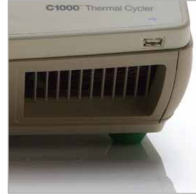
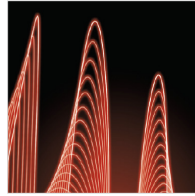


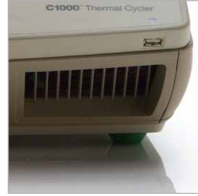
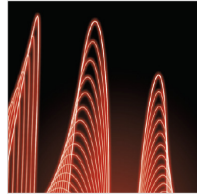
Relative Quantification: Data Management & Analysis Settings

Ray Meng, Ph.D.
International Field Applications Specialist
Gene Expression Division
Bio-Rad Laboratories



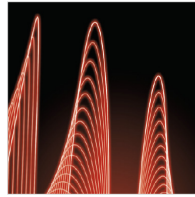
Gene Expression

- Assay design is the key to success
- Design primers to bind to different exons or if possible over exon/exon junctions
 - Promotes amplification of cDNA over contaminating genomic DNA
- Avoid Pseudo genes
- Treat samples when possible using RNase free DNase
- Design primers for target and reference to have similar properties
- Use the right reference gene(s)



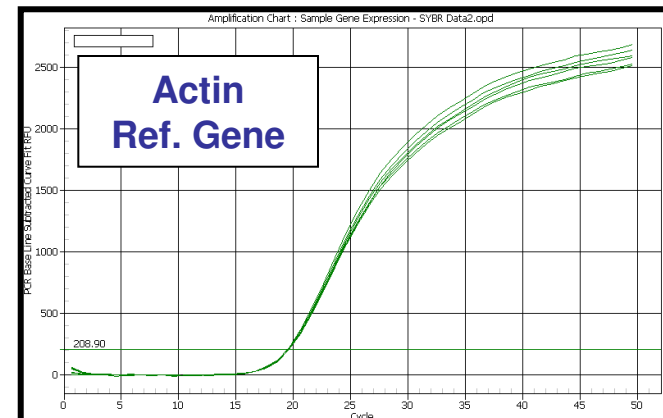
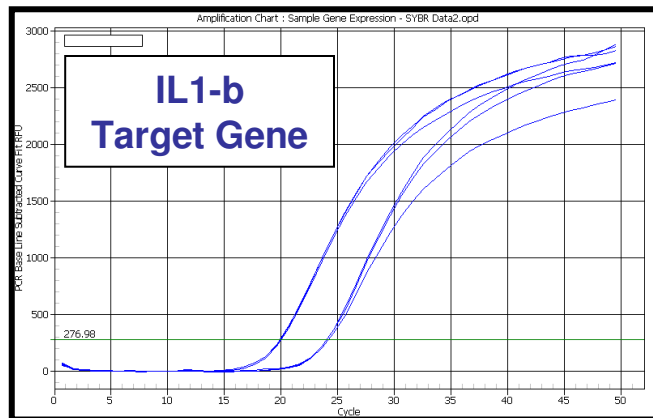
Reference Genes

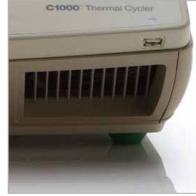
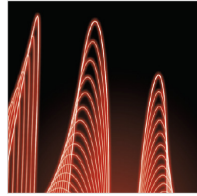
- The correct selection of the reference gene for your quantitative real-time PCR reactions is essential.
- Due to the complexity of biological systems, no single reference gene will serve the purpose of normalization for all assays.
- The reference gene selected must have consistent expression in the experimental system being studied.



Reference Genes

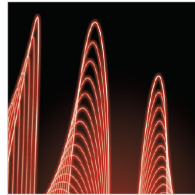
- Test and validate reference genes in your system
- Examples : β -Actin, GAPDH, 18s rRNA, cyclophilins





Reference Gene Validation

- “Normalize” input sample by some means and test expression consistency
 - Input RNA concentration
 - Cell number
 - Tissue weight
 - Input cDNA
- CT or calculated concentration of reference message should remain the same

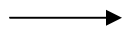
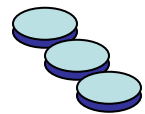


Reference Gene Validation

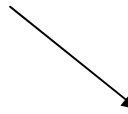
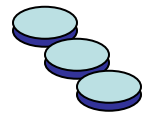
All cells plated **same time** from **same suspension**

 = Culture Dish

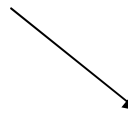
Control



Count Cells

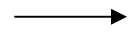
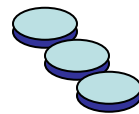


Quantitate RNA

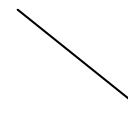
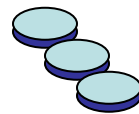


RT-qPCR

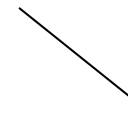
Experimental



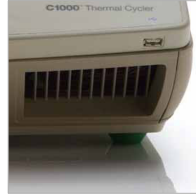
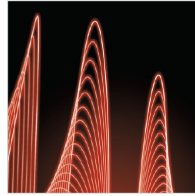
Count Cells



Quantitate RNA



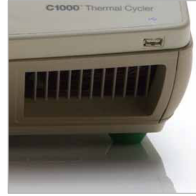
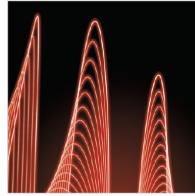
RT-qPCR



Reference Gene Validation

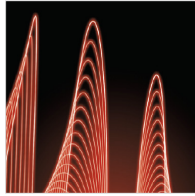
	Cell Number	ng RNA	CT
Control	1X 10e8	200ng	13
Experimental	1 X10e8	200ng	13

	Cell Number	ng RNA	CT
Control	1X 10e8	200ng	13
Experimental	1 X10e8	200ng	10



Comparative C_T

- Relative Quantity (ΔC_T)
 - Not normalized
 - Normalization accomplished via equal loading of samples
 - Post analysis normalization
- Normalized Expression ($\Delta\Delta C_T$)
 - Accounts for loading differences
 - Usually normalize to reference gene
 - Relative quantity of GOI is normalized by the relative quantity of the reference genes



Normalized Expression ($\Delta\Delta CT$)

$\Delta\Delta CT$

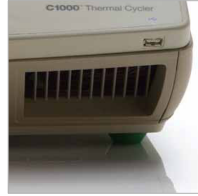
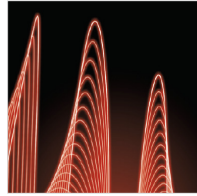
- Assume 100% efficiency
- Only one Ref Gene
- Pfaffl Modification
 - Accounts for efficiency differences
 - Only one Ref Gene
- Vandesompele Method
 - Accounts for efficiency differences
 - Allows multiple reference genes for normalization

Simple



Complex

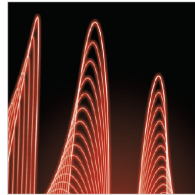
AMPLIFICATION



Relative Quantification - ΔCt

	GOI
Tissue #1:	22
Tissue #2:	24
<hr/>	
Delta Ct:	$24 - 22 = 2$

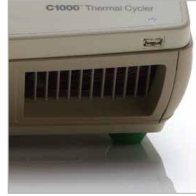
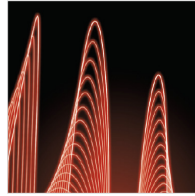
$$\text{Fold induction} = 2^2 = 4$$



Comparative Ct Method ($2^{-\Delta\Delta Ct}$)

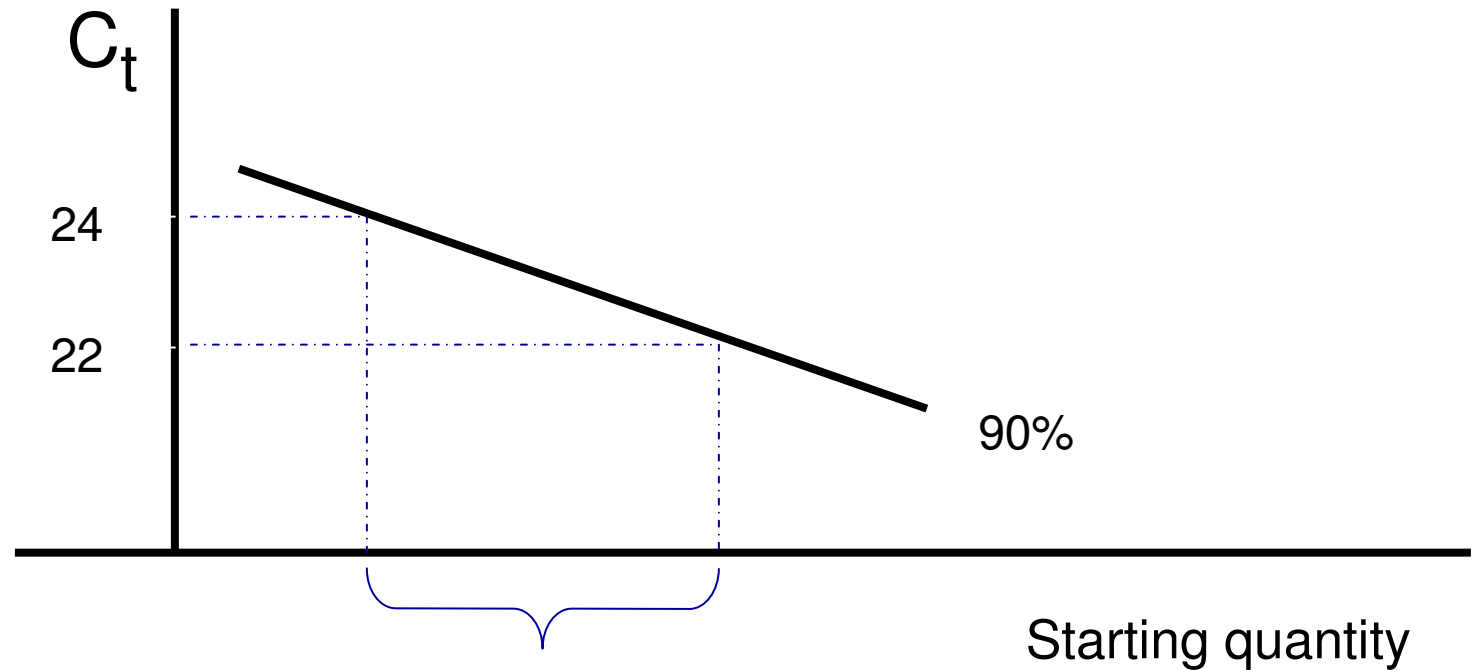
	Reference	GOI
Tissue #1:	21	22
Tissue #2:	20	24
<hr/>		
1 st Delta	Delta Ct #1:	22-21 = 1
	Delta Ct #2:	24-20 = 4
<hr/>		
2 nd Delta	Delta Ct:	4-1 = 3

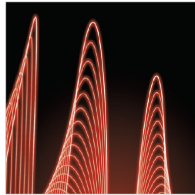
$$\text{Fold induction} = 2^3 = 8$$



Relative Quantification

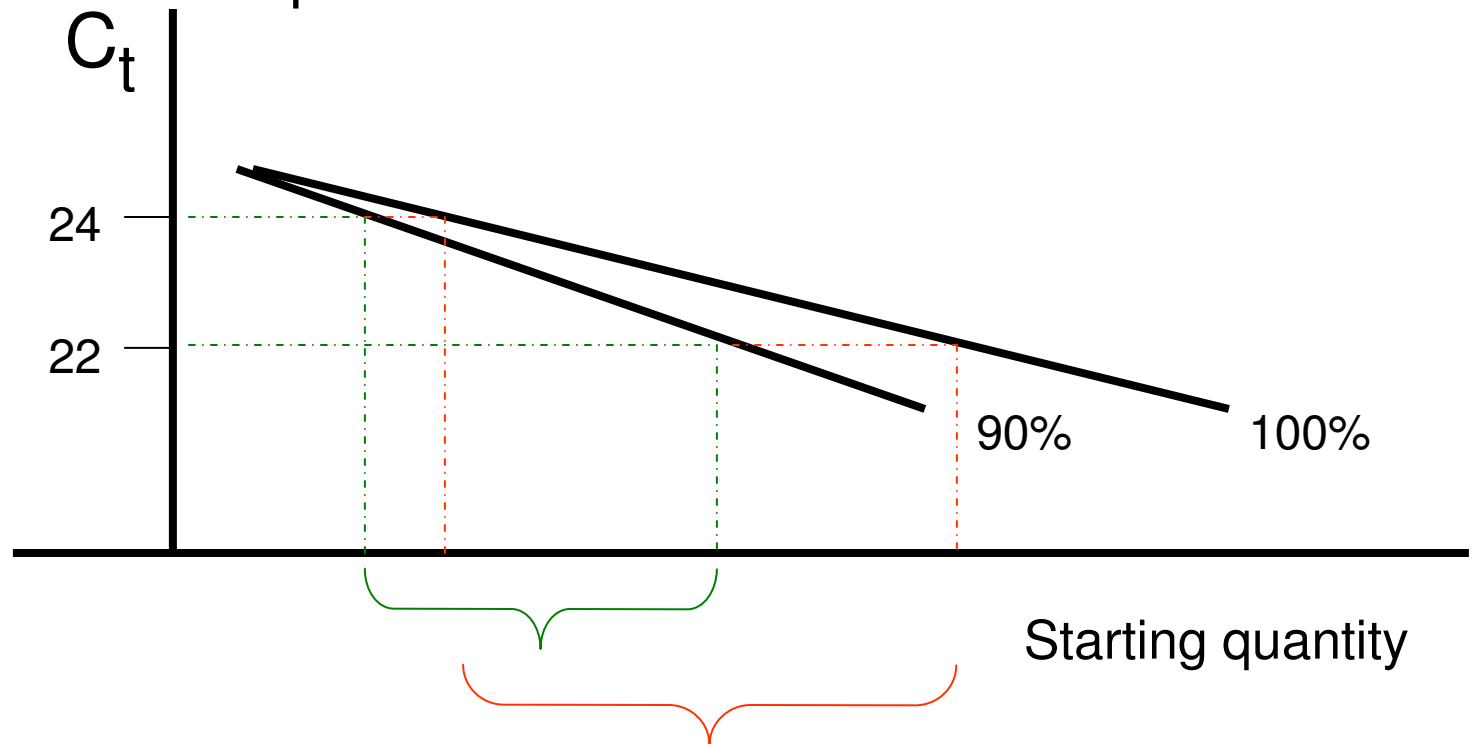
❖ Problem with the $\Delta\Delta CT$

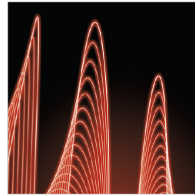




Relative Quantification

- ❖ Problem with the $\Delta\Delta CT$
- ❖ Slopes are not parallel



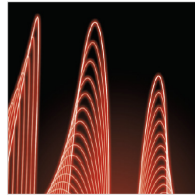


Relative Quantification

$$\text{Fold induction} = \frac{\text{Efficiency}_{\text{target}}^{\text{deltaCt}_{\text{target}} \text{ (control-sample)}}}{\text{Efficiency}_{\text{reference}}^{\text{deltaCt}_{\text{reference}} \text{ (control-sample)}}$$

(Pfaffl, 2001; Nucleic Acid Research)

$$\text{Efficiency} = 10^{-1/\text{slope}}$$



Relative Quantification – Pfaffl Modification

Primer set #1 Reference

Primer set #2 GOI

Tissue #1:

21

22

Tissue #2:

20

24

(From Standard curve)

Efficiency:

90% = 1.9

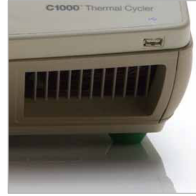
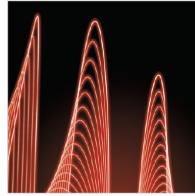
100% = 2

Delta Ct:

20-21 = -1

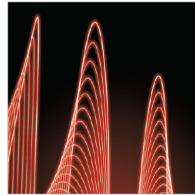
24-22 = 2

$$\text{Fold induction} = \frac{2^{\text{deltaCt}_{\text{target}} (24-22 = 2)}}{1.9^{\text{reference}}_{\text{deltaCt}_{\text{reference}} (20-21 = -1)}} = \frac{4}{0.53} = 7.5$$



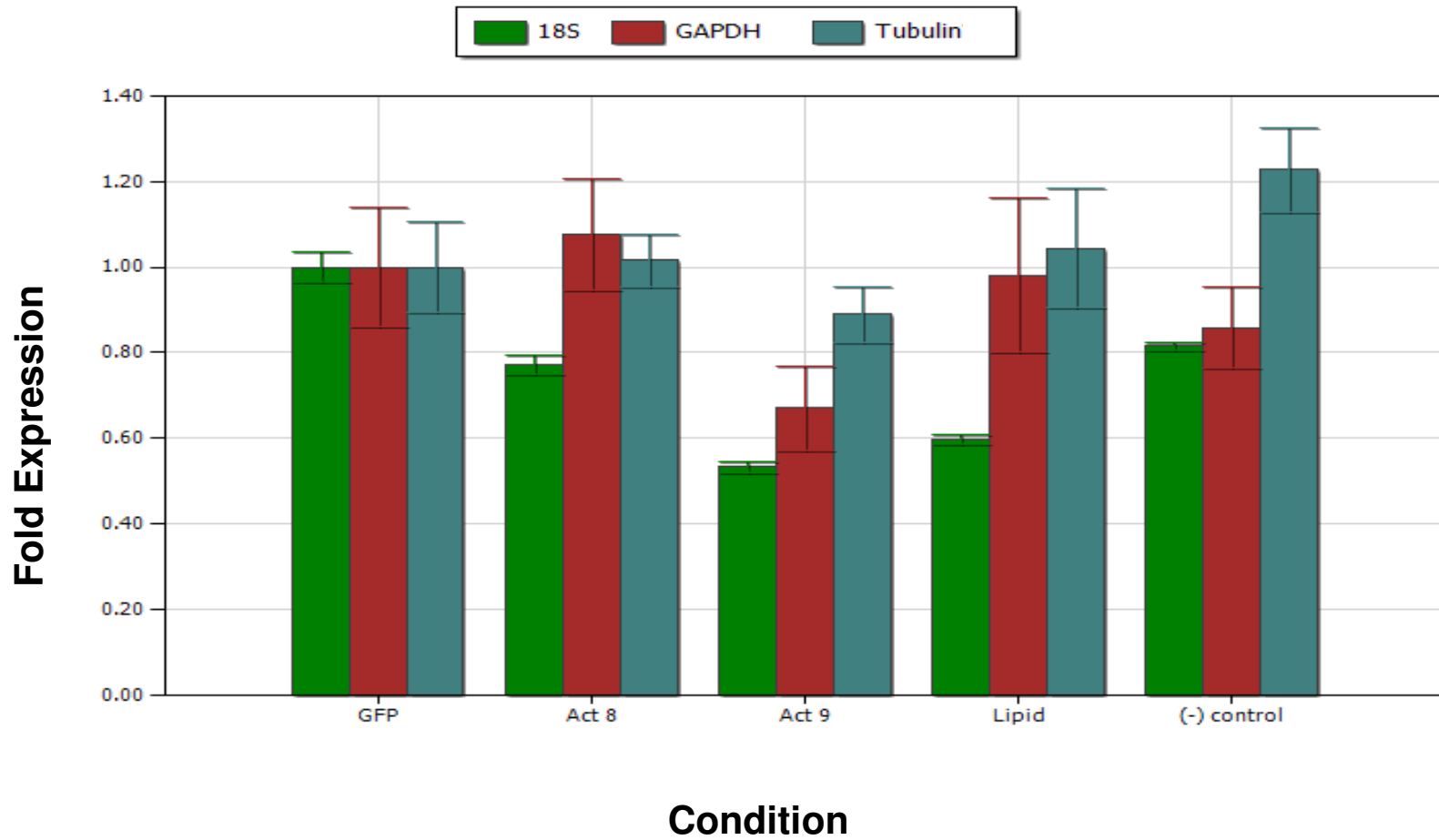
Methods Comparison

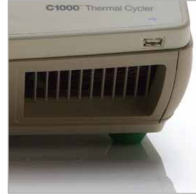
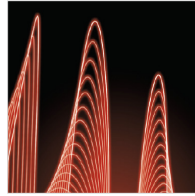
- ΔC_t method: (no reference gene)
 - Fold induction : 4
- $\Delta\Delta C_t$ method: (reference gene)
 - Fold induction : 8
- Pfaffl modification: (reference gene and efficiency)
 - Fold induction : 7.5



Multiplex RT-qPCR Results

Different reference genes

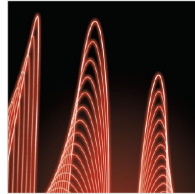




Multiple Reference Genes

- Using a single reference gene leads to erroneous normalization, up to 3.0-fold and 6.4-fold in 25% and 10% of the cases, respectively, with sporadic values above 20-fold
- Using multiple reference genes is a good idea to obtain more accurate results
 - <http://medgen.ugent.be/~jvdesomp/genorm/>
 - Method uses geometric mean of multiple reference genes to normalize
 - Fold-expression standard deviation derived from standard deviation of Ct values

AMPLIFICATION



geNorm

geNorm normalization of real-time PCR expression data

File Edit View History Bookmarks Window Help

http://medgen.ugent.be/~jvdesomp/genorm/ Genorm

Yahoo! Bio-Rad DNA tools Fun finance Reference News (4223) Popular scarlet

geNorm

[introduction] [download] [geNorm detection kits] [geNorm citations] [discussion] [feedback] [reference genes] [RTPrimerDB] [qBase]

[introduction]

geNorm is a collection of VBA macros for Microsoft Excel to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel. From this, a gene expression normalization factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes.

The underlying principles and formulas are described in Vandesompele et al., Genome Biology, 2002, 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes'. The full article can be read at <http://genomebiology.com/2002/3/7/research/0034/> [n° 5 in ranking of all time most-viewed articles published by BioMed Central]


[download] [7267 geNorm downloads in 101 countries]

geNorm is freely available for non-commercial, academic research to be conducted at a non-profit institution. Rights to use the software outside the [license agreement](#) (e.g. commercial use) can be obtained through [PrimerDesign Ltd.](#)

- the [geNorm](#) VBA applet for Microsoft Excel (Windows version, v3.5)
- a beta version of [geNorm for Mac](#) (thanks to Bastian Peter, Switzerland)
- [geNorm user manual](#)
- an [Excel file](#) containing example calculations (normalization and error propagation)

[geNorm detection kits]

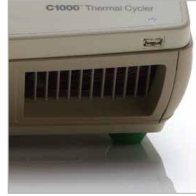
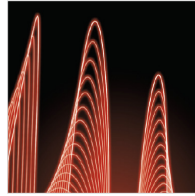
geNorm based detection kits are available commercially from [PrimerDesign Ltd.](#) Currently available are primer sets to detect a wide variety of Homo, Mus, Rattus, Caenorhabditis, Xenopus, Arabidopsis and Ovis normalising genes. For commercial entities, kits purchased from PrimerDesign Ltd. come with a limited licence to use the geNorm software in conjunction with the kit.



[geNorm citations]

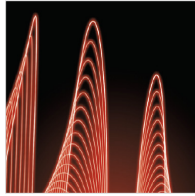
- How to choose best reference gene (s)
- How to do the calculations
- Frequently used ref genes are listed
- Linked to [RTPrimerDB](#)
- You can submit your favorite ref gene(s)

AMPLIFICATION



Switch to CFX software and demonstrate gene expression utilities and Gene Study functionality.

AMPLIFICATION



- Thank You!

- Questions?