Estimation of Power and Analysis of qPCR Data with Normal Mixed Models

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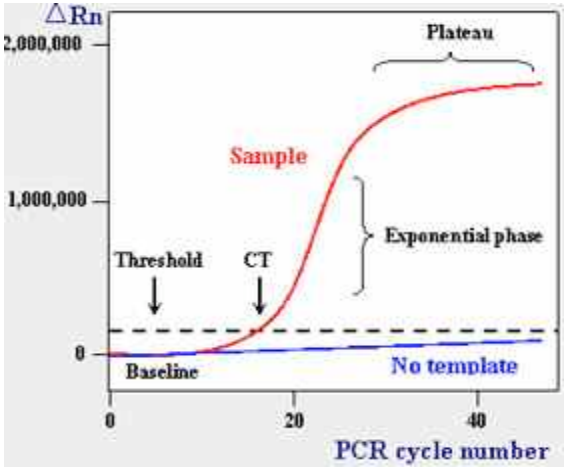
qPCR

- Is used to quantify DNA or messenger RNA (mRNA) in a sample
- When combined with reverse transcriptase (RT-PCR), relative gene expressions between tissues or genes can be compared
- Measure fluorescence reporter



http://www.rt-pcr.com/

http://www.gene-quantification.info/

Standardizing measurements

- Significant problems caused by
  - variability of RNA templates, assay designs and protocols
  - various data normalization
  - data analysis strategies
- Which are tried to control by
  - consistently using standard chemistries, protocols and reaction conditions
  - pipetting robot
  - repeated measurements (technical repeats)
  - all measurements (to be compared) at the same time
  - controlling efficiency in each run

Aim of study and Study design

- **Aim of study is to evaluate relative expression ratio between genes and treatments**
- **18 animal tissue samples in 4 groups**
  1. control (n=4)
  2. disease model (n=5)
  3. disease model with study drug treatment (n=4)
  4. disease model with reference drug treatment (n=5)
- Expression levels of **3 target genes** and **12** potential **reference genes** were analyzed by quantitative RT-PCR in **3 replicates** for each sample.

Study design

	Original data		Selected data		**Analysis data**
Samples	18		8		**8**
Treatments	4		2		**2**
Genes	3 targets 12 references	X 3 technical repeats	1 target 3 references	X 3 technical repeats	**1 target** **Mean* over references**	**Mean* over technical repeats**
N	810		96			**16**

*) Arithmetic mean

Analysis Data

Treatment	Gene	Sample	Ct	Subgroup
Control	Target A	28	35.6	1
		32	38.1	1
		33	37.3	1
		34	35.7	1
Control	Reference	28	24.7	2
		32	28.3	2
		33	26.5	2
		34	25.1	2
Study drug treatment	Target A	66	37.1	3
		69	36.3	3
		70	36.6	3
		71	37.1	3
Study drug treatment	Reference	66	24.5	4
		69	24.9	4
		70	23.8	4
		71	25.4	4

Definitions

- For reference gene: mean $^{r}_{control}$ and mean $^{r}_{treatment}$
- ΔCt_{ref} = mean $^{r}_{control}$ – mean $^{r}_{treatment}$
- For target gene: mean $^{t}_{control}$ and mean $^{t}_{treatment}$
- ΔCt_{target} = mean $^{t}_{control}$ – mean $^{t}_{treatment}$
- $\Delta\Delta Ct = \Delta Ct_{ref} - \Delta Ct_{target}$
- Expression ratio = $\frac{(E_{target})^{\Delta Ct_{target}}}{(E_{ref})^{\Delta Ct_{ref}}} = 2^{-\Delta\Delta Ct\_adjusted}$

PAE and adjusted ΔΔCt

- qPCR data analysis is based on the assumption that PCR products double each cycle (AE=2).
- When the AE (Amplification Efficiency) is not 2, Ct -values are recommended to be adjusted.
- We used percentile AE (PAE) instead of AE

$$AE = 2^{PAE}$$

$$PAE = \log_2(AE)$$

- $\Delta\Delta Ct_{adjusted} = PAE_{ref} * \Delta Ct_{ref} - PAE_{target} * \Delta Ct_{target}$
- Efficiency can be estimated for a group of reactions or a single reaction by simple regression model.

Normal Mixed Model with Gene as a repeated factor

- **Effects**
  - gene (target, reference)
  - treatment (study drug, control)
  - gene by treatment interaction
  - sample (sample number)
  - residual

- **Mixed Model in SAS**

```
PROC MIXED; CLASS gene treatment sample;
MODEL   Ct   =   gene   treatment   gene*treatment;
REPEATED  gene   /   SUBJECT  =  sample   TYPE = UN;
```

Estimation of ΔΔCt based on Mixed Model parametrization (A) and (B) ?

Mixed Model in SAS (parametrization A)

```
PROC MIXED; CLASS gene treatment sample;
MODEL   Ct   =   gene   treatment   gene*treatment;
REPEATED   gene   /  SUBJECT  =  sample   TYPE = UN;
```

ΔΔCt: `ESTIMATE  gene*treatment  +1  -1  -1  +1;`

Mixed Model in SAS (parametrization B)

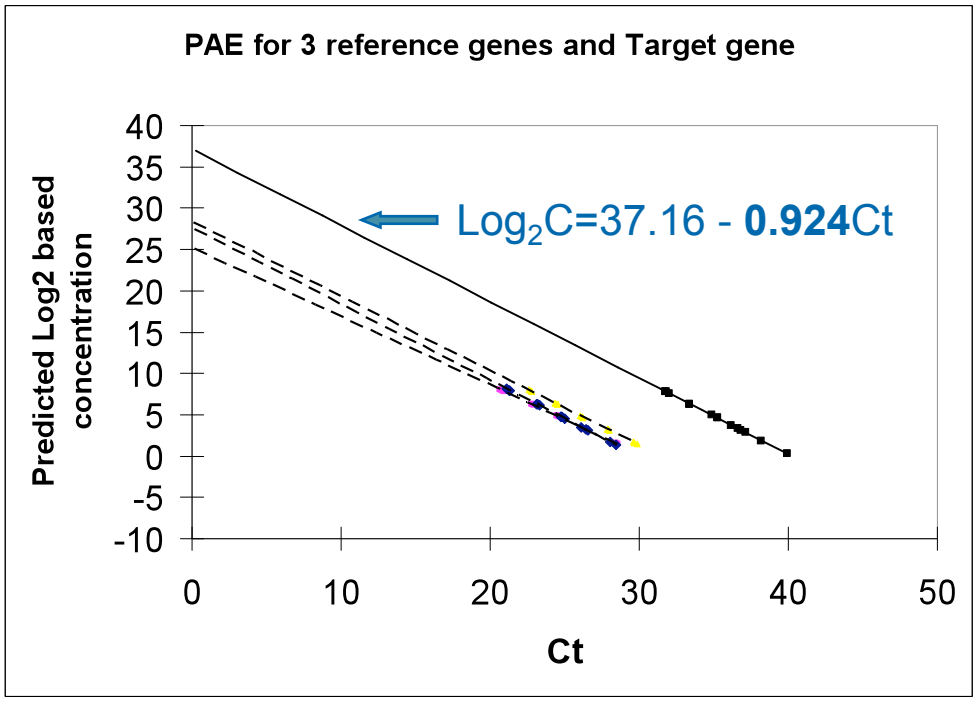
```
PROC MIXED; CLASS subgroup sample;
MODEL   Ct   =   subgroup;
REPEATED   /   SUBJECT  =  sample   TYPE = UN;
```

$\Delta\Delta Ct_{adjusted}$: `ESTIMATE  subgroup '+`$PAE_{target}$ `-`PAE_{target} `-`PAE_{ref} `+`PAE_{ref}`';`

- (subgroup is a categorical variable with 4 classes)

Estimation of PAE

PAE estimates are based on the data of 5 different dilutions (and 3 technical repeats per dilution) over the pooled samples treated by 4 treatments.



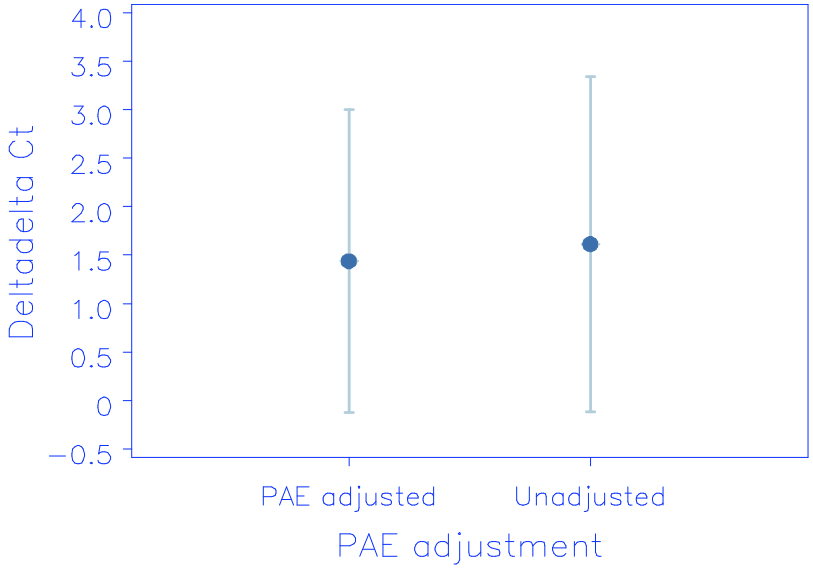
Gene	PAE	
Reference 1	0.831	Mean PAE of reference genes: 0.888
Reference 2	0.929	
Reference 3	0.903	
Target A	**0.924**	

Table with descriptive ΔΔCt and model estimates

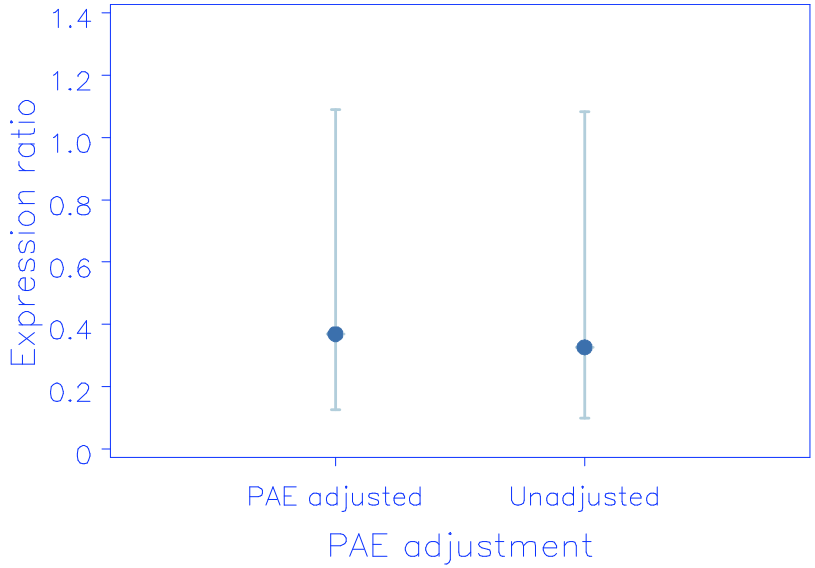
	ΔΔCt(SE)	
	Descriptive statistics	**Normal mixed model**
PAE Adjusted	1.437 (0.708)	1.439 (0.729)
Unadjusted	1.613 (0.786)	1.614 (0.806)

ΔΔCt and expression ratio with 95% CIs

ΔΔCts



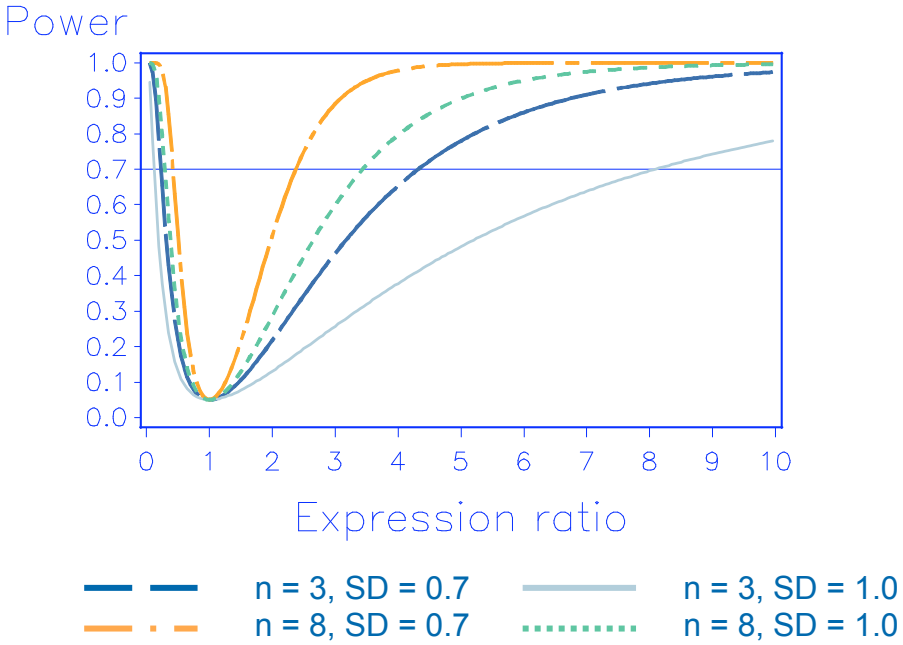
Expression ratios



Expression ratio = $2^{-\Delta\Delta Ct}$

Power

Power curves for the expression ratio between 0 and 10 when type I error is 0.05



Power calculation is based on

- noncentral t-distribution and the log2 transformed expression ratio (ΔΔCt)
- variance was estimated as the sum of four equal variances
- degrees of freedom was estimated as a sum of n subtracted by a number of groups

Conclusion

- Importance of power calculation in study planning phase.
- Normal mixed model works well with qPCR data and enables the dependence between genes.
- With balanced, complete data these two methods give similar results.
- Mixed model gives more accurate estimates with unbalanced data.
- Data transfer from instrument to analysis software is challenging.

References

- [1] Brown, H. and Prescott, R. (2006): Applied mixed models in medicine.
- [2] Yuan, J., Reed, A., Chen, F. and Stewart, C. N.: Statistical analysis of real-time PCR data. BMC Bioinformatics 2006, 7:85.
- [3] Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D. and Schabenberger O. (2006): SAS® for Mixed Models, Second Edition. Cary, NC: SAS Institute Inc.
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Thank you!

Questions?