

Introduction

- RT-PCR involves a complex mathematical processing of a large volume of raw numerical data.
- Although RT-PCR is the most precise available method for measurement of gene expression, the coefficients of variation in RT-PCR replicates are still often higher than 10%.
- This requires improving in the noise filtering during mathematical processing.
- Computer simulation predicts the same positions of plateau in different samples (see Figure 1 and Appendix).
- However, in practice the differences in plateau positions are pronounced (even in replicates) and still are not being filtered (see Figure 2).
- Amplitude normalization should therefore be added into current RT-PCR data processing to filter the plateau scattering.

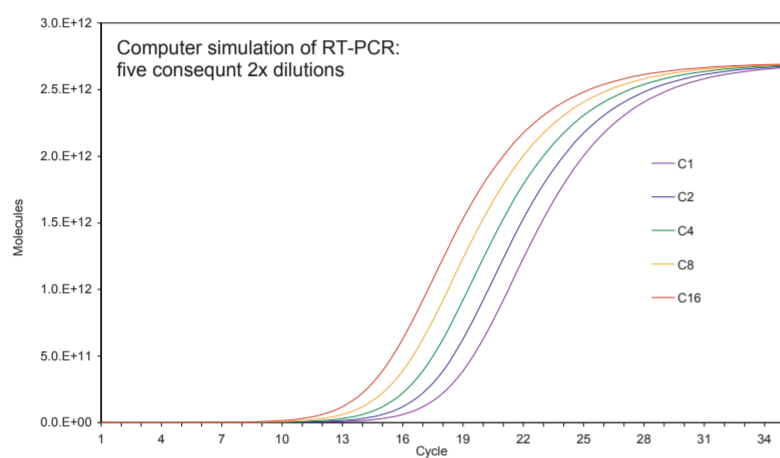


Figure 1: Positions of plateau predicted by computer simulation

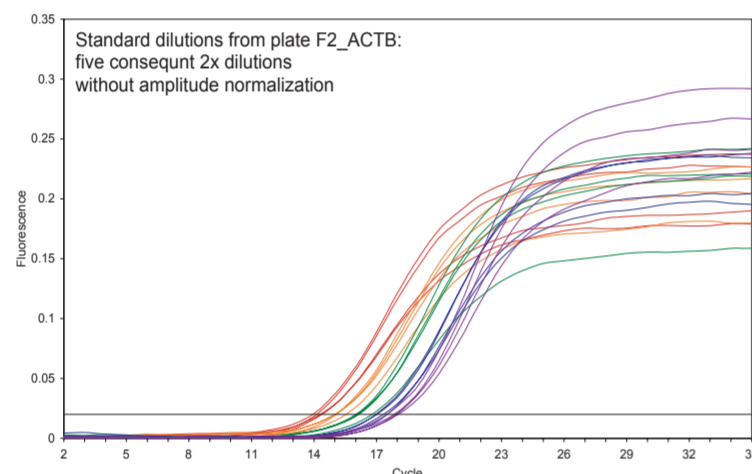


Figure 2: Positions of plateau actually observed in RT-PCR

Aim

- To validate amplitude normalization in RT-PCR data processing

Materials and methods

- Amplitude normalization was tested while measuring expression of 6 genes in 42 biopsies of breast cancer.
- RNeasy Mini Kit (Qiagen), SuperScript RT (Invitrogen) were used for RNA extraction and reverse transcription; QuantiTect SYBR Green PCR kit (Qiagen) and Opticon Monitor PCR machine (MJ Research) were used to run PCR.
- PCR was performed in quadruplicates with standard curve on each plate.
- Raw readings exported by Opticon Monitor software were analyzed with home made application developed using VBA for Excel (Microsoft).
- Division of each reading in the cell by the maximal reading in this cell through the run was used for amplitude normalization.
- The amplitude normalization was applied after smoothing and baselining, before threshold selection and crossing point calculation.

Results

- Table 1 summarizes the effect of amplitude normalization on the parameters of three consequent steps of RT-PCR data processing: average coefficients of variation for crossing points (CP CV), coefficients of determination for standard curves (R2) and average coefficients of variation for final results (Final CV).

Table 1: Amplitude normalization in RT-PCR data processing

Plate	Without amplitude normalization			After amplitude normalization			Ratio No normalization / After normalization	
	CP CV	R2	Final CV	CP CV	R2	Final CV	CP CV	Final CV
F1_1_ACTB	1.10%	0.988	12%	0.37%	0.994	4%	3.0	2.8
F1_1_CB	0.59%	0.984	7%	0.27%	0.999	4%	2.2	1.7
F1_1_CDC	0.58%	0.985	10%	0.30%	0.983	7%	1.9	1.5
F1_1_MAM	0.65%	0.990	10%	0.31%	0.996	6%	2.1	1.7
F1_1_PLE	0.56%	0.991	7%	0.28%	0.995	3%	2.1	2.2
F1_2_ACTB	0.92%	0.995	8%	0.16%	0.998	2%	5.6	4.1
F1_2_CB	0.71%	0.973	9%	0.42%	0.998	6%	1.7	1.4
F1_2_CDC	0.72%	0.965	10%	0.33%	0.957	6%	2.2	1.6
F1_2_MAM	1.26%	0.970	12%	0.17%	0.976	3%	7.5	4.0
F1_2_PLE	0.61%	0.992	7%	0.29%	0.992	4%	2.1	2.0
F1_D_ACTB	1.34%	0.986	20%	0.26%	0.995	5%	5.2	4.0
F1_D_GAPD	0.81%	0.966	16%	0.24%	0.996	5%	3.4	3.3
F2_ACTB*	1.19%	0.965	15%	0.26%	0.999	3%	4.5	4.6
F2_CB	0.83%	0.964	14%	0.21%	0.998	4%	4.0	3.8
F2_CDC	1.05%	0.961	19%	0.50%	0.995	8%	2.1	2.3
F2_GAPD	1.13%	0.971	15%	0.22%	0.998	3%	5.2	4.4
F2_MAM	1.06%	0.935	18%	0.30%	0.997	5%	3.5	3.5
F2_PLE	0.87%	0.925	18%	0.38%	0.994	8%	2.3	2.3
Weighted mean	0.92%	0.973	13%	0.29%	0.992	5%	3.1	2.8

Note: * - details from plate F2_ACTB are shown on figures 2, 3, 5 and 6.

Data extracted from row readings look close to the mathematical prediction

- Compare Figure 1 and Figure 3

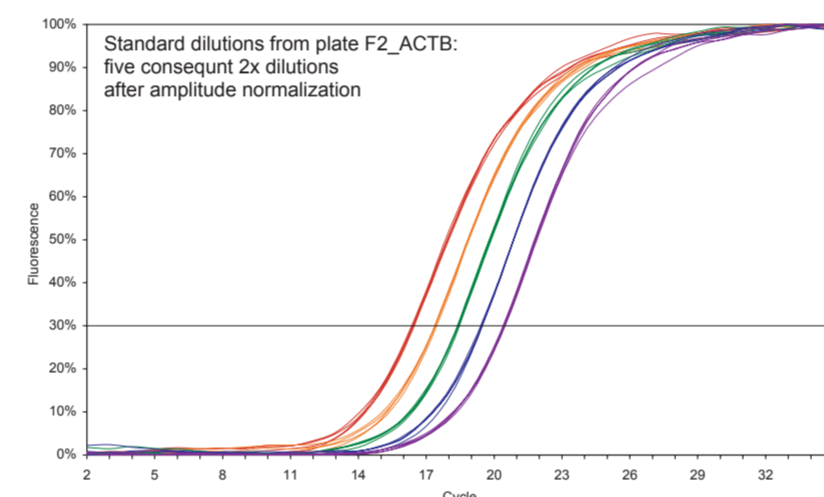


Figure 3: Positions of plateau after amplitude normalization

Dispersion in crossing points goes down

- Average coefficient of variation for crossing points decreased by 3.1 times: from 0.92% to 0.29% (see Table 1).
- Figure 4 summarises effect of amplitude normalization on crossing points distribution around mean in 1312 quadruplicates from the present study.

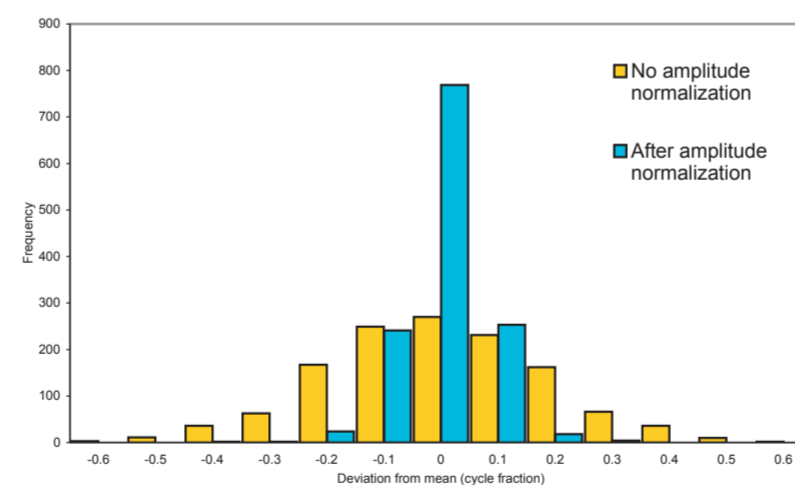


Figure 4: Effect of amplitude normalization on distribution of crossing points around mean

Better fit in standard curves

- After amplitude normalization average coefficient of determination (R²) increased from 0.973 to 0.992 (see Table 1 and Figure 5, standard curves with maximal R² were chosen automatically).

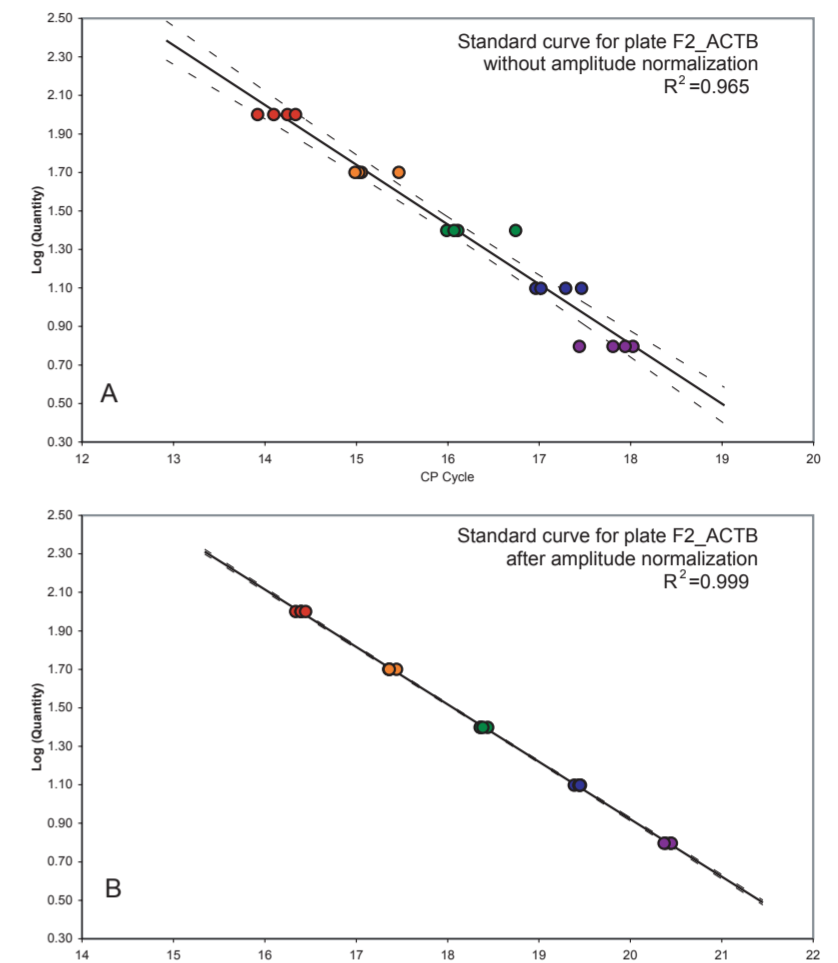
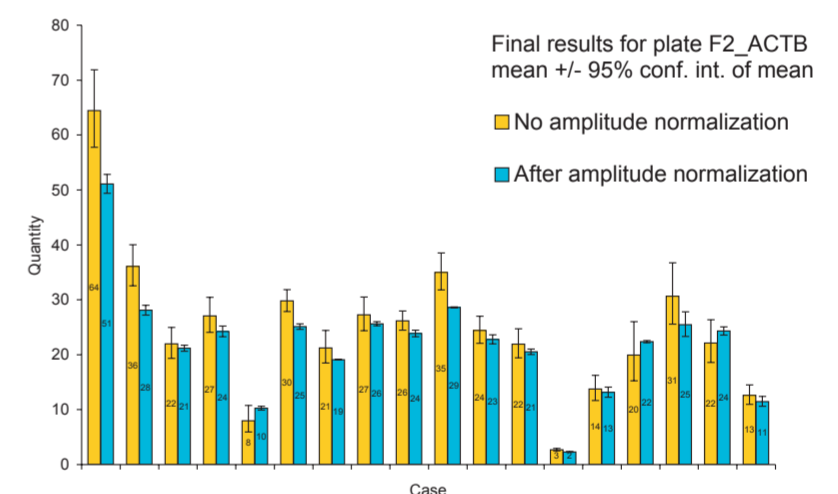


Figure 5: Effect of amplitude normalization on standard curves

Less dispersion in final results

- With amplitude normalization the mean coefficient of variation propagated to final results was decreased by 2.8 times: from 13% to 5% (see Table 1).
- However, amplitude normalization usually did not affect significantly the mean values (see Figure 6).



Possible limitations

- Amplitude normalization can be used only when all samples achieved undoubted plateau.
- The amplitude normalization improved parameters only when true replicates were used: mixing all components before dispensing samples to the plate. When "replicas" were made by adding cDNA separately from other pre-mixed components the dispersion was usually higher and amplitude normalization did not improve the results significantly.

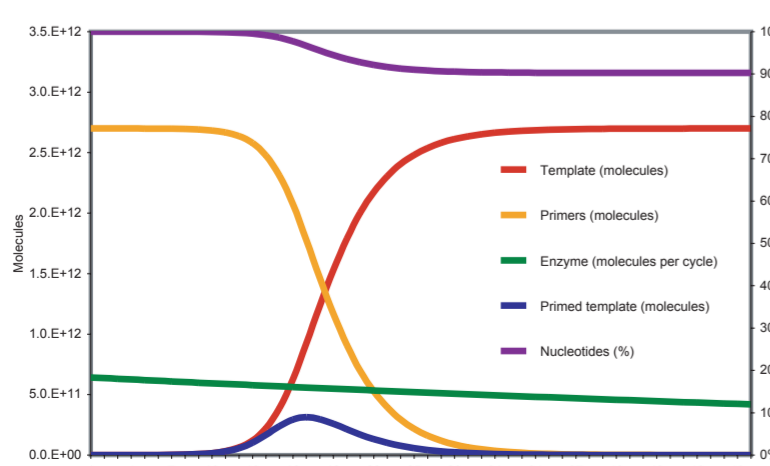
Conclusions

- Amplitude normalization is an effective mathematical procedure to filter non-specific noise during RT-PCR data processing.
- Amplitude normalization decreased coefficient of variation in crossing points as well in final results by about 3 times.
- Amplitude normalization may be used to improve precision of RT-PCR providing correct procedures were used during RT-PCR set-up.

Appendix: used computer simulation of RT-PCR

Main formulas used for simulation:

$$\begin{aligned} \text{Enzyme after cycle} &= \text{Enzyme before cycle} - \text{Enzyme degraded during cycle} \\ \text{dNTP after cycle} &= \text{dNTP before cycle} - \text{dNTP consumed during cycle} \\ \text{Primers after cycle} &= \text{Primers before cycle} - \text{Primers consumed during cycle} \\ \text{Product after cycle} &= \text{Product before cycle} + \text{Product formed during cycle} \\ \text{Product formed during cycle} &= \text{Min (Primed template, Limits from enzyme or primers or dNTP)} \\ \text{Primed template} &= a \times \text{Template} \times \frac{\text{Primers}}{\text{Primers} + b \times \text{Template}} \end{aligned}$$



Kinetics of main PCR components predicted by simple computer simulation

PCR simulation was performed using step by step calculation in MS Excel. Main formulas are presented at the left. Nucleotides and primers consumed during the cycle were calculated out of product formed during the cycle. Figure illustrates kinetics of main PCR components predicted by the simulation for typical starting conditions. The term for primed template is based on the simple combinatory sense: during annealing for each molecule of template the number of combinations leading to primed template is proportional to the concentration of primers; at the same time the total number of all possible annealing combinations is proportional to the summed concentration of primers and templates. Coefficient *b* reflects the fact that template-template pairs are more stable than template-primer pairs (because of their length). Coefficient *a* may account for the proportion of template engaged into any pair formation. In general form the amount of primed template can also be estimated from the system of the Michael-Mentis equations for primer-template and template-template equilibriums. However, this is not a trivial task. So for the simple model the simplified term was used.