

# Absolute Quantification in Real-time PCR by Nonlinear Regression Analysis of Fluorescence Data

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## BACKGROUND

Absolute quantification by real-time PCR requires allocation of up to 1/8 of the wells of a 96 well plate for the standard curve. Furthermore, the cycle threshold method assumes equal PCR efficiency in all reactions, which is not always the case (e.g. in fecal samples). Previous attempts to generate alternative algorithms for absolute quantitation by curve fitting or regression analysis have shown variable success, and have been complicated to perform(1;2). Primarily, an alternative method should have a precision comparable to the cycle threshold (CT) method. Secondly, a simple hands-on procedure is necessary, as few have access to an experienced statistician on a daily basis.

## AIMS

To develop and evaluate an automated nonlinear regression (NLR) algorithm capable of generating batch production regression analysis, with precision and characteristics comparable to the CT method.

## METHODS

Total RNA samples extracted from human gastric mucosa were reverse transcribed and analysed for TNF-A, IL18 and Beta-actin by TaqMan™ real-time PCR on an ABI-prism 7900 system. Fluorescence tracings were analysed by regular CT method with a standard curve, and by NLR with a positive control for optical calibration. Eleven separate regression models were tested, including combinations of log-transformation, weighted analysis, baseline correction, etc. Output data were subjected to Altman-Bland analysis(3).

## BASE FORMULA(1)

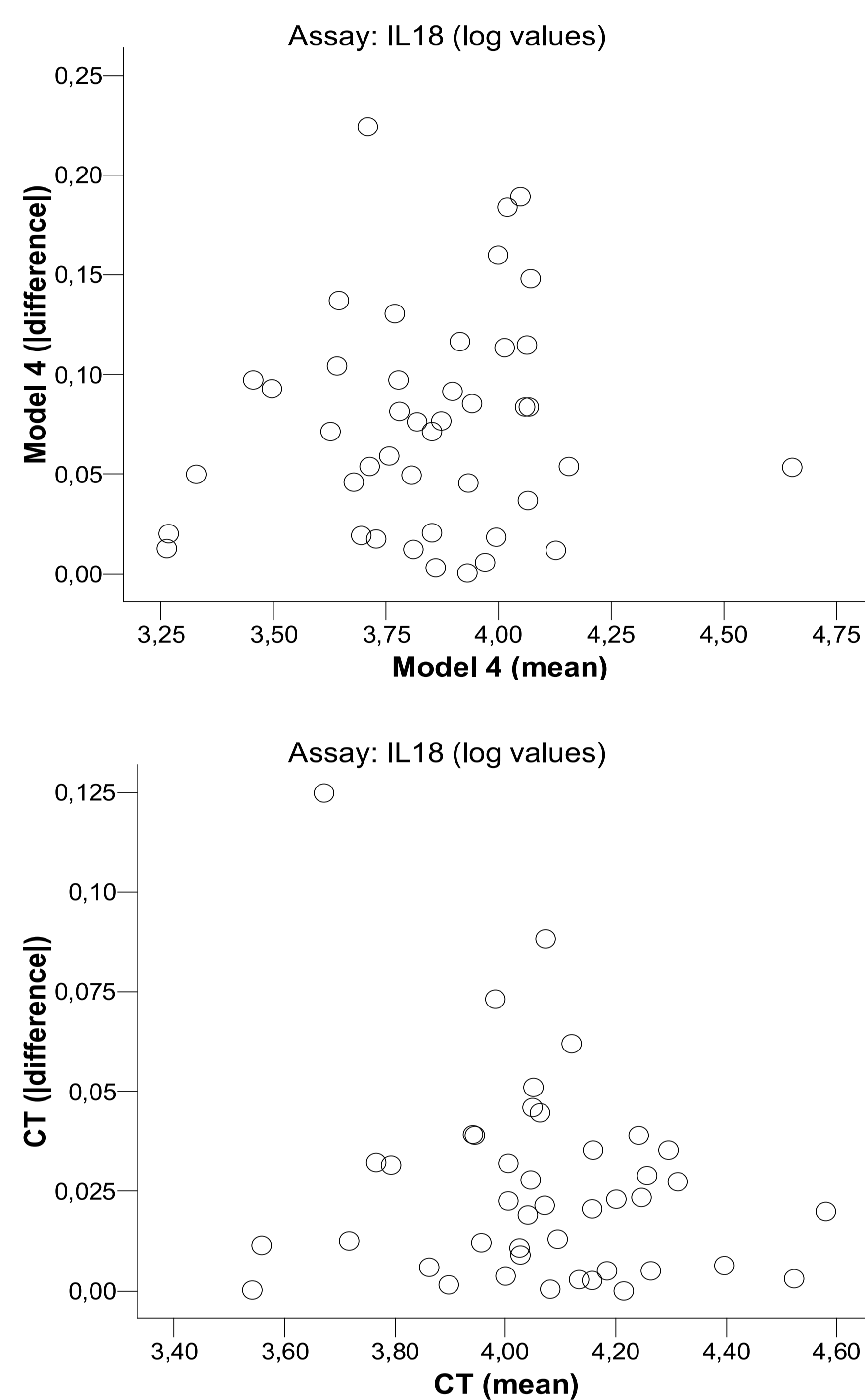
$$F_C = \frac{F_{\max}}{1 + e^{-\left(\frac{C - C_{1/2}}{k}\right)}} + F_b$$

## REFERENCES

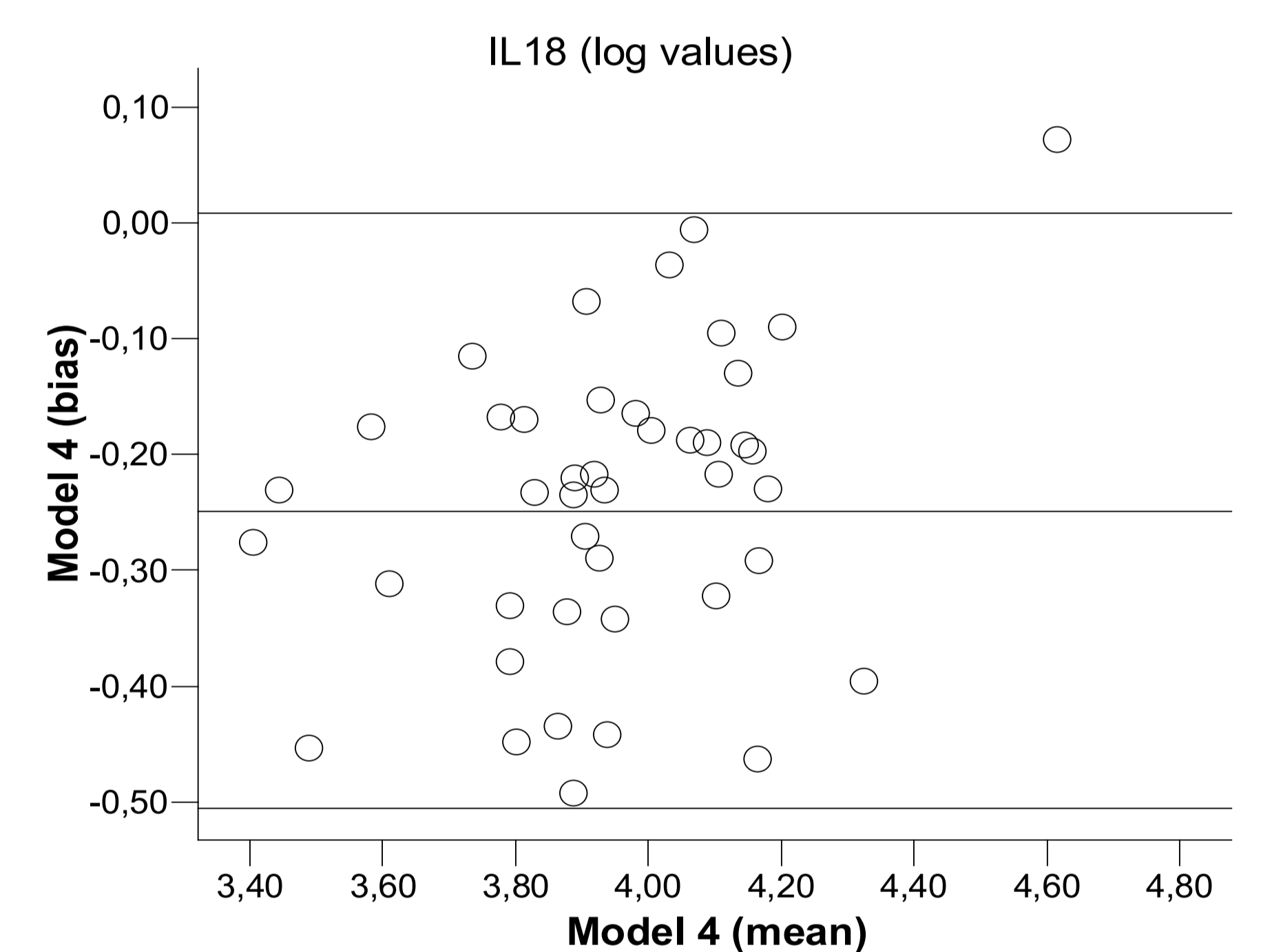
- (1) Rutledge RG. Sigmoidal curve-fitting redefines quantitative real-time PCR with the prospective of developing automated high-throughput applications. *Nucleic Acids Res* 2004; 32(22):e178.
- (2) Liu W, Saint DA. Validation of a quantitative method for real time PCR kinetics. *Biochem Biophys Res Commun* 2002; 294(2):347-353.
- (3) Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 1(8476):307-310.

## RESULTS

An automated algorithm was written in SPSS syntax. A 96 well plate could be analysed in less than 2 minutes. In three models one or more of the 96 regressions turned out with a “bad fit” and these models were excluded from further analysis. The Altman-Bland analysis showed that the best regression model had higher intra-assay variation of 60% vs 24% for the CT method, and a mean bias of 30% when the individual values were compared to the values by CT.



**Figure 1:** Altman-Bland plots showing intraassay variation in duplicate samples. The intra assay variation is independent on mean value. The variation in the NLR (A) data is approximately twice as high as for the CT (B) data.



**Figure 2:** Altman-Bland plot of the bias for the IL18 assay (mean, 95% CI for individual values), NLR compared to CT method. The bias is significantly different from 0.

Regression Model					Results		
	ROX Corr.	Log <sub>10</sub> Transform	Weight	Baseline Drift Corr.	Mean R <sup>2</sup>	Intra-assay Variation	Bias
1	N	N	N	N	0.9987	66%	21%
2	N	N	N	Y	0.9988	97%	24%
3	N	N	<=	Y	0.9982	85%	33%
4	N	Y	N	N	0.9987	60%	30%
5	N	Y	N	Y	0.9991	89%	34%
7	N	Y	=>	Y	0.9985	97%	49%
8	Y	N	<=	N	0.9978	101%	40%
CT	Y					24%	

## CONCLUSIONS

The CT method is more precise for absolute quantification, but further development of the NLR algorithm may decrease intra assay variability. The observed bias is an indication that the calibration of the regression method can be improved. However, the versatility depends on the level of precision required, and in some settings, the increased cost effectiveness of NLR may justify a marginally lower precision. When inhibition is an issue, NLR may be more precise than CT, as PCR efficiency is included in the estimate.