Quantitation using Real-Time PCR

Jennifer McMahon PhD

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What is the aim of the Study?

To determine differences in gene expression levels between a control group and an experimental group(s).

Experiment considerations

Appropriate selection of Controls

Needs to be determined statistically

What are the groups being investigated?

Patients, treatments, time points

What genes are being targeted?

Based on micro-arrays, literature, physiological studies

Do I need a normalizer gene?

Can you control for the variation in starting template amount?
Can you rely on your U.V spectrophotometric results?
If not you need a normalizer

Statistics

Are you running a valid number of samples per group?
Are you running enough biological replicates per sample?

Choice of statistical test

- Student t-test (comparing two groups)
- 2 way ANOVA (number of groups over a number of variables)
- Repeated measures ANOVA (time course)
- Correlation analysis (relationship)

Outlier detection (Grubb's test)

Reverse Transcription

Convert total RNA to cDNA or just messenger RNA (mRNA)

- Use of Random hexamer (RNA total) or OligodT primers (mRNA)
- Avoid using oligodT primers if the target is at the 5' end of the RNA
- Can't use oligodT if using 16S because it doesn't have a poly A tail

Choice of Enzymes available

- RNAse H +/-
- some work better on low copy template
- one-step or two step reverse transcriptase real-time PCR?

Reaction Setup

General PCR rules apply e.g. don't set up reactions in electrophoresis areas

UNG - Uracil-DNA Glycosylase can prevent product contamination (not appropriate if product is to be used downstream)

NTCs - use no template controls to monitor contamination issues

RT-ve controls - use controls without reverse transcriptase to check for presence of DNA if using cDNA. Consider designing primers spanning exon/exon boundaries so that DNA can't be amplified

Normalizer or "Housekeeping" Genes

- Normalize for variation in sample amount
  - Samples may vary in RNA extraction efficiency, RNA quality, cDNA synthesis, RNA (or DNA) concentration, pipetting of template
- MUST be unregulated under experimental conditions
  - If amount of RNA ↑ then amount of normaliser must ↑
    - i.e. normalizer must not change in response to treatment
- Preferable to have similar abundance of normalizer & GOI
  - e.g. rRNA (18S) is more stable than mRNA and highly abundant so may not correlate well with low copy number genes
- No such thing as a universal normalizer
  - Different experiments may affect normalizer expression
- Selecting a normalizer
  - Check the literature, compare prospective normalizers, some people use 2 normalizers for all analysis

Vandestomple, J., DePreter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data bygeometric averaging of multiple internal control genes, *Genome Biology* **3(7)**

Choosing a Calibrator

Calibrator will be used for comparisons

Need a starting point or "normal"

GOI may be up regulated or down regulated as a result of "treatments" compared to the "normal"

Allows comparison of samples from various "treatments" over a number of runs – calibrator is like an internal control for run variation

Example of calibrator

Tissue culture experiment – untreated cells v treated cells

Pairwise normal tissue v tumour tissue – either choose one sample as a "normal" or pool a little from all "normals" and use as calibrator

Need a lot of calibrator

Calibrator will be used in every run to allow comparisons between runs

What do I use to calculate efficiency?

Important that the target used to calculate efficiency is similar to the samples

e.g. plasmid may amplify better than extracted material because it is cleaner (no inhibitors) and more pure (no other sequences present) for cross reactions

Serially dilute the target, plot and calculate the slope

The software does this for you, see next slide

Efficiency of PCR

Linear regression model

Serial dilution of the template

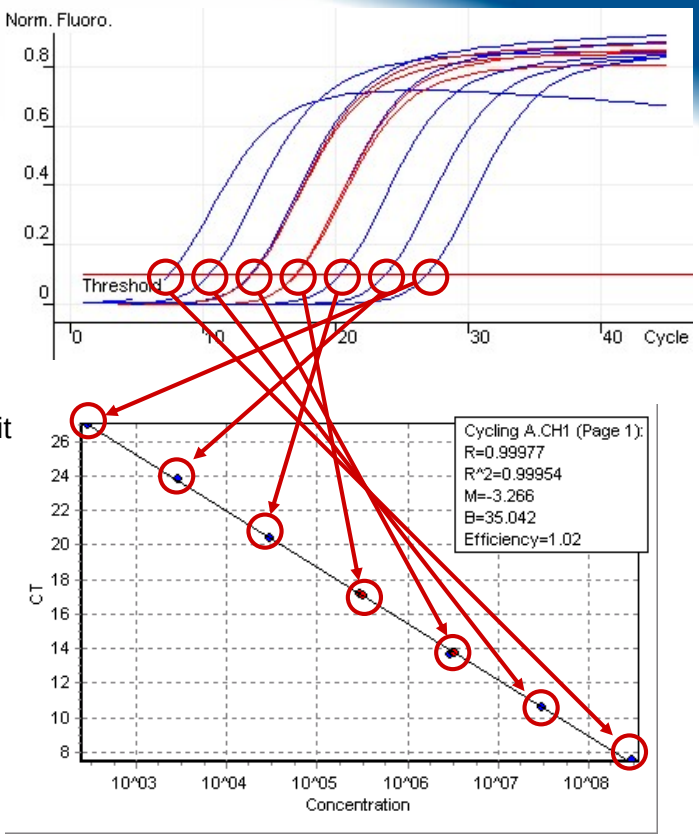
Plot the C_T vs log of concentration of template

Calculate the formula for the line of best fit

Efficiency is related to the slope (m)

$E = (10^{-1/m}) - 1$

E= 1 = 100%



Quantitation

Relative to a Standard Curve

1. Absolute standard curve - results are in numbers
2. Relative standard curve - results are as ratios (comparative)

Relative to a reference sample (calibrator)

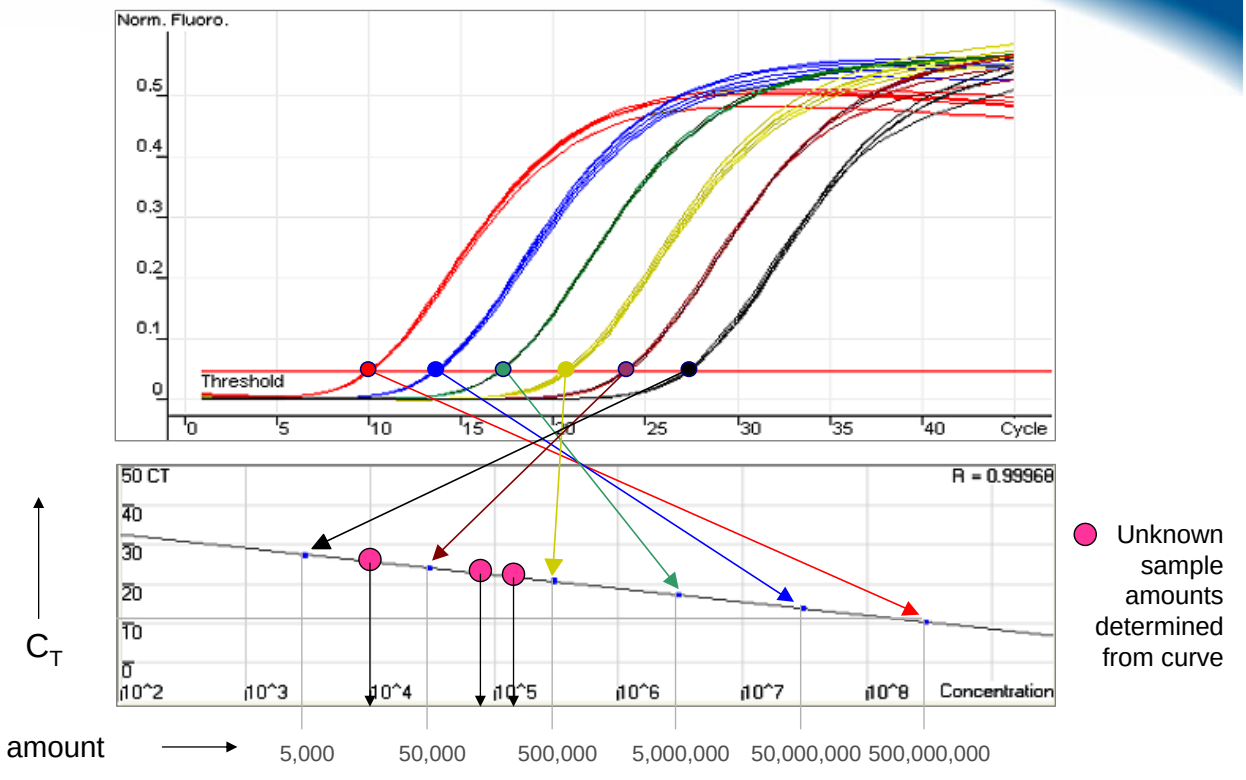
Results are in ratios (up or down relative to calibrator)

Normalizer used to correct for amount of template added

The most powerful and widely used method

Several formulae available

Absolute Quantitation
Standard curve can normalize C_Ts to input amount



Comparative Quantitation

Available in Rotor-Gene Software only

Amplification Plot based Efficiency calculation

- Based on the fluorescence history of each reaction
- Software uses a second derivative of the raw amplification data
- Software determines the “take off” point of a reaction – no need to draw a threshold
- The slope of the line from the take off point until exponential amplification stops is used to calculate the amplification efficiency
- Values are out of 2 – 2 is doubling. Anything above 1.6 generally OK

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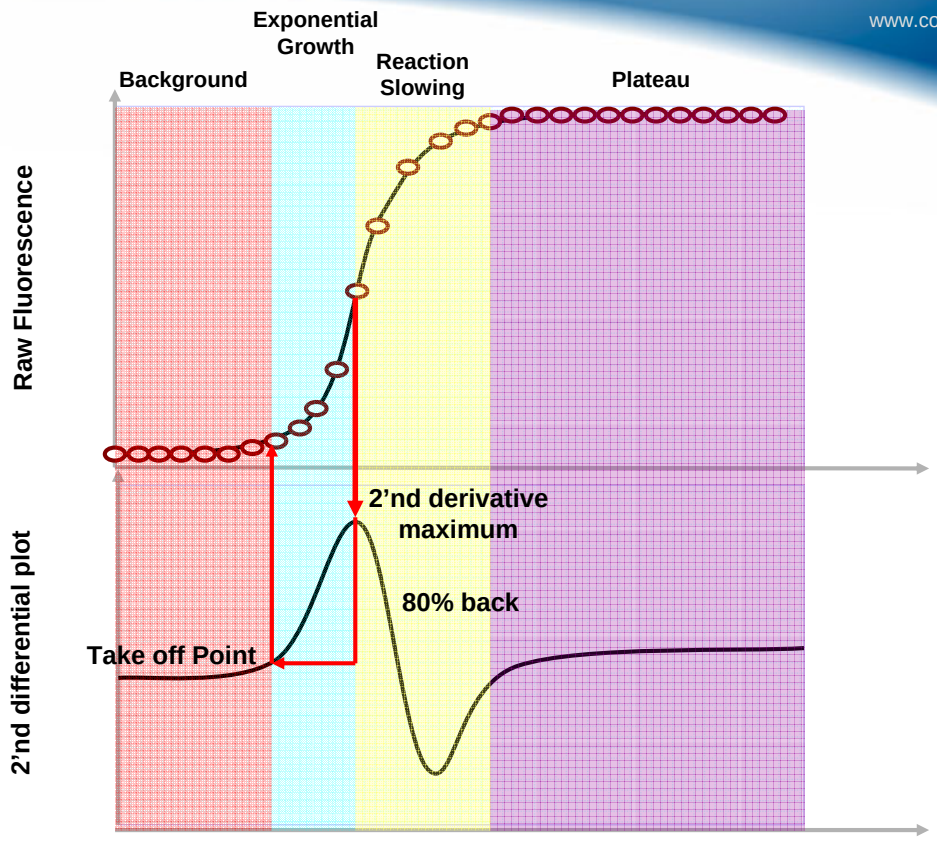

Notes

Amplification Value (Efficiency) of each reaction calculated

Average Amplification value used for analysis

- Make sure you are looking at one gene at a time in case amplification efficiencies vary between genes

Variation in the Average Amplification value must be minimal

- Switch off all NTCs and samples that have failed

Good assay quality control

Using Comparative Quantitation

No.	C	Name	Take Off	Amplification	Comparative Conc.	Rep. Takeoff	Rep. Takeoff (95% CI)	Rep. Amp.	Rep. Amp. (95% CI)	Rep. Conc.
A2		Calibrator 6	17.1	1.86	9.83E-01	17.1	[16.9 , 17.2]	1.78	[1.59 , 1.96]	1.00E+00
A3		Calibrator 6	17.1	1.75	9.83E-01					
A4		Calibrator 6	17.0	1.72	1.03E+00					
A5		Sample 2	17.9	1.97	6.51E-01	17.8	[17.4 , 18.1]	1.82	[1.33 , 2.31]	6.98E-01
A6		Sample 2	17.6	1.60	7.60E-01					
A7		Sample 2	17.8	1.89	6.86E-01					
A8		Sample 3	17.7	1.84	7.22E-01	17.4	[16.6 , 18.1]	1.83	[1.55 , 2.11]	8.57E-01
B1		Sample 3	17.1	1.71	9.83E-01					
B2		Sample 3	17.3	1.94	8.87E-01					
B3		Calibrator 5	18.6	1.79	4.54E-01	18.5	[18.3 , 18.7]	1.71	[1.51 , 1.91]	4.78E-01

Click on comp quant tab

Switch off NTCs and any samples that haven't worked as this will affect amplification value and std deviation

No.	C	Name	Take Off	Amplification	Rep. Takeoff	Rep. Amp.	Rep. Conc.	Rep. Calibrat
A2		Calibrator 6	17.1	1.86	17.1	1.78	1.00E+00	Calibrator
A3		Calibrator 6	17.1	1.75				
A4		Calibrator 6	17.0	1.72				
A5		Sample 2	17.9	1.97	17.8	1.82	6.72E-01	
A6		Sample 2	17.6	1.60				
A7		Sample 2	17.8	1.89				
A8		Sample 3	17.7	1.84	17.4	1.83	8.44E-01	
B1		Sample 3	17.1	1.71				
B2		Sample 3	17.3	1.94				
B3		Calibrator 5	18.6	1.79	18.5	1.71	4.44E-01	
B4		Calibrator 5	18.4	1.63				
B5		Calibrator 5	18.5	1.71				
B6		Sample 5	17.7	1.65	17.6	1.68	7.39E-01	
B7		Sample 5	17.3	1.68				
B8		Sample 5	17.8	1.71				
C1		Sample 6	18.0	1.96	17.8	1.71	6.60E-01	
C2		Sample 6	17.7	1.53				
C3		Sample 6	17.7	1.62				
C4		Sample 7	17.4	1.69	17.6	1.80	7.53E-01	

Calibrator Replicate

(A2) Calibrator 6

Results

Average Amplification 1.76±0.12

Want amplification values >1.6, SD as low as possible

Choose calibrator from drop down menu at side (defaults to first sample)

Export “Rep Conc” column to excel

Repeat analysis and calibrator selection for HK

Divide GOI by HK to correct for variation in starting amount

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Relative Quantitation
Two standard curve method

2 Standard Curve Method

Generate a standard curve

for the GOI and for the HKG separately

use different channels or different pages to separate the curves

Calculate concentration

read values for the HKG and GOI of samples from their respective standard curves, divide one by the other

Standard Curves required every run

Rotor-Gene software can do analysis within a run but not between runs

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Relative Quantitation

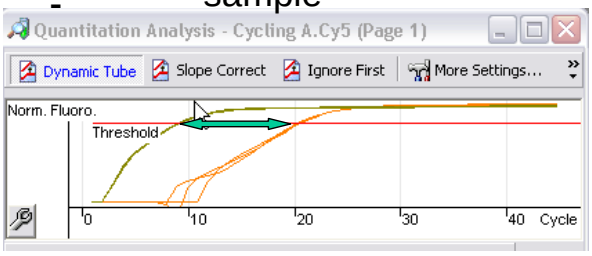
Comparative Ct ($\Delta\Delta$Ct Method)

$\Delta\Delta C_t$ formula - $R = 2^{-\Delta\Delta CT}$

ΔC_t = change in C_t,

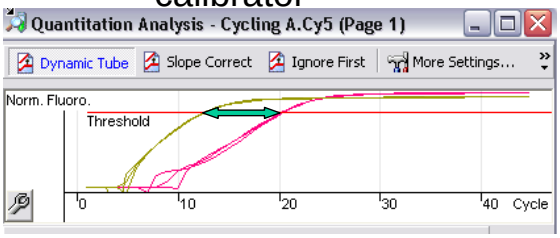
Compares the Ct difference for calibrator (GOI minus HK) and the sample (GOI minus HK)

- sample



C_THK 9.47; C_TGOI 20.84; ΔC_T sample 11.37

calibrator



C_T HK13.1: C_TGOI 20.66; ΔC_T 7.56

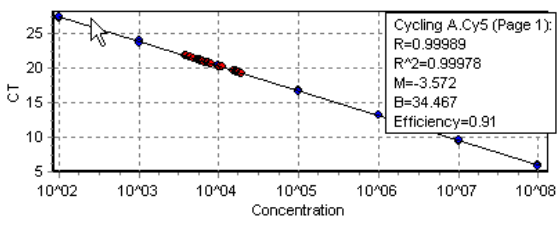
Livak, J. K., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method, *Methods* **25** p402-408

Using $\Delta\Delta C_T$ method

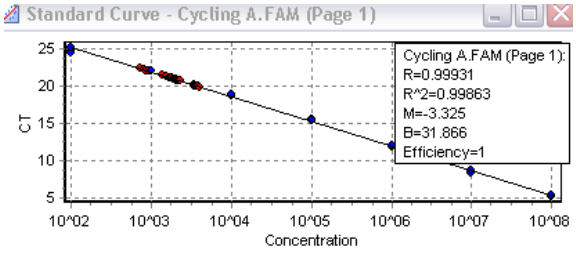
run standard curves for each gene at beginning of experiment

compare efficiencies of PCRs

efficiencies must be equal for this method



standard curve HKG

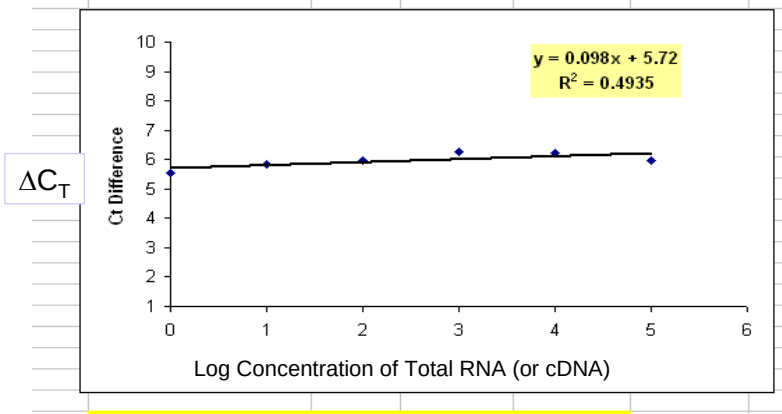


standard curve GOI

Assay validation

Validation Analysis of Standard Curves for ΔΔ Ct Method

Concentration	Ct Gene of Interest	Ct Housekeeper	Log Concentration	ΔC_T Ct Difference
GAPDH Std. conc: 100000	15.77	9.82	5	5.95
GAPDH Std. conc: 10000	19.47	13.25	4	6.22
GAPDH Std. conc: 1000	22.77	16.52	3	6.25
GAPDH Std. conc: 100	25.85	19.87	2	5.98
GAPDH Std. conc: 10	29.14	23.29	1	5.85
GAPDH Std. conc: 1	32.35	26.81	0	5.54



The gradient of the line is < 0.1 and therefore analysis is viable.

Rules and Assumptions

Formula assumes 100% efficiency

- 2 = doubling every cycle

Amplification efficiencies of GOI and HKG must be near identical

- validation must be performed before using this method
- assume thereafter that efficiency is constant so don't run standard curves every run
- consider revalidation on semi-regular basis or with a new batch of reagents

Available in the Rotor-Gene software within a run but not between runs

Relative Quantitation
REST method (Pfaffl)

REST Method (Pfaffl)

REST = **R**elative **E**xpression **S**oftware **T**ool

Formula

$$R = \frac{\text{Efficiency GOI}^{\Delta CP(\text{calibrator-sample})}}{\text{Efficiency HK}^{\Delta CP(\text{calibrator-sample})}}$$

Pfaffl efficiency out of 2, according to the formula $E=10^{(-1/-m)}$

Rotor-Gene efficiency out of 1, according to the formula $E=(10^{(-1/-m)}) - 1$

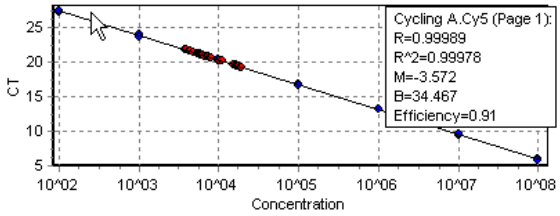
To convert Rotor-Gene efficiency to Pfaffl efficiency add 1

Using REST Method (Pfaffl)

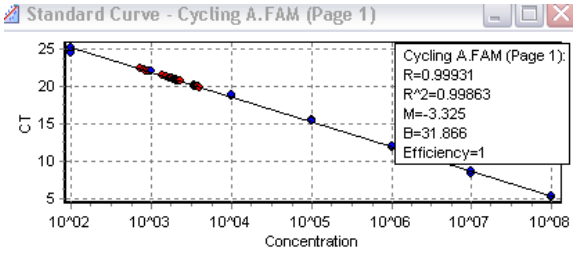
run standard curves for each gene at beginning of experiment

use software to calculate efficiency for HKG and GOI

compare efficiencies of PCRs – efficiencies can differ



standard curve housekeeper



standard curve GOI

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Rules and Assumptions

Formula works on true efficiency

- doubling not required or assumed

Amplification efficiencies of GOI and HKG can be different

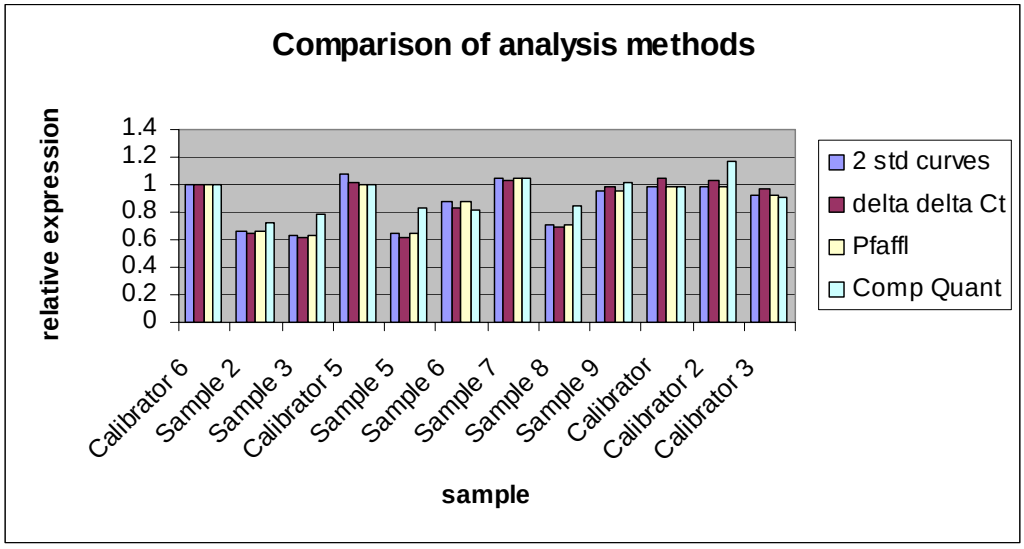
- efficiencies must be calculated prior to the analysis
- assume thereafter that efficiency is constant so don't run standard curves every run
- consider revalidation on semi-regular basis or with a new batch of reagents

Not available in the Rotor-Gene software –use spreadsheet or REST-RG

http://www.gene-quantification.de/download.html#rest-2005

Relative Quantitation
Comparative Quantitation

Comparison of 4 methods of analysis



Reading

Livak, J. K., Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method, *Methods* **25** p402-408

Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Research*, **29** p2002-2007

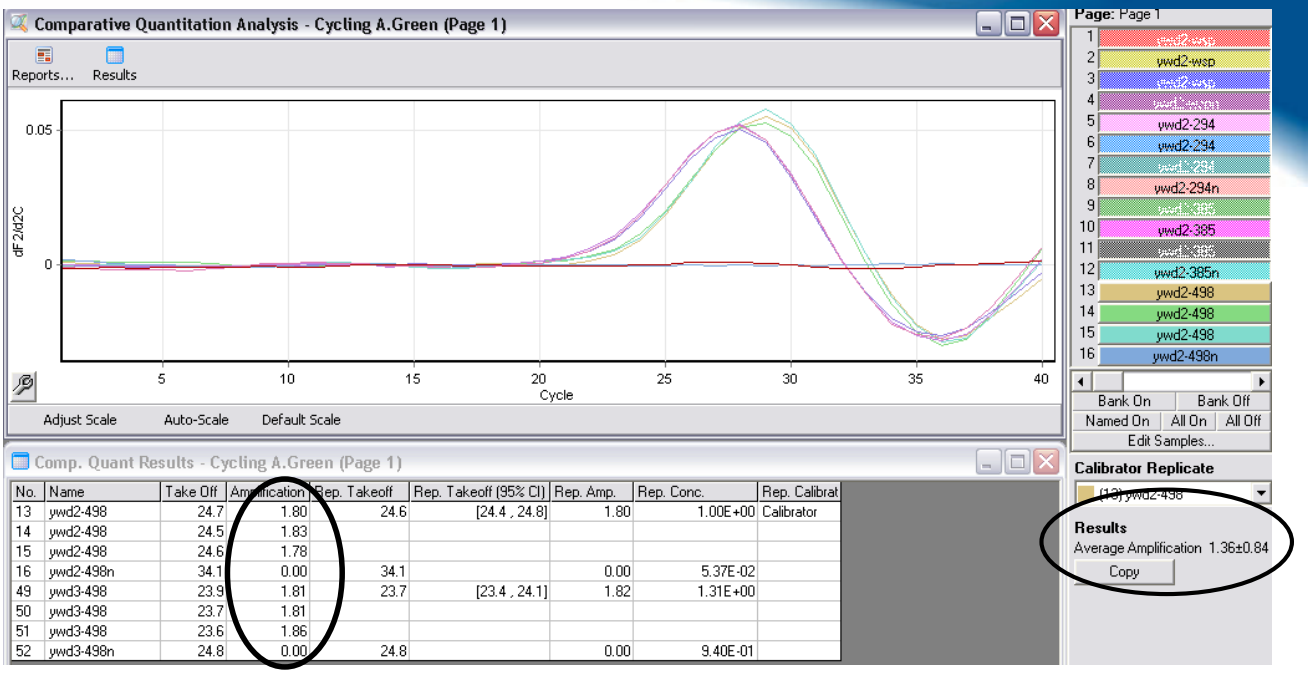
Pfaffl, M. W. (2002) Relative expression software tool (REST®) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, *Nucleic Acid Research*, **30** p 2-10

Ramakers, C Ruijter, J. M., Lekanne Deprez, R. H., Moorman, A. F. M (2003)Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data, *Nuroscience Letters* **339** p62-66

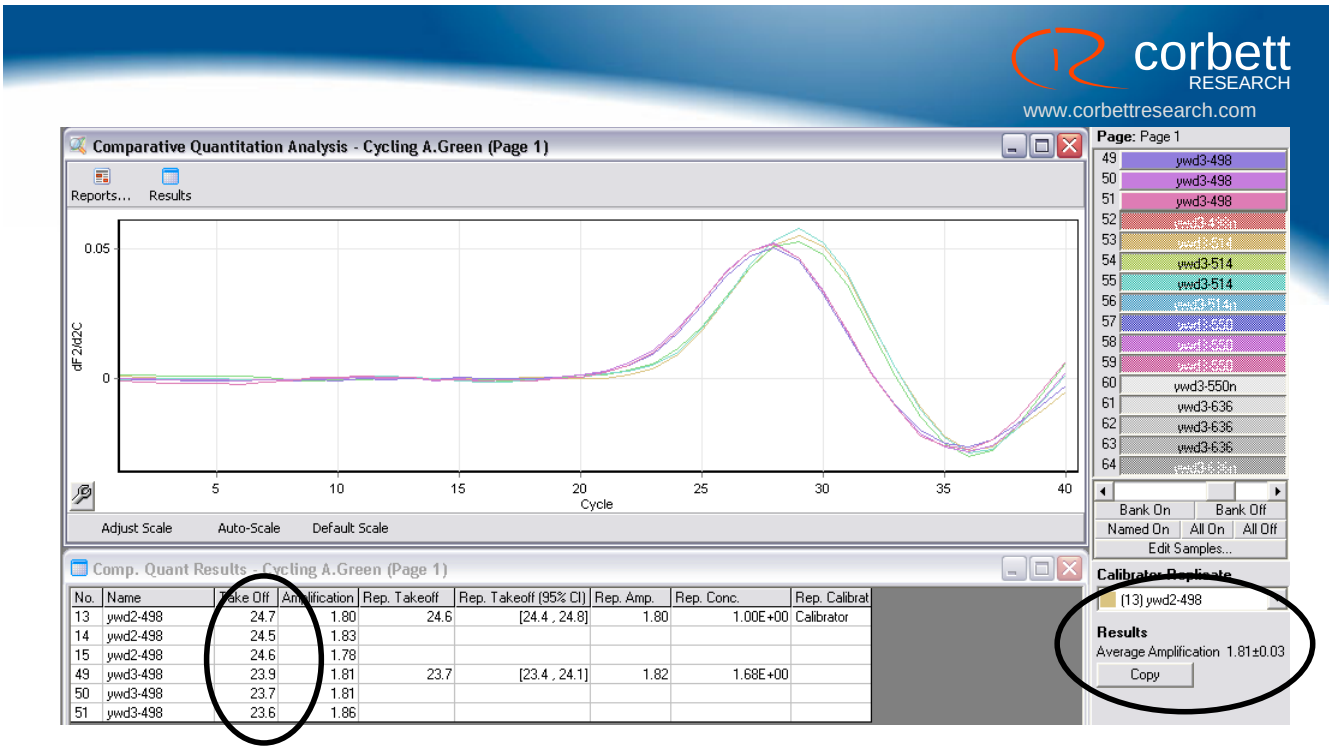
Vandestomple, J., DePreter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data bygeometric averaging of multiple internal control genes, *Genome Biology* **3(7)** p

Analyzing a real run file…first steps

- Test various primer sets on two samples
  - Test two samples (incase one sample poorly extracted)
  - Confirm that the primers do amplify the product (should run on a gel after amplification)
- Check for efficiency and specificity
  - Use comp quant to look at efficiency of each reaction
  - Do melt analysis to compare true product and primer-dimer
- Preliminary analysis using comp quant
  - Can get an idea of whether the genes are up or down regulated between the two samples

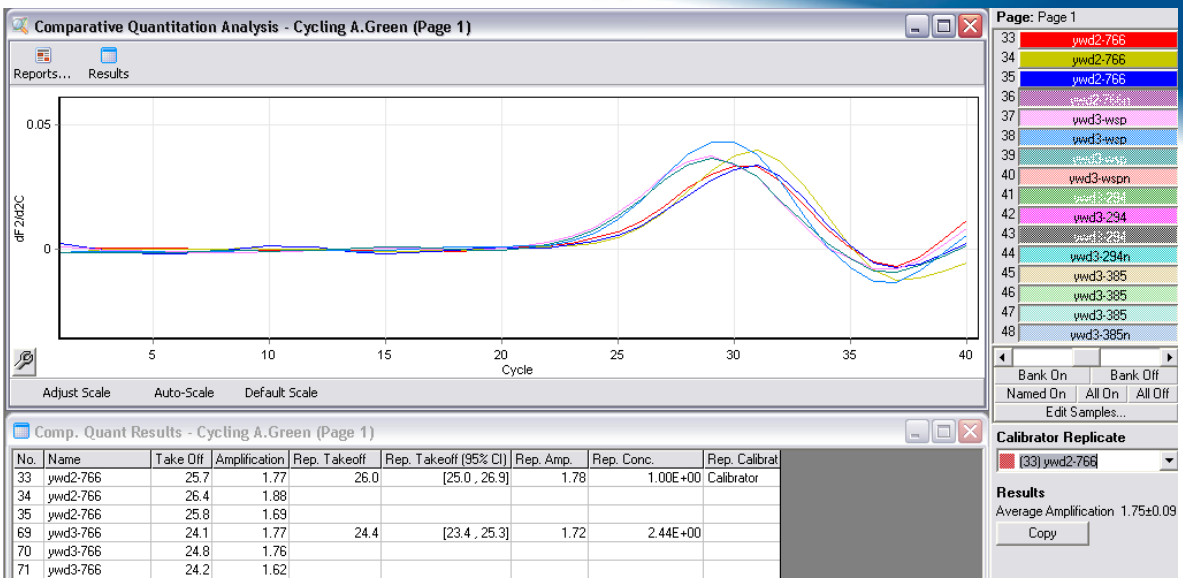


Both samples have amplified well for this gene so can use the data
Need to remove NTCs from the analysis (lanes 16 & 52, amplification value of 0) to get tighter standard deviation for amplification value



Standard deviation for amplification value for gene 498 is good 0.03
Take off points are close (<0.3 cycles) = replicates are close.
Choose ywd2 as calibrator (drop down menu) or “1”; ywd3 has 1.68 fold more expression relative to ywd2 (as shown in the “Rep Conc” box).

Gene 550 has amplified well, standard deviation good, replicates close.
If choose ywd2 as as calibrator then ratio is 1:1.68, exactly the same as for the last gene.
If gene 498 was the HKG and gene 550 the GOI then there would be no difference in expression of 550 between the samples



Gene 766 has amplified well, standard deviation good, replicates close but a bigger spread than genes 498 and 550 (0.7 cycle spread for 766 compared with 0-0.4 cycles spread for 498 and 550)
If choose ywd2 as a calibrator then ratio is 1:2.44, different to the ratio with genes 498 and 550 (1:1.68) but not a significant difference due to cycle spread.

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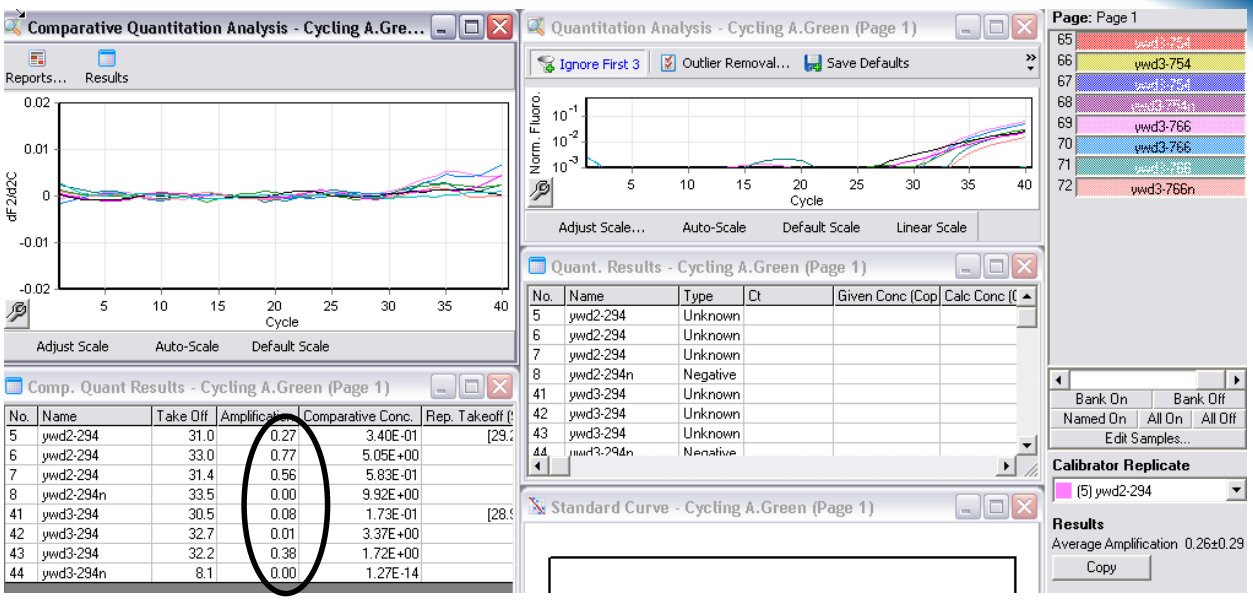
Poor amplification



Both samples have amplified very poorly for this gene so can't use the data

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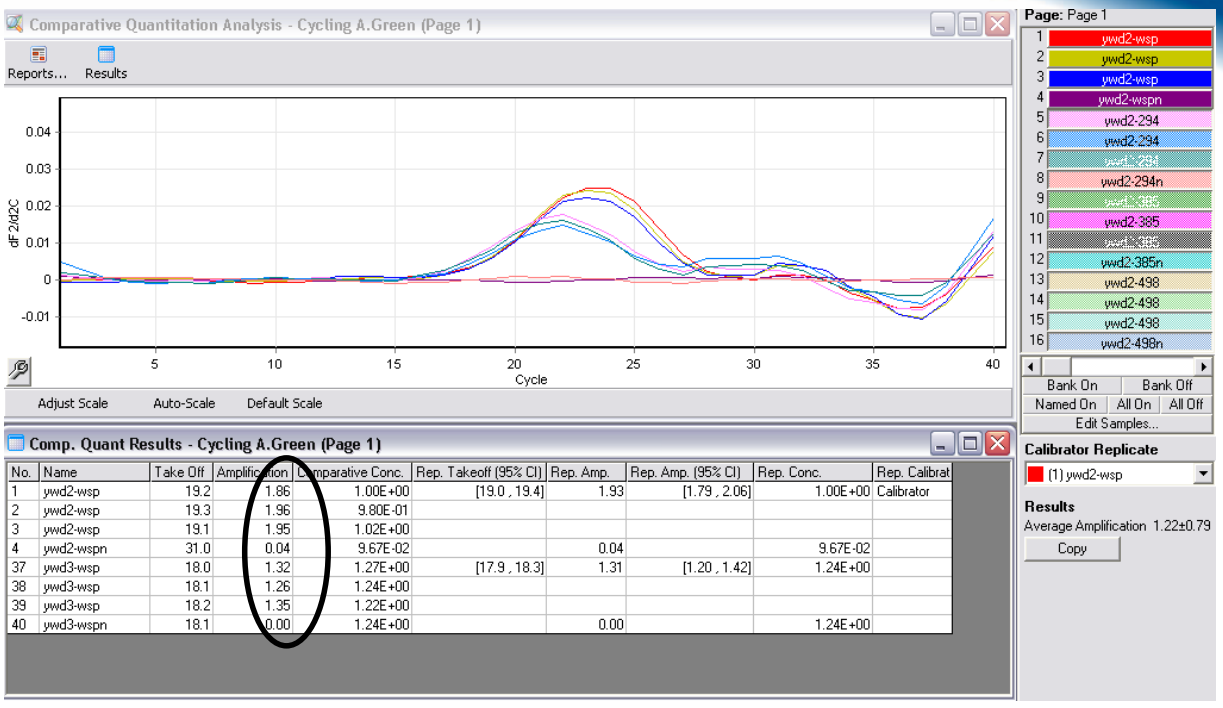
Unequal amplification between samples



Ywd2 has amplified far better than Ywd3 for this gene so can't use the data