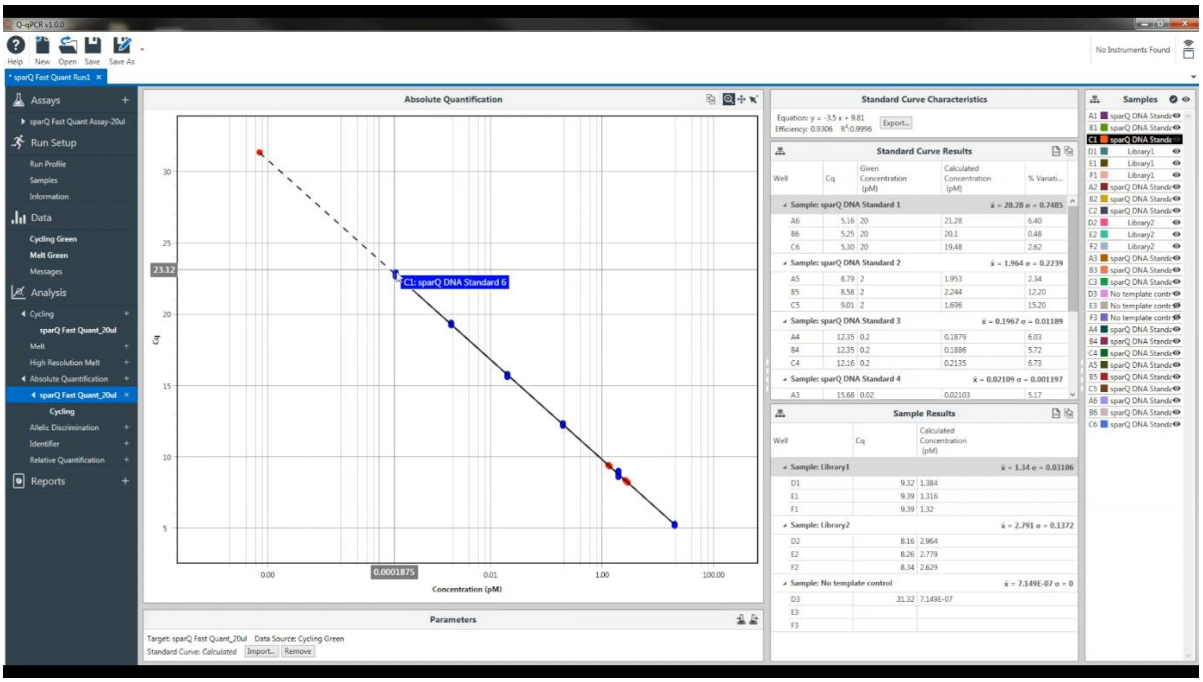
A Quick Guide to Analysis of sparQ Fast Library Quantification Data

Introduction

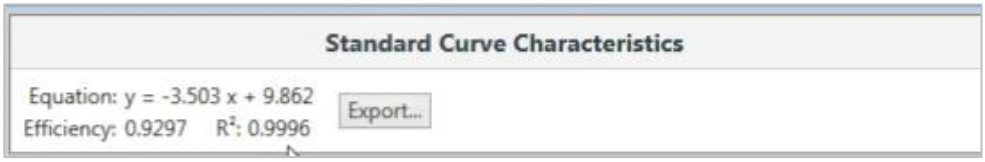
The purpose of this document is to describe how to view and analyze the data output by the Q-qPCR software following an amplification run using the sparQ Fast Library Quant Kit. The procedures described within are relevant to both runs set up using a template file or set up manually without template.

Examining Data in the Q-qPCR Software

Quantification data from the Q run is viewed by choosing [Absolute Quantification] in the Analysis section of the Q software. Clicking on the assay name used for the run brings up the Absolute Quantification standard curve plot. The sparQ DNA Standards are shown as Blue markers and the unknown library samples as Red markers. The diluted library values should always fall within the concentration range of the sparQ DNA standards (20 pM to 0.0002 pM) for reliable quantification.



The Standard Curve Characteristics panel gives information about efficiency and slope of the standard curve. Efficiency values above 0.90 are considered excellent.



The Standard Curve Results panel gives the Cq values for each standard reaction and shows given concentration vs concentration determined using the standard curve equation.

Standard Curve Results

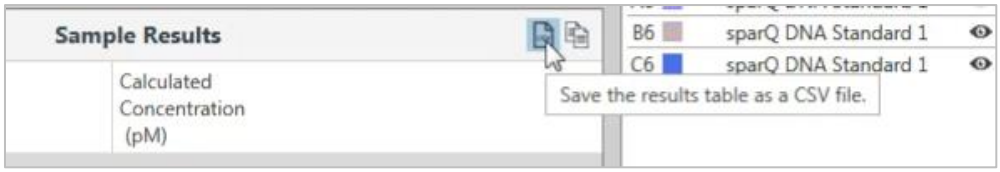
Well	Cq	Given Concentration (pM)	Calculated Concentration (pM)	% Variation
Sample: sparQ DNA Standard 1				$\bar{x}$ = 20.3 σ = 0.7658
A6	5.21	20	21.31	6.56
B6	5.30	20	20.11	0.56
C6	5.35	20	19.46	2.69
Sample: sparQ DNA Standard 2				$\bar{x}$ = 1.958 σ = 0.213
A5	8.86	2	1.935	3.24
B5	8.64	2	2.23	11.48
C5	9.05	2	1.709	14.53
Sample: sparQ DNA Standard 3				$\bar{x}$ = 0.1973 σ = 0.01234
A4	12.40	0.2	0.1881	5.93
B4	12.40	0.2	0.189	5.51
C4	12.20	0.2	0.2147	7.37
Sample: sparQ DNA Standard 4				$\bar{x}$ = 0.02101 σ = 0.001229
A3	15.74	0.02	0.02094	4.68

The Sample Results panel gives the Cq values and concentrations of the diluted library samples calculated from the standard curve equation.

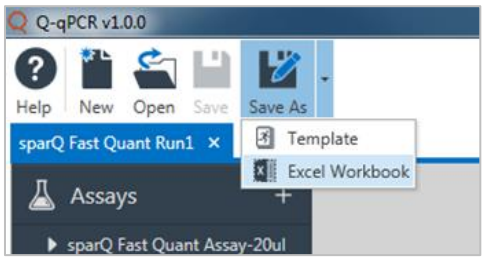
Sample Results

Well	Cq	Calculated Concentration (pM)
Sample: Library1		$\bar{x}$ = 1.34 σ = 0.03169
D1	9.37	1.384
E1	9.45	1.315
F1	9.44	1.319
Sample: Library2		$\bar{x}$ = 2.797 σ = 0.1402
D2	8.20	2.974
E2	8.30	2.785
F2	8.39	2.631
Sample: No template control		
E3		
F3		

Clicking one of the Icons at the top right side of a panel allows data to be saved to the clipboard or to a .csv file.

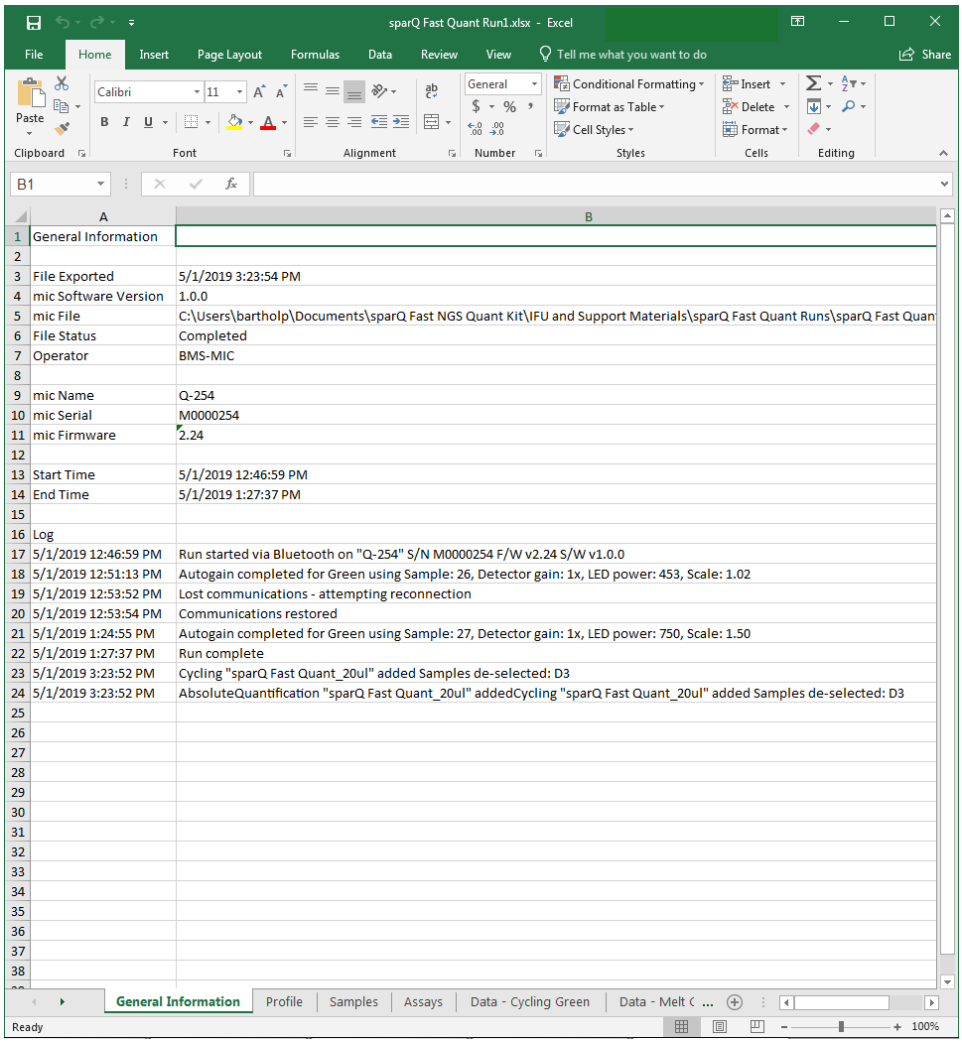


Clicking on the [Save As] icon allows all the data to be saved in an Excel Workbook



Examining Data in the Excel Workbook

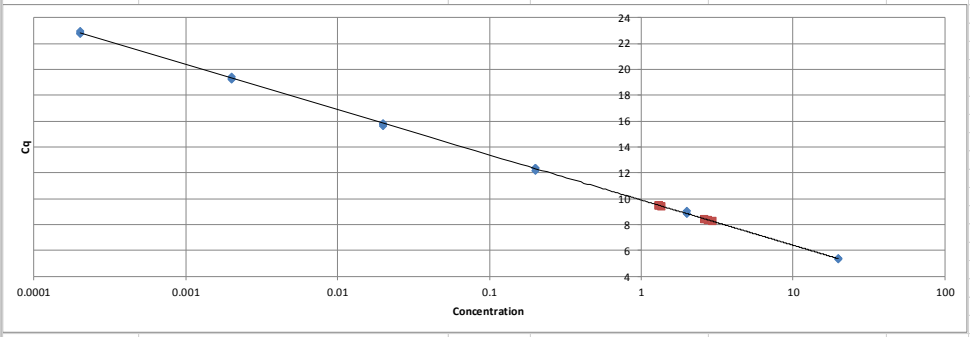
The resulting Excel workbook contains multiple worksheets. The first few worksheets provide information about the run profile, samples, and assays. The next few show the raw fluorescence data for each sample at each collection point. The remaining worksheets show the normalized cycling data, Cq values, and absolute quantification results.



	A	B
1	General Information	
2		
3	File Exported	5/1/2019 3:23:54 PM
4	mic Software Version	1.0.0
5	mic File	C:\Users\bartholp\Documents\sparQ Fast NGS Quant Kit\IFU and Support Materials\sparQ Fast Quant Runs\sparQ Fast Quan
6	File Status	Completed
7	Operator	BMS-MIC
8		
9	mic Name	Q-254
10	mic Serial	M0000254
11	mic Firmware	2.24
12		
13	Start Time	5/1/2019 12:46:59 PM
14	End Time	5/1/2019 1:27:37 PM
15		
16	Log	
17	5/1/2019 12:46:59 PM	Run started via Bluetooth on "Q-254" S/N M0000254 F/W v2.24 S/W v1.0.0
18	5/1/2019 12:51:13 PM	Autogain completed for Green using Sample: 26, Detector gain: 1x, LED power: 453, Scale: 1.02
19	5/1/2019 12:53:52 PM	Lost communications - attempting reconnection
20	5/1/2019 12:53:54 PM	Communications restored
21	5/1/2019 1:24:55 PM	Autogain completed for Green using Sample: 27, Detector gain: 1x, LED power: 750, Scale: 1.50
22	5/1/2019 1:27:37 PM	Run complete
23	5/1/2019 3:23:52 PM	Cycling "sparQ Fast Quant_20ul" added Samples de-selected: D3
24	5/1/2019 3:23:52 PM	AbsoluteQuantification "sparQ Fast Quant_20ul" addedCycling "sparQ Fast Quant_20ul" added Samples de-selected: D3

The Absolute Quantification worksheet shows the standard curve characteristics and plot as well as the Cq and concentration values of the diluted libraries and sparQ DNA standards.

Settings									
Target	sparQ Fast Quant Assay-20ul → sparQ Fast Quant_20ul								
Standard Curve Results									
Efficiency	0.929711499								
R^2	0.999625989								
Gradient	-3.502720398								
Y Intercept	9.861676103								



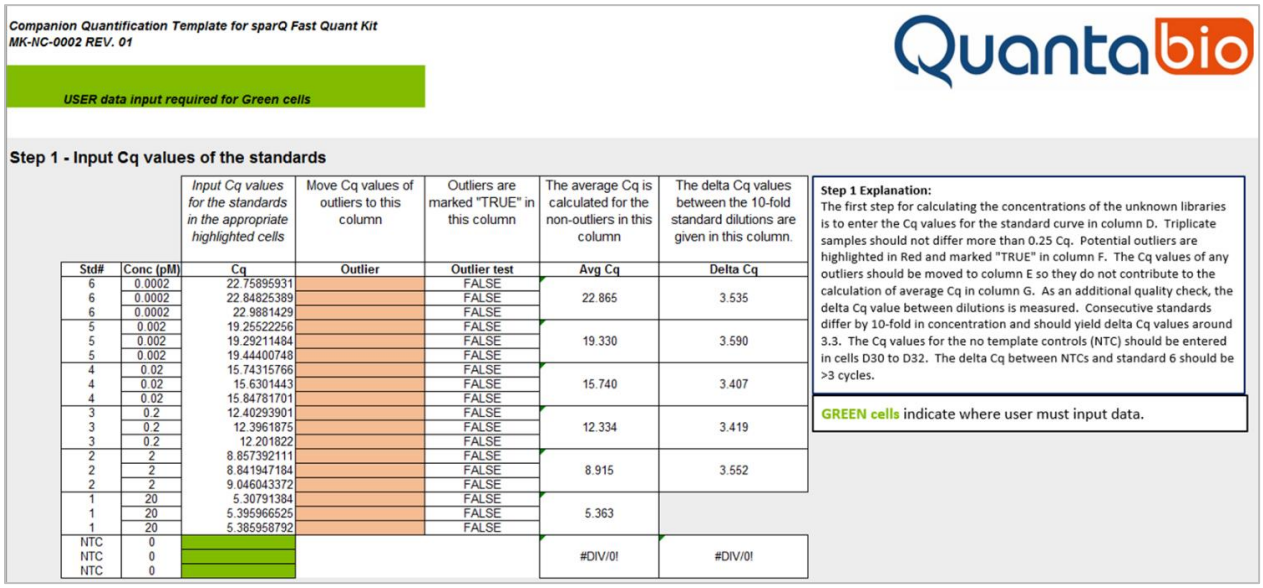
Standards Results								
Well	Sample Name	Cq	Given Concentration (pM)	Calculated Concentration (pM)	% Variation		Visible Cq	Visible Conc
A1	sparQ DNA Standard 6	22.75895931	0.0002	0.000222061	11.03070618		22.75895931	0.0002
B1	sparQ DNA Standard 6	22.84825389	0.0002	0.000196079	1.96053303		22.84825389	0.0002
C1	sparQ DNA Standard 6	22.9881429	0.0002	0.000178852	10.57402331		22.9881429	0.0002
A2	sparQ DNA Standard 5	19.25522256	0.002	0.002080722	4.036090679		19.25522256	0.002
B2	sparQ DNA Standard 5	19.29211484	0.002	0.002030867	1.543366961		19.29211484	0.002
C2	sparQ DNA Standard 5	19.44400748	0.002	0.001837881	8.105955409		19.44400748	0.002
A3	sparQ DNA Standard 4	15.74315766	0.02	0.020935426	4.677130209		15.74315766	0.02
B3	sparQ DNA Standard 4	15.6301443	0.02	0.022549985	12.74992479		15.6301443	0.02
C3	sparQ DNA Standard 4	15.84781701	0.02	0.019543501	2.282497008		15.84781701	0.02
A4	sparQ DNA Standard 3	12.40293901	0.2	0.188143314	5.928342761		12.40293901	0.2
B4	sparQ DNA Standard 3	12.3961875	0.2	0.188980196	5.509901958		12.3961875	0.2
C4	sparQ DNA Standard 3	12.201822	0.2	0.214736647	7.368323672		12.201822	0.2
A5	sparQ DNA Standard 2	8.857392111	2	1.935153554	3.242322298		8.857392111	2
B5	sparQ DNA Standard 2	8.841947184	2	2.229581324	11.47906622		8.841947184	2
C5	sparQ DNA Standard 2	9.046043372	2	1.70945209	14.5273955		9.046043372	2
A6	sparQ DNA Standard 1	5.30791384	20	21.31148373	6.557418672		5.30791384	20
B6	sparQ DNA Standard 1	5.395966525	20	20.1129288	0.564643987		5.395966525	20
C6	sparQ DNA Standard 1	5.385958792	20	19.46269176	2.686541195		5.385958792	20
Sample Results								
Well	Sample Name	Cq	Calculated Concentration (pM)				Visible Cq	Visible Conc
D1	Library1	9.367055	1.384237333				9.367055	1.384237333
E1	Library1	9.445327526	1.314814086				9.445327526	1.314814086
F1	Library1	9.43996879	1.319453916				9.43996879	1.319453916
D2	Library2	8.203624989	2.974136978				8.203624989	2.974136978
E2	Library2	8.303348338	2.785420131				8.303348338	2.785420131
F2	Library2	8.389866904	2.631420891				8.389866904	2.631420891
E3	No template control							
F3	No template control							

Using the Companion Quantification Template

Due to differences in SYBR Green binding to DNA, size adjustment calculations must be performed. A longer amplicon at a lower concentration may produce the same amount of signal as a shorter amplicon at a higher concentration. The Q-qPCR does not factor the size adjustment or dilution factor into the concentrations calculations. For this reason, we provide the Companion Quantification Template.

The Companion Quantification Template for sparQ Fast Library Quant Kit includes detailed explanations on where to input data and provides guidance on how to assess the quality of the data. The first step is to input the Cq values for each sparQ DNA standard assay. This can be done manually by typing values displayed in

the Q-qPCR software or by copy-and-pasting values from the Absolute Quantification worksheet of the exported Excel workbook.



Companion Quantification Template for sparQ Fast Quant Kit
MK-NC-0002 REV. 01

USER data input required for Green cells

Step 1 - Input Cq values of the standards

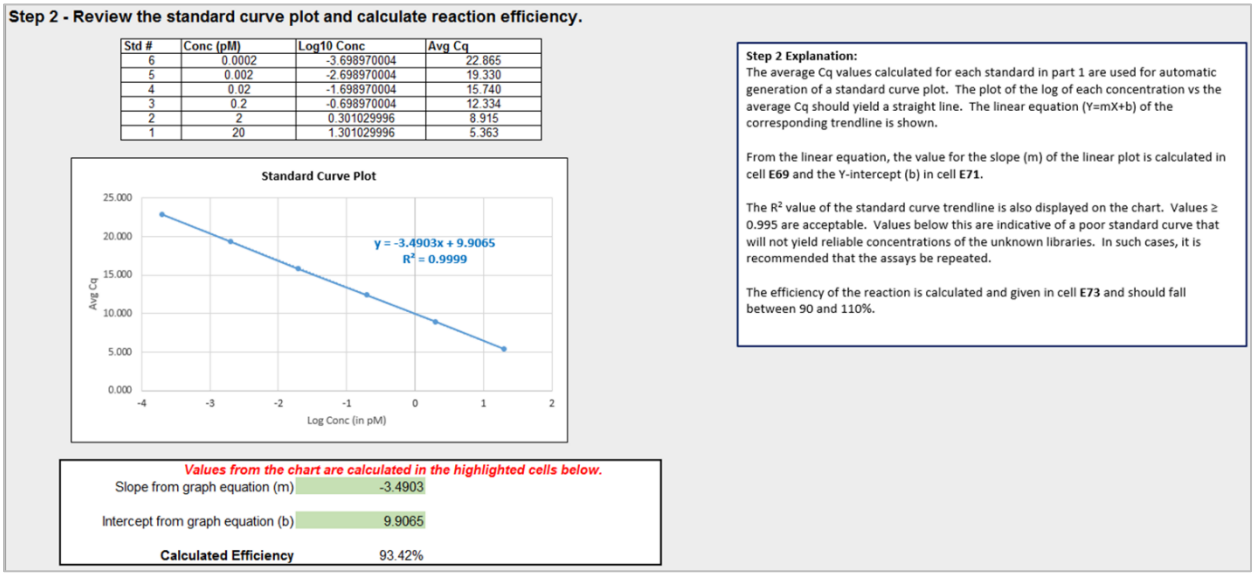
		Input Cq values for the standards in the appropriate highlighted cells	Move Cq values of outliers to this column	Outliers are marked "TRUE" in this column	The average Cq is calculated for the non-outliers in this column	The delta Cq values between the 10-fold standard dilutions are given in this column.
Std#	**Conc (pM)**	**Cq**	**Outlier**	**Outlier test**	**Avg Cq**	**Delta Cq**
6	0.0002	22.75895931		FALSE		
6	0.0002	22.84825389		FALSE	22.865	3.535
6	0.0002	22.9881429		FALSE		
5	0.002	19.25522256		FALSE		
5	0.002	19.29211484		FALSE	19.330	3.590
5	0.002	19.44400748		FALSE		
4	0.02	15.74315766		FALSE		
4	0.02	15.6301443		FALSE	15.740	3.407
4	0.02	15.84781701		FALSE		
3	0.2	12.40293901		FALSE		
3	0.2	12.3961875		FALSE	12.334	3.419
3	0.2	12.201822		FALSE		
2	2	8.857392111		FALSE		
2	2	8.841947184		FALSE	8.915	3.552
2	2	9.046043372		FALSE		
1	20	5.30791384		FALSE		
1	20	5.395966525		FALSE	5.363	
1	20	5.385958792		FALSE		
NTC	0					
NTC	0				#DIV/0!	#DIV/0!
NTC	0					

Step 1 Explanation:
The first step for calculating the concentrations of the unknown libraries is to enter the Cq values for the standard curve in column D. Triplicate samples should not differ more than 0.25 Cq. Potential outliers are highlighted in Red and marked "TRUE" in column F. The Cq values of any outliers should be moved to column E so they do not contribute to the calculation of average Cq in column G. As an additional quality check, the delta Cq value between dilutions is measured. Consecutive standards differ by 10-fold in concentration and should yield delta Cq values around 3.3. The Cq values for the no template controls (NTC) should be entered in cells D30 to D32. The delta Cq between NTCs and standard 6 should be >3 cycles.

GREEN cells indicate where user must input data.

Follow the guidelines in the explanation text box if any outliers are detected. Any delta Cq with values larger or smaller than expected could indicate poor sample handling. In such cases, it is recommended to repeat the run rather than continue with absolute quantification using an inaccurate standard curve.

The average Cq values of the replicate sparQ DNA standards are calculated and automatically populate the table in step 2, which already contains the concentration and $\log_{10}$ of the concentration values. The standard curve is plotted and another table is automatically populated with values for the slope and intercept. The slope is then used in calculation of the overall efficiency. The explanation text box for this section provides guidance on values considered acceptable for reliable absolute quantification analysis.



Step 2 - Review the standard curve plot and calculate reaction efficiency.

Std #	Conc (pM)	Log10 Conc	Avg Cq
6	0.0002	-3.698970004	22.865
5	0.002	-2.698970004	19.330
4	0.02	-1.698970004	15.740
3	0.2	-0.698970004	12.334
2	2	0.301029996	8.915
1	20	1.301029996	5.363

Values from the chart are calculated in the highlighted cells below.

Slope from graph equation (m)	-3.4903
Intercept from graph equation (b)	9.9065
Calculated Efficiency	93.42%

Step 2 Explanation:
The average Cq values calculated for each standard in part 1 are used for automatic generation of a standard curve plot. The plot of the log of each concentration vs the average Cq should yield a straight line. The linear equation (Y=mX+b) of the corresponding trendline is shown.

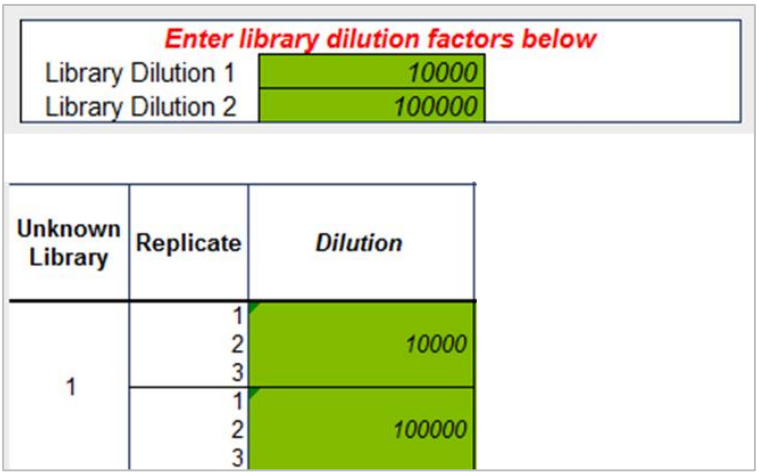
From the linear equation, the value for the slope (m) of the linear plot is calculated in cell **E69** and the Y-intercept (b) in cell **E71.**

The R^2 value of the standard curve trendline is also displayed on the chart. Values ≥ 0.995 are acceptable. Values below this are indicative of a poor standard curve that will not yield reliable concentrations of the unknown libraries. In such cases, it is recommended that the assays be repeated.

The efficiency of the reaction is calculated and given in cell **E73** and should fall between 90 and 110%.

Unless outliers were identified and removed specifically in this companion template, the values obtained to this point should exactly match those seen in the Q-qPCR software results.

Step 3 involves entering information about the library names, average fragment sizes, and dilutions used for the absolute quantification. If the same dilution factor(s) were used for all unknown libraries in the run, the values can be entered in the upper table, which will automatically populate the dilution column of the lower table.



Enter library dilution factors below	
Library Dilution 1	10000
Library Dilution 2	100000

Unknown Library	Replicate	*Dilution*
1	1 2 3	10000
	1 2 3	100000

Alternatively, if the dilutions factors differ between the different libraries, the upper table can be left blank and the appropriate dilution factors can be entered directly in the lower chart.

Next, enter the Cq values for each library amplification reaction in the lower main chart. This can be done manually by typing values displayed in the Q-qPCR software or by copy-and-pasting values from the Absolute Quantification worksheet of the exported Excel workbook. Follow the guidelines in the text box to remove any outliers from the analysis.

Input Cq values for the libraries in the appropriate highlighted cells	Move Cq values of outliers to this column	Outliers are marked "TRUE" in this column
Cq	**Outlier**	**Outlier test**
8.37		FALSE
8.57		FALSE
8.44		FALSE
11.93		FALSE
12.18		FALSE
12.08		FALSE
8.74		FALSE
8.85		FALSE
8.69		FALSE
12.14		FALSE
12.21		FALSE
12.14		FALSE

The final step is to enter the average fragment sizes for each library in the appropriate cells. These values are necessary for size adjustment calculations, which utilize the following formula:

$$\text{Adjusted concentration} = \text{raw concentration} \times \left[\frac{\text{size of sparQ DNA standard}}{\text{avg size of library fragments}} \right]$$

Once all information is entered, the average Cq, average raw (uncorrected) concentration (in pM), and size adjusted concentrations are automatically calculated and fill the table. Furthermore, the concentrations of the original, undiluted libraries are back-calculated using dilution factor(s) input for each sample. Values are given as pM, nM, and ng/ul and are indicated by the red arrow.

			Enter average fragment length values for the libraries in the appropriate highlighted cells.	*Input Cq values for the libraries in the appropriate highlighted cells*	Move Cq values of outliers to this column	Outliers are marked "TRUE" in this column					***Calculated Concentrations***		
Unknown Library	**Replicate**	***Dilution***	***Average fragment length in base pairs***	***Cq***	**Outlier**	**Outlier test**	**Avg Cq**	**Log Conc**	**Avg Conc (pM)**	**Size-adjusted Conc (pM)**	**Conc of undiluted library (pM)**	**Conc of undiluted library (nM)**	**Conc of undiluted library (ng/ul)**
1	1	*10000*	*496*	*8.37*		FALSE	8.46	0.414435	2.596782	2.230301	22303.01	22.30301	6.8364958
	2			*8.57*		FALSE							
	3			*8.44*		FALSE							
	1	*100000*		*11.93*		FALSE	12.06333	-0.61794	0.241025	0.207009	20700.94	20.70094	6.3454179
	2			*12.18*		FALSE							
	3			*12.08*		FALSE							
2	1	*10000*	*470*	*8.74*		FALSE	8.76	0.328484	2.130512	1.93106	19310.6	19.3106	5.6089559
	2			*8.85*		FALSE							
	3			*8.69*		FALSE							
	1	*100000*		*12.14*		FALSE	12.16333	-0.64659	0.225638	0.204514	20451.42	20.45142	5.9403183
	2			*12.21*		FALSE							
	3			*12.14*		FALSE							
3	1	*10000*	*481*	*8.04*		FALSE	8.113333	0.513757	3.264053	2.890825	28908.25	28.90825	8.5932078
	2			*8.09*		FALSE							
	3			*8.21*		FALSE							
	1	*100000*		*11.69*		FALSE	11.57	-0.4766	0.333737	0.295576	29557.6	29.5576	8.7862334
	2			*11.52*		FALSE							
	3			*11.5*		FALSE							

The calculated concentrations provide an accurate and reliable measure for subsequent library dilutions to concentrations appropriate for input on Illumina platform flow cells.