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


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 $C_\text{T}$ 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 $C_T$s to input amount

![Graph showing absolute quantitation](image)
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
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

Click on comp quant tab
Switch off NTCs and any samples that haven’t worked as this will affect amplification value and std deviation
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

### Relative Quantitation

**Two standard curve method**

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<td>17.1</td>
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<td>17.1</td>
<td>1.78</td>
<td>1.062</td>
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<td>B1</td>
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<td>1.71</td>
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<td>1.71</td>
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<td>C</td>
<td>Sample 7</td>
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<td>1.89</td>
<td>17.6</td>
<td>1.00</td>
<td>7.536</td>
<td>3.1</td>
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</tbody>
</table>
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

Relative Quantitation
Comparative Ct (ΔΔCt Method)
\[ \Delta\Delta C_t \text{ formula } - R = 2^{-\Delta\Delta C_T} \]

\[ \Delta C_t = \text{change in } C_t, \]

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

Sample:

\[ C_T\text{HK} = 9.47; \ C_T\text{GOI} = 20.84; \ \Delta C_T\text{ sample} = 11.37 \]

Calibrator:

\[ C_T\text{HK} = 13.1; \ C_T\text{GOI} = 20.66; \ \Delta C_T = 7.56 \]


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

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Ct Gene of Interest</th>
<th>Ct Homologous</th>
<th>Log Concentration</th>
<th>ΔCt</th>
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<tr>
<td>GAPDH Std. cec. 10000</td>
<td>16.77</td>
<td>13.35</td>
<td>4</td>
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<td>GAPDH Std. cec. 1000</td>
<td>22.77</td>
<td>16.62</td>
<td>3</td>
<td>6.15</td>
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<td>GAPDH Std. cec. 100</td>
<td>24.86</td>
<td>19.87</td>
<td>2</td>
<td>5.99</td>
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<td>GAPDH Std. cec. 10</td>
<td>29.14</td>
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<td>32.36</td>
<td>26.81</td>
<td>0</td>
<td>5.54</td>
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</tbody>
</table>

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

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 = Relative Expression Software Tool

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**

---

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

Comparison of analysis methods

- 2 std curves
- delta delta Ct
- Pfaffl
- Comp Quant

Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8
Sample 9
Calibrator 1
Calibrator 2
Calibrator 3

Relative expression
Reading


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.
Poor amplification

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

Unequal amplification between samples

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