

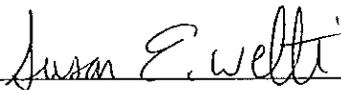
District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

Quantitation Internal Validation

Quantitation Internal Validation

Plexor® HY System

This validation is reviewed and approved by:



Susan Welti, Forensic Biology Unit Technical Leader

120215

Date

*minor edit checked 010516
JW 010516*

Studies in this validation were conducted and reviewed by:



Jessica Skillman, Forensic Scientist III

120215

Date

**Minor edit made to page 24
on 010516. JW 010516*

Studies in this validation were conducted and written by Élise Caron, Forensic Biology Unit Intern.

Table of Contents

1. Introduction.....	3
2. Precision Study.....	4
3. Reproducibility Study.....	9
4. Accuracy Study.....	14
5. Sensitivity and Reproducibility Study.....	19
6. Mixture Study.....	28
7. Contamination	33
8. Standard Curve	35
9. Performance Check.....	39
10. Amplification Calculation.....	43
11. Final Conclusions.....	45
12. References.....	46
13. Appendix.....	47

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

1. Introduction

This report describes the internal validation of the Promega Corporation Plexor® HY System performed according to the Quality Assurance Standards of Forensic DNA Testing Laboratories (2011) issued by the Federal Bureau of Investigation (FBI) and the revised validation guidelines issued by the Scientific Working Group on DNA Analysis Methods (SWGDM, 2012).

To verify the performance of the Plexor® HY System, the following studies were performed as part of the internal validation: Precision, Reproducibility, Accuracy, Sensitivity, Mixture, Contamination and Standard Curve.

2. Precision Study

2.1. Objective

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

2.2. Materials and Methods

Before using the Plexor® HY System, a background calibration was performed as recommended by the instrument manufacturer. Then, the instrument was calibrated for fluorescein (FL), CAL Fluor® Orange 560 (CO 560), CAL Fluor® Red 610 (CR 610) and IC5 as recommended by the kit manufacturer. Monthly instrument maintenance was carried out throughout the validation study, as needed. See Appendix for a copy of the instrument maintenance log.

Precision A (020614EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 020614EC Case Number: Plexor HY Precision A
 Analyst: EC Date: 2/6/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	85	89.25
Water, Amplicon Grade	7.0	59.5	62.475
Plexor HY 20X Primer IPC Mix	1.0	8.5	89.25
total reaction volume	20.0		
number of reactions	85		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1											
B	STND 2											
C	STND 3											
D	STND 4											
E	STND 5											
F	STND 6											
G	STND 7											
H	NTC											

Plexor HY Kit Lot #: 93927 Master Mix Lot #: 80941 Primer IPC Mix Lot #: 55948
 Plexor HY Kit Expiration Date: 12/27/2015 DNA standard Lot #: 92056 H₂O, amp. grade Lot #: 88091
 TE Buffer Lot #: T022028L1101
 DNA Standard Batch #: 020614EC (A1-G10) 020614EC (A11-G12)
 7500 Used: B

Following quantification, all extracts have been returned to (Temperature/Location):

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Precision B (033114EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 033114EC

Case Number: Plexor HY Precision B

Analyst: EC

Date: 3/31/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	55¢	\$92.5
Water, Amplicon Grade	7.0	23¢	\$21.75
Plexor HY 20X Primer IPC Mix	1.0	85¢	\$2.25
total reaction volume	20.0		
number of reactions	85		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1											
B	STND 2											
C	STND 3											
D	STND 4											
E	STND 5											
F	STND 6											
G	STND 7											
H	NTC											

Plexor HY Kit Lot #: 104525
 Plexor HY Kit Expiration Date: 07/15/16
 TE Buffer Lot #: T022024L1101
 DNA Standard Batch #: 033114EC
 7500 Used: B

Master Mix LN: 93555
 DNA standard LN: 104248
 Primer IPC Mix LN: 93522
 H₂O, amp. grade LN: 95505

Following quantification, all extracts have been returned to (Temperature/Location):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

2.3. Experimental Setup

A serial dilution ranging from 50ng/μl to 3.2pg/μl of the supplied Plexor® HY System was prepared according to the manufacturer's instructions. This dilution series was pipetted in 2μl aliquots as columns across a 96 well plate (see plate documents shown above). Two plates were run by the same analyst with two separately prepared dilution series sets.

2.4. Data Analysis

For both plates (020614EC and 033114EC), data was collected with the 7500 SDS software and analyzed with the Plexor® Analysis Software.

2.5. Results

In order to assess the precision of the Plexor® HY System on the 7500 Real-time PCR instrument, two plates of standards were run by the same analyst. C_q values were evaluated and compared to determine the precision of the kit and instrument.

Within a plate, the precision of each standard's quantities is important in establishing a reliable standard curve with which to determine a sample's concentration prior to amplification. According to the results listed below the highest observed standard deviations in Human C_q values were in the 0.016 and 0.0032ng/μl standards. The general trend was for standard deviation to increase as standard quantity decreased. This trend was also observed in the standard deviations associated with the Male C_q values.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

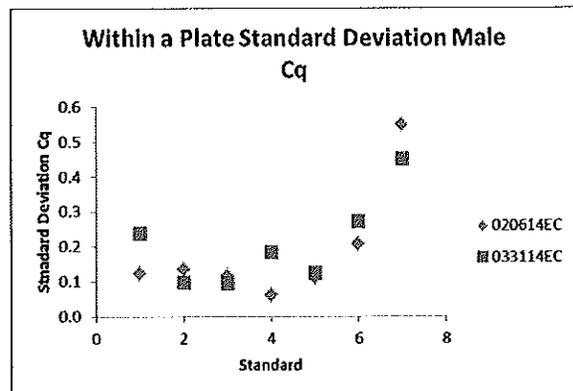
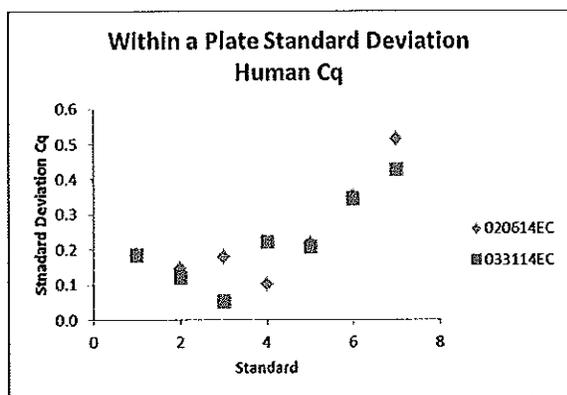
The standard deviations observed in the IPC C_q's were below 0.25, with the exception of standard 1 of 033114EC plate which contained an outlier. IPC C_q values showed no dependence upon the quantity of DNA present in the associated sample.

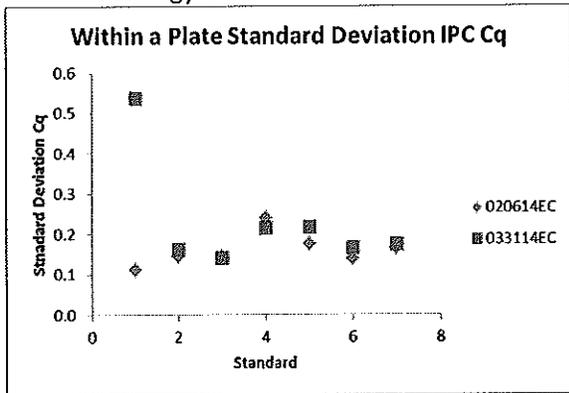
020614EC

STND	Average Cq Human	StdDev Cq Human	Average Cq IPC	StdDev Cq IPC	Average Cq Male	StdDev Cq Male
STND 1	16.766	0.183	21.497	0.113	18.114	0.125
STND 2	19.286	0.147	20.784	0.146	20.572	0.135
STND 3	21.667	0.180	20.703	0.143	22.935	0.116
STND 4	24.263	0.101	20.740	0.240	25.646	0.061
STND 5	26.790	0.217	20.831	0.175	28.185	0.112
STND 6	28.875	0.352	20.752	0.138	30.549	0.208
STND 7	31.798	0.514	20.881	0.166	32.746	0.548

033114EC

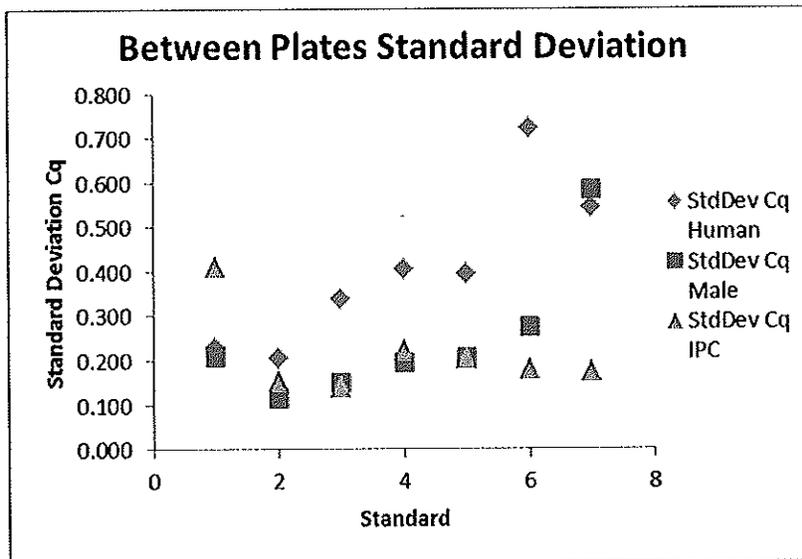
STND	Average Cq Human	StdDev Cq Human	Average Cq IPC	StdDev Cq IPC	Average Cq Male	StdDev Cq Male
STND 1	17.044	0.183	21.288	0.536	18.275	0.239
STND 2	19.611	0.119	20.752	0.161	20.563	0.098
STND 3	22.288	0.052	20.743	0.140	23.145	0.095
STND 4	24.983	0.223	20.782	0.215	25.909	0.184
STND 5	27.448	0.208	20.718	0.217	28.519	0.124
STND 6	30.126	0.345	20.565	0.165	30.812	0.271
STND 7	32.373	0.428	20.789	0.175	33.382	0.452

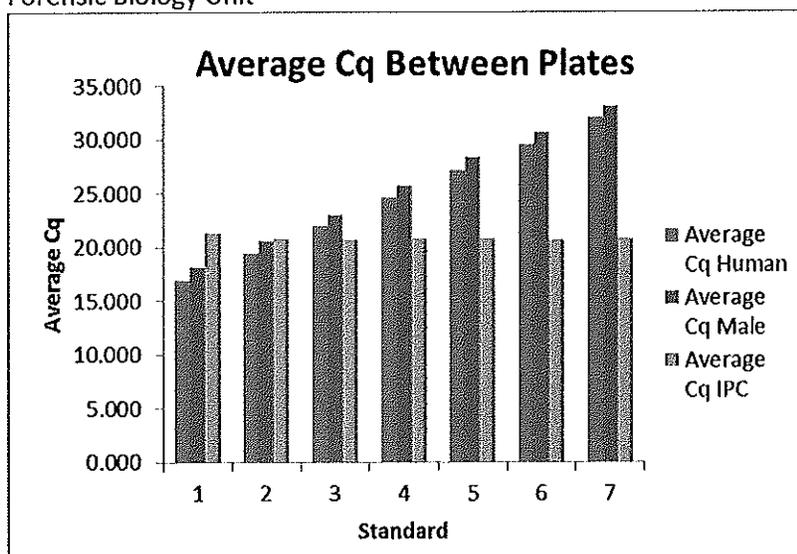




From plate to plate, the precision of each standard's quantity is important in maintaining a consistent standard curve for determining sample concentrations prior to amplification. Higher standard deviation values were obtained for the 16 and 3.2pg/ μ l standards and the lowest standard deviation values were obtained from the 50, 10 and 2ng/ μ l standards.

STND	Human		IPC		Male	
	Average C _q	StdDev C _q	Average C _q	StdDev C _q	Average C _q	StdDev C _q
STND 1	16.918	0.228	21.383	0.409	18.202	0.208
STND 2	19.452	0.206	20.766	0.152	20.567	0.114
STND 3	22.005	0.340	20.725	0.139	23.050	0.148
STND 4	24.655	0.406	20.763	0.222	25.790	0.193
STND 5	27.149	0.394	20.770	0.203	28.367	0.206
STND 6	29.557	0.722	20.650	0.177	30.692	0.274
STND 7	32.112	0.543	20.831	0.173	33.093	0.584





2.6. Conclusions

According to this study, standard deviations of the standards increase as concentration decreases with values ranging from 0.206 to 0.722. Using the following equation,

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

a standard deviation of 0.206 in C_q value could result in a possible 1.15 fold difference in concentration. A standard deviation of 0.722 could result in a possible 1.65 fold difference in concentration. Therefore, a 10 ng/ μ l sample could quantify as high as 11.5 ng/ μ l or as low as 8.7 ng/ μ l. A 16 pg/ μ l sample could quantify as high as 26.4 pg/ μ l or as low as 9.7 pg/ μ l. The pattern of higher standard deviations in the lower concentration standards is present in both the Human C_q and Male C_q standard values. According to the Plexor® HY System manual, it is expected to see a higher amount of variation in the lower concentration standards, especially the 3.2pg/ μ l standard, due to the stochastic effects in the low concentrations of DNA during PCR.

Also demonstrated in this study was the reliability of the IPC C_q value despite the concentration of DNA in the sample. The IPC C_q 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_q was 20.84 with a standard deviation of 0.092. Using three standard deviations, the normal range of IPC C_q values should be between 21.116 and 20.564.

The range of IPC C_q values will continue to be evaluated in the subsequent plates of this validation to determine how the range may change with different sample types and concentrations.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

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 Study

3.1. Objective

To demonstrate reproducibility, two full plates consisting of an individually prepared standard dilution series of the Plexor® HY 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 curves with which to determine each sample's quantities, excluding columns 11 and 12 from 020614EC. 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.

3.2. Materials and Methods

Reproducibility A (020614EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 020614EC Case Number: Plexor HY Precision A
 Analyst: EC Date: 2/6/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	450	\$92.5
Water, Amplification Grade	7.0	595	624.75
Plexor HY 20X Primer IPC Mix	1.0	85	\$9.25
total reaction volume	20.0		
number of reactions	85		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1											
B	STND 2											
C	STND 3											
D	STND 4											
E	STND 5											
F	STND 6											
G	STND 7											
H	NTC											

Plexor HY Kit Lot #: 93927 Master Mix Lot #: 87941 Primer IPC Mix Lot #: 55948
 Plexor HY Kit Expiration Date: 12/27/2015 DNA Standard Lot #: 92956 H₂O, amp. grade Lot #: 85/91
 TE Buffer Lot #: 10220241101
 DNA Standard Batch #: 020614EC (A1-G10), 020614EC (A11-G12)
 7500 Used: B

Following quantification, all extracts have been returned to (Temperature/Location):

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Reproducibility B (033114EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 033114EC

Case Number: Plexor HY Protocol B

Analyst: EC

Date: 3/31/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5% m/m
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	\$50	\$92.5
Water, Amplicon grade	7.0	\$9	\$101.4
Plexor HY 20X Primer IPC Mix	1.0	\$1	\$102.4
total reaction volume	20.0		
number of reactions	83		

Plate Setup Type in your sample names in the appropriate well header

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1											
B	STND 2											
C	STND 3											
D	STND 4											
E	STND 5											
F	STND 6											
G	STND 7											
H	NTC											

Plexor HY Kit Lot #: 104525
 Plexor HY 2X Expiration Date: 07/15/16
 TE Buffer Lot #: 10220281101
 DNA Standard Batch #: 033114EC
 7500 Used: B

Master Mix Lot #: 93555
 DNA standard Lot #: 104248
 Primer IPC Mix Lot #: 93522
 H₂O, amp. grade Lot #: 95505

Following quantification, all extracts have been returned to (Temperature/Lot/Kit):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

3.3. Experimental Setup

A serial dilution ranging from 50 ng/μl to 3.2 pg/μl of the supplied Plexor® HY DNA Standard was prepared according to the manufacturer's instructions. This dilution series was pipetted in 2 μl aliquots as columns across a 96 well plate (see plate document above). Two plates were run by the same analyst with two separately prepared dilution series sets.

3.4. Data Analysis

For both plates (020614EC and 033114EC), data was collected with the 7500 SDS software and analyzed with the Plexor® Analysis Software.

3.5. Results

In order to assess the reproducibility of the Plexor® HY System on the 7500 Real-Time PCR instrument, two plates of standards were run by the same analyst. Sample quantities were evaluated using different columns set as standards, excluding columns 11 and 12 from 020614EC. This data was compared to determine the ability of the kit and instrument to reproducibly obtain quantities of different concentrations of samples.

Within a plate, an average quantity and standard deviation was obtained for each set of samples of the same concentration. A percent difference was calculated to determine how the actual quantities

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

obtained compared to the expected quantities. Within a plate and between plates, up to an approximate 24% difference was observed in the human quantities and up to 21% difference was observed in the male quantities. Between plates and in both the male and human detector, these higher percentages of difference in quantity were observed in standards 6 (16pg/ μ L) and 7 (3.2pg/ μ L) and lower percentages of difference in quantity were observed in standard 1 (50ng/ μ L).

Within a Plate (Human)

020614EC

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	50.64	6.30	1.29
STD 2	10	9.816	0.920	-1.84
STD 3	2	2.093	0.232	4.64
STD 4	0.4	0.3869	0.0248	-3.28
STD 5	0.08	0.07546	0.01033	-5.67
STD 6	0.016	0.01978	0.00416	23.64
STD 7	0.0032	0.003052	0.000932	-4.63

033114EC

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	52.18	6.00	4.36
STD 2	10	10.62	0.88	6.17
STD 3	2	1.963	0.063	-1.86
STD 4	0.4	0.3685	0.0551	-7.87
STD 5	0.08	0.07906	0.01016	-1.18
STD 6	0.016	0.01509	0.00325	-5.66
STD 7	0.0032	0.003760	0.000884	17.49

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Within a Plate (Male)

020614EC

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	50.74	3.85	1.49
STD 2	10	10.23	0.89	2.26
STD 3	2	2.190	0.168	9.48
STD 4	0.4	0.3730	0.0148	-6.76
STD 5	0.08	0.07129	0.005271	-10.88
STD 6	0.016	0.01536	0.002015	-3.98
STD 7	0.0032	0.003882	0.001462	21.30

033114EC

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	47.53	6.59	-4.94
STD 2	10	11.06	0.70	10.59
STD 3	2	2.156	0.130	7.79
STD 4	0.4	0.3761	0.0448	-5.97
STD 5	0.08	0.07193	0.00564	-10.09
STD 6	0.016	0.01706	0.002936	6.64
STD 7	0.0032	0.003432	0.000855	7.24

Between Plates (Human)

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	51.56	6.12	3.11
STD 2	10	10.29	0.89	2.92
STD 3	2	2.015	0.131	0.77
STD 4	0.4	0.3760	0.0428	-6.01
STD 5	0.08	0.07757	0.01023	-3.04
STD 6	0.016	0.01700	0.00362	6.22
STD 7	0.0032	0.003476	0.000905	8.62

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Between Plates (Male)

	[Expected]	Average [Observed]	SD	% Difference from Expected
STD 1	50	48.83	5.48	-2.34
STD 2	10	10.72	0.78	7.21
STD 3	2	2.170	0.146	8.48
STD 4	0.4	0.3748	0.0327	-6.29
STD 5	0.08	0.07164	0.00549	-10.45
STD 6	0.016	0.01637	0.00256	2.33
STD 7	0.0032	0.003613	0.001103	12.91

3.6. Conclusions

According to this study, up to approximately 24% difference may be observed from an expected human quantity especially samples with DNA concentrations below 80pg/ μ l. Up to an approximate 21% difference may be observed from an expected male quantity especially in samples below 16pg/ μ l. An average percent difference in expected quantity is 1.80% in the human detector and 1.69% in the male detector. The data also indicates that the locations of the columns of standards on the plate will not affect the standard curve generated.

This study used the Plexor[®] HY System standard to assess reproducibility. This standard is generated from pooled human male genomic DNA. The reproducibility of the Plexor[®] HY System on the 7500 Real-Time PCR instrument will be further evaluated with mock samples and various dilutions of single source samples in the sensitivity study to determine if similar values are obtained.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
4. Accuracy Study

4.1. Objective

To demonstrate accuracy, two plates consisting of two columns of a serial dilution of the Plexor® HY System DNA Standard, samples previously quantified with Quantifiler Duo, and NIST's 2372 Quantitation Standards (NIST A, B, and C) were prepared. The average concentrations and standard deviations obtained were compared to their expected values to determine the accuracy of the Plexor® HY System.

4.2. Materials and Methods

Accuracy A (022014EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 022014EC Case Number: Plexor HY Accuracy A
 Analyst: EC Date: 2/20/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 556
sample	200	0.5	n/a
Plexor HY 2X Master Mix	10.0	810	830.5
Water, Amplification Grade	7.0	567	595.5
Plexor HY 20X Primers/PC Mix	1.0	81	83.05
total reaction volume	20.0		
number of reactions	81		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 2	STND 2	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C			
B	STND 1	STND 1	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C	STD 2	STD 2	
C	STND 3	STND 3	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C	STD 3	STD 3	
D	STND 4	STND 4	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50	STD 4	STD 4	
E	STND 5	STND 5	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50	STD 5	STD 5	
F	STND 6	STND 6	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50	STD 6	STD 6	
G	STND 7	STND 7		STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 7	STD 7	
H	NTC			STD 2	STD 3	STD 4	STD 5	STD 6	STD 7			

Plexor HY Kit Lot #: 53772 Master Mix Lot #: 93355 Primers/PC Mix Lot #: 93322
 Plexor HY Kit Expiration Date: 07/15/2016 DNA standard Lot #: 56280 H₂O, amp. grade Lot #: 95504
 TE Buffer Lot #: T02202811101
 DNA Standard Batch #: 022014EC (A1-G2), 020614EC (B10-G11), 021114S1 (G4-FE)
 7500 Used: B

Following quantification, all extracts have been returned to (Temperature/Location):

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Accuracy B (040114EC1)

Plexor HY Quantitation Set-Up Worksheet

Quantitative Batch Name: 040114EC1

Case Number: Plexor HY Accuracy B

Analyst: EC

Date: 4/2/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	57.0	59.85
Water, Amp. Modification Grade	7.0	39.0	418.50
Plexor HY 20X Primers/PC Mix	1.0	5.0	59.85
total reaction volume	20.0		
number of reactions	57		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C			
B	STND 2	STND 2	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C			
C	STND 3	STND 3	Q11	Q13	Q19	Q28	NIST_A	NIST_B	NIST_C			
D	STND 4	STND 4	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50			
E	STND 5	STND 5	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50			
F	STND 6	STND 6	Q12	Q14	Q23	Q31	NIST_A_1:50	NIST_B_1:50	NIST_C_1:50			
G	STND 7	STND 7										
H	NTC											

Plexor HY Kit Lot #: 104525
 Plexor HY KR Expiration Date: 07/15/16
 TE Buffer Lot #: J02202SL1101
 DNA Standard Batch #: 040114EC
 7500 Used: B

Master Mix Lot #: 93555
 DNA standard Lot #: 101348
 Primer/PC Mix Lot #: 93522
 H₂O, amp. grade Lot #: 95925

Following quantification, all extracts have been returned to (Temperature/Location):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

4.3. Experimental Setup

A serial dilution ranging from 50ng/μl to 3.2pg/μl of the Plexor® HY System DNA Standard was prepared according to the manufacturer's instructions. NIST A, B and C were diluted to obtain a 1:50 ratio (1.14, 1.22, and 1.18ng/μL respectively). Q11, Q12, Q13, Q14, Q19, Q23, Q28, and Q31 are non-probative samples from previously analyzed proficiency tests, extracted using organic extraction or the EZ1 Advanced XL instrument. All samples were pipetted in 2μL aliquots in 18μL Master Mix (see plate documents shown above). The NIST samples and previously quantified samples were tested in triplicate. Two plates were run by the same analyst with two separately prepared dilution series sets for each standard.

4.4. Data Analysis

For both plates (022014EC and 040114EC1), data was collected with the 7500 SDS software and analyzed with the Plexor® Analysis Software.

4.5. Results

In order to evaluate the accuracy of the Plexor® HY System on the 7500 Real-Time PCR instrument, two plates were run with serial dilutions of the Plexor Human DNA standard, NIST A, B, C, their respective 1:50 dilutions and 8 non-probative samples. Results were analyzed using the two columns of the Plexor®

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 HY System dilution series as standards to compare the concentrations obtained to the concentrations expected.

Shown below are tables representing the observed and expected quantities for the NIST and 8 non-probative samples and their observed/expected ratios.

Human (022014EC)

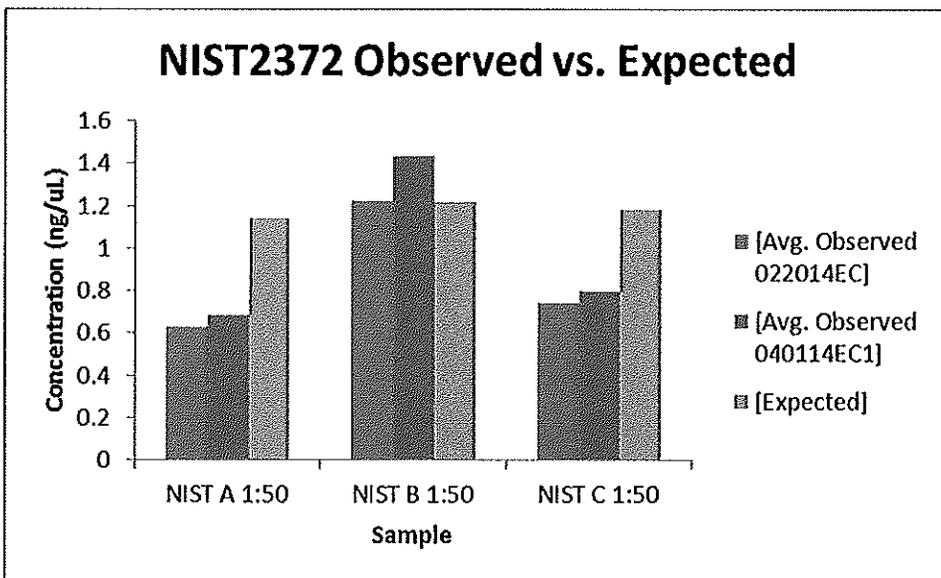
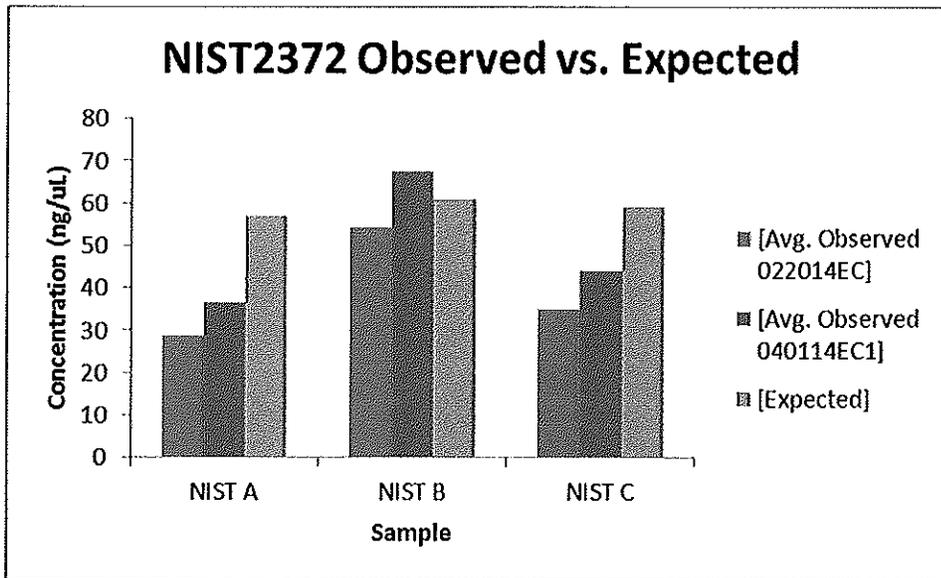
Sample	Average Observed Concentration	Expected Concentration	Observed/Expected
NIST A	28.70	57.00	0.50
NIST A 1:50	0.63	1.14	0.55
NIST B	54.43	61.00	0.89
NIST B 1:50	1.23	1.22	1.01
NIST C	35.16	59.00	0.60
NIST C 1:50	0.74	1.18	0.63
Q11	1.76	4.00	0.44
Q12	1.33	2.58	0.52
Q13	4.26	2.83	1.51
Q14	1.74	3.07	0.57
Q19	2.26	3.41	0.66
Q23	1.57	2.19	0.72
Q28	3.91	3.88	1.01
Q31	8.11	4.03	2.01

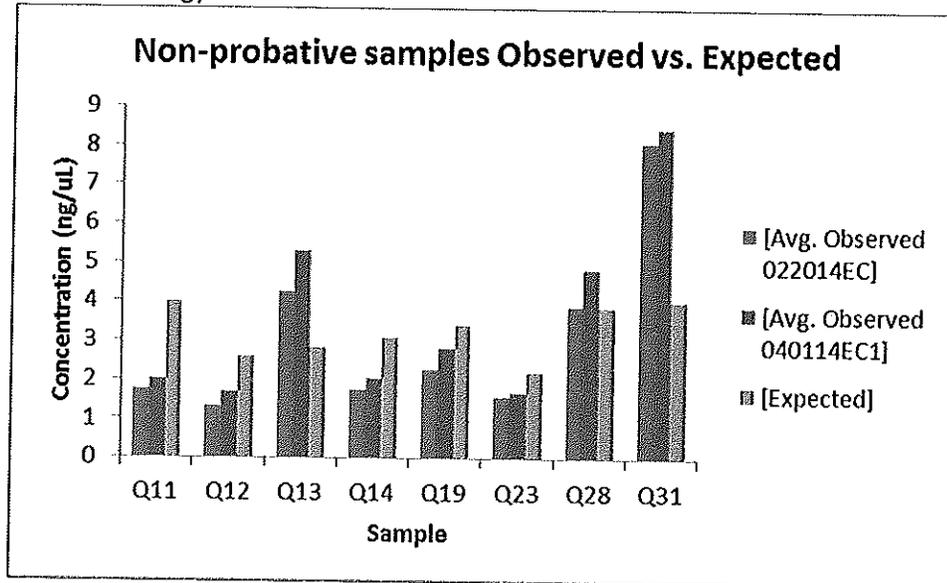
Human (040114EC1)

Sample	Average Observed Concentration	Expected Concentration	Observed/Expected
NIST A	36.73	57.00	0.64
NIST A 1:50	0.68	1.14	0.60
NIST B	67.60	61.00	1.11
NIST B 1:50	1.43	1.22	1.17
NIST C	44.18	59.00	0.75
NIST C 1:50	0.80	1.18	0.68
Q11	2.02	4.00	0.50
Q12	1.68	2.58	0.65
Q13	5.31	2.83	1.88
Q14	2.04	3.07	0.67
Q19	2.81	3.41	0.82
Q23	1.67	2.19	0.76
Q28	4.83	3.88	1.25
Q31	8.46	4.03	2.10

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Shown below are bar graphs of the observed and expected concentrations for the NIST and non-probative samples. The observed values were significantly lower than the expected values for NIST A and C, which is also seen in their 1:50 dilutions. NIST B quantitated closer to its expected value in both undiluted and diluted samples. The observed values of 5 out of 8 non-probative samples were significantly lower than the expected quantities. Two samples were significantly higher and one sample was close to its expected values. Overall, there was minimum variability between replicates across the plate.





4.6. Conclusions

According to this study, although it appears that the concentrations obtained were significantly different than the expected values, an average difference of 0.76 in the NIST samples and 1.00 in the non-probative samples were observed. These values are within the calculated possible difference between plates observed in the precision study. Also, minimal variability was observed between replicates within a plate and between plates, which is consistent with the results obtained in the reproducibility study. For the majority of the non-probative samples, concentrations were lower than the expected values, which were obtained using Quantifiler® Duo. This underestimation could result in overamplification at the current amplification target range of 0.5-1.0ng. A decrease in the amplification target range may need to be considered.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

5. Sensitivity and Reproducibility Study

5.1. Objective

For sensitivity, serial dilutions of four samples (ranging from 1.0 to 0.001ng/μl) were prepared and used to assess the ability of the Plexor® HY System to accurately quantify samples, with particular consideration to low level DNA samples.

For Reproducibility, serial dilutions of two samples (ranging from 1.0 to 0.001ng/μl) were prepared and used to assess the ability of the Plexor® HY System to reliably reproduce quantitation values over a range of human DNA concentrations within a plate and between plates.

5.2. Materials and Methods

Dilution Setup Worksheet

Sensitivity (female samples)				Sensitivity (male samples)			
Tube Name	Concentrations (ng/uL)	Q32 (50.02ng/uL)	TE buffer (uL)	Tube Name	Concentrations (ng/uL)	Q37 (21.04ng/uL)	TE buffer (uL)
Q32.A	1	2 uL of Q32	98	Q37.A	1	4.75 uL of Q37	95.25
Q32.B	0.5	40 uL of A	40	Q37.B	0.5	40 uL of A	40
Q32.C	0.25	40 uL of B	40	Q37.C	0.25	40 uL of B	40
Q32.D	0.125	40 uL of C	40	Q37.D	0.125	40 uL of C	40
Q32.E	0.0625	40 uL of D	40	Q37.E	0.0625	40 uL of D	40
Q32.F	0.03215	40 uL of E	40	Q37.F	0.03215	40 uL of E	40
Q32.G	0.015625	40 uL of F	40	Q37.G	0.015625	40 uL of F	40
Q32.H	0.0078125	40 uL of G	40	Q37.H	0.0078125	40 uL of G	40
Q32.I	0.00390625	40 uL of H	40	Q37.I	0.00390625	40 uL of H	40
Q32.J	0.001953125	40 uL of I	40	Q37.J	0.001953125	40 uL of I	40
Q32.K	0.0009765625	40 uL of J	40	Q37.K	0.0009765625	40 uL of J	40
Dilutions	Concentrations (ng/uL)	Q15 (30.71ng/uL)	TE buffer (uL)	Tube Name	Concentrations (ng/uL)	Q35 (39.00ng/uL)	TE buffer (uL)
Q15.A	1	3.25 uL of Q15	96.75	Q35.A	1	2.56 uL of Q35	97.44
Q15.B	0.5	40 uL of A	40	Q35.B	0.5	40 uL of A	40
Q15.C	0.25	40 uL of B	40	Q35.C	0.25	40 uL of B	40
Q15.D	0.125	40 uL of C	40	Q35.D	0.125	40 uL of C	40
Q15.E	0.0625	40 uL of D	40	Q35.E	0.0625	40 uL of D	40
Q15.F	0.03215	40 uL of E	40	Q35.F	0.03215	40 uL of E	40
Q15.G	0.015625	40 uL of F	40	Q35.G	0.015625	40 uL of F	40
Q15.H	0.0078125	40 uL of G	40	Q35.H	0.0078125	40 uL of G	40
Q15.I	0.00390625	40 uL of H	40	Q35.I	0.00390625	40 uL of H	40
Q15.J	0.001953125	40 uL of I	40	Q35.J	0.001953125	40 uL of I	40
Q15.K	0.0009765625	40 uL of J	40	Q35.K	0.0009765625	40 uL of J	40

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Sensitivity A (020714EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 020714EC

Case Number: Plexor HY Sensitivity A

Analyst: EC

Date: 2/7/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	200	n/a	n/a
Plexor HY 2X Master Mix	1000	982	687
Water, Amplicon Grade	700	651	690.5
Plexor HY 20X Primer IPC Mix	1.0	91	93.7
Total reaction volume	2000		
number of reactions	91		

*no. of master mix for second STND 7 (020414EC) replicate

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
B	STND 2	STND 2	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
C	STND 3	STND 3	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
D	STND 4	STND 4	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
E	STND 5	STND 5	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
F	STND 6	STND 6	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
G	STND 7	STND 7	Q32A	Q32A	Q32A	Q15A	Q15A	Q15A	STND 1	STND 1	STND 2	STND 2
H	NTC		STND 3	STND 3	STND 4	STND 4	STND 5	STND 5	STND 6	STND 6	STND 7	

Plexor HY Kit Lot #: 93927
 Plexor HY Kit Expiration Date: 12/27/2015
 TE Buffer Lot #: T02292811101
 DNA Standard Batch #: 020714EC (A1-G2) (020414EC (09-G12) (H3-H11))
 7500 Used: B

Master Mix LN: 82941
 DNA standard LN: 92996
 Primer IPC Mix LN: 58948
 H₂O, amp. grade LN: 84021

Following quantification, all extracts have been returned to (Temperature/Location):

Sensitivity B (021114JS1)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 021114JS1

Case Number: Plexor HY Sensitivity B

Analyst: JS

Date: 2/11/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	200	n/a	n/a
Plexor HY 2X Master Mix	1000	930	976.5
Water, Amplicon Grade	700	651	683.55
Plexor HY 20X Primer IPC Mix	1.0	93	97.65
Total reaction volume	2000		
number of reactions	93		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
B	STND 2	STND 2	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
C	STND 3	STND 3	Q32B	Q32C	Q32D	Q32E	Q32F	Q32G	Q32H	Q32I	Q32J	Q32K
D	STND 4	STND 4	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
E	STND 5	STND 5	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
F	STND 6	STND 6	Q15B	Q15C	Q15D	Q15E	Q15F	Q15G	Q15H	Q15I	Q15J	Q15K
G	STND 7	STND 7	Q32A	Q32A	Q32A	Q15A	Q15A	Q15A	Q15H	Q15I	Q15J	Q15K
H	NTC	Q32G	STND 3	STND 3	STND 4	STND 4	STND 5	STND 5	STND 6	STND 6	STND 7	STND 7

Plexor HY Kit Lot #: 93927
 Plexor HY Kit Expiration Date: 12/27/2015
 TE Buffer Lot #: T02292811101
 DNA Standard Batch #: 021114JS1 (A1-G2) (020414EC (011-G12) (H3-H12))
 7500 Used: B

Master Mix LN: 82941
 DNA standard LN: 92996
 Primer IPC Mix LN: 58948
 H₂O, amp. grade LN: 84021

Following quantification, all extracts have been returned to (Temperature/Location):

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Sensitivity C (040214EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 040214EC

Case Number: Plexor HY Sensitivity C

Analyte: EC

Date: 4/2/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount ± 5%
sample	2.0	80	80
Plexor HY 2X Master Mix	10.0	810	850.5
Water, Amplicon Grade	7.0	567	595.35
Plexor HY 20X Primer/PC Mix	1.0	81	85.05
total reaction volume	20.0		
number of reactions	81		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	Q37A	Q37B	Q37C	Q37D	Q37E	Q37F	Q37G	Q37H	Q37I	Q37J
B	STND 2	STND 2	Q37A	Q37B	Q37C	Q37D	Q37E	Q37F	Q37G	Q37H	Q37I	Q37J
C	STND 3	STND 3	Q37A	Q37B	Q37C	Q37D	Q37E	Q37F	Q37G	Q37H	Q37I	Q37J
D	STND 4	STND 4	Q35A	Q35B	Q35C	Q35D	Q35E	Q35F	Q35G	Q35H	Q35I	Q35J
E	STND 5	STND 5	Q35A	Q35B	Q35C	Q35D	Q35E	Q35F	Q35G	Q35H	Q35I	Q35J
F	STND 6	STND 6	Q35A	Q35B	Q35C	Q35D	Q35E	Q35F	Q35G	Q35H	Q35I	Q35J
G	STND 7	STND 7	Q37K	Q37K	Q37K	Q35K	Q35K	Q35K				
H	NTC											

Plexor HY Kit Lot #: 104625
 Plexor HY Kit Expiration Date: 07/15/16
 TE Buffer Lot #: T02802H1101
 DNA Standard Batch #: 040214EC
 7500 Used: B

Master Mix Lot #: 93855
 DNA standard Lot #: 104248
 Primer/PC Mix Lot #: 93522
 H₂O, amp. grade Lot #: 95505

Following quantification, all extracts have been returned to (Temperature/Location):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

5.3. Experimental Setup

A serial dilution ranging from 50ng/μl to 3.2pg/μl of the DNA standard from Plexor® HY System was prepared according to the manufacturer's instructions. Four DNA samples in serial dilutions ranging from 1.0 to 0.001ng/μl were prepared based on an initial quantitation using Quantifiler Duo and quantitated in replicates of 3. All samples were pipetted in 2μl aliquots in 18μl of master mix. The two female samples were quantitated by separate analysts on 2 quantitation plates, 020714EC and 021114JS1 (for a total of 6 replicates of each sample). The two male samples were quantitated on one plate (040214EC) for a total of 3 replicates of each dilution.

5.4. Data Analysis

For all the plates (020714EC, 021114JS1, and 040214EC), data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

5.5. Results

Sensitivity

To assess the sensitivity of the Plexor® HY System, serial dilutions of four DNA samples were quantitated at the following approximate concentrations: 1.0(A), 0.5(B), 0.25(C), 0.125(D), 0.063(E), 0.032(F), 0.016(G), 0.008(H), 0.004(I), 0.002(J), and 0.001(K)ng/μl. Quantities (ng/μl) were evaluated to determine the sensitivity of the Plexor®HY System chemistry.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

The average quantities (ng/μl), standard deviations and variability coefficients are displayed in the tables below. The variability coefficient was calculated by dividing the standard deviation by the quantity, which means greater variability between replicates will result in higher variability coefficient. The highest variability coefficients were observed in samples ranging from 0.008 to 0.001 ng/μl, the lowest quantities tested, and the lowest values were observed in 1.0, 0.5 and 0.25ng/μl samples. The general trend was for the variability coefficient to increase as sample quantity decreased for both human and male detectors.

020714EC (Sensitivity A)

Sample Name	Avg. Human Qty	Human Qty Std.Dev.	Variability Coefficient
Q15A	0.516	0.0213	0.041
Q15B	0.161	0.00822	0.051
Q15C	0.0854	0.0120	0.141
Q15D	0.0352	0.00230	0.065
Q15E	0.0169	0.00255	0.151
Q15F	0.00702	0.00150	0.213
Q15G	0.00219	0.000387	0.177
Q15H	0.00168	0.000336	0.200
Q15I	0.00141	0.000444	0.316
Q15J	0.000248	0.000305	1.228
Q15K	0.000408	0.000216	0.530
Q32A	0.379	0.0357	0.094
Q32B	0.0959	0.00597	0.062
Q32C	0.0654	0.00105	0.016
Q32D	0.0286	0.00301	0.105
Q32E	0.0127	0.00159	0.126
Q32F	0.00544	0.000962	0.177
Q32G	0.00297	0.000573	0.193
Q32H	0.00111	0.000777	0.699
Q32I	0.00112	0.000520	0.463
Q32J	0.000566	0.000453	0.801
Q32K	0.000088	0.0000812	0.927

021114JS1 (Sensitivity B)

Sample Name	Avg. Human Qty	Human Qty Std.Dev.	Variability Coefficient
Q15A	0.524	0.0386	0.074
Q15B	0.181	0.00266	0.015
Q15C	0.0815	0.00381	0.047
Q15D	0.0324	0.00230	0.071
Q15E	0.0160	0.000881	0.055
Q15F	0.00523	0.00144	0.275
Q15G	0.00249	0.000597	0.239
Q15H	0.00142	0.000353	0.248
Q15I	0.00113	0.000544	0.483
Q15J	0.000398	0.0000851	0.214
Q15K	0.000140	0.000121	0.869
Q32A	0.426	0.0190	0.045
Q32B	0.0738	0.00452	0.061
Q32C	0.0534	0.00366	0.069
Q32D	0.0283	0.00258	0.091
Q32E	0.00954	0.00113	0.119
Q32F	0.00383	0.000329	0.086
Q32G	0.00181	0.0000229	0.013
Q32H	0.000506	0.000225	0.444
Q32I	0.000837	0.000373	0.446
Q32J	0.0000467	0.0000809	1.732
Q32K	0.000130	0.000224	1.732

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 040214EC (Sensitivity C)

Sample Name	Avg. Human Qty	Human Qty Std.Dev.	Variability Coefficient	Avg. Male Qty	Male Qty Std.Dev.	Variability Coefficient
Q35A	0.591	0.0814	0.138	0.716	0.0580	0.081
Q35B	0.194	0.00747	0.038	0.214	0.00252	0.012
Q35C	0.0697	0.00565	0.081	0.0846	0.00528	0.062
Q35D	0.0243	0.00454	0.187	0.0264	0.000896	0.034
Q35E	0.00893	0.000598	0.067	0.0106	0.00112	0.106
Q35F	0.00380	0.000385	0.101	0.00430	0.000841	0.196
Q35G	0.000508	0.000327	0.644	0.00182	0.000649	0.357
Q35H	0.000546	0.000111	0.204	0.000938	0.000743	0.793
Q35I	0.000321	0.000286	0.890	0.000590	0.000906	1.536
Q35J	0.0000747	0.000129	1.732	0	0	0
Q35K	0.000108	0.000131	1.211	0.000190	0.000204	1.074
Q37A	1.07	0.211	0.197	0.592	0.0358	0.061
Q37B	0.392	0.0420	0.107	0.223	0.0286	0.128
Q37C	0.154	0.0247	0.160	0.0792	0.00554	0.070
Q37D	0.0531	0.00251	0.047	0.0263	0.00077	0.029
Q37E	0.0217	0.00280	0.129	0.0123	0.00158	0.129
Q37F	0.00905	0.000762	0.084	0.00520	0.00153	0.294
Q37G	0.00213	0.000374	0.176	0.00135	0.000607	0.448
Q37H	0.00127	0.000337	0.265	0.000739	0.000400	0.542
Q37I	0.000373	0.000381	1.024	0.0000743	0.000129	1.732
Q37J	0.000324	0.000229	0.706	0	0	0
Q37K	0.000417	0.000493	1.184	0	0	0

As seen in the table below, the greatest variability in average quantities between all six replicates tested for samples Q15 and Q32 was seen at the lower concentrations. The variability coefficient is inversely proportional to the concentration. Loss of detection of the sample by Plexor® HY System was observed at both 0.002(J) and 0.001(K) ng/μl levels. It is important to note that both of these quantities fall outside of the Plexor® HY System standard curve range (<0.0032ng/μl).

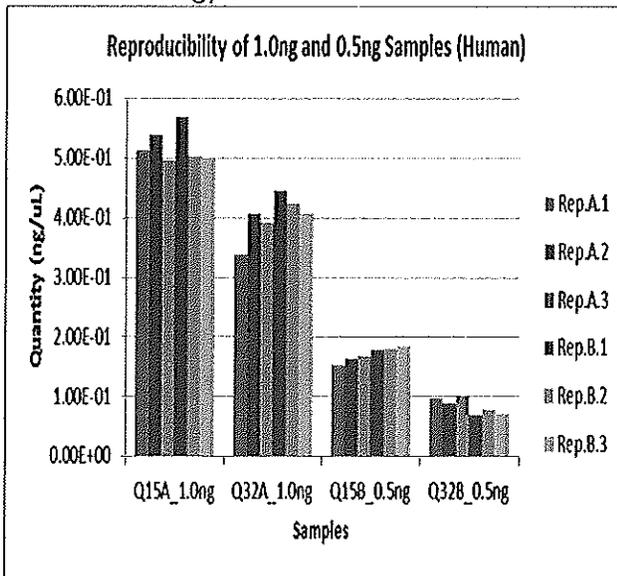
District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Sensitivity A and B Average

Sample Name	Avg. Human Qty	Human Qty Std.Dev.	Variability Coefficient
Q15A	0.520	0.0282	0.054
Q15B	0.343	0.201	-0.586
Q15C	0.0834	0.00828	0.099
Q15D	0.0338	0.00257	0.076
Q15E	0.0164	0.00176	0.107
Q15F	0.00613	0.00164	0.268
Q15G	0.00234	0.000480	0.205
Q15H	0.00155	0.000338	0.218
Q15I	0.00127	0.000470	0.371
Q15J	0.000323	0.000216	0.669
Q15K	0.000274	0.000215	0.785
Q32A	0.402	0.0363	0.090
Q32B	0.0848	0.0130	0.153
Q32C	0.0594	0.00702	0.118
Q32D	0.0284	0.00251	0.088
Q32E	0.0111	0.00212	0.191
Q32F	0.00463	0.00109	0.235
Q32G	0.00239	0.000730	0.306
Q32H	0.000809	0.000609	0.754
Q32I	0.000979	0.000433	0.443
Q32J	0.000306	0.000407	1.329
Q32K	0.000109	0.000153	1.406

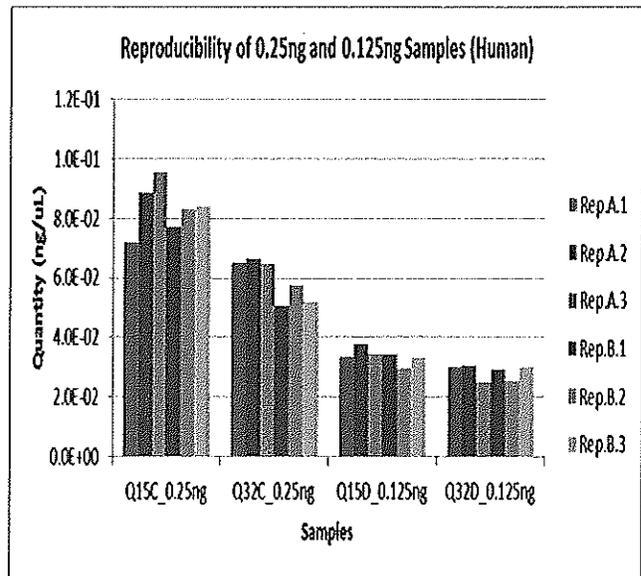
Reproducibility

To assess the ability of the Plexor® HY System to accurately and reliably obtain reproducible results both within and across plates, serial dilutions of two female DNA samples (1.0(A), 0.5(B), 0.25(C), 0.125(D), 0.063(E), 0.032(F), 0.016(G), 0.008(H), 0.004(I), 0.002(J), and 0.001(K)ng/μl) were quantitated on two plates by two different analysts. The replicates of each sample were evaluated within a plate and between plates to determine if the results were reproducible.

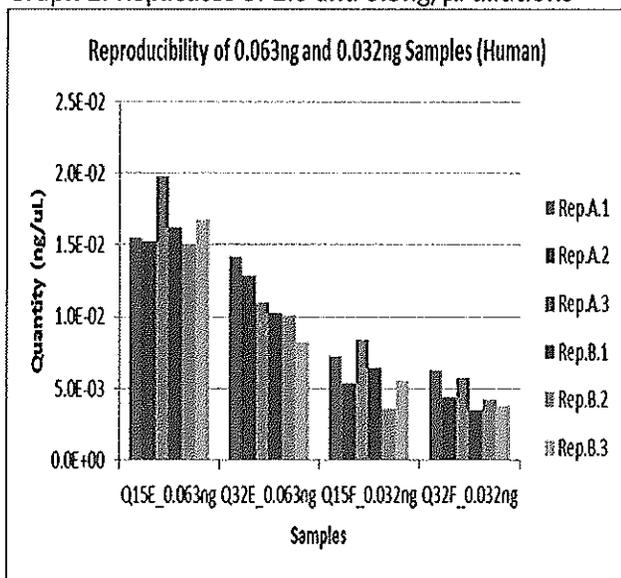
Sample Q15 generated quantitative values for all six replicates of concentrations from 1.0 to 0.004ng/μl. One replicate of the 0.002 and 0.001ng/μl dilutions yielded no quantitation data. Sample Q32 also showed results at all six replicates for concentrations 1.0 to 0.004ng/μl. Two replicates of the 0.002ng/μl dilution and three replicates of the 0.001ng/μl dilution yielded no quantitative data. The 1.0, 0.5, 0.25, 0.125, 0.063, 0.032, 0.016, 0.008, 0.004, 0.002, and 0.001ng/μl replicates are displayed in Graphs 1,2,3,4 and 5. The higher DNA concentrations showed the greatest amount of reproducibility between replicates, both within a plate and between plates. The lower DNA concentrations, in addition to seeing a loss of data, showed greater variation in the Plexor® HY System. Significant variability between replicates and between plates appears at around 2 pg/μL, which corresponds to the 16 pg/μl (G) sample, indicating that the system works best and produces reliable results for concentrations above 2pg/μl.



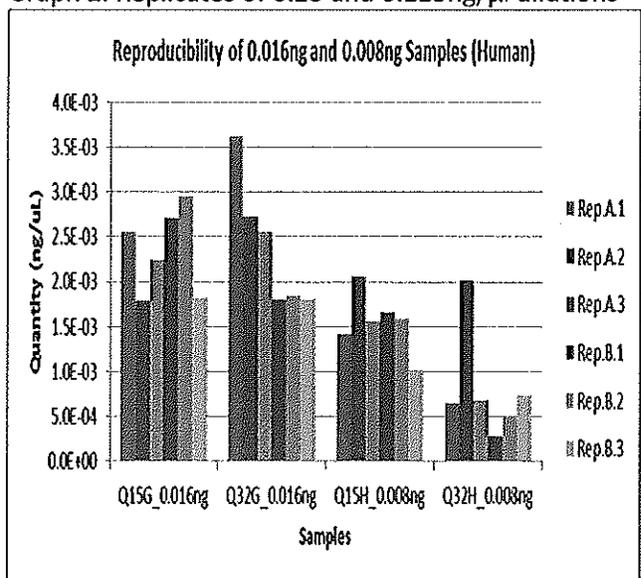
Graph 1. Replicates of 1.0 and 0.5ng/ μ l dilutions



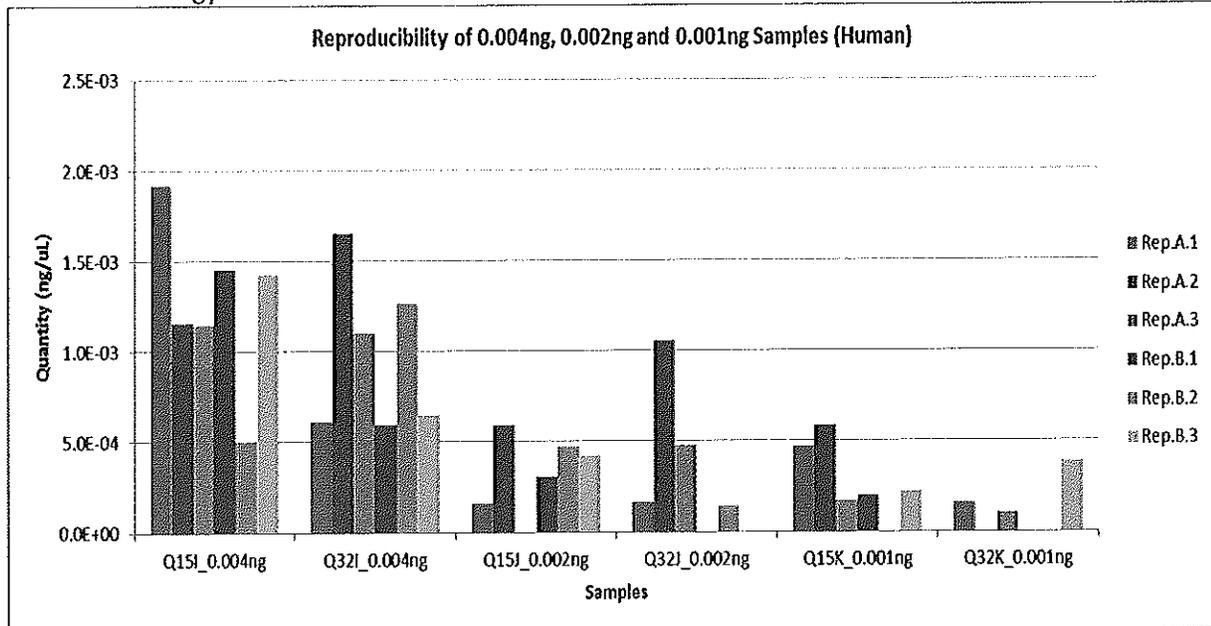
Graph 2. Replicates of 0.25 and 0.125ng/ μ l dilutions



Graph 3. Replicates of 0.063 and 0.032ng/ μ l dilutions



Graph 4. Replicates of 0.016 and 0.008ng/ μ l dilutions



Graph 5. Replicates of 0.004, 0.002 and 0.001ng/μl dilutions

5.6. Conclusions

Sensitivity

In this study, the ability of Plexor® HY 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 C_q values between replicates was seen at lower concentrations. The variability coefficient was noted to be inversely proportional to the concentration. Loss of detection of the sample by Plexor® HY was observed in both 1(K) and 2(J) pg samples. It is important to note that these quantities fall outside the Plexor® HY standard curve range. The human and male detectors follow the expected results pattern, with an increase in the variability coefficient as the sample concentration decreases.

Based on this study, it is seen that Plexor® HY is able to detect low level DNA samples, as low as 0.865 pg/μL, which corresponds to the 4pg/μL(I) sample. 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 (<3.2pg/μl) should be interpreted with caution.

In general, Plexor® HY obtained much lower values than the targeted concentrations obtained from Quantifiler® Duo. Therefore, this underestimation in the DNA concentration could result in a decrease in the established amplification target range.

Reproducibility

In this study, the ability of Plexor® HY to yield reproducible values across multiple replicates and multiple plates was evaluated. The greatest variability is seen at the lower level concentrations. Based

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

on this study, Plexor® HY is capable of yielding reproducible results both within and between plates.
However, as the sample concentration decreases the variability of the quantitation results increases.

6. Mixture Study

6.1. Objective

In order to demonstrate the ability of Plexor® HY System with the 7500 Real-Time PCR instrument to estimate the ratio of human to male DNA present in mixed samples, two mixture sets were prepared, quantified and run in triplicate. The ratio of male to female DNA was calculated from the data obtained from the quantitation step.

6.2. Materials and Methods

Dilution Setup Worksheet

Dilutions						
Sample	Tube Name	Initial [Conc.] ng/uL	Final [Conc.] ng/uL	Vol. Sample (uL)	TE buffer (uL)	Total Volume (uL)
Q37	Q37.1	21.04	1.0	2.1	42.9	45
	Q37.2	21.04	0.2	1.1	118.9	120
Q34	Q34.1	20.49	1.0	6.8	133.2	140
	Q34.2	20.49	2.0	6.8	63.2	70
Q35	Q35.1	39.00	1.0	1.3	48.7	50
	Q35.2	39.00	0.2	1.0	199.0	200
Q32	Q32.1	50.02	1.0	3.6	176.4	180
	Q32.2	50.02	2.0	2.0	48.0	50

Mixtures									
Tubes	Ratios (M:F)	Initial [M]	Final [M]	Q37.1 [M] (uL)	Initial [F]	Final [F]	Q34.1 [F] (uL)	TE buffer	Total Volume
M1.A	1:1	1.0	0.5	20.00	1.0	0.5	20.0	0.0	40
M1.B	1:5	1.0	0.2	8.00	1.0	0.8	32.0	0.0	40
M1.C	1:10	1.0	0.1	4.00	1.0	0.9	36.0	0.0	40
M1.D	1:20	1.0	0.05	2.00	1.0	0.95	38.0	0.0	40
Tubes	Ratios (M:F)	Initial [M]	Final [M]	Q37.2 [M] (uL)	Initial [F]	Final [F]	Q34.2 [F] (uL)	TE buffer	Total Volume
M1.E	1:40	0.2	0.025	5.00	2.0	0.975	19.5	15.5	40
M1.F	1:80	0.2	0.0125	2.50	2.0	0.9875	19.8	17.8	40
M1.G	1:160	0.2	0.00625	1.25	2.0	0.99375	19.9	18.9	40

Tubes	Ratios (M:F)	Initial [M]	Final [M]	Q35.1 [M] (uL)	Initial [F]	Final [F]	Q32.1 [F] (uL)	TE buffer	Total Volume
M2.A	1:1	1.0	0.5	20.00	1.0	0.5	20.0	0.0	40
M2.B	1:5	1.0	0.2	8.00	1.0	0.8	32.0	0.0	40
M2.C	1:10	1.0	0.1	4.00	1.0	0.9	36.0	0.0	40
M2.D	1:20	1.0	0.05	2.00	1.0	0.95	38.0	0.0	40
M2.E	1:40	1.0	0.025	1.00	1.0	0.975	39.0	0.0	40
Tubes	Ratios (M:F)	Initial [M]	Final [M]	Q35.2 [M] (uL)	Initial [F]	Final [F]	Q32.2 [F] (uL)	TE buffer	Total Volume
M2.F	1:80	0.2	0.0125	2.50	2.0	0.9875	19.8	17.8	40
M2.G	1:160	0.2	0.00625	1.25	2.0	0.99375	19.9	18.9	40

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Mixtures A (022414EC)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 022414EC

Case Number: PlexorHYMixtures A

Analyte: EC

Date: 2/24/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	816	\$99.5
Water, Amplification Grade	7.0	567	\$95.39
Plexor HY 20X Primers/PC Mix	1.0	81	\$5.05
total reaction volume	20.0		
number of reactions	81		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F	STD 2	STD 2	
B	STND 2	STND 2	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F	STD 3	STD 3	
C	STND 3	STND 3	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F	STD 4	STD 4	
D	STND 4	STND 4	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G	STD 5	STD 5	
E	STND 5	STND 5	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G	STD 6	STD 6	
F	STND 6	STND 6	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G	STD 7	STD 7	
G	STND 7	STND 7	STND 2	STND 3	STND 4	STND 5	STND 6	STND 7				
H	NTC		STND 2	STND 3	STND 4	STND 5	STND 6	STND 7				

Plexor HY Kit Lot #: 95772
 Plexor HY Kit Expiration Date: 07/15/2016
 TE Buffer Lot #: T022028L1101
 DNA Standard Batch #: 022414EC (A1-G2), 022014EC (A11-F12), 021114US1 (G3-H5)
 7500 Used: B

Master Mix L/N: 93855
 DNA standard L/N: 96289
 Primers/PC Mix L/N: 93522
 H₂O, amp. grade L/N: 95905

Following quantification, all extracts have been returned to (Temperature/Location):

Mixtures B (040114EC2)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 040114EC2

Case Number: PlexorHYMixtures B

Analyte: EC

Date: 4/1/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	n/a	n/a
Plexor HY 2X Master Mix	10.0	576	\$98.5
Water, Amplification Grade	7.0	399	\$48.99
Plexor HY 20X Primers/PC Mix	1.0	57	\$9.89
total reaction volume	20.0		
number of reactions	57		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F			
B	STND 2	STND 2	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F			
C	STND 3	STND 3	MI.A	MI.C	MI.E	MI.G	MI.B	MI.D	MI.F			
D	STND 4	STND 4	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G			
E	STND 5	STND 5	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G			
F	STND 6	STND 6	MI.B	MI.D	MI.F	MI.A	MI.C	MI.E	MI.G			
G	STND 7	STND 7										
H	NTC											

Plexor HY Kit Lot #: 104525
 Plexor HY Kit Expiration Date: 07/15/16
 TE Buffer Lot #: T022028L1101
 DNA Standard Batch #: 040114EC
 7500 Used: B

Master Mix L/N: 93855
 DNA standard L/N: 104243
 Primers/PC Mix L/N: 93522
 H₂O, amp. grade L/N: 95905

Following quantification, all extracts have been returned to (Temperature/Location):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

6.3. Experimental Setup

Two mixture sets including the ratios listed in the worksheets above were created using samples previously quantified on the 7500 Real-Time PCR instrument with Quantifiler® Duo. These samples were

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

not quantified in multiples; therefore the mixtures created are approximate. The mixtures were pipetted in 2µL aliquots in 18µL Master Mix as columns across two 96 well plates (022414EC and 040114EC2) and run in replicates of 3 using the Plexor® HY System on the 7500 Real-Time PCR instrument.

6.4. Data Analysis

For both plates (022414EC and 040114EC2), data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

6.5. Results

The results listed below contain the average values of observed human to male ratios obtained from the Plexor® HY System and the expected human to male ratios calculated from the Quantifiler® Duo.

022414EC (Mixture 1)

Samples	[Auto]	[Y]	Observed [Auto]/[Y]	Average Observed [Auto]/[Y]	Observed [Auto]/[Y] SD	Expected [Auto]/[Y]
M1.A	0.977	0.088060	11.10	10.36	0.6440	1
M1.A	0.905	0.091150	9.929			
M1.A	0.899	0.089410	10.05			
M1.B	0.851	0.021150	40.23	43.25	3.624	5
M1.B	0.964	0.022800	42.26			
M1.B	1.260	0.026650	47.27			
M1.C	0.644	0.009260	69.58	83.51	15.69	10
M1.C	0.819	0.008146	100.5			
M1.C	0.760	0.009449	80.44			
M1.D	0.823	0.005588	147.2	172.0	24.22	20
M1.D	0.880	0.004498	195.6			
M1.D	0.708	0.004091	173.1			
M1.E	0.728	0.001592	457.1	334.8	108.4	40
M1.E	0.653	0.002199	296.7			
M1.E	0.616	0.002457	250.7			
M1.F	0.683	0.002918	234.1	280.7	72.31	80
M1.F	0.669	0.002740	244.0			
M1.F	0.851	0.002339	364.0			
M1.G	0.817	0.000694	1177	1016	439.1	160
M1.G	0.843	0.000624	1351			
M1.G	0.733	0.001414	518.6			

040114EC2 (Mixture 1)

Samples	[Auto]	[Y]	Observed [Auto]/[Y]	Average Observed [Auto]/[Y]	Observed [Auto]/[Y] SD	Expected [Auto]/[Y]
M1.A	0.977	0.092642	10.54444055	9.57	1.53	1
M1.A	0.720	0.092289	7.802227571			
M1.A	0.893	0.086223	10.36244445			
M1.B	0.799	0.023579	33.88693093	37	4	5
M1.B	0.927	0.026125	35.48748885			
M1.B	1.162	0.027519	42.22354803			
M1.C	1.069	0.011419	93.5789509	80	14	10
M1.C	0.924	0.011403	81.0106632			
M1.C	0.747	0.011296	66.10738786			
M1.D	0.993	0.006733	147.5234049	179	29	20
M1.D	1.059	0.005796	182.7610061			
M1.D	0.997	0.004851	205.6282054			
M1.E	1.091	0.003770	289.3209223	313.48	67.22	40
M1.E	1.142	0.002932	389.4473067			
M1.E	1.110	0.004240	261.6800149			
M1.F	0.841	0.002571	327.2412298	394.07	58.36	80
M1.F	0.757	0.001741	434.9644677			
M1.F	0.784	0.001867	420.0059297			
M1.G	1.074	0.001127	952.9518655	2691.44	2799.70	160
M1.G	1.240	0.000209	5921.097689			
M1.G	1.090	0.000908	1200.261784			

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

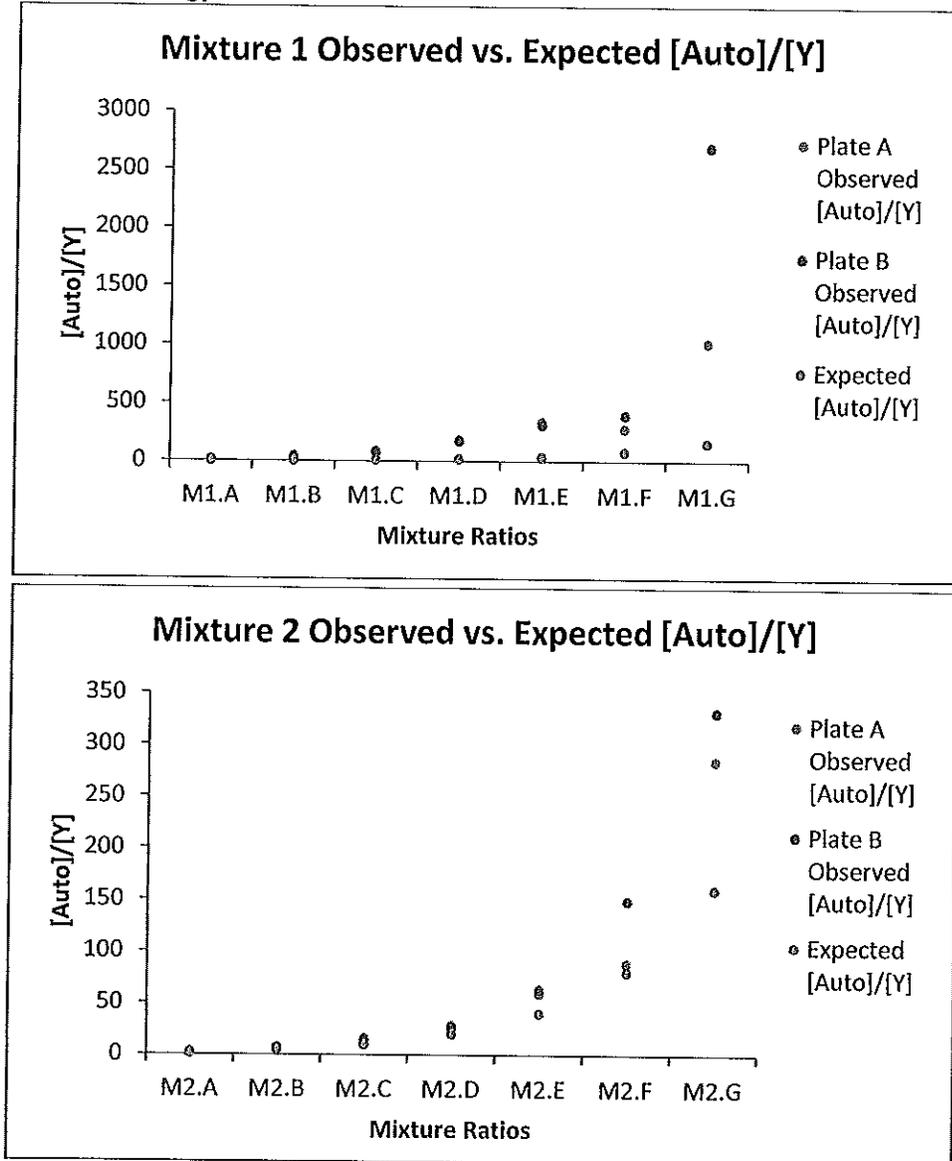
022414EC (Mixture 2)

Samples	[Auto]	[Y]	Observed [Auto]/[Y]	Average Observed [Auto]/[Y]	Observed [Auto]/[Y] SD	Expected [Auto]/[Y]
M2.A	0.208	0.100200	2.080	2.077	0.1105	1
M2.A	0.216	0.110000	1.965			
M2.A	0.219	0.100000	2.186			
M2.B	0.212	0.027140	7.826	7.030	0.9941	5
M2.B	0.187	0.025380	7.349			
M2.B	0.176	0.029760	5.916			
M2.C	0.110	0.011980	9.215	10.98	1.607	10
M2.C	0.151	0.013260	11.36			
M2.C	0.130	0.010510	12.36			
M2.D	0.188	0.006830	27.54	28.31	1.448	20
M2.D	0.140	0.005106	27.41			
M2.D	0.139	0.004630	29.98			
M2.E	0.187	0.002802	66.86	59.27	10.18	40
M2.E	0.189	0.002980	63.26			
M2.E	0.161	0.003365	47.70			
M2.F	0.114	0.001633	69.83	88.70	27.78	80
M2.F	0.108	0.001431	75.67			
M2.F	0.126	0.001048	120.6			
M2.G	0.109	0.000616	177.4	284.6	188.7	160
M2.G	0.119	0.000685	173.9			
M2.G	0.130	0.000259	502.5			

040114EC2 (Mixture 2)

Samples	[Auto]	[Y]	Observed [Auto]/[Y]	Average Observed [Auto]/[Y]	Observed [Auto]/[Y] SD	Expected [Auto]/[Y]
M2.A	0.232	0.093014	2.49478962	2	0	1
M2.A	0.183	0.087806	2.078925038			
M2.A	0.190	0.095004	1.998204776			
M2.B	0.180	0.031741	5.677788644	5.61	0.47	5
M2.B	0.166	0.032418	5.108034467			
M2.B	0.183	0.030269	6.03193657			
M2.C	0.155	0.012329	12.60366588	15.29	2.41	10
M2.C	0.171	0.009899	17.26325746			
M2.C	0.173	0.010811	16.00170491			
M2.D	0.127	0.003832	33.23167799	26	7	20
M2.D	0.125	0.004809	26.08724963			
M2.D	0.132	0.007062	18.63337982			
M2.E	0.155	0.003835	40.47757122	63	23	40
M2.E	0.150	0.002417	62.05587652			
M2.E	0.173	0.001991	86.69245378			
M2.F	0.167	0.000998	166.911102	149.04	34.24	80
M2.F	0.132	0.000774	170.6472222			
M2.F	0.112	0.001020	109.5546635			
M2.G	0.089	0.000149	596.8282659	331.36	231	160
M2.G	0.117	0.000675	173.7885063			
M2.G	0.127	0.000569	223.4543772			

Shown below are graphs representing the observed and expected human to male ratios for mixtures 1 and 2 of both plates. Mixture 1 shows an overall higher ratio than expected which may be attributed to the accuracy of initial quantity of the samples used to prepare the mixtures. Both mixtures demonstrate values which are close to the expected values until a ratio of 1:80 where variability increases between the plates and the expected ratio, however the overall observed values follow the trend of the expected values. This study demonstrates that Plexor® HY System has the ability to detect mixtures even at low concentrations.



6.6. Conclusions

In this study, Plexor® HY's ability to detect mixtures at low concentrations was evaluated. Although the observed [auto]/[male] ratios were higher than the expected values, it was expected that these ratios may be different considering the samples were previously quantitated using the Quantifiler® Duo. However, the general trend was followed for both mixtures in both plates.

Plexor® HY was able to detect male concentrations as low as 0.61 pg/μL in the mixture samples, obtained by averaging the male concentrations of the six G samples of plate 040114EC2, while it detected down to 0.84 pg/μL in the male sensitivity samples (see sensitivity study). Therefore, Plexor® HY can reliably detect male DNA in the presence of a considerably large amount of female DNA as well as accurately assess the male to female ratio in a mixture sample.

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

7. Contamination

7.1. Objective

Demonstrated in this study are the laboratory's procedures to minimize contamination that would compromise the integrity of results. Not only was the instrument calibration 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_q 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.

7.2. Materials and Methods

(Plates previously discussed: 020614EC, 033114EC, 022014EC, 040114EC1, 020714EC, 021114JS1, 040214EC, 022414EC, and 040114EC2)

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

7.3. Experimental Setup

A "Non-Template Control" was run on each plate consisting of reaction mix (master mix and primer/IPC mix) and 2 μ L TE Buffer.

7.4. Data Analysis

For all plates, data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

7.5. Results

Non-Template Control	Human C_q	Male C_q	IPC C_q
020614EC	Undetermined	Undetermined	20.44
033114EC	Undetermined	Undetermined	20.72
022014EC	Undetermined	Undetermined	20.13
040114EC1	Undetermined	Undetermined	20.50
020714EC	Undetermined	Undetermined	20.91
021114JS1	Undetermined	Undetermined	20.51
040214EC	Undetermined	Undetermined	21.13
022414EC	Undetermined	Undetermined	20.41
040114EC2	Undetermined	Undetermined	20.29

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

In all plates, the Non-Template Control (NTC) sample produced C_q values of "N/A" for human and male detectors and no melt curve was observed. IPC C_q values ranged from 20.13 to 21.13.

7.6. Conclusions

The human and male C_q 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. True negative results have to be confirmed by the absence of melt curve. In this study, no melt curve was observed in any of the NTC, confirming that no DNA was detected. The IPC C_q values were consistent with the range of IPC C_q 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 ensure proper maintenance and control of the instrument. An NTC has been demonstrated to ensure the detection of possible contamination in the quantification process. While all results were "N/A" for the NTC in this validation, the Plexor® HY System Technical Manual states the following:

"There should be no amplification product (i.e., $<1.0\text{pg}/\mu\text{l}$ with a $2\mu\text{l}$ input volume) detected in the NTC reaction. Amplification of $>1.0\text{pg}/\mu\text{l}$ of DNA in the NTC reaction indicates nonspecific amplification or the presence of contaminating DNA.

Note: The Plexor® HY System is extremely sensitive. The NTC reaction may show amplification products in the subpicogram range."

Based on this information, samples can be re-quantitated if a quantity value is given to the NTC, however it may not be necessary.

8. Standard Curve

8.1. Objective

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 Plexor®HY System validation and four plates ran during the amplification cutoff. The IPC C_q range was evaluated using the IPC C_q values of all samples run on all the plates for the Plexor® HY System validation. These values were used to establish an acceptable range of values which should be expected within a lot and between lots of Plexor® HY System on the 7500 Real-Time PCR instrument. Additional standard curves were run throughout the validation to assess the quality of the standard curve over a period time and, therefore, determine how long standards can be used for.

8.2. Materials and Methods

Plates from this validation: 020614EC (L/N 93927), 033114EC (L/N 104625), 022014EC (L/N 98772), 040114EC1 (L/N 104625), 020714EC (L/N 93927), 021114JS1 (L/N 93927), 040214EC (L/N 104625), 022414EC (L/N 98772), 040114EC2 (L/N 104625), 042114EC1 (L/N 107091) and 042114EC2 (L/N 107091).

Plates from the amplification cutoff validation: 022614JS (L/N 93927), 042114JS-QT1 (L/N 107091), 042114JS-QT2 (L/N 107091) and 042814JS-QT1 (L/N 107091).

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)

Instrument Serial Number: 275006459

Monthly calibrated

8.3. Experimental Setup

All plates consisted of at least two columns of a serial dilution from the Plexor® HY System standard supplied in the kit and described in the User's manual.

8.4. Data Analysis

For all plates, data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

8.5. Results

The tables below represent the human and male standard curve ranges calculated by adding and subtracting three standard deviations to the average value for the slope, y-intercept and R² value, respectively.

Human Standard Curve					Male Standard Curve				
Std. Name	Plate Name	Slope	Y-Intercept	R ²	Std. Name	Plate Name	Slope	Y-Intercept	R ²
020614EC	020614EC	-3.55	22.80	0.996	020614EC	020614EC	-3.53	24.13	0.997
033114EC	033114EC	-3.69	23.37	0.997	033114EC	033114EC	-3.64	24.35	0.997
022014EC	022014EC	-3.55	22.51	0.998	022014EC	022014EC	-3.45	24.38	0.999
040114EC	040114EC1	-3.56	22.87	0.999	040114EC	040114EC1	-3.55	24.38	0.996
020714EC	020714EC	-3.54	22.97	0.999	020714EC	020714EC	-3.54	24.66	0.997
021114JS1	021114JS1	-3.56	23.29	0.997	021114JS1	021114JS1	-3.63	25.28	0.996
040214EC	040214EC	-3.50	22.99	0.999	040214EC	040214EC	-3.44	24.43	0.999
022414EC	022414EC	-3.47	22.39	0.995	022414EC	022414EC	-3.47	24.23	0.997
040114EC	040114EC2	-3.47	22.61	0.997	040114EC	040114EC2	-3.49	24.61	0.996
042114EC	042114EC1	-3.59	22.91	0.999	042114EC	042114EC1	-3.62	24.28	0.998
042114EC	042114EC2	-3.58	22.62	0.998	042114EC	042114EC2	-3.42	24.46	0.998
022614JS	022614JS	-3.73	23.69	0.998	022614JS	022614JS	-3.53	24.78	0.999
042114JS	042114JS-QT1	-3.83	23.09	0.997	042114JS	042114JS-QT1	-3.66	24.49	0.995
042114JS	042114JS-QT2	-3.71	23.28	0.998	042114JS	042114JS-QT2	-3.58	25.32	0.995
042814EC	042814JS-QT1	-3.51	22.85	0.998	042814EC	042814JS-QT1	-3.47	24.30	0.997
	Average	-3.59	22.95	0.998		Average	-3.53	24.54	0.997
	SD	0.1043666	0.3527740	0.0011751		SD	0.0779988	0.3515855	0.0013345
	Avg + 3 SD	-3.2762336	24.0076555	1.0011921		Avg + 3 SD	-3.3006703	25.5934232	1.0010702
	Avg - 3 SD	-3.9024330	21.8910112	0.9941412		Avg - 3 SD	-3.7686630	23.4839101	0.9930631

The table below shows the range of the IPC C_q values, including ± 3 standard deviations from the average value.

All Samples	
Plate Name	IPC C _q
Average	20.661
SD	0.382
Avg + 3 SD	21.808
Avg - 3 SD	19.514

The tables below represent the standard curve values obtained from standards ran over a period of time.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Human Standard Curve

Days old	Std. Name	Plate Name	Slope	Y-intercept	R ²
2	020414EC	020614EC	-3.51	22.71	0.994
3	020414EC	020714EC	-3.53	22.84	0.997
4	022014EC	022414EC	-3.45	22.56	0.998
7	020414EC	021114JS1	-3.50	23.11	0.998
9	021114JS1	022014EC	-3.80	23.13	0.997
13	021114JS1	022414EC	-3.62	22.92	0.996
14	020614EC	022014EC	-3.72	22.90	0.996
Average			-3.59	22.88	0.997
SD			0.1288410	0.2042408	0.0013973
Avg + 3 SD			-3.2034770	23.4941508	1.0007633
Avg - 3 SD			-3.9765230	22.2687063	0.9923796

Male Standard Curve

Days old	Std. Name	Plate Name	Slope	Y-intercept	R ²
2	020414EC	020614EC	-3.47	24.02	0.999
3	020414EC	020714EC	-3.65	24.61	0.997
4	022014EC	022414EC	-3.56	24.21	0.998
7	020414EC	021114JS1	-3.51	24.96	0.998
9	021114JS1	022014EC	-3.70	24.94	0.998
13	021114JS1	022414EC	-3.64	24.74	0.995
14	020614EC	022014EC	-3.76	24.52	0.997
Average			-3.61	24.57	0.997
SD			0.1041976	0.3545151	0.0012724
Avg + 3 SD			-3.3002643	25.6349739	1.0012458
Avg - 3 SD			-3.9254500	23.5078833	0.9936113

8.6. Conclusions

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 standard 7) or re-performing the quantification should be considered. The range obtained for the human detector is in the range while the range obtained for the male detector is slightly larger than suggested in the Plexor® HY System User's Manual. This manual suggests a slope range of -3.2 to -4.0 for the human detector and -3.0 to -3.6 for the male detector, with an R² greater than or equal to 0.990. Even though no range is listed for the y-intercept, it is suggested to establish it during the internal validation. The range of the y-intercept was determined in order to maintain consistency in the quantitation values between analysts.

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

The IPC C_q range was found to be 19.514 to 21.808. The user's manual states that "if the IPC value of an unknown is several cycles higher than that of DNA standards with similar total DNA amounts, inhibition may have occurred and the quantitation data is in doubt. High levels of total human DNA ($\geq 10\text{ng}/\mu\text{L}$) can also cause a slight delay in the IPC crossing the amplification threshold (1-2cycles)." Therefore, inhibition and high levels of total human DNA can be evaluated by assessing the IPC C_q values.

The standard curve values of the standards tested over a period of 14 days indicated that standards can be used up to 14 days after preparation. Although, there was a slight decrease in the slope values after 9 days, the slope, y-intercept and R^2 values were well within the ranges observed previously. Therefore, fresh standards should be prepared after 14 days.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

9. Performance Check

9.1. Objective

This study describes the performance check conducted on the Applied Biosystems 7500 Real-Time PCR System (7500A). The purpose is to demonstrate the laboratory's adherence to guideline 6.2 of the SWGDAM Validation Guidelines for DNA Analysis Methods (2012) which states the following:

"After an internal validation has been performed on a critical instrument, each additional critical instrument of the same make and model shall require a performance check. The performance check should demonstrate that results are reproducible on the new critical instrument and that values from the internal validation can still be obtained. For example, the performance check of a new critical instrument should demonstrate that the sensitivity level is consistent with the sensitivity level obtained from an internal validation, but need not demonstrate whether or not the new critical instrument is more sensitive."

For this performance check, to demonstrate the reproducibility and sensitivity level obtained from the internal validation, six non-probative casework samples of varying concentrations were prepared and run on instrument A and B simultaneously.

9.2. Materials and Methods

Before using instrument A, it was calibrated for fluorescein (FL), CAL Fluor® Orange 560 (CO 560), CAL Fluor® Red 610 (CR 610) and IC5 as recommended by the kit manufacturer. Monthly instrument maintenance was carried out throughout the validation study, as needed. See Appendix for a copy of the instrument maintenance log.

Performance Check Instrument A (042114EC1)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 042114EC1 Case Number: Performance Check Instrument A
 Analyst: EC Date: 4/21/2014

Plexor Reaction Mix	amount per rxn	total amount	total amount + 5%
sample	2.0	1%	2%
Plexor HY 2X Master Mix	10.0	33%	34.5%
Water, Amplification Grade	7.0	23%	24.5%
Plexor HY 20X Primers/PC Mix	1.0	3%	3.6%
total reaction volume	20.0		
number of reactions	33		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	STND 1	STND 1	Q37.B	Q32.C	Q11							
B	STND 2	STND 2	Q37.E	Q32.F	Q15							
C	STND 3	STND 3	Q37.G	Q32.G	Bad							
D	STND 4	STND 4	Q37.H	Q32.H	Q19							
E	STND 5	STND 5	Q37.I	Q32.I								
F	STND 6	STND 6	Q37.J	Q32.J								
G	STND 7	STND 7	Q37.K	Q32.K								
H	NTC											

Plexor HY Kit Lot #: 107091 Master Mix Lot #: 101304 Primers/PC Mix Lot #: 93522
 Plexor HY Kit Expiration Date: 07-15-16 DNA standard Lot #: 104248 H₂O, amp. grade Lot #: 100880
 TE Buffer Lot #: 102207SL1101
 DNA Standard Batch #: 042114EC
 7500 Used: A

Following quantification, all extracts have been returned to (Temperature/Location):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit
 Biosystems) and Plexor® Analysis Software (Promega)
 Instrument Serial Number: 275002560
 Monthly calibrated.

Performance Check Instrument B (042114EC2)

Plexor HY Quantitation Set-Up Worksheet

Quantification Batch Name: 042114EC2

Case Number: Performance Check Instrument B

Analyst: EC

Date: 4/21/2014

Plexor Reaction Mix	Amount per rxn	Total amount	Total amount + 5%
sample	2.0	0.2	0.2
Plexor HY 2X Master Mix	10.0	3.0	34.65
Water, AmpErase Inhib Grade	7.0	2.31	242.55
Plexor HY 20X Primers/PC Mix	1.0	3.9	34.65
Total reaction volume	20.0		
number of reactions	33		

Plate Setup Type in your sample names in the appropriate well location

	1	2	3	4	5	6	7	8	9	10	11	12
A	SIND 1	SIND 1	Q37 B	Q32 C	Q11							
B	SIND 2	SIND 2	Q37 E	Q32 E	Q15							
C	SIND 3	SIND 3	Q37 G	Q32 G	Box D							
D	SIND 4	SIND 4	Q37 H	Q32 H	Q19							
E	SIND 5	SIND 5	Q37 J	Q32 J								
F	SIND 6	SIND 6	Q37 L	Q32 L								
G	SIND 7	SIND 7	Q37 K	Q32 K								
H	NTC											

Plexor HY Kit Lot #: 107091
 Plexor HY Kit Expiration Date: 07-15-16
 TE Buffer Lot #: T022025L1101
 DNA Standard Batch #: 042114EC
 7500 Used: B

Master Mix Lot #: 101901
 DNA standard Lot #: 104248
 Primer/PC Mix Lot #: 93522
 H₂O, amp grade Lot #: 106889

Following quantification, all extracts have been returned to (Temperature/Centigrade):

Instrumentation and Software: 7500 Real-Time PCR System and SDS Software Version 1.2.3f2 (Applied Biosystems) and Plexor® Analysis Software (Promega)
 Instrument Serial Number: 275006459
 Monthly calibrated

9.3. Data Analysis

For both plates (042114EC1 and 042114EC2), data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

9.4. Results

Standard Curve values obtained from 042114EC1:

	Human	Male
Slope	-3.59	-3.62
Y-Intercept	22.91	24.28
R ²	0.999	0.998

Standard Curve values obtained from 042114EC2:

	Human	Male
Slope	-3.58	-3.42
Y-Intercept	22.62	24.46
R ²	0.998	0.998

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Standard curve values from both plates are within the acceptable range determined during the standard curve assessment (see standard curve section). The standard curve values are within two standard deviations for the male detector and within one standard deviation for the human detector for both plates.

The tables below summarize the autosomal and male concentrations obtained for each of the samples tested on both instruments. The acceptable ranges are relative to instrument B concentrations and were calculated using the maximal average difference of 1.65 found during the precision study. Most of the samples tested were within the acceptable range, except for the samples of lower concentrations. It is expected that greater variation is observed in samples of very low concentration especially those that fall outside the standard curve range, which is the case for samples G through K. Instrument A was able to detect samples as low as 0.18 pg/ μ L, which is well below the 0.865 pg/ μ L sensitivity found during the sensitivity study.

[Auto]

Sample Name	Instrument A	Instrument B	Acceptable Range		
BucD	1.175E+01	1.411E+01	2.328E+01	8.552E+00	Yes
Q11	1.857E+00	1.839E+00	3.034E+00	1.115E+00	Yes
Q15	2.293E+01	2.463E+01	4.064E+01	1.493E+01	Yes
Q19	2.341E+00	2.201E+00	3.632E+00	1.334E+00	Yes
Q32.C	2.785E-02	2.233E-02	3.684E-02	1.353E-02	Yes
Q32.E	4.357E-03	5.422E-03	8.946E-03	3.286E-03	Yes
Q32.G	1.441E-03	1.457E-03	2.404E-03	8.830E-04	Yes
Q32.H	6.976E-04	4.046E-04	6.676E-04	2.452E-04	No
Q32.I	1.816E-04	9.397E-04	1.551E-03	5.695E-04	No
Q32.J	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.K	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q37.B	3.085E-01	3.661E-01	6.041E-01	2.219E-01	Yes
Q37.E	1.888E-02	1.973E-02	3.255E-02	1.196E-02	Yes
Q37.G	1.482E-03	1.705E-03	2.813E-03	1.033E-03	Yes
Q37.H	1.239E-03	7.226E-04	1.192E-03	4.379E-04	No
Q37.I	1.938E-04	4.750E-04	7.838E-04	2.879E-04	No
Q37.J	1.785E-04	4.863E-04	8.024E-04	2.947E-04	No
Q37.K	4.455E-04	0.000E+00	0.000E+00	0.000E+00	N/A

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

[Male]

Sample Name	Instrument A	Instrument B	Acceptable Range		
BucD	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q11	2.211E+00	2.755E+00	4.546E+00	1.670E+00	Yes
Q15	3.653E-04	3.375E-04	5.569E-04	2.045E-04	Yes
Q19	1.531E+00	1.718E+00	2.835E+00	1.041E+00	Yes
Q32.C	1.717E-04	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.E	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.G	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.H	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.I	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.J	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q32.K	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q37.B	1.700E-01	1.655E-01	2.731E-01	1.003E-01	Yes
Q37.E	1.003E-02	5.258E-03	8.676E-03	3.187E-03	No
Q37.G	1.873E-03	1.167E-03	1.926E-03	7.073E-04	Yes
Q37.H	6.310E-04	4.134E-04	6.821E-04	2.505E-04	Yes
Q37.I	2.094E-04	3.934E-04	6.491E-04	2.384E-04	No
Q37.J	0.000E+00	0.000E+00	0.000E+00	0.000E+00	N/A
Q37.K	0.000E+00	1.806E-04	2.980E-04	1.095E-04	N/A

9.5. Conclusions

This study demonstrates that Instrument A can obtain reproducible results that are in concordance with the results obtained during this internal validation. Also, the sensitivity level of instrument A is consistent with the sensitivity obtained in the sensitivity study.

10. Amplification Calculation

10.1. Objective

To evaluate the accuracy of the amplification calculations performed by the Plexor® Analysis Software, manual calculations were performed to determine if the same results were obtained.

10.2. Materials and Methods

Samples 1-6, 2-10, 5-37, BUCCALB-6, BUCCALD-5, Q11, Q15, and Q16 from plate 022614JS1 quantitated during the amplification cutoff validation, and samples Q15A, Q15B, Q15C, Q15D, Q15E, Q15F, Q32A, Q32B, Q32C, Q32D, Q32E, and Q32F from plate 020714EC quantitated during the sensitivity study were evaluated.

10.3. Data Analysis

For both plates (022614JS1 and 020714EC), data was collected with the 7500 SDS software 1.2.3f2 and analyzed with the Plexor® Analysis Software.

10.4. Results

The table below represents the calculations performed manually to determine if the analysis software can reliably calculate a target concentration for amplification. All the calculations manually performed rounded to 0.07ng/μL, which was the targeted concentration entered in the analysis software.

District of Columbia
 Department of Forensic Sciences
 Forensic Science Laboratory
 Forensic Biology Unit

Sample name	[Auto] ng/ μ L	Auto STR Volume (μ L)	Auto STR Dilution Factor	Calculation	[Targeted] ng/ μ L
1-6	29.25	-	420	$\frac{29.25 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{420 \mu\text{L}} = 0.0696 \text{ ng}/\mu\text{L}$	0.07
2-10	9.294	-	130	$\frac{9.294 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{130 \mu\text{L}} = 0.0715 \text{ ng}/\mu\text{L}$	0.07
5-37	30.03	-	430	$\frac{30.03 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{430 \mu\text{L}} = 0.0698 \text{ ng}/\mu\text{L}$	0.07
BUCCALB-6	58.23	-	830	$\frac{58.23 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{830 \mu\text{L}} = 0.0702 \text{ ng}/\mu\text{L}$	0.07
BUCCALD-5	17.83	-	250	$\frac{17.83 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{250 \mu\text{L}} = 0.0713 \text{ ng}/\mu\text{L}$	0.07
Q11	2.511	-	36	$\frac{2.511 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{36 \mu\text{L}} = 0.0698 \text{ ng}/\mu\text{L}$	0.07
Q15	26.98	-	390	$\frac{26.98 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{390 \mu\text{L}} = 0.0692 \text{ ng}/\mu\text{L}$	0.07
Q16	25.24	-	360	$\frac{25.24 \text{ ng}/\mu\text{L} \times 1 \mu\text{L}}{360 \mu\text{L}} = 0.0701 \text{ ng}/\mu\text{L}$	0.07
Q15A	0.5143	1.4	-	$\frac{0.5143 \text{ ng}/\mu\text{L} \times 1.4 \mu\text{L}}{10 \mu\text{L}} = 0.0720 \text{ ng}/\mu\text{L}$	0.07
Q15B	0.1518	4.6	-	$\frac{0.1518 \text{ ng}/\mu\text{L} \times 4.6 \mu\text{L}}{10 \mu\text{L}} = 0.0698 \text{ ng}/\mu\text{L}$	0.07
Q15C	0.07208	9.7	-	$\frac{0.07208 \text{ ng}/\mu\text{L} \times 9.7 \mu\text{L}}{10 \mu\text{L}} = 0.0699 \text{ ng}/\mu\text{L}$	0.07
Q15D	0.03335	10	-	-	-
Q15E	0.01550	10	-	-	-
Q15F	0.00727	10	-	-	-
Q32A	0.3385	2.1	-	$\frac{0.3385 \text{ ng}/\mu\text{L} \times 2.1 \mu\text{L}}{10 \mu\text{L}} = 0.0711 \text{ ng}/\mu\text{L}$	0.07
Q32B	0.09839	7.1	-	$\frac{0.09839 \text{ ng}/\mu\text{L} \times 7.1 \mu\text{L}}{10 \mu\text{L}} = 0.0699 \text{ ng}/\mu\text{L}$	0.07
Q32C	0.06492	10	-	-	-
Q32D	0.03017	10	-	-	-
Q32E	0.01418	10	-	-	-
Q32F	0.006237	10	-	-	-

10.5. Conclusions

In this study, the Plexor[®] Analysis Software was shown to reliably calculate amplification dilutions based on a specific target concentration, as all the calculations performed manually resulted in 0.07ng/ μ L which was the targeted concentration set by the analyst.

11. Final Conclusions

Based on the studies and tests performed in this validation, the following conclusions regarding the use of the Plexor® HY System and 7500 instrument on casework can be made:

- The precision of the instrument is sufficient for the purposes of accurately and reliably quantifying DNA extracts.
- 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.
- The most variability was observed in concentrations below the standard curve range.
- The kit can detect samples as low as 0.865 pg/μL, although reproducibility within a plate and between plates decreases considerably below 2pg/μL.
- 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	-3.90 to -3.28	-3.77 to -3.30
Y-intercept	21.89 to 24.01	23.48 to 25.59
R ²	≥0.994	≥0.993

- Prepared standards should be stored 2°C to 8°C and should expire after no more than two weeks.
- Male DNA quantity can be detected to a level of 0.84 pg despite male to female ratio.
- The Plexor® HY Analysis Software can reliably calculate amplification dilutions using a specific target concentration set by the analyst
- Overall, Plexor® HY System obtained lower quantitation values compare to Quantifiler® Duo, which may result in a lower amplification target.
- Based on this validation, Plexor® HY is capable of reliably detecting samples of 0.0125 ng or lower, which is the amplification target at which dropout (0.125ng) was observed in the ID Plus validation.

District of Columbia
Department of Forensic Sciences
Forensic Science Laboratory
Forensic Biology Unit

12. References

Plexor® HY System Technical Manual. Promega Corporation. 2013.

Scientific Working Group on DNA Analysis Methods (SWGDM), Validation Guidelines for DNA Analysis Methods. 2012.

District of Columbia Department of Forensic Sciences

7500 Instrument Maintenance Log

7500 Used:	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Year: 2014												
Check Lamp Status	✓ Good	✓ Good	✓ Good	✓ Good								
Perform Background Calibration	3/11/14	3/11/14	3/11/14	3/11/14								
Run Disk Cleanup & Defragment	3/11/14	3/11/14	3/11/14	3/11/14								
Check Disk Space/Back-up	3/11/14	3/11/14	3/11/14	3/11/14								
Power Off Computer	3/11/14	3/11/14	3/11/14	3/11/14								
Clean Instrument Surface	3/11/14	3/11/14	3/11/14	3/11/14								
ROI Calibration	3/11/14	3/11/14	3/11/14	3/11/14								
Optical Calibration	3/11/14	3/11/14	3/11/14	3/11/14								
Dye Calibration	3/11/14	3/11/14	3/11/14	3/11/14								
Annual Preventative Maintenance												
Date, Initial, Pass/Fail (to be performed by AB Service Engineer)												

7500 Instrument Maintenance Log
 Document Control Number: 1469
 Revision: 4

UNCONTROLLED WHEN PRINTED

Page 1 of 1
 Approved By: Christopher Maguire
 Issue Date: 12/2/2013 12:51:39 PM

District of Columbia Department of Forensic Sciences

7500 Instrument Maintenance Log

7500 Used: B

Year:	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
2014												
Check Lamp Status	✓ Good	✓ Good	✓ Good	✓ Good								
Perform Background Calibration	3/1/14	3/1/14	3/1/14	3/1/14								
Run Disk Cleanup & Defragment	3/1/14	3/1/14	3/1/14	3/1/14								
Check Disk Space/Back-up	3/1/14	3/1/14	3/1/14	3/1/14								
Power Off Computer	3/1/14	3/1/14	3/1/14	3/1/14								
Clean Instrument Surface	3/1/14	3/1/14	3/1/14	3/1/14								
ROI Calibration	3/1/14	3/1/14	3/1/14	3/1/14								
Optical Calibration	3/1/14	3/1/14	3/1/14	3/1/14								
Dye Calibration	3/1/14	3/1/14	3/1/14	3/1/14								
Annual Preventative Maintenance	3/1/14	3/1/14	3/1/14	3/1/14								
Date, Initial, Pass/Fail (to be performed by AB Service Engineer)												

* = 020414 PASS 08 - 105, 00500, 02610, F-

7500 Instrument Maintenance Log
 Document Control Number: 1469
 Revision: 4

Page 1 of 1
 Approved By: Christopher Maguire
 Issue Date: 12/2/2013 12:51:39 PM

UNCONTROLLED WHEN PRINTED

Page 4