Using In-Reaction Standard Curves to Quantify Multiplexed qPCR Experiments

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Abstract

Real time qPCR experiments rely on external standard curves to perform the quantification. The external standard curves are derived from previous experiments with known quantities of targets and then are applied to reactions with unknown quantities. However the external standard curve approach does not take into account the natural variability of PCR reactions. With the application of STAR (Scalable Target Amplification Routine) technology, that combines qPCR with capillary electrophoresis, we have the ability to quantify dozens of targets in a single reaction. This approach allows us to incorporate internal calibration standards into each individual reaction and build the internal standard curve that represents the particular multiplexed PCR reaction. The use of internal standard curves mitigates the effects of experimental variability and results in the improved precision of the quantification.

ViraQuant Assay

We developed a multiplexed qPCR assay to address the need to detect viral infections of transplant patients. These patients are prescribed immunosuppressant drugs to decrease the chance of organ rejection and this makes them more susceptible to infections. The ViraQuant assay simultaneously detects and quantifies five viruses that are common infections in transplant patients:

1. Cytomegalovirus (CMV)
2. Eppstein-Barr virus (EBV)
3. Polyomavirus (BKV)
4. Herpesvirus 6 (HHV6)
5. Herpesvirus 7 (HHV7)

The reagents of the ViraQuant PCR reaction consists of Qiagen® Multiplex PCR buffer system with additional 5U of HotStart Taq polymerase (Qiagen, Valencia, CA), gene specific primer pairs with one primer of the pair labeled with 5,6-FAM fluorophore, quantification calibrators, and detection sensitivity controls. The primers are designed to ensure that amplified DNA fragments for each target are of unique size and therefore easily distinguishable by capillary electrophoresis. Samples are overlaid with mineral oil and PCR amplified under the following conditions:

1. Initial denaturation at 95ºC for 15 min.
2. Touchdown PCR protocol of 3 cycles each at 62ºC, 60ºC, and 58ºC annealing temperature.
3. Final 33 cycles of denaturation at 95ºC for 30 seconds, annealing at 57ºC for 90 seconds, extension at 72ºC for 60 seconds

Two-microliter aliquots of the multiplex PCR reaction are removed after every other cycle starting at cycle 20 and ending at cycle 42. The aliquots are deposited into 13uL formamide (Teknova, Hollister, CA) containing ROX-labeled DNA standards(MM1000, Bioventures, Murfreesboro, TN) After collecting all aliquots, samples are denatured at 95ºC for 5 minutes and separated by capillary electrophoresis in POP-7 polymer (ABI Genetic Analyzer 3730XL, Global Medical Instrumentation Inc., Ramsey, Minnesota)

Data Analysis

We use GeneMapper 3.0 software (Applied Biosystems, Foster City, CA) to de-convolute the raw signal into electropherograms for FAM and ROX dyes, to detect peaks in the electopherograms, and to determine the height and area of the peaks.
ROX peaks are associated with the corresponding size standards and the sizes of FAM peaks are then determined using the Local Southern method. Peaks within ±3bp of the expected size are collected into points of amplification curves.

The following heuristics is used to find the log-linear portion of the amplification curve. All non-decreasing sets of points are evaluated and the set that maximizes the weighted fitness score FS is selected:

$$FS = w_r\, FS_r + w_n\, FS_n + w_t\, FS_t$$

where w_r, w_n, w_t are the weights and the components of the score are:

$$FS_r = R^2 - 0.8$$

$$FS_n = \max(5, N)$$

$$FS_t = \begin{cases} T - C_{\min} & \text{if } T < C_{\min} \\ 1.0 & \text{if } C_{\min} \le T \le C_{\max} \\ C_{\max} - T & \text{if } C_{\max} < T \end{cases}$$

where R^2 is the correlation correlation coefficient, N is the number of selected points, T (=4.4) is the log(RFU) threshold, $C_{\max}$ and $C_{\min}$ are the minimal and maximal log(RFU) values for the selected points.

We use internal (or external) standard curve with slope s and intercept a to compute the log of copy number Q from the corresponding cycle threshold c_T:

$$\log(Q) = (c_T - a) / s$$

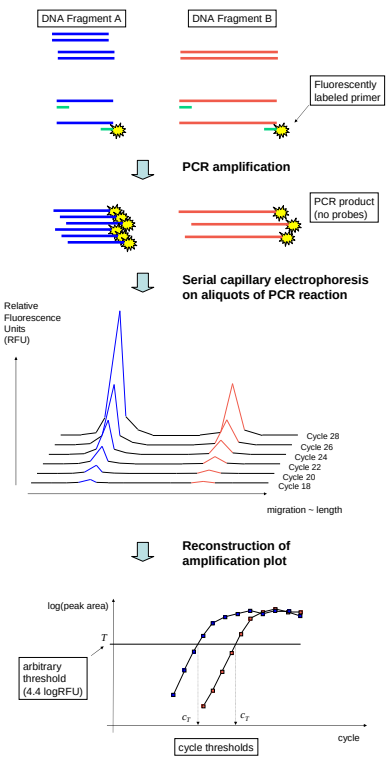
The variability of the assay is measured in terms of coefficient of variation

$$CV = S/X,$$

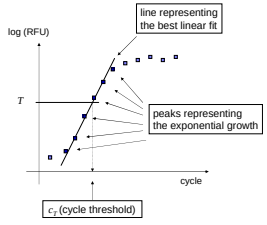
where S is standard deviation of the measured copy numbers and X is the average of the measured copy numbers. We multiply the coefficient by 100 to express it as a percentage (%CV). To determine the components of variability we separately compute the within run standard deviation S_r, run-to-run standard deviation S_{rr}, and day-to-day standard deviation S_{dd}. The total standard variation is then expressed as

$$S_T = \sqrt{S_r^2 + S_{rr}^2 + S_{dd}^2}$$

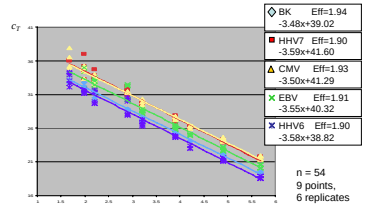
Multiplex PCR



Computation of c_T

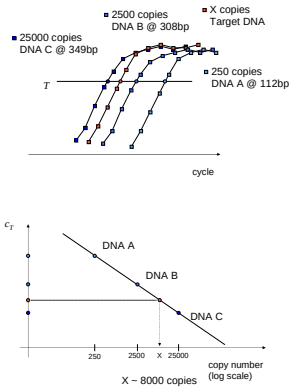


External Standard Curves

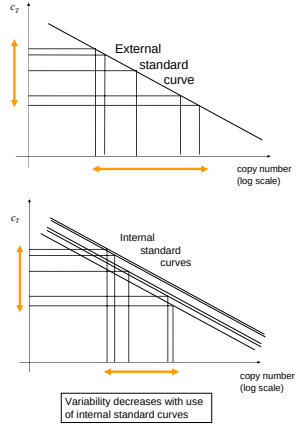


Note that intercept values have no effect on the resulting %CV

Internal Standard Curves

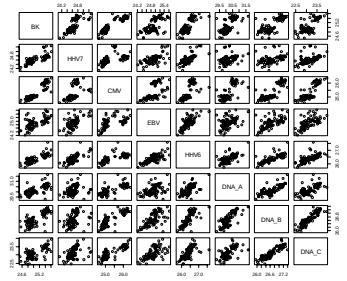


Comparison of Quantifications



Variability decreases with use of internal standard curves

Pairwise c_T Correlations Plots



Precision Study

To evaluate the precision we run the assay with all target DNAs spiked in plasma at three different levels, high (~16000 copies/reaction), medium (~8000 copies/reaction), and low (~400). Also included are negative controls with no spiked target DNA. Two runs a day were performed for 20 days with two replicates in a run for the total of 80 replicates. The variability of measured copy numbers per reaction are expressed as %CV and shown in the table below.

Comparison of %CV using internal and external standards

Target	Level	%CV Stored	%CV Internal	Delta
BK	High	17.84	13.64	4.20
HHV7	High	10.64	18.91	-8.27
CMV	High	31.07	21.82	9.25
EBV	High	22.57	18.68	3.90
HHV6	High	13.99	13.10	0.90
BK	Medium	21.63	14.24	7.39
HHV7	Medium	20.58	22.78	-2.20
CMV	Medium	29.30	18.16	11.14
EBV	Medium	24.95	17.50	7.44
HHV6	Medium	17.99	14.88	3.11
BK	Low	22.03	18.39	3.64
HHV7	Low	18.19	23.30	-5.11
CMV	Low	34.49	24.64	9.85
EBV	Low	26.99	26.52	0.47
HHV6	Low	26.78	28.18	-1.40
			average delta	2.95

Conclusions

- Use of internal standards increases precision of multiplex PCR assays
- Internal standards allow compensation of run-to-run variability
- Use on internal standard curve decreases variability in situations where it is difficult to achieve assay consistency.