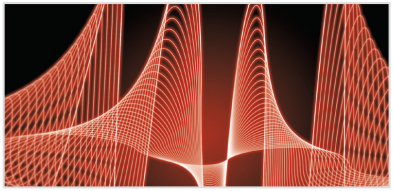
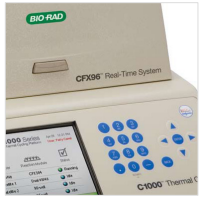
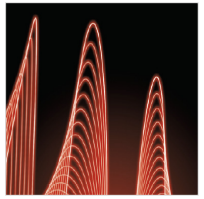


Relative Quantification: Data Management & Analysis Settings

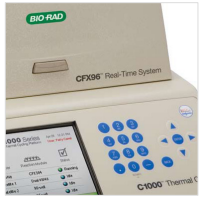
Ray Meng, Ph.D.
International Field Applications Specialist
Gene Expression Division
Bio-Rad Laboratories

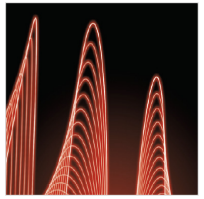




Gene Expression

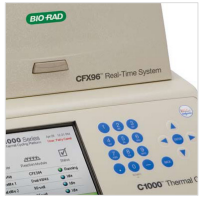
- Assay design is the key to success
- Design primers to bind to different exons or if possible over exon/exon junctions
  - Promotes amplification of cDNA over contaminating genomic DNA
- Avoid Pseudo genes
- Treat samples when possible using RNase free DNase
- Design primers for target and reference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

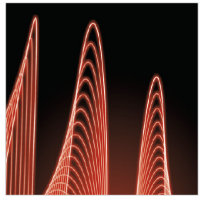




Reference Genes

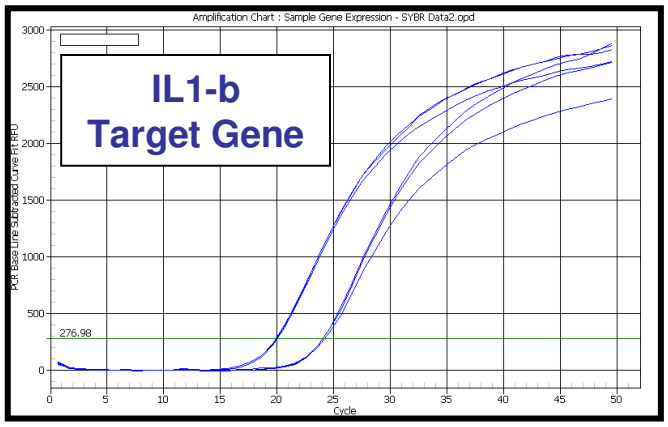
- The correct selection of the reference gene for your quantitative real-time PCR reactions is essential.
- Due to the complexity of biological systems, no single reference gene will serve the purpose of normalization for all assays.
- The reference gene selected must have consistent expression in the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

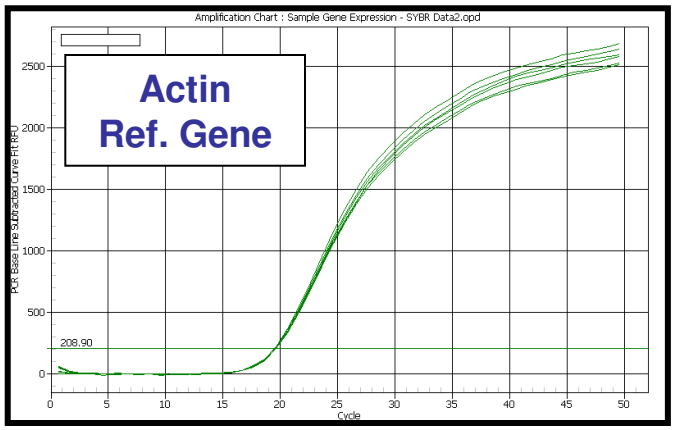


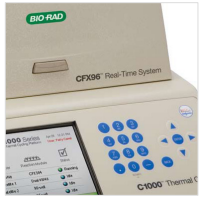


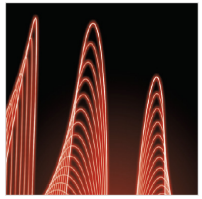
Reference Genes

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



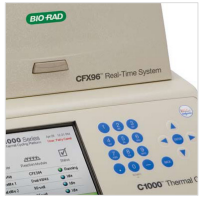


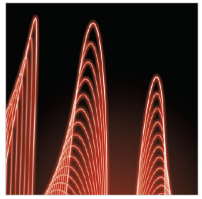




Reference Gene Validation

- "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

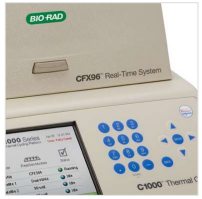
RT-qPCR

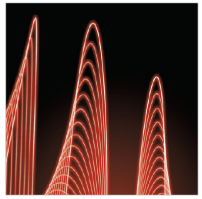
Experimental

Count Cells

Quantitate RNA

RT-qPCR

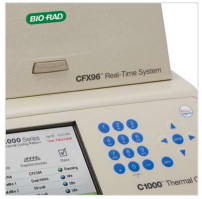


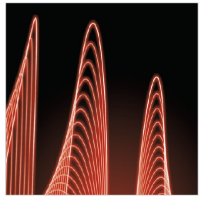


Reference Gene Validation

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

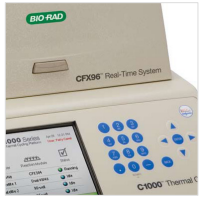
	Cell Number	ng RNA	CT
Control	1X 10e8	200ng	13
Experimental	1 X10e8	200ng	10

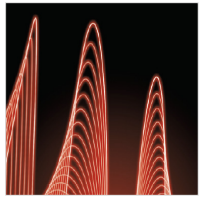




Comparative C_T

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





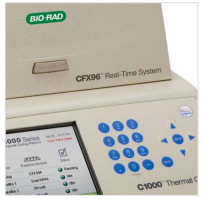
Normalized Expression (ΔΔCT)

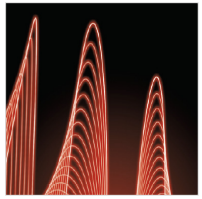
ΔΔCT
- Assume 100% efficiency
- Only one Ref Gene

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

Simple

Complex

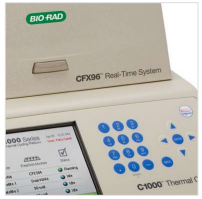


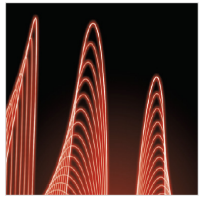


Relative Quantification - ΔCt

	GOI
Tissue #1:	22
Tissue #2:	24
Delta Ct:	24-22 = 2

Fold induction = 2^2 = 4

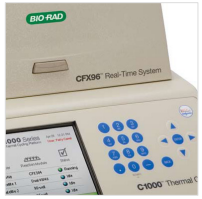


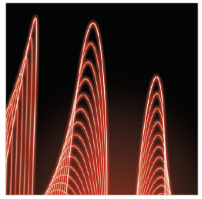


Comparative Ct Method ($2^{-\Delta\Delta Ct}$)

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

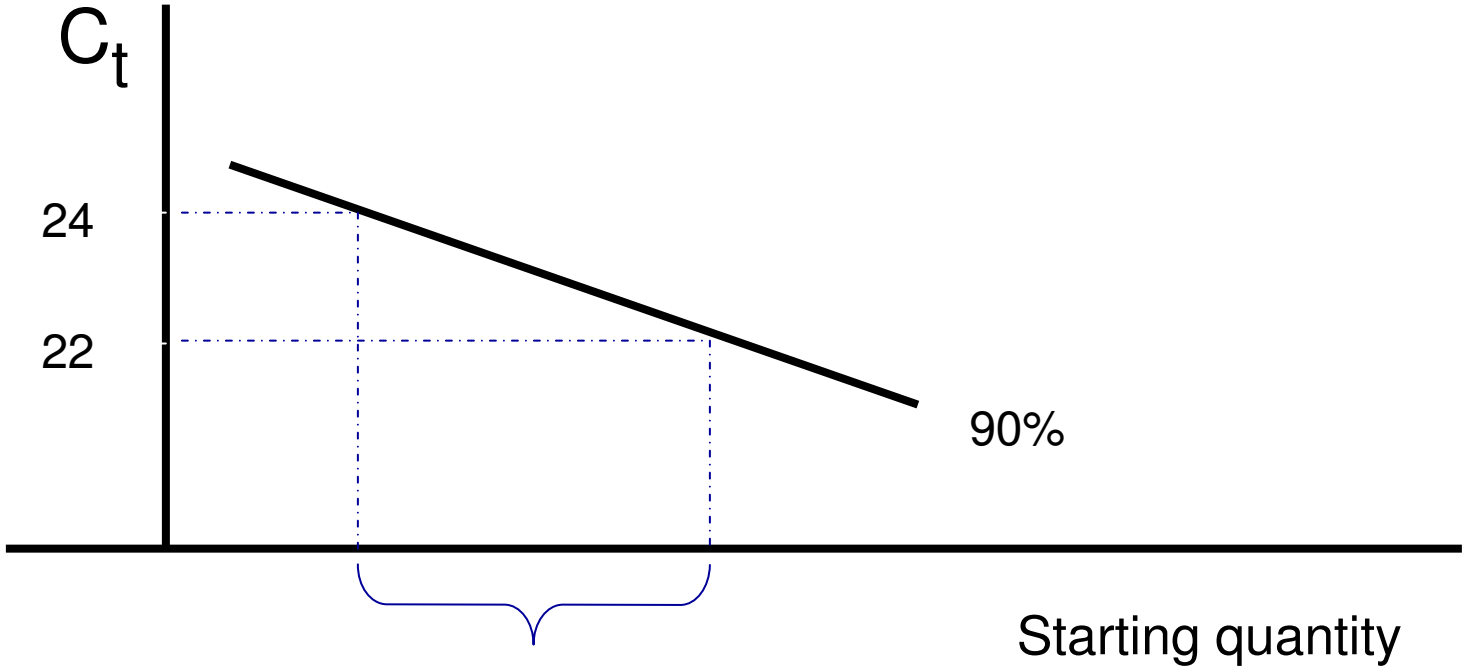
Fold induction = 2^3 = 8

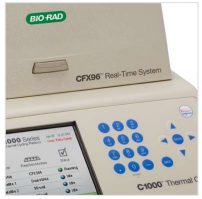


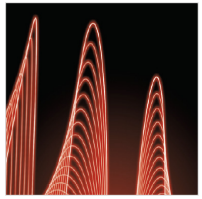


Relative Quantification

- Problem with the ΔΔCT

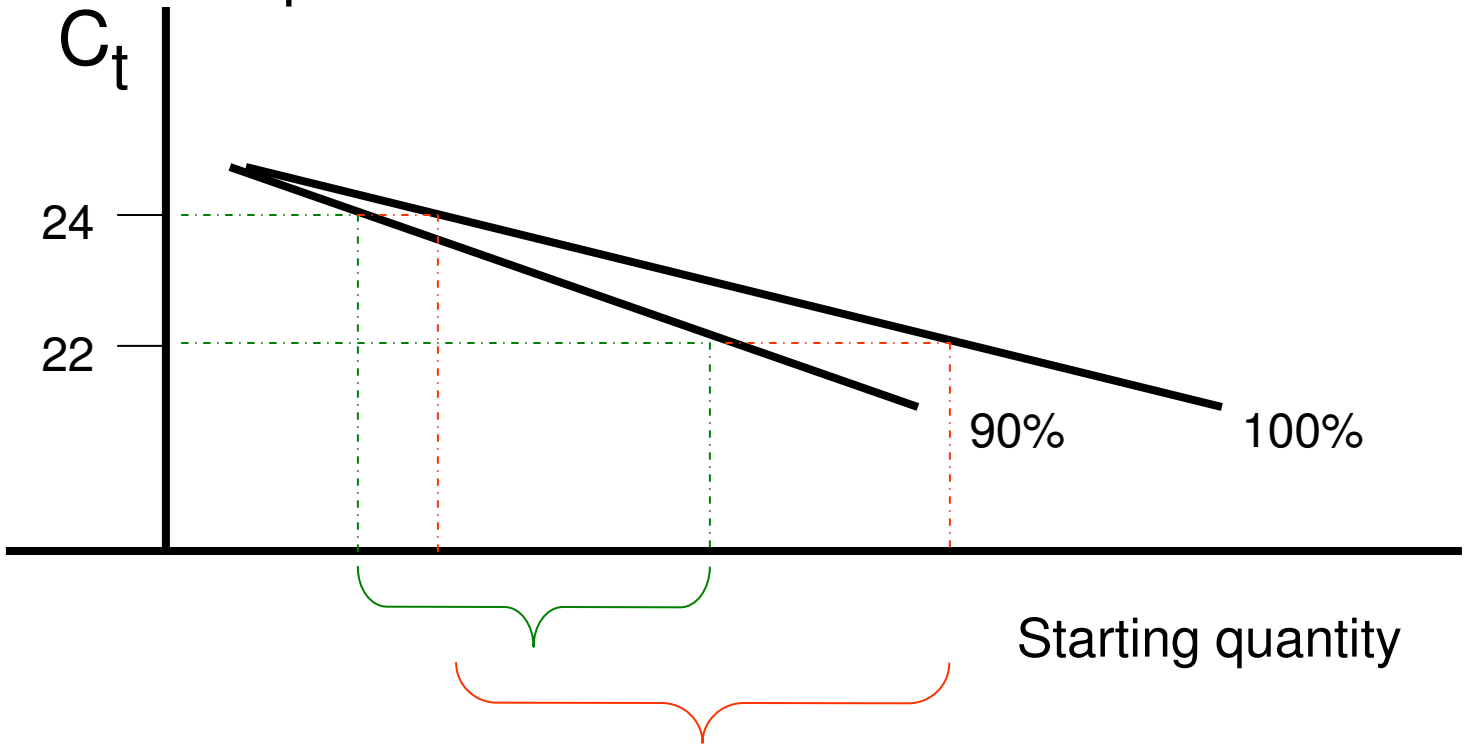


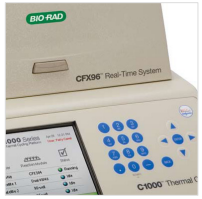


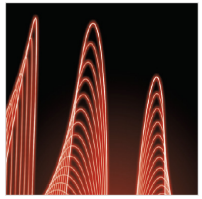


Relative Quantification

- Problem with the ΔΔCT
- Slopes are not parallel





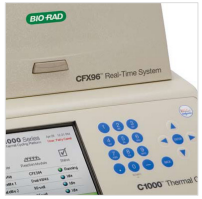


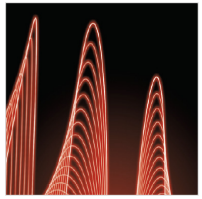
Relative Quantification

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

(Pfaffl, 2001; Nucleic Acid Research)

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

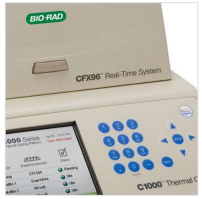


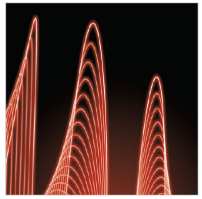


Relative Quantification –Pfaffl Modification

	Primer set #1Reference	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

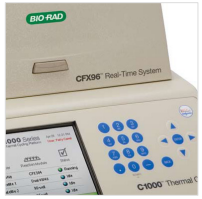
$$\text{Fold induction} = \frac{2_{\text{target}}^{\text{deltaCt }_{\text{target}}\ (\mathbf{24-22 = 2})}}{1.9_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\ (\mathbf{20-21 = -1})}} = \frac{4}{0.53} = 7.5$$

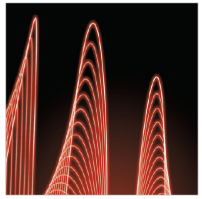




Methods Comparison

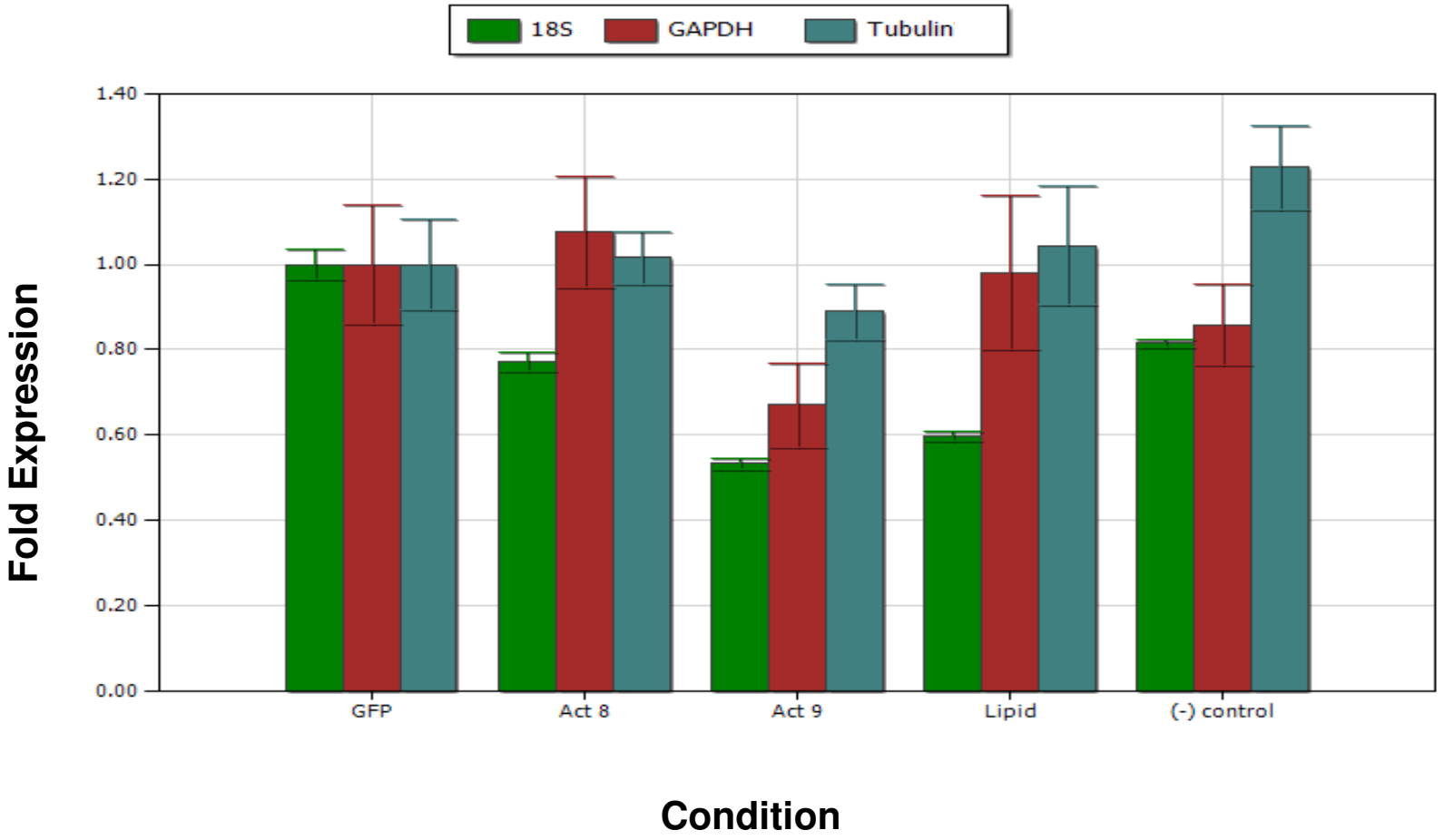
- ΔCt method: (no reference gene)
  - Fold induction : 4
- ΔΔCt method: (reference gene)
  - Fold induction : 8
- Pfaffl modification: (reference gene and efficiency)
  - Fold induction : 7.5



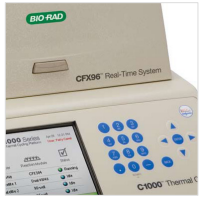


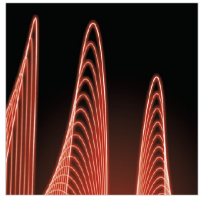
Multiplex RT-qPCR Results

Different reference genes



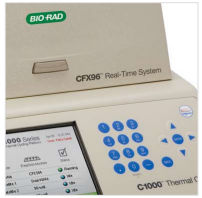
BIO-RAD

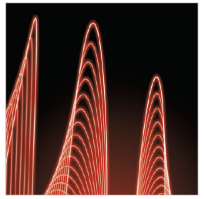




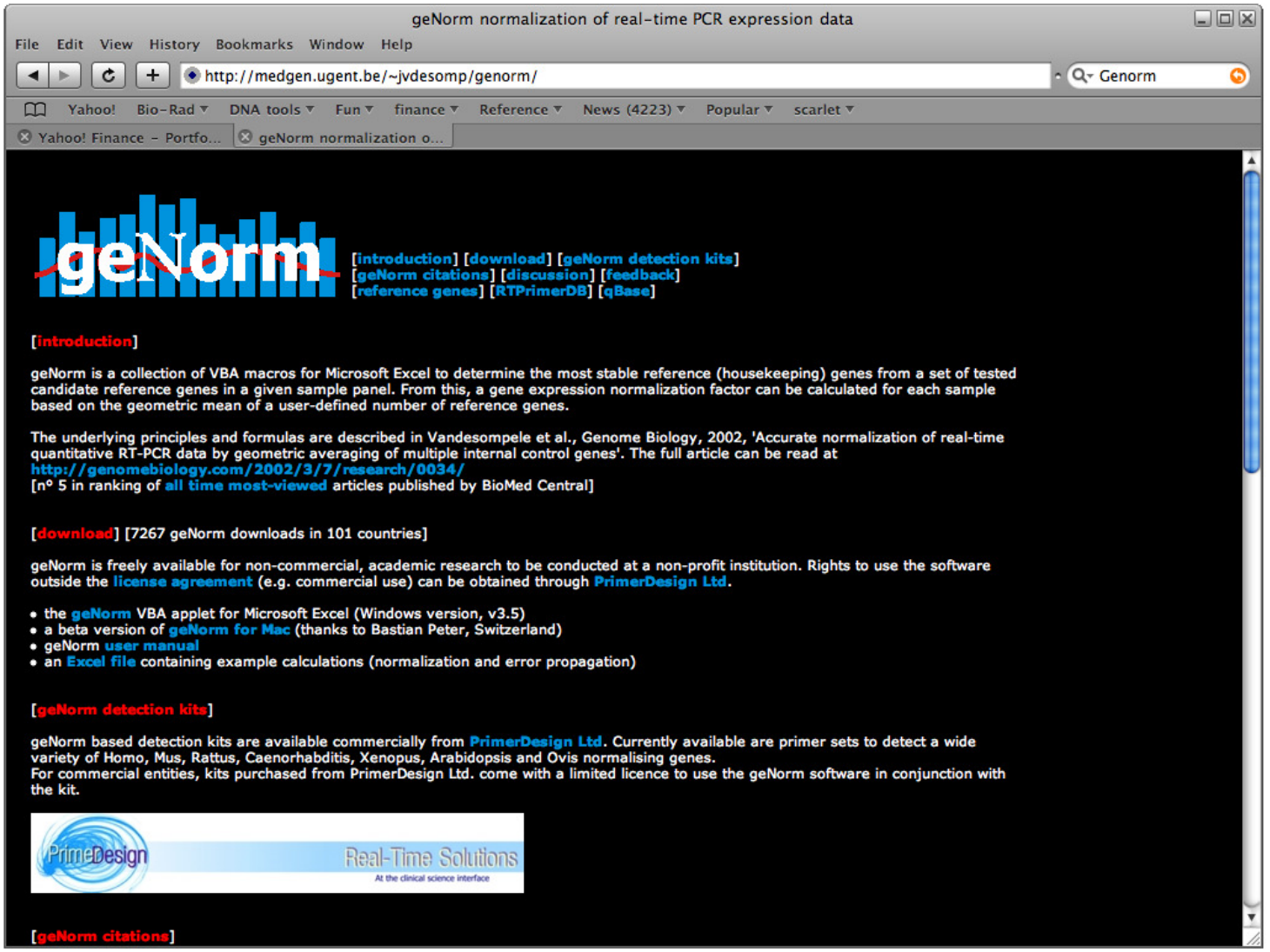
Multiple Reference Genes

- 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

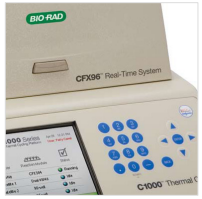


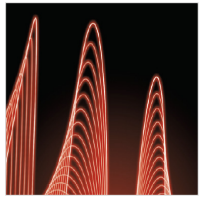


geNorm

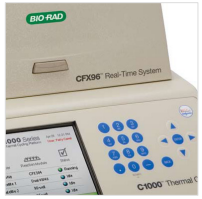


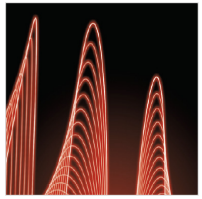
- How to choose best reference gene (s)
- How to do the calculations
- Frequently used ref genes are listed
- Linked to RTPrimerDB
- You can submit your favorite ref gene(s)





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





- Thank You!

- Questions?