53297	6.6	0.40410	-19 2	0.55648	11.3	0.53550	7.1	0.36752	-26.5	0.45626	-8.7	0.37763	-24.5
0.1	0 08333	-16.7	0.10740	7.4	0.12445	24.5	0.09544	-4.6	0.12827	28.3	0.13520	35.2	0.08792	-12.1	0.10440	4.4	0.10567	5.7
0.09	0 07025	-21.9	0.09250	2.8	0.11651	29.5	0.07659	-14 9	0.11041	22.7	0.11979	33.1	0.06873	-23.6	0.08394	-6.7	0.09569	6.3
0.07	0 05418	-22.6	0.07967	13.8	0.10983	56.9	0.07768	11.0	0.10107	44.4	0.13110	87.3	0.05848	-16.5	0.07519	7.4	0.06782	-3.1
0.05	0 03357	-32.9	0.04646	-7.1	0 05238	4 8	0.04542	-9.2	0.05750	15.0	0.05022	0.4	0.03041	-39.2	0.03950	-21.0	0.03315	-33.7
0.03	0 01906	-36.5	0.02510	-16.3	0 02913	-2 9	0.02372	-20 9	0.03104	3.5	0.03598	19.9	0.01678	-44.1	0.02045	-31.8	0.02557	-14.8
0.01	0 00898	-10.2	0.01146	14.6	0 01457	45.7	0.01172	17.2	0.01321	32.1	0.01511	51.1	0.00942	-5.8	0.00957	-4.3	0.01337	33.7
0.009	0 00815	-9.4	0.01009	12.1	0 01543	71.4	0.01008	12.0	0.01152	27.9	0.01234	37.1	0.00724	-19.5	0.00791	-12.1	0.00974	8.3
0.008	0 00768	-4.0	0.00922	15.2	0 01249	56.2	0.01025	28.1	0.01051	31.3	0.01435	79.3	0.00744	-7.0	0.00897	12.1	0.01147	43.3
0.007	0 00684	-2.3	0.00769	9.9	0 01013	44.7	0.00958	36.9	0.00939	34.1	0.00703	0.5	0.00563	-19.6	0.00602	-13.9	0.00863	23.3
0.006	0 00597	-0.6	0.00681	13.5	0 00658	9.6	0.00638	6.4	0.00730	21.6	0.00939	56.6	0.00390	-35.1	0.00417	-30.5	0.00534	-11.0
0.005	0 00582	16.4	0.00487	-2.5	0 00806	61.3	0.00735	47.0	0.00611	22.2	0.00964	92.8	0.00444	-11.2	0.00445	-11.1	0.00507	1.5
0.004	0 00397	-0.7	0.00431	7.7	0 00313	-21.8	0.00421	5.2	0.00382	-4.6	0.00328	-18.0	0.00315	-21.1	0.00281	-29.7	0.00146	-63.5
0.003	0 00299	-0.4	0.00317	5.6	0 00339	13.0	0.00340	13.4	0.00286	-4.7	0.00518	72.7	0.00177	-41.0	0.00155	-48.2	0.00185	-38.4
0.002	0 00215	7.6	0.00267	33.6	0 00291	45.5	0.00246	22.8	0.00202	1.2	0.00223	11.4	0.00108	-46.1	0.00063	-68.4	0.00093	-53.5
0.001	0 00103	3.1	0.00096	-4.0	0 00166	65.6	0.00155	55.0	0.00095	-4.7	0.00197	96.9	0.00081	-19.3	0.00057	-43.1	0.00084	-16.5
0 0001	0 00028	181.6	0.00030	198 0	0 00019	92.9	0.00015	47.0	0.00017	67.6	0.00019	90.8	0.00015	52.3	0.00022	116 2	0.00056	461.2
Average % Inacc.		1.1		16.4		36.1		12.9		20.8		44.1		-18.6		-9.8		19.4

Note: Cells shaded in green indicate a higher accuracy comparing the three instruments

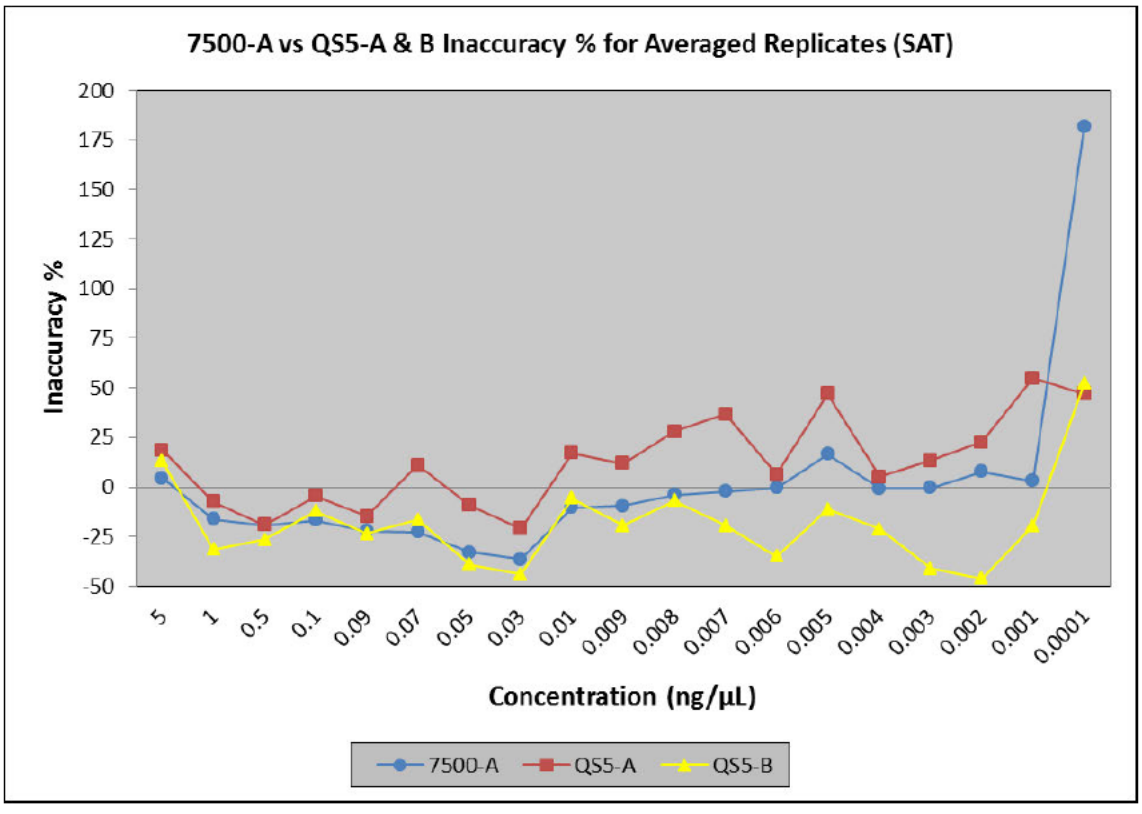

Figure 1: Percent inaccuracy for SAT

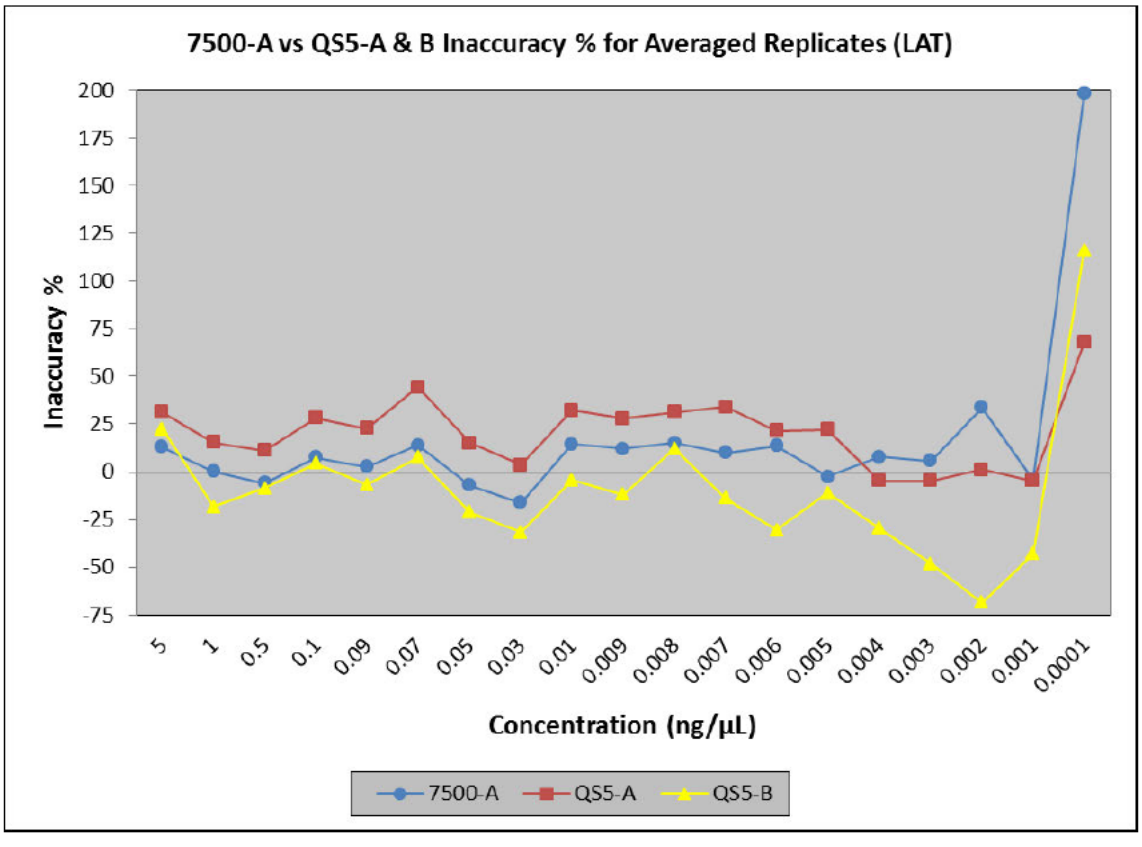

Figure 2: Percent inaccuracy for LAT

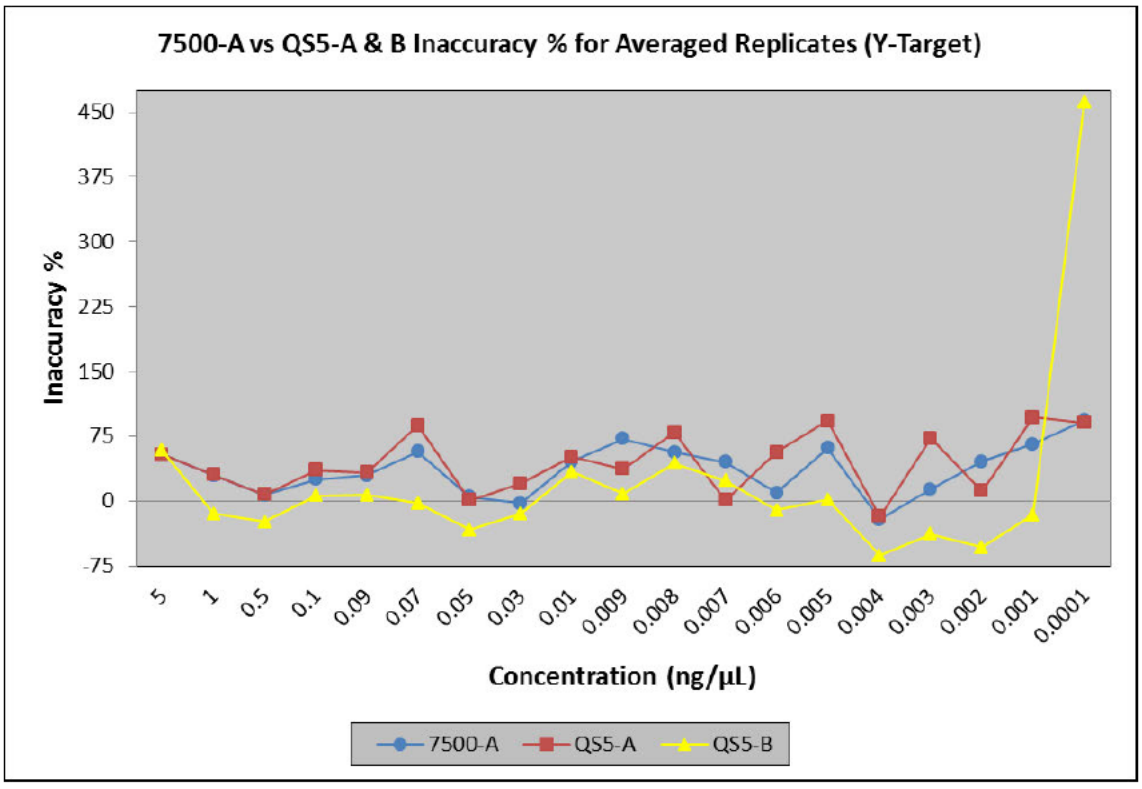

Figure 3: Percent inaccuracy for Y-Target

Discussion

The percent inaccuracy for 7500-A and the QS5s for all quantification targets (SAT, LAT and Y-Target) were similar for most dilutions, although the difference in inaccuracy was greater for some dilutions which is to be expected considering the observations of previous studies [2], and the inherent variation that is routinely observed between replicates using the Quant Trio kit.

The lowest dilution for which all replicates gave a quantification result for all targets on the 7500-A and QS5-A was 0.001 ng/µL. On QS5-B one replicate each of NIST A SAT, NIST B LAT and NIST C Y-target showed quantification values of undetermined at the 0.001 ng/µL dilution.

At the 0.0001 ng/µL dilution, 8/16 replicates gave an undetermined result for the 7500-A compared to 6/16 replicates for QS5-A and 11/16 for QS5-B (data not shown). This suggests the LOD for the QS5s are comparable to the 7500-A.

The large disparity between the 7500-A and the QS5s observed for SAT and LAT at 0.0001 ng/µL (Figures 1 & 2) supports the recommendations of previous studies [2] that the LOD for Quant Trio on the 7500s should be set at 0.001 ng/µL. This suggests the QS5s may be more accurate than the 7500-A at concentrations between 0.001 ng/µL and 0.0001 ng/µL.

Acceptance Criteria

The results indicate the LOD for Quant Trio on the QS5s is as good or better than the 7500A. Considering all the results, it is recommended the LOD for Quant Trio on the QS5 for SAT, LAT and Y-Target be set at 0.001 ng/µL.

Experiment 2: Comparison of QS5s and 7500

Purpose

To compare the performance of the two instrument types, the Student *t*-test (two-tailed distribution, paired) was performed to determine if there was a significant difference in quantification results across the entire dilution series. Student *t*-tests were performed separately for SAT, LAT and Y-Targets specific to each of the NIST standards using both replicates for each instrument. Only NIST A and C were used for Y-Target results. The two QS5s were compared to 7500-A using separate *t*-tests.

Results

The *t*-test results indicate that there is no significant difference between the quantification values between 7500-A and the QS5 instruments at quantification targets SAT, LAT and Y-Target as shown in Table 4.

Table 4: Student's *t*-test P-values for comparison of QS5-A and QS5-B with 7500-A.

Standard	Instruments compared	SAT	LAT	Y-Target
NIST A	QS5-A & 7500-A	0.70050	0.06813	0.42519
	QS5-B & 7500-A	0.44247	0.77529	0.19765
NIST B	QS5-A & 7500-A	0.05212	0.06054	N/A
	QS5-B & 7500-A	0.19258	0.15191	
NIST C	QS5-A & 7500-A	0.23834	0.09180	0.39582
	QS5-B & 7500-A	0.52538	0.45386	0.32165

Note: P-values < 0.05 indicate a significant difference between results produced by the two instruments.

Discussion

The results indicate the difference between quantification values for 7500-A and the QS5s are not significant for the SAT, LAT and Y-Targets for both the QS5s. The difference in LAT values for the QS5-A comparison was observed to be higher than for QS5-B, however the opposite trend was evident for the Y-Target comparison, where the QS5-B comparison showed a greater difference. The difference between SAT values showed no specific trend with QS5-A showing a greater difference than QS5-B for NIST B and C, but not for A.

As the LAT region component of the Quant Trio kit is designed to provide only an approximate estimation of the level of degradation for samples, it is expected quantification values for this target would vary over time and with freeze/thaw cycles since the target is more than twice the size of the SAT and Y-Targets [1]. The LAT and degradation index is currently not used by Forensic DNA Analysis.

Acceptance Criteria

The comparison of the QS5s and 7500-A quantification results using student *t*-tests indicates there is no significant difference in the ability to quantify SAT, LAT and Y-Targets, therefore both the QS5 instruments are comparable to 7500-A for these parameters.

Experiment 3a: Repeatability

Purpose

To assess whether the QS5s produce the same results when one sample set is processed in duplicate by one user under the same conditions. The results from plates 1 and 2 (Tables 1 and 2 respectively) for the entire dilution series were compared using percentage change between the two replicates for SAT, LAT and Y-Target for each of the NIST standards.

A Student *t*-test (two-tailed distribution, paired) was performed separately for all NIST standards for SAT, LAT and Y-Target (NIST A only) to compare the repeatability results for the two replicates of QS5-A and QS5-B. The analysis was performed across all of the 19 dilutions for each standard.

Results

The percentage change between replicates for NIST A, B and C dilution series are shown in Figures 4, 5 and 6 respectively for QS5-A, and in Figures 7, 8 and 9 for QS5-B. The percentage change for QS5-A NIST A (Figure 4) targets appear to be variable and show no specific trends. QS5-B NIST A targets appeared to exhibit less variability at lower dilutions (eg. 0.09 ng/µL and 0.009 ng/µL), however higher levels of percentage change than QS5-A were observed at 0.002 ng/µL.

Similarly, the percentage changes for NIST B and C also do not exhibit continuous trends (i.e. one target having a consistently lower change than another, or one target fluctuating more than another) for both QS5s.

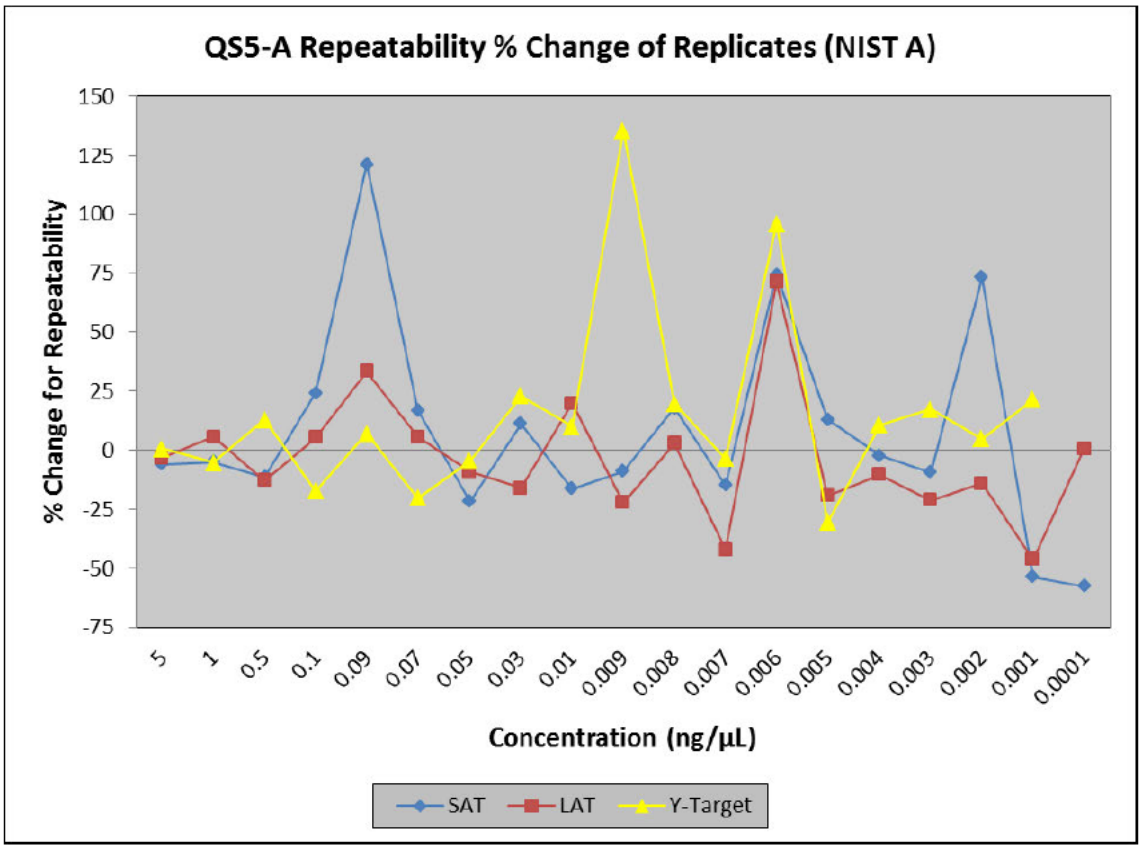

Figure 4: Percent change in repeatability for NIST A
One 0.0001 ng/µL replicate for Y-Target produced an undetermined result, this data point could not be included.

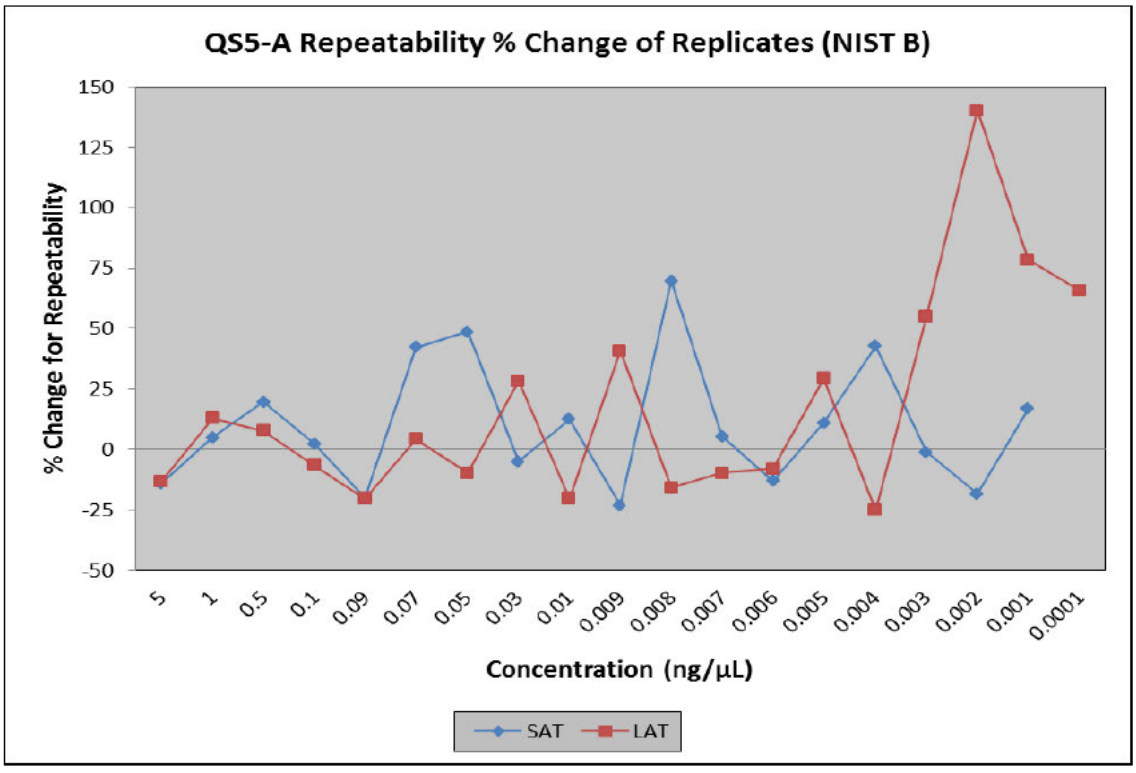

Figure 5: Percent change in repeatability for NIST B
Both 0.0001 ng/µL replicates for SAT produced an undetermined result, this data point could not be included.

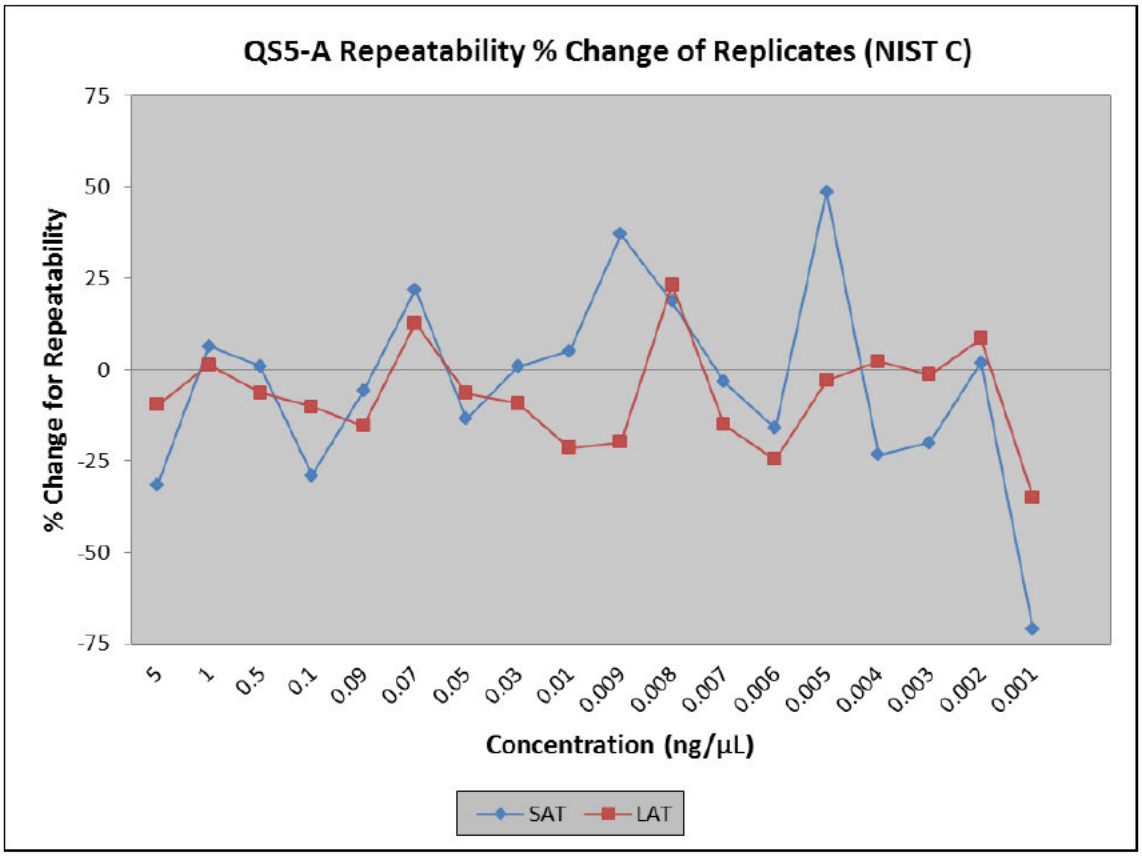

Figure 6: Percent change in repeatability for NIST C
One 0.0001 ng/µL replicate for SAT and both replicates for LAT produced an undetermined result, these data points could not be included.

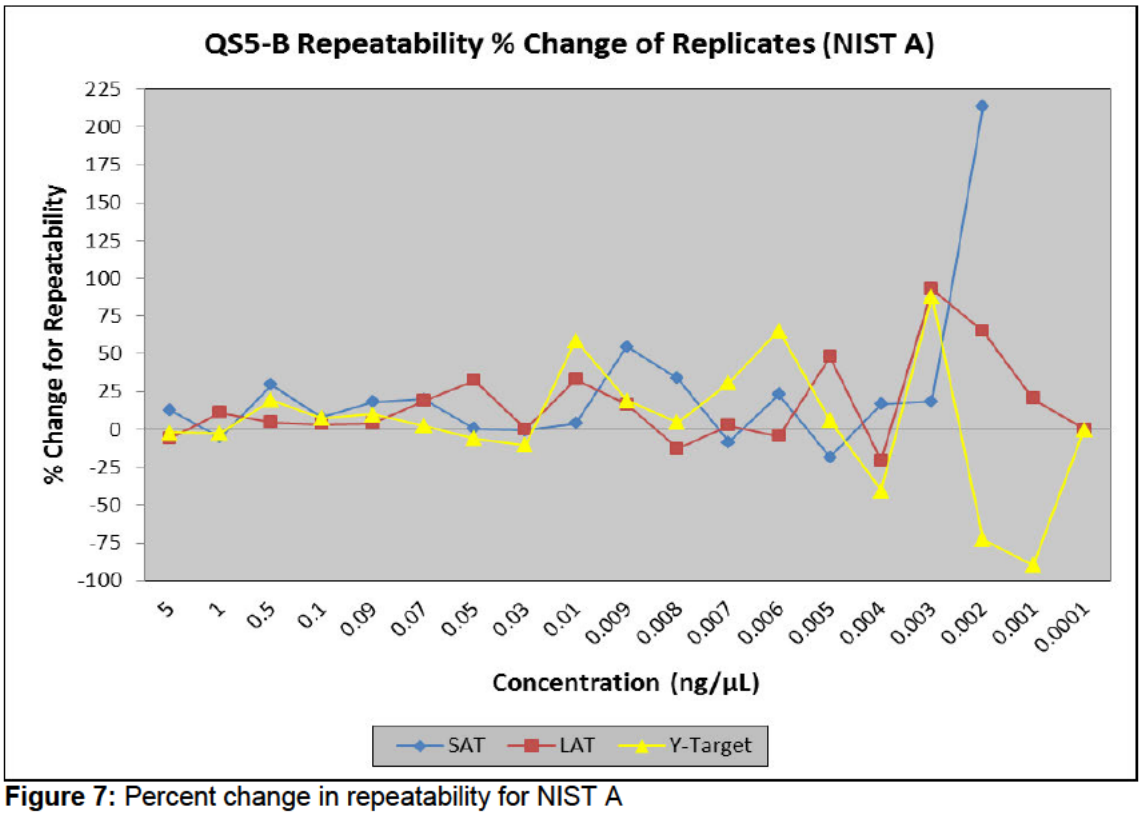

Figure 7: Percent change in repeatability for NIST A
One 0.001 ng/µL replicate and both 0.0001 ng/µL replicates for SAT produced undetermined results, these data points could not be included.

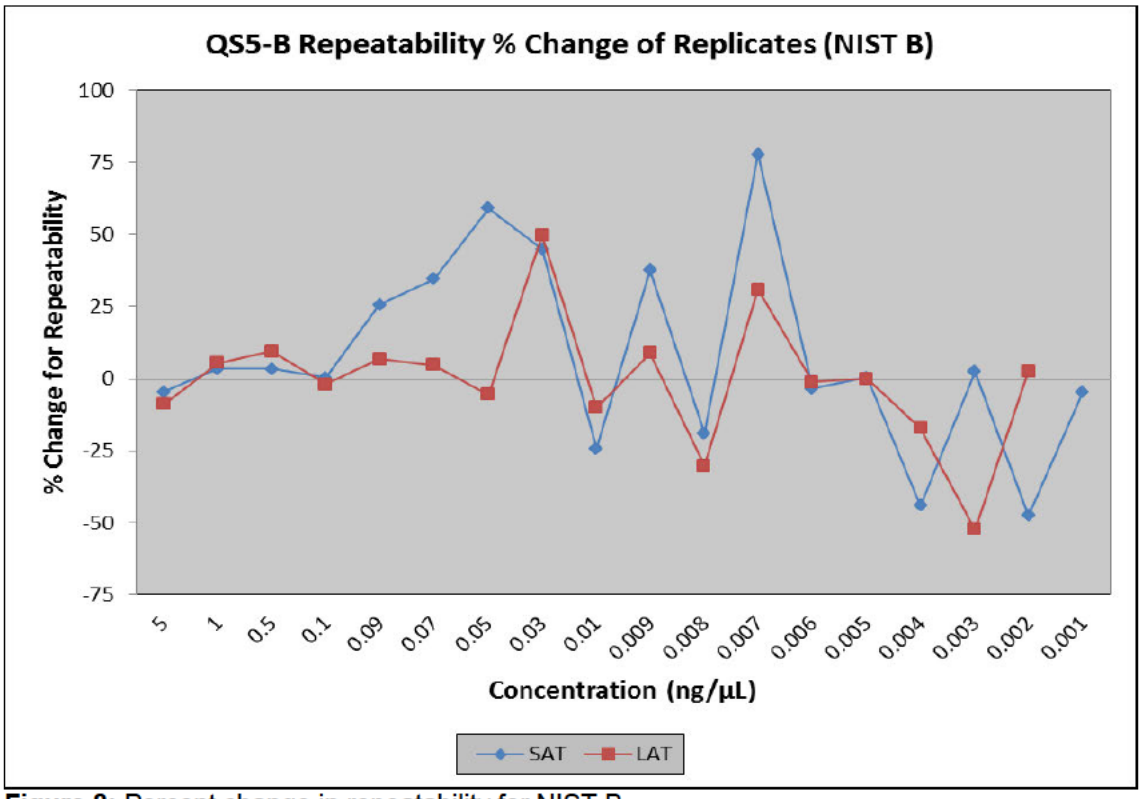

Figure 8: Percent change in repeatability for NIST B
One 0.001 ng/µL replicate for LAT and both 0.0001 ng/µL replicates for SAT and LAT produced an undetermined result, these data points could not be included.

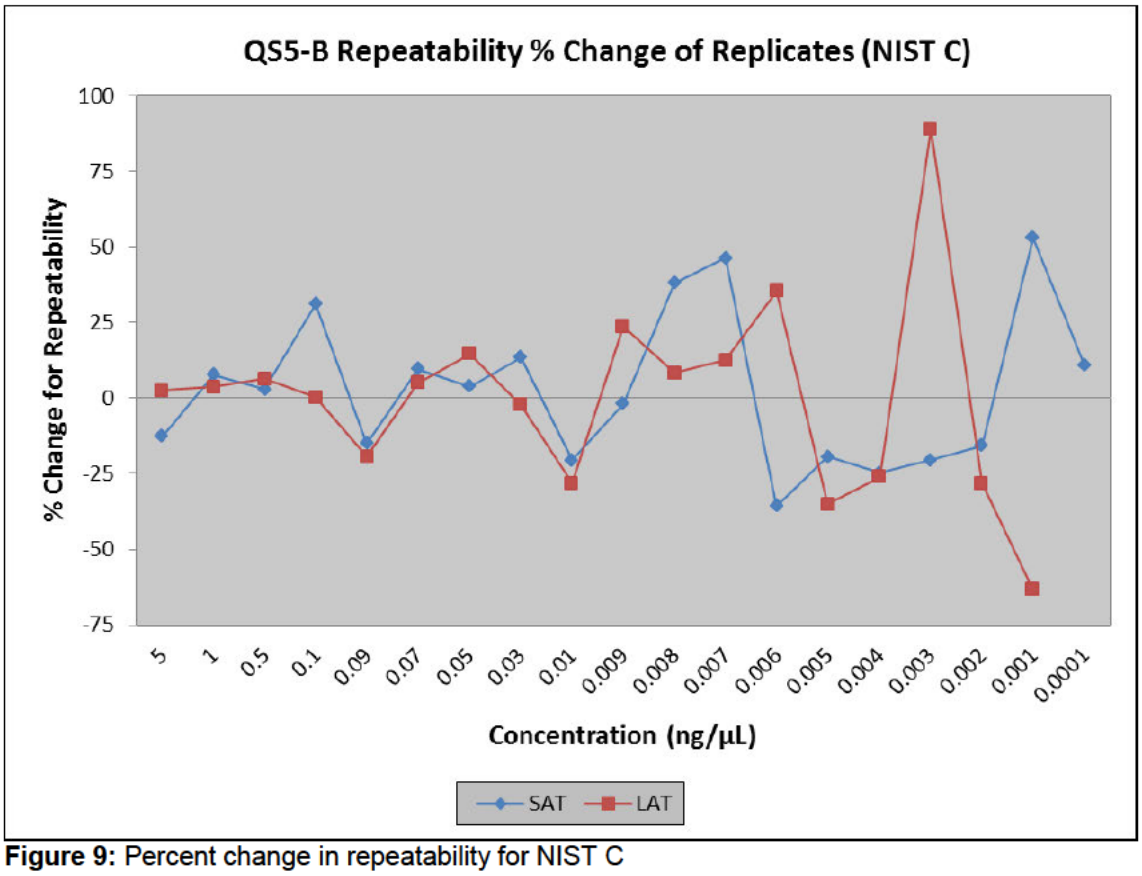

Figure 9: Percent change in repeatability for NIST C
Both 0.0001 ng/µL replicates for LAT produced an undetermined result, this data point could not be included.

Table 5: Student's *t*-test scores for QS5-A and QS5-B repeatability

	QS5-A SAT P-Value	QS5-B SAT P-Value	QS5-A LAT P-Value	QS5-B LAT P-Value	QS5-A Y-Target P-Value	QS5-B Y-Target P-Value
NIST A	0.32981	0.28357	0.45117	0.53489	0.86460	0.64993
NIST B	0.43151	0.52119	0.42641	0.43049		
NIST C	0.33981	0.39797	0.29143	0.19407		

Note: Y-Target analysis was only performed on NIST A (Male only DNA). Values < 0.05 indicate a significant difference between results produced by the two instruments.

Variability in quantification result repeatability for both QS5s across targets and NIST standards is apparent as can be seen from the P-Values in Table 5, which reflects results in Figures 4-9. Regardless, the Student's *t*-test scores shows that no significant differences were observed between the two replicates for each of the NIST standards using the SAT and LAT targets, and the Y-Target for NIST A (Table 4) for both the QS5s across the entire dilution series.

Discussion

The variability observed between targets at specific dilutions, and across the entire dilution series for each NIST standard provides evidence that a degree of variation is present in the Quantifiler Trio system's ability to produce repeatable results. This is

particularly evident for QS5-A NIST A (Figure 4) where the percentage change for SAT and Y-Target at 0.09 ng/µL and 0.009 ng/µL varies noticeably, whereas at 5 ng/µL and 0.006 ng/µL all targets are relatively similar despite the vast difference in concentration. This is also evident for QS5-B as can be seen for NIST-B at 0.05 ng/µL and 0.002 ng/µL where SAT and LAT percentage change varies, but at 0.1 ng/µL and 0.005 ng/µL they are similar. Variability in quantification result repeatability using Quantifiler Trio has also been documented in previous studies [2], and as in the current study they were not at significant levels and did not correlate to a specific dilution range.

The repeatability *t*-test results (Table 5) shows that repeatability results were not significantly different between replicates for all NIST standards using SAT and LAT targets, and for NIST A using the Y-Target across all dilutions for both QS5s.

Acceptance Criteria

Repeatability across all dilutions for both QS5 were shown to not differ significantly between replicates. These findings indicate that the QS5s have produced results that are comparable to the original Quantifiler® Trio validation using the 7500 instrument [2], which also showed no significant differences between replicates.

Experiment 3b: Reproducibility

Purpose

To assess whether the QS5s produce the same results when one sample set is processed by different operators under different conditions. The average of the two replicates from plate 1 (Table 1) for dilution series 5 – 0.005 ng/µL were compared to the corresponding averages of a reproduced plate 1 using percentage change.

A Student *t*-test (two-tailed distribution, paired) was performed separately for all NIST standards for SAT, LAT and Y-Targets (NIST A only) for the averages of both replicates at each examined dilution series to determine if there was a significant difference between reproduced results.

Results

The percentage change between reproduced replicates (averaged) for NIST A, B and C dilution series are shown in Figures 10, 11 and 12 respectively for QS5-A, and in figures 13, 14 and 15 for QS5-B. The percentage change for QS5-A NIST A (Figure 10) appear to be similar between targets at higher concentrations (5-0.1 ng/µL), however similarities can also be seen at the 0.005 ng/µL dilution which indicates variability and no specific trends for the three targets across the dilution series.

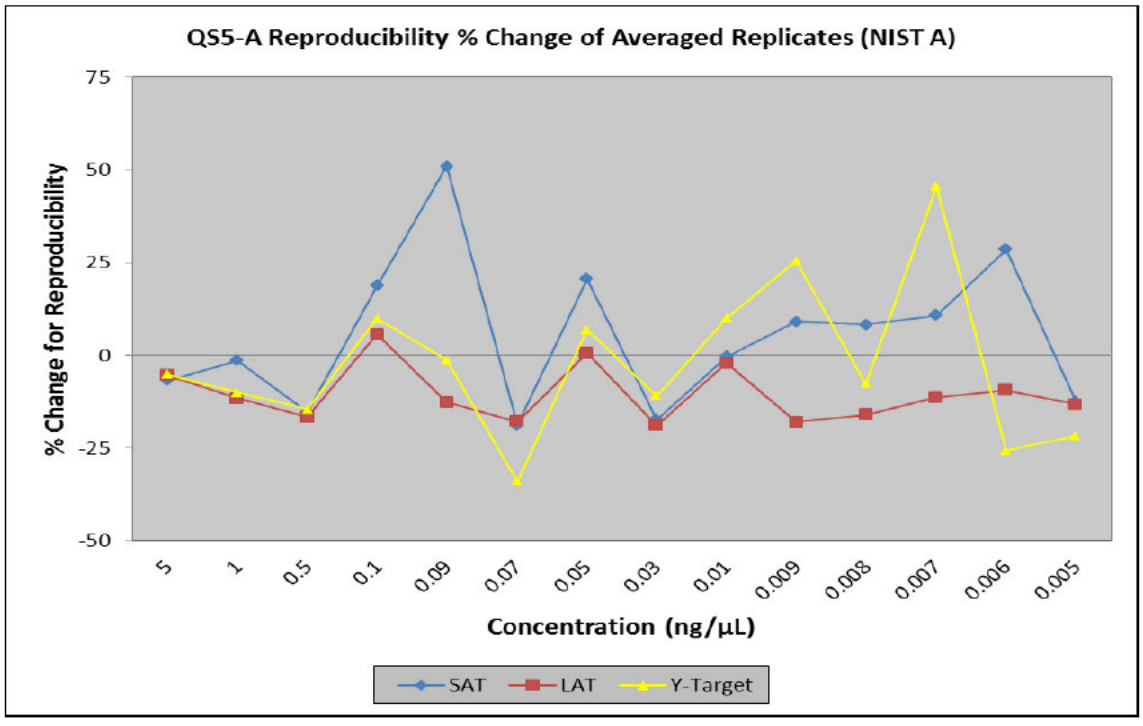

Figure 10: Percent change in reproducibility for NIST A

Similarly, the percentage changes for QS5-A NIST B and C (Figure 11 and 12) also do not exhibit continuous trends (i.e. one target having a consistently lower % change than another, or one target fluctuating more than another). The lack of a distinct trend for QS5-B NIST B and C (Figures 14 and 15) is also evident.

Both SAT and LAT targets for QS5-A NIST B and C appear to not vary by more than +/- ~30%, which is similar to QS5-B with the exception of NIST B 0.01 ng/µL LAT and 0.009 ng/µL SAT. One replicate of QS5-A dilution 0.07 ng/µL for NIST C SAT on the reproduced plate produced a quantification value of ~6.5 ng/µL, this sample was quantified again in duplicate using identical consumables, reagents and instruments, and the new results used for Figure 12 and Table 6.

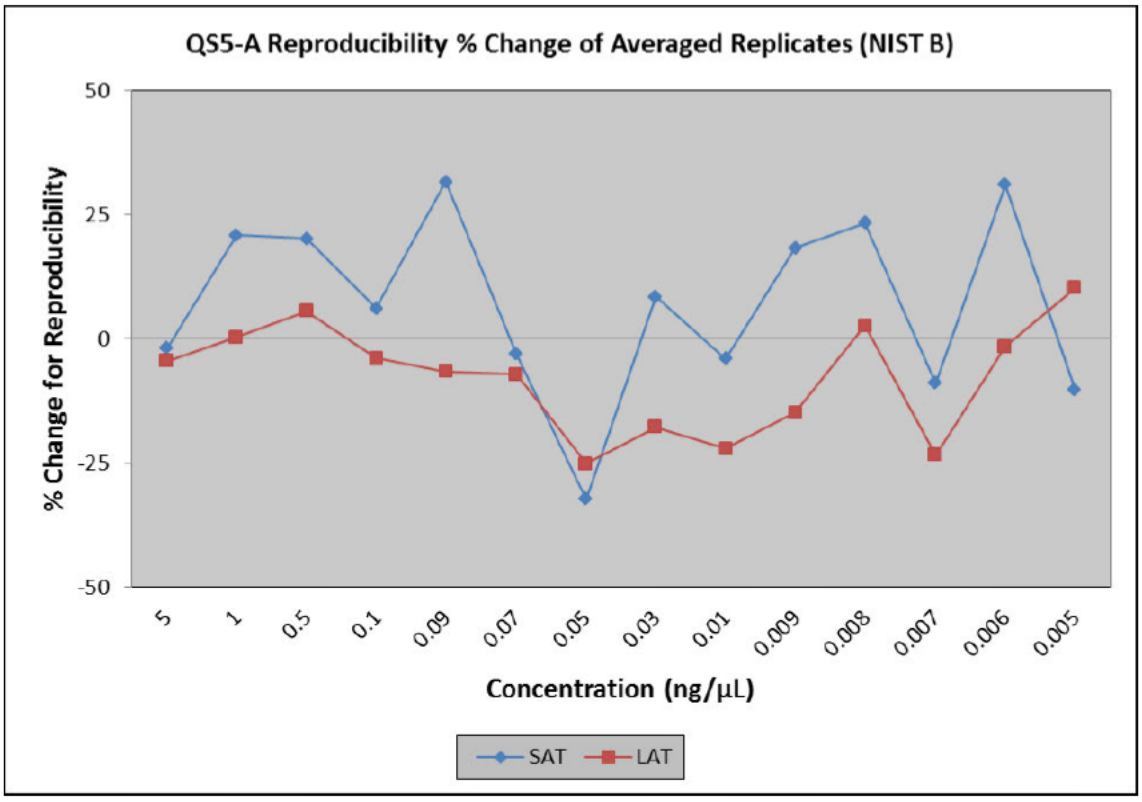

Figure 11: Percent change in reproducibility for NIST B

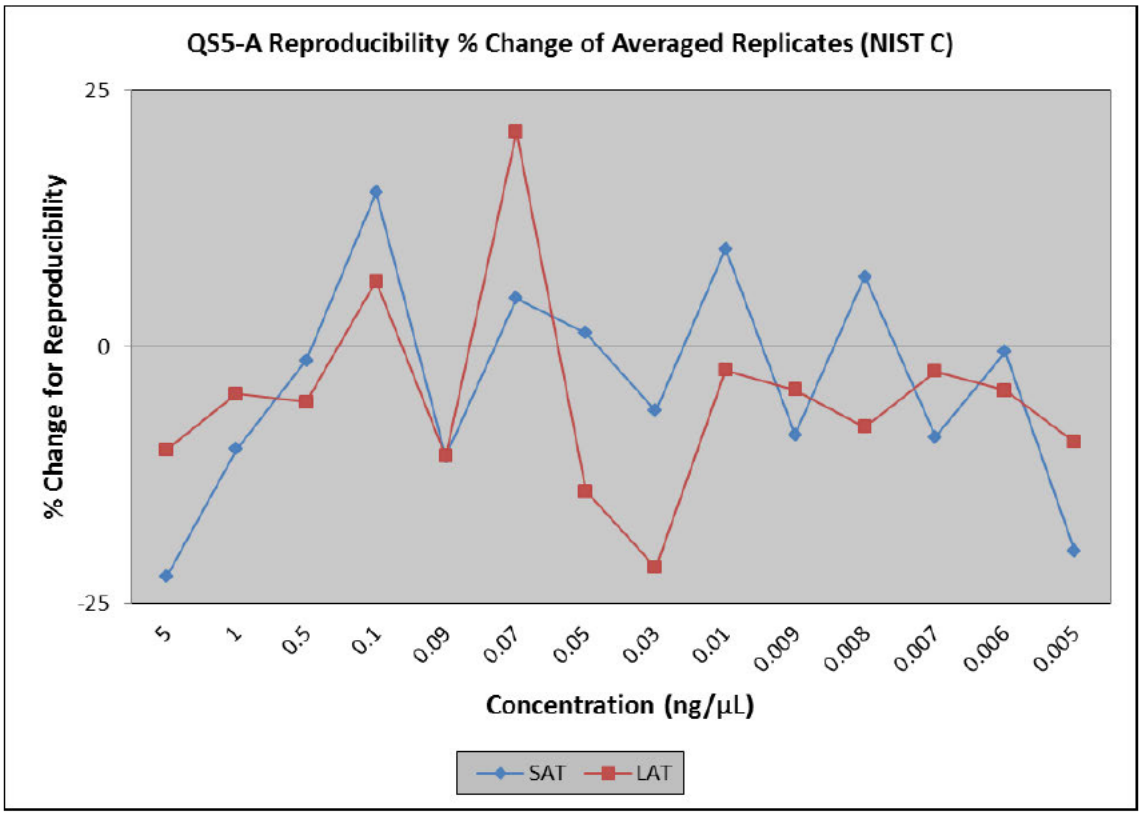

Figure 12: Percent change in reproducibility for NIST C

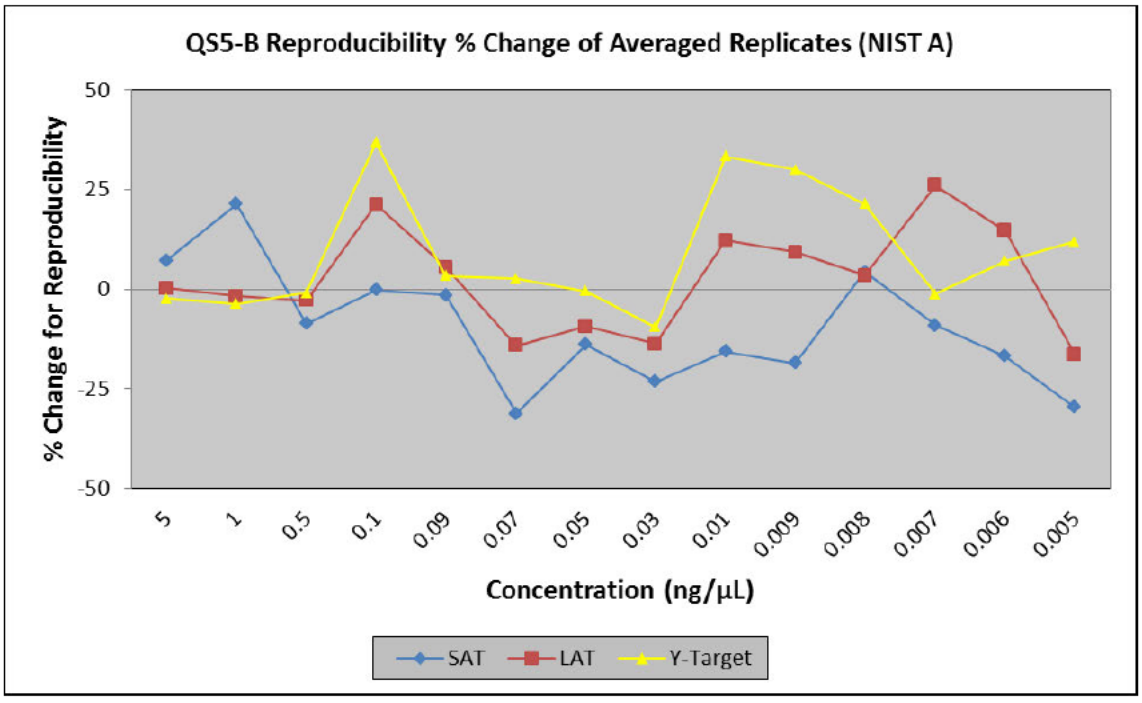

Figure 13: Percent change in reproducibility for QS5-B NIST A

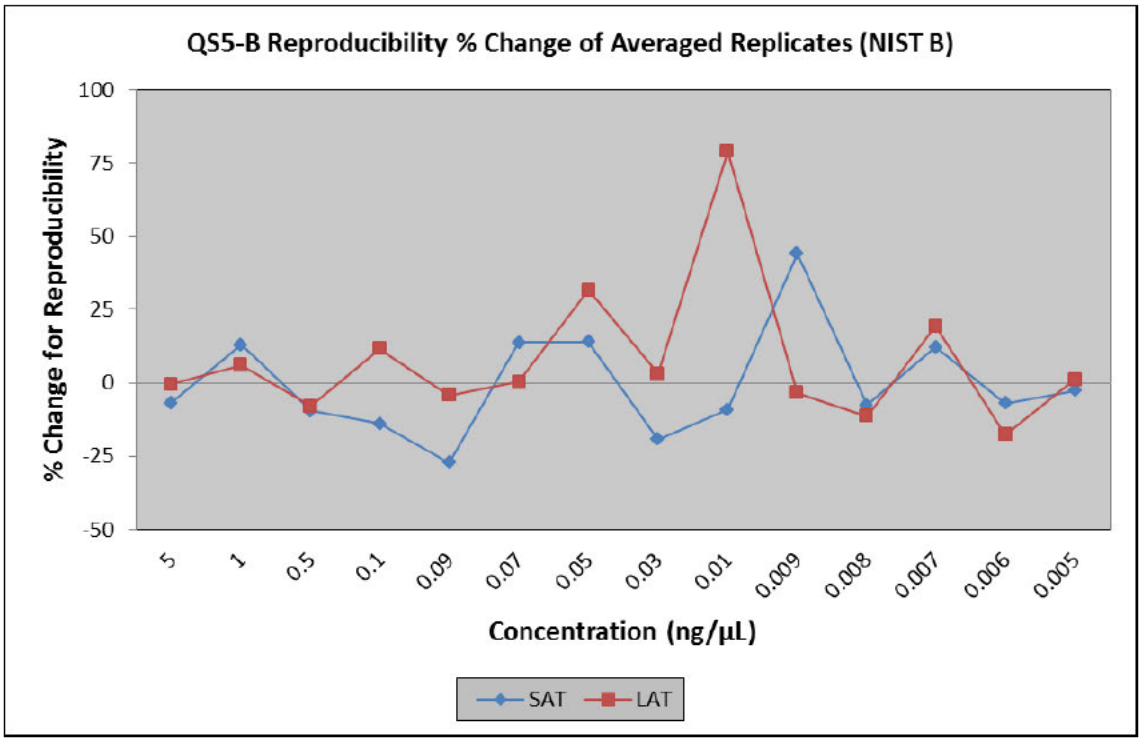

Figure 14: Percent change in reproducibility for QS5-B NIST B

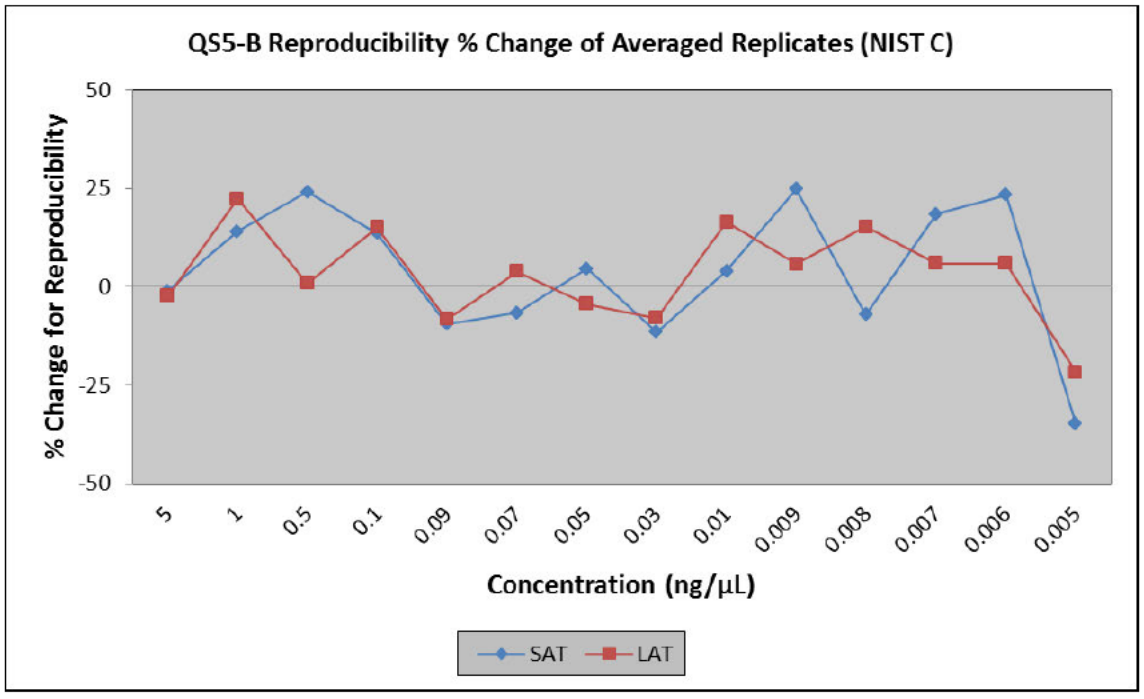

Figure 15: Percent change in reproducibility for QS5-B NIST C

Table 6: Student's *t*-test score for QS5-A & QS5-B reproducibility

	QS5-A SAT P-Value	QS5-B SAT P-Value	QS5-A LAT P-Value	QS5-B LAT P-Value	QS5-A Y-Target P-Value	QS5-B Y-Target P-Value
NIST A	0.31434	0.28732	0.11717	0.88733	0.14081	0.39869
NIST B	0.47476	0.38778	0.32552	0.89879		
NIST C	0.30332	0.33023	0.27463	0.77289		

Note:Y-Target analysis was only performed on NIST A (Male only DNA)

The Student's *t*-test scores shows that no significant differences were observed between the reproduced replicate averages for each of the NIST standards using the SAT and LAT targets, and the Y-Target for NIST A (Table 6) for both QS5s.

Discussion

As for experiment 3a (repeatability), the variability observed between targets at specific dilutions, and across the tested dilution series for each NIST standard supports the premise that a degree of variation is present in the Quantifiler Trio system's ability to generate reproducible results. An example of this is evident for QS5-A NIST-A (Figure 10) where the percentage change for all targets are relatively similar at 0.5 ng/µL and 0.005 ng/µL despite the large difference in concentration, however at 0.09 ng/µL and 0.007 ng/µL the difference in change observed between targets is considerably higher. The observations do not indicate a correlation between template concentration and percent change in reproducibility for any of the targets.

The greatest percentage change observed for the reproducibility data was approximately 79%, whereas the results from the repeatability experiment produced figures >125%. This is possibly due to the repeatability percentage being calculated from two replicates whereas the reproducibility is calculated using the averages of two replicates, therefore reducing the overall impact of outlying quantification values.

As for experiment 3a (repeatability), variability in the reproducibility of quantification results using both QS5s across all targets and NIST standards can be seen from the P-Values in Table 6, reflecting the results in figures 10-15. Despite this inherent propensity for the Quantifiler Trio system to produce variable results was has been observed in previous studies[2], overall there were no significant differences observed for reproduced replicate averages observed across the 5 – 0.005 ng/µL dilution series.

Acceptance Criteria

Reproducibility across the 5 – 0.005 ng/µL dilution series for both QS5s were shown to not differ significantly between replicate averages. These findings indicate that QS5 has produced results that are comparable to the original Quantifiler® Trio validation using the7500 instrument [2], which also showed no significant differences between reproduced quantification results.

Experiment 4: Y-Intercept Thresholds

Purpose

To determine the Y-Intercept thresholds for the SAT, LAT and Y-Targets, the values from eight plates run on the QS5s (Plate 1 (QS5-A & B), Plate 2 (QS5-A & B), reproduced Plate 1 (QS5-A & B), re-quant of dilution 0.07 ng/µL NIST C QS5-A, and QS5-B standards only) were used. The current ranges [5] will be used for the implementation of the two QS5 instruments with Quantifiler® Trio if the calculated Y-intercept values fall within these ranges.

Results

The average Y-intercept values taken from the eight plates ran on the QS5s +/- 3 x standard deviations was calculated and compared to the current Y-Intercept thresholds [5] as shown in Table 7.

Table 7: Y-Intercept ranges calculated for QS5A compared to current ranges.

	QS5 Y-Int. Range	Current Y-Int. Range
LAT	24.66 – 25.19	24.28 – 26.30
SAT	26.56 – 27.46	26.36 – 28.63
Y-Target	25.62 – 26.24	25.51 – 28.11

The QS5 Y-Intercept ranges for SAT, LAT and Y-Target all fall into the current ranges outlined in the Quantification SOP [5].

Discussion

The newly calculated Y-Intercept ranges for QS5 are considerably narrower than the current ranges, which is in part due to the relatively small number of plates used to calculate them. It is important to consider that calculated thresholds are instrument and kit specific so variation is to be expected. As more plates are processed after implementation, the cumulative data will be used to recalculate these ranges over time.

Acceptance Criteria

Since the newly calculated QS5 Y-Intercept ranges are relatively narrow but fall within the current ranges, the QS5 implementation will utilise the current ranges until more data is available to allow recalculation for QS5.

Conclusion

The results of experiment 1 showed the LOD for QS5 is similar to that of 7500 and possibly even more sensitive although more studies are required to confirm this. These findings support the recommendations of the original Quantifiler® Trio validation that the LOD be set to 0.001 ng/µL.

Independently comparing the results of both QS5 instruments to those produced by 7500-A showed no significant differences in SAT, LAT and Y-Target quantification results demonstrating comparability between 7500 and QS5.

Both QS5 instruments showed no significant differences in repeatability results across all dilutions series demonstrating comparability to the 7500 instrument which produced repeatable results in the original Quantifiler® Trio validation.

The QS5 instruments were able to demonstrate no significant difference between results reproduced by different operators on different days at the specific dilutions examined. These results are comparable to the findings for the original Quantifiler® Trio validation using the 7500.

The Y-intercept ranges calculated from the values obtained from all eight QS5 plates produced in this study all fall within the ranges that are currently in use. Given the ranges calculated for QS5 are considerably narrower than current ranges, it is recommended that the current ranges be used for QS5 implementation, and the thresholds revised every 2 weeks for the first 3 months once the data set is expanded.

Recommendations

1. QuantStudio™ 5 Real-Time PCR systems A and B be implemented for DNA quantification using the Quantifiler® Trio DNA quantification kit, and thus replacing the two 7500 Real-Time PCR systems.
2. Y-Intercept data for SAT, LAT and Y-Targets are to be collated and used to recalculate/monitor ranges over time after implementation of the QS5s.

References

[1] Thermo Fisher Scientific, Quantifiler® HP and Trio DNA Quantification Kits UserGuide, Publication Number 4485354, Revision A. Publication Number 4485354, Revision A ed2014.

[2] Validation of Quantifiler® Trio. P. Acedo, M. Mathieson, L. Ryan, C. Allen. September 2015. Forensic DNA Analysis.

[3] Certificate of Analysis – Standard Reference Material® 2372 Human DNA Quantitation Standard. National Institute of Standards & Technology.

[4] Project Proposal #185 – Validation of the QuantStudio™ Real-Time PCR Systems (June 2017).

[5] Operation and Maintenance of the Microlab STARlet and LabElite Integrated I.D.Capper. QIS 34050.

[6] Quantification of Extracted DNA using the Quantifiler® Trio DNA Quantification Kit. QIS 33407.