Improved Accuracy of DNA Quantitation Using a Chemistry Model-Based Method for Estimating Nucleic Acid Quantities in PCR Amplified Samples

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Introduction and Purpose

Real-time polymerase chain reaction (PCR) technology is a powerful tool, enabling the detection and quantitation of virus particles in clinical specimens. We have developed a method for quantitative analysis of PCR data based on an idealized chemical model. The main reaction modeled in the chemical-model based PCR analysis (CMPA) is the binding of polymerase to primer-target duplex. The method does not depend on a set detection threshold, requires no ad hoc curve fitting and estimates amounts of target nucleic acid using cycles from near the first cycle before amplification is apparent to several cycles beyond where a typical threshold would be set. All parameters of the model (determined by non-linear least squares) represent physical attributes of PCR chemistry. Replication efficiency is not a parameter used in the model, but because the model is recursive, efficiency is computed for every individual reaction at every cycle from cycle 1 to the last cycle modeled. Accuracy and precision of estimated amounts of target nucleic acid are as good as or better than derived by threshold based methods, particularly when reaction conditions cause replication efficiency to vary. Also, as the parameters represent actual attributes of the reaction, they serve as useful checks of validity and have potential utility in the development and optimization of assays.

Features of the chemical model-based PCR analysis method

- No threshold required for data analysis
- No assumptions regarding reaction efficiency
- Target amounts estimated from 10 cycles, not threshold crossing
- First cycle efficiency estimated independently for every reaction
- Calibration parameters correspond to actual reaction attributes

Advantages of the chemical model-based PCR analysis method

- Target amounts estimated accounting for efficiency in each reaction
- Predicted quantity amounts corrected for bias
- Linearity statistics generated for every calibration
- Individual efficiency estimate and fit statistics are validity checks
- Instrument independence of all but one parameter facilitates calibration transfer
- Potential utility of parameters corresponding to polymerase binding and activity for assay development

Methods

Assay Conditions: Real-time PCR assays designed to specifically detect and quantify Epstein Barr Virus, human Cytomegalovirus, or BK virus were used for all comparative analyses. Each assay was carried out with 5 µL of template nucleic acid (either purified and quantified amplicon or purified viral DNA from clinical specimens) and 5 µL of PCR mastermix. PCR was run on the the 3M Integrated Cycler, using cycling conditions as follows: initial denaturation for 2 min at 97°C, thermalcycling protocol was 45 cycles of denaturation at 97°C for 10 s, and annealing/extension at 60°C for 30 s. Fluorescence measurements were taken during the annealing phase. Data was analyzed using both the CMPA, and conventional threshold methods.

Methods (cont.)

Linearity/Method Comparison: A series of quantified calibrators (four replicates) was run for assays targeting each of the 3 selected analytes. A 10 member calibration panel was used for analysis of the BKV assay, and 5 member panels were used for the EBV and CMV assays. Linearity was assessed from the Ct standard curve or a plot of log predicted vs. log true.

Inhibition Studies: CMV amplicons were spiked with diluted sodium heparin recovered from heparin blood collection tubes, with an estimated 1X concentration of 143 USP Units/mL. The 1/60, 1/40, 1/20, and 1/10 dilutions were made for estimated final reactions concentrations of 0.2, 0.4, 0.7, and 1.4 mUnits/mL. Samples were assayed, and expected and observed quantity values obtained using the CMPA method and the threshold method were compared.

Results

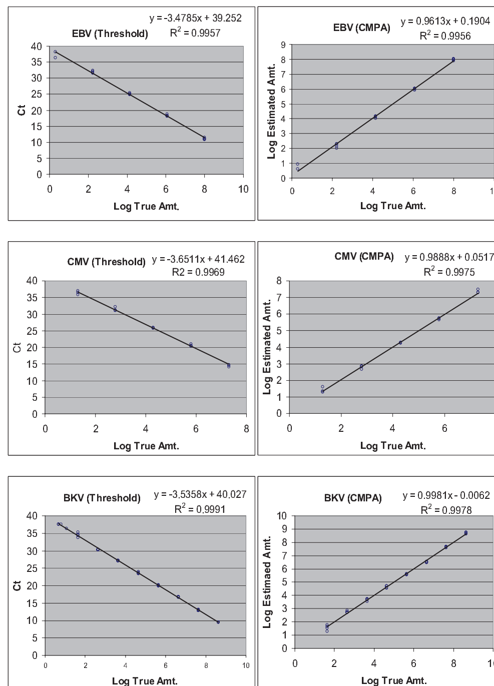

Figure 1. Linearity comparisons between Threshold and CMPA methods of quantitation.

Results (Cont.)

Linearity and Method Comparison: Linearity of Ct standard curves and CMPA linearity plot as measured by R^2 was > 0.995 in all 3 assays (Figure 1). Both methods also appeared to perform equally well at both high and low quantity extremes. Although performance was roughly equivalent, the CMPA method was found to have superior performance and better accuracy in some situations. For example, for clinical samples with less than expected amplification efficiency (likely due to inhibition), the CMPA model was able to detect the inefficiency and provide a more accurate quantity determination than the threshold method (Figure 2).

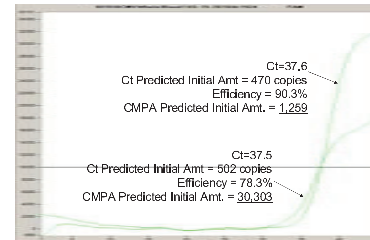

Figure 2: Analysis of 2 CMV positive specimens extracted from blood. The CMP algorithm identifies lower amplification efficiency in the second, and estimates a higher initial copy number in that sample compared to the Ct method.

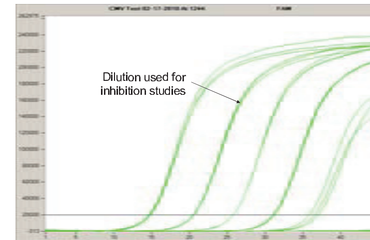

Figure 3: 5 equally spaced CMV amplicon calibrators spanning the linear range of CMV Quantitation from $2x10^7$ to $2x10^1$ copies per reaction

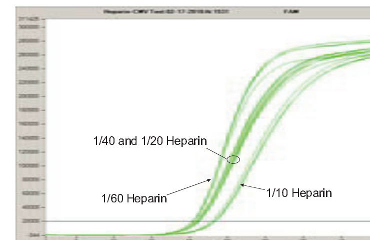

Figure 4: Amplification curves from samples with increasing concentrations of Heparin, showing reaction inhibition and delayed Ct values.

Results (Cont.)

Inhibition Studies: Spiking samples with increasing amounts of sodium heparin delayed Ct values (Figure 4 and Table 1) and resulted in lower predicted target quantities estimated by a threshold method. The CMPA method was less affected (due to accounting for individual reaction efficiencies), and predicted quantities had lower % error.

Table 1: Comparison of threshold and CMPA quantity predictions in the presence of increasing amounts of heparin (which causes PCR inhibition).

	Threshold Predictions		CMPA Predictions	
Inhibitor Level	Ct	Predicted Quantity	Predicted Quantity	Cycle 1 Efficiency
1 to 60 Heparin	19.4	589,475	615,874	88.5%
1 to 60 Heparin	19.4	594,379	604,816	88.5%
1 to 60 Heparin	19.5	555,958	619,410	87.8%
1 to 60 Heparin	19.4	599,038	641,632	88.2%
	True Value:	631,000	631,000	
	Mean Predicted:	584,713	620,433	
	% Error	-7.3	-1.7	
	% CV	3.1	2.5	
1 to 40 Heparin	19.8	457,307	675,450	85.2%
1 to 40 Heparin	19.8	472,238	697,199	85.1%
1 to 40 Heparin	19.8	469,887	712,178	84.8%
1 to 40 Heparin	19.8	453,921	678,098	84.8%
	True Value:	631,000	631,000	
	Mean Predicted:	463,338	690,731	
	% Error	-26.6	9.5	
	% CV	1.4	2.5	
1 to 20 Heparin	20.1	393,094	653,768	84.2%
1 to 20 Heparin	20.1	374,273	654,283	83.9%
1 to 20 Heparin	20.1	389,817	657,040	84.2%
1 to 20 Heparin	20.1	373,604	663,538	83.8%
	True Value:	631,000	631,000	
	Mean Predicted:	382,697	657,157	
	% Error	-39.4	4.1	
	% CV	1.6	0.7	
1 to 10 Heparin	22.0	119,282	334,745	80.8%
1 to 10 Heparin	22.0	112,552	317,248	80.8%
1 to 10 Heparin	21.5	154,063	383,887	81.2%
1 to 10 Heparin	22.1	110,260	297,936	81.1%
	True Value:	631,000	631,000	
	Mean Predicted:	124,039	333,454	
	% Error	-80.3	-47.2	
	% CV	3.2	11.0	

Summary/Conclusions

- The Chemistry Model-based method for quantitative analysis requires no assumptions about efficiencies at different calibrator levels, within calibrator replicates or between calibrators and unknown samples
- Accuracy and precision performance of the Chemistry Model-based method matches Gold Standard threshold based methods for quality data, and compensates (partially or better) for reduced polymerase activity when altered by inhibition or non-ideal reaction conditions
- Replication efficiency is estimated for every individual PCR reaction
- Efficiency, polymerase activity parameter and R^2 are validity checks for each reaction
- Polymerase binding parameter and polymerase activity parameters are potentially useful for assay development and optimization

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