AN ALTERNATIVE WAY FOR REAL TIME PCR DATA ANALYSIS

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INTRODUCTION

Real Time PCR is a methodology with increasing applications in the clinical laboratory. This new and revolutionary method combines the PCR chemistry with the fluorescent probe/dye detection of the amplified product, all in the same reaction tube. Since the equipment used can record the emission of fluorescence during all the cycles of amplification, a significant increase of the PCR product is directly linked with the initial amount of target DNA. In Real Time PCR, we can determine a fixed fluorescent threshold, above the background. When the PCR product that we want to detect cross this threshold, we can determine a parameter named Cycle Threshold (Ct). All the equipment used in Real Time PCR experiments have some kind of software to analyze the data, namely the analysis of the expression of Ct value relatively to the log[DNA]. However, this software doesn't give much details regarding the linear regression: it only calculates the ***Slope***, ***Y Intercept*** and ***Coefficient of Determination*** (R^2). As an accreditated laboratory, we have to monitor the performance of our methods. In Clinical Chemistry, the performance is measured with the determination of Total Error (TE) and Six Sigma (6S), but until now it was never applied to Real Time PCR.

AIM

Development of an Excel sheet that calculates several parameters regarding the linear regression and assay validation/calibration.
Use of the data generated by this Excel sheet to monitor the performance of the In House Assays.

METHODOLOGY

We have implemented the Viral Load assays, using *In House* technology, for the quantification of Cytomegalovirus (CMV), Virus Epstein-Barr (EBV), Human Herpes Virus Type 6 (HHV6), Parvovirus B19 (B19) and BK Virus BKV). This techniques were described elsewhere (1, 2, 4)). The methodology for the determination of Detection and Quantification Limits were described by Schwarz et al, 2004.

RESULTS

We have developed an Excel sheet that uses the data from calibrators (4 different concentrations of Virus) in order to determine the following parameters: Slope, Y Intercept, Coefficient of Determination (R^2), Efficiency Amplification (E), Detection and quantification limit (analytical and method), Standard Error (RMSE) and P-Value associated with the linear regression (validation).
Relatively to the linear regression parameters, we have introduced the Analysis of Variance (***ANOVA***) in order to determine the ***Sum of Squares of***: ***Regression***, ***Residual***, ***Lack of Fit*** and ***Pure Error***. With this approach we can make an objective analyses of the goodness of fit, residuals, determine outliers and the confidence interval of the samples with viral load. In the next tables we can observe the constitution of the Excel Sheet, relatively to the CMV. The Excel sheet has 4+1 pages :

- Sample identification (1)
- Reagents (2)
- Real Time PCR Calculations (3)
- Sample Evalutarion (4)
- ANOVA analyses (includes residuals) (5)

Since our laboratory is accredited (ISO 17025: 2005), it also helps in the maintenance of records relatively to batch/expire date of DNA/RNA extraction kit, primers, probes, master mix, internal control (amplification) and the reaction plates. All the data obtained is introduced in an Access database, in order perform the monitorization of the assays. The Ct values obtained with the different dilutions of calibrator are used for:

- Precision of the assay, for different concentrations
- Determination of Total Error
- Determination of 6S(performance)

In the table below we have the performance of our CMV assay. For the determination of 6S, we have set the Total Error Allowed (TEa) as 25%.

SPECIFICATION: (TEA)	25%	PRECISION	TOTAL ERROR	SIGMA
Decision Level (15 copies/ul)	**3,05**	1,8%	21,2%	4,12
Decision Level (150 copies/ul)	**4,05**	1,6%	20,8%	4,63
Decision Level (1500 copies/ul)	**5,05**	1,9%	21,4%	3,90

CONCLUSION

Our Excel sheet is a very good alternative to the data analysis of the standard software present in the various Real Time PCR equipments. It also allows the continuous monitorization of the data generated (internal quality control), together with the External Quality Assessment programs, can be used to monitor the performance of our In House assays.

REFERENCES

1. Machida, U., M. Kami, et al. (2000). "Real-time automated PCR for early diagnosis and monitoring of cytomegalovirus infection after bone marrow transplantation." J Clin Microbiol **38**(7): 2536-42.
2. Niesters, H. G., J. van Esser, et al. (2000). "Development of a real-time quantitative assay for detection of Epstein-Barr virus." J Clin Microbiol **38**(2): 712-5.
3. Schwarz, G., S. Baumler, et al. (2004). "Determination of detection and quantification limits for SNP allele frequency estimation in DNA pools using real time PCR." Nucleic Acids Res **32**(3): e24.
4. Watzinger, F., M. Suda, et al. (2004). "Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients." J Clin Microbiol **42**(11): 5189-98.
5. Westgard J. O. "Six Sigma Quality Design and Control", 2nd Edition, 2006.

Assay CMV
Data 27-02-2009

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	1	2	3	4	5	6	7	8	9	10	11	12
A	1600	1600	1600	160	160	160	16	16	16	1,6	1,6	1,6
B	1258005	1258005	1258005	1259015	1259015	1259015	1259103	1259103	1259103	1259196	1259196	1259196
C	1259203	1259203	1259203	1259215	1259215	1259215	1259254	1259254	1259254	1259262	1259262	1259262
D	1259266	1259266	1259266	1259269	1259269	1259269	1259273	1259273	1259273	1259277	1259277	1259277
E	1259280	1259280	1259280	1259284	1259284	1259284	1259288	1259288	1259288	1259291	1259291	1259291
F	1259296	1259296	1259296	1259301	1259301	1259301	1259306	1259306	1259306	1259690	1259690	1259690
G	1259861	1259861	1259861	NTC	NTC	NTC						
H												

DNA Extraction: Luís Banha **DNA Amplification:** Luís Banha **Validation :** Carmo Ornelas

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Reagents		Brand	Lot	Expires in
Liquid Extraction	EZ1 Virus Mini Kit v2.0	QIAGEN	130190254	12-12-2009
	Reagents Cartridges	QIAGEN	130188702	
	Carrier RNA	QIAGEN	130188213	
Amplification:	Master Mix	Applied Biosystem	k12020	30-09-2009
	Controlo Interno	Applied Biosystem	0804117	-
	H_2O	Braun	8276B04	Jun-13
Vírus CMV	Primer Rev.	Applied Biosystem	-	Reconstitution: 09-01-2009
	Primer Forw.	Applied Biosystem	-	Reconstitution: 16-01-2008
	Probe	Applied Biosystem	-	Reconstitution: 03-02-2009
	Calibrator	ABI	8C0015	Mar-09
Detection	96 Well Plate	Applied Biosystem	SG4437-5A	-
	Cover	Applied Biosystem	20080302	-

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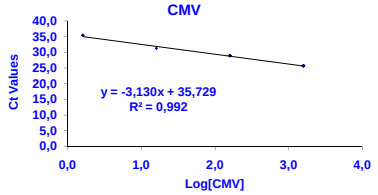
Calculations [Calibrator]	Log [Calibrator]	Ct	Variation		Grubs Test (Outlier)		Aver.Ct	SD Ct	%CV	Variation (Copies/ul)		Expect Value	Variation (Ct)
1600	3,20	25,73	1565,08	2,2%	1,155	Remove							
1600	3,20	25,70	1600,00	0,0%	0,577	Accept	25,71	0,02	0,07%	992,6	-38,0%	-	
1600	3,20	25,70	1600,00	0,0%	0,577	Accept							
800	2,90		ND	ND	ND	ND							
800	2,90		ND	ND	ND	ND	ND	ND	ND	ND	ND	-	
800	2,90		ND	ND	ND	ND							
160	2,20	29,00	141,19	11,8%	0,718	Accept							-0,11%
160	2,20	28,97	144,34	9,8%	0,424	Accept	28,93	0,10	0,35%	91,0	-43,1%	29,0	-0,21%
160	2,20	28,81	162,37	1,5%	1,142	Accept							-0,76%
80	1,90		ND	ND	ND	ND							
80	1,90		ND	ND	ND	ND	ND	ND	ND	ND	ND	-	
80	1,90		ND	ND	ND	ND							
16	1,20		ND	ND	ND	ND							
16	1,20		ND	ND	ND	ND	31,29	ND	ND	15,7	-1,7%	32,2	
16	1,20	31,29	26,19	63,7%	ND	ND							-2,97%
1,6	0,20		ND	ND	ND	ND							
1,6	0,20		ND	ND	ND	ND	35,44	ND	ND	0,7	-54,9%	34,6	
1,6	0,20	35,44	1,24	22,7%	ND	ND							2,39%



Regression Equation

		Range		
Slope:	-3,13	-3,10	-3,70	OK
Y Intercept:	35,73	35	40	OK
R^2:	0,992	0,99	1	OK
Amp. Efficiency:	109%	0,8	1	
RMSE(S_{xy}):	0,322			
Analitycal DL:	2,0	Copies/tube		
Analitycal QL:	10,7	Copies/tube		
Method DL:	61	Copies/ml		
Method QL	320	Copies/ml		
P Value:	1,52E-07			

N°: 8 CI 0,95

Threshold: 0,4

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Samples	Type of Sample	Ct Values R1	R2	R3	Average	SD	Copies/tube	Inicial Volume	Final Volume	Dilution	Copies/ml	Confidence Interval	
1258005	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259015	Plasma	39,02	38,85		38,94	0,12	0,05	400	60	6,7	8	6	11
1259103	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259196	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259203	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259215	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259254	Plasma	31,55	32,04	31,29	31,63	0,38	12,25	220	60	3,7	3341	3339	3344
1259262	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259266	Plasma		42,42	45,63	44,03	2,27	0,00	400	60	6,7	0	-2	3
1259269	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259273	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259277	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259280	Plasma		38,17		38,17	ND	0,09	400	60	6,7	14	12	17
1259284	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259288	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259291	Plasma	35,98	36,92	36,53	36,48	0,47	0,33	400	60	6,7	50	48	53
1259296	Plasma	38,06	36,98	37,14	37,39	0,58	0,17	400	60	6,7	25	23	28
1259301	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259306	Plasma		39,17		39,17	ND	0,05	400	60	6,7	7	4	9
1259690	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
1259861	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
NTC	H2O				ND	ND	ND	400	60	6,7	ND	ND	ND
	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND
	Plasma				ND	ND	ND	400	60	6,7	ND	ND	ND

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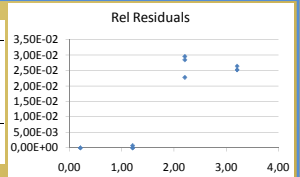
	SS	DL	MS	F	p Value
Regression (SS_{Reg})	78,38	1	78,38	757,00	1,52E-07
Residuals (SS_R)	0,62	6	0,1035		
"Lack of Fit" (SS_L)	0,64	2	0,3206	6,41E+01	9,15E-04
Pure Error (SS_{PE})	2,00E-02	4	5,00E-03		
Total	709,47	7			

Good Fit