Evaluation of Virtual Standard Curve Functionality of the HID Real-time PCR Analysis Software by Comparison to Assay Specific Standard Curves and an External Standard Curve Generated In-house

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INTRODUCTION

The goal of this research project was to examine two alternative methods in determining the concentration of DNA in questioned or reference samples that do not require a standard curve to be performed on each assay. The two alternative methods that can be used by laboratories are an in-house external standard curve, which utilizes a single standard curve over the course of multiple runs, and an instrument applied virtual standard curve. A comparison of the two methods was performed on the QuantStudio® 5 Real-Time PCR Instrument using the Quantifiler® Trio DNA Quantification Kit with full scale reactions. The new HID Real-Time PCR Analysis Software v1.3 that is compatible with the QuantStudio® 5 Real-Time PCR Instrument from Applied Biosystems enables data analysis with a virtual standard curve feature. Utilizing an external or virtual curve method can help laboratories optimize workflows, decrease costs, create more efficient workflows, and help decrease time in backlogs, such as in sexual assault cases. The outcome of these results will help the forensic community in determining criteria and parameters for each method that can be applied to adopting a workflow that includes a virtual or external quantitation curve to develop a more efficient quantitation workflow in their laboratories.

METHODS: PART I

Standard Curve Preparation

•Utilized Quantifiler® Trio DNA Quantification Kit and Quantifiler® THP DNA Standard (recorded as 100 ng/µL)

•Quantified DNA standard to determine true concentration

•Standard curves were prepared from a serial dilution using the true concentration to include a 50, 5, 0.5, 0.05, and 0.005 ng/µL standard

Method 1: Utilizing An Assay Specific Standard Curve

•Followed the instructions listed in standard curve preparation and utilized the HID Software to calculate the standard curve with the following formula:

$$DNA\ Concentration\left(\frac{ng}{\mu L}\right) = 10^{\frac{Cycle\ Threshold\ -Intercept}{X-Variable}}$$

Method 2: Generation of Excel Based External Standard Curve

•Created a combined linear relationship between the C_T and Log(Concentration of DNA) using a linear regression to obtain the line of "best" fit of all standard values generated for each curve for a given variable

•$C_T = m[\log(Qty)] + b$).

•Performed for Large Autosomal, Small Autosomal, and Y DNA targets

Method 3: HID Virtual Standard Curve

•Averaged the slopes and Y-intercept for each curve for a given variable and set as the virtual standard curve parameters for analysis for each Target

Variables Evaluated

•Different Lot Numbers

•Different Analyst Preparations

•Before and After Instrument Calibration

•Calibrations included temperature verification readings, cleaning block, region of interest (ROI), background, optical, and all dye calibrations including ABY-HID and JUN-HID

•Statistical Significance was determined between variables for the ESC/VSC Data and the Assay Specific Data using an ANOVA or TUKEY test

RESULTS: PART I

Table 1. Examples of Slope and Y-Int Comparisons of the ESC and VSC

Analyst 1	External Standard Curve	Virtual Curve
Slope	-3.2425	-3.243
Y-Intercept	27.061	27.061
Analyst 2		
Slope	-3.2189	-3.219
Y-Intercept	27.1052	27.105
Before Calibration		
Slope	-3.1741	-3.174
Y-Intercept	27.0443	27.044

Table 2. Statistical Comparison between Variables Evaluated

Variables Compared	Standard Concentration	Large Auto. Target	Small Auto. Target	Y Target
Analyst 1 v. Analyst 2	50	A1<A2		
	5	A1<A2		
	0.5	A1<A2		
	0.05	A1<A2		
	0.005			
Analyst 1 v. Analyst 2 v. Combined	50			
	5			
	0.5			
	0.05			
	0.005			
Kit v. Kit 2	50	Kit 1 > Kit 2		
	5	Kit 1 > Kit 2		
	0.5	Kit 1 > Kit 2		
	0.05	Kit 1 > Kit 2		
	0.005		Kit 1 > AC	

Key: **Kit 1** – First Lot, **A1** – Analyst 1, **A2** - Analyst 2, **C** – Combining A1 and A2, **AC** – After Calibration, **BC** – Before Calibration

No Statistical Significance – Statistical Significance –

Table 3. Example of hypothetical amplification values between Analyst 1 and Analyst 2

Expected DNA Conc. (ng/µL)	Analyst 1 Results (ng/µL)	Analyst 2 Results (ng/µL)	Expected DNA conc. to amplify (ng)	Analyst 1 Total DNA Amplified (ng/µL)	Analyst 2 Total DNA Amplified (ng/µL)	DNA Amplified Dilution 1	DNA Amplified Dilution 1	Volume Needed Analyst 1	Volume Needed Analyst 2
50	53.96	50.78	500	539.6	507.8	0.539	0.508	1.855	1.969
50	48.96	46.11	500	489.6	461.1	0.489	0.461	2.045	2.169
5	4.752	4.554	50	47.53	45.54	0.475	0.455	2.105	2.198
5	5.044	4.831	50	50.44	48.31	0.504	0.483	1.984	2.070
0.5	0.4955	0.4829	5	4.956	4.829	0.496	0.483	2.016	2.070
0.5	0.5718	0.5566	5	5.718	5.566	0.572	0.557	1.748	1.795
0.05	0.0443	0.0439	0.5	0.4431	0.4396	-	-	2.257	2.275
0.05	0.0485	0.0480	0.5	0.4846	0.4803	-	-	2.064	2.082
0.005	0.0061	0.0062	0.05	0.0618	0.0622	-	-	16.18	16.08
0.005	0.0068	0.0068	0.05	0.0678	0.0684	-	-	14.71	14.62

METHODS: PART II

Comparison of quantitation values for unknowns between the assay specific values and the HID Virtual Standard Curve

•Analyzed two standard curve plates as unknowns using the Virtual Curve Function and compared to the Assay specific quantitation values

RESULTS: PART II

Table 4. Statistical Comparison of Quantitation Values Generated from the Assay Specific Curve vs. the Virtual Standard Curve

Variables Compared	Standard Concentration	Large Auto. Target	Small Auto. Target	Y Target
Analyst 1 v. Analyst 2 v. Combined v. Assay Specific	50	AS<A1<C<A2		AS<A1<A2<C
	5	AS<A1<C<A2		
	0.5			
	0.05			
	0.005	AS<A1<C<A2		
Kit 1) v. Before Calibration (Kit 2) v. Assay Specific	50	Kit 1>BC>AS		BC>Kit 1>AC
	5	Kit 1>BC>AS		
	0.5			
	0.05			
	0.005	Kit 1>AS>BC		
Combined (Kit 1) v. After Calibration (Kit 2) v. Assay Specific	50	Kit 1>AC>AS		Kit 1>AC>AS
	5	Kit 1>AC>AS		
	0.5			
	0.05			
	0.005	Kit 1>AS>AC		

Key: **Kit 1** – First Lot, **A1** – Analyst 1, **A2** - Analyst 2, **C** – Combining A1 and A2, **AC** – After Calibration, **BC** – Before Calibration, **AS** –Assay Specific

No Statistical Significance – Statistical Significance –

CONCLUSION

• The external standard curve/virtual standard curve showed there was no statistically significant differences between creating a curve for quantitation analysis between instrument calibration and between kit lots.

• However, there were significant differences between pipetting from different analysts, which is why it is necessary to have as many analysts as possible pipetting to create a VSC/ESC to account for pipetting variability.

• If more variables are introduced, such as a new kit lot like shown in this project, in between calibrations, then a new virtual standard curve needs to be generated.

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