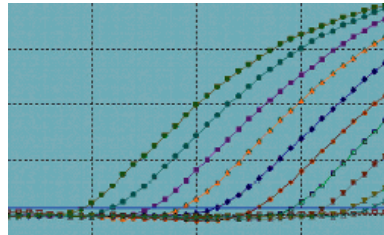
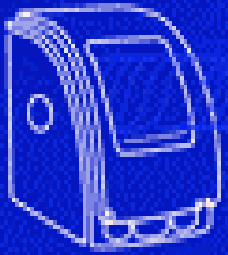


Application specific Design, Data Analysis and Troubleshooting



Dr. Steffen Müller

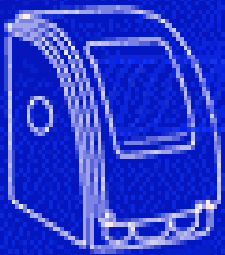
Field Application Scientist



Seminar Outline

Fast Track – QPCR Education

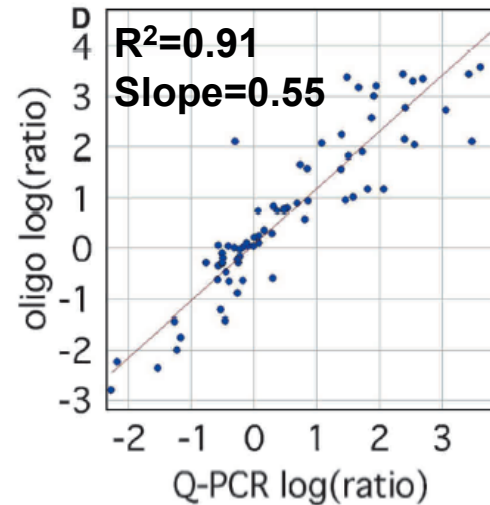
- Application specific Design
- Quantification
- Comparative Quantification
- Primer on Statistics
- Troubleshooting



Application specific Design

Microarray Validation/Gene Expression

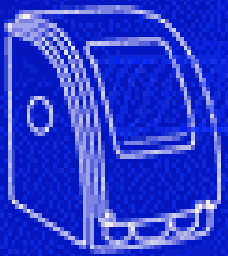
- Adjust your assay design (amplicon, primers and probes) to the type of target sequence used on array.
- Validation of specific transcript variants will require avoiding 3' end.
- Use SYBR when quantifying large numbers of genes
- Even when performing a DNase Digest: Design exon-junction overlapping primers



Expect to see differences in fold-changes comparing array and QPCR!

Arrays...

- Tend to underestimate positive/ overestimate negative regulation
- Have a smaller dynamic range 4 vs. 10 orders of magnitude

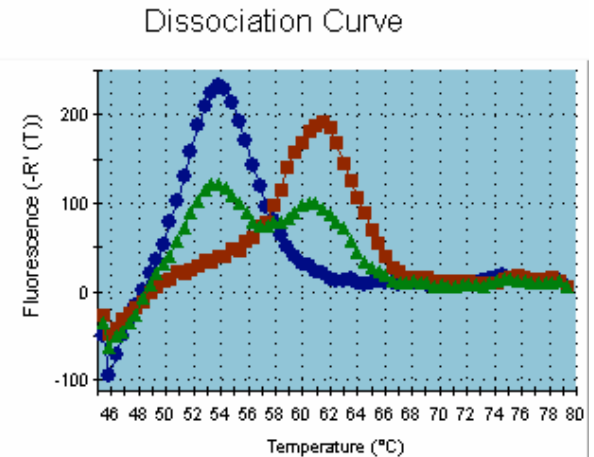


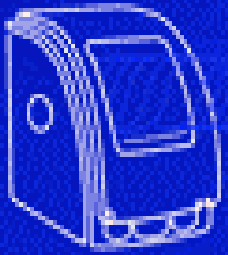
Application specific Design

Allelic Discrimination/SNP Detection

➔ Using SYBR Green looking at melt curves

- You need a T_m difference of at least 2°C for reproducible discrimination
- Use of very small amplicons 70-80 bp
- It is essential to have the same amount of template for each reaction as the T_m is dependent on dye/template ratio
- SYBR is a non-saturating dye which can interfere with discrimination. Consider alternative saturating dyes.



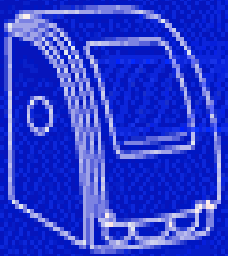


Application specific Design

Allelic Discrimination/SNP Detection

➔ Using a probe based chemistry

- Allows multiplexing of both alleles
- The shorter the probe the better the discrimination
- Position the mismatch in the center of the probe
- Beacons work better than Taqman probes
 - ➔ Ability to determine optimal annealing temperature of the Beacon to your perfect match allele

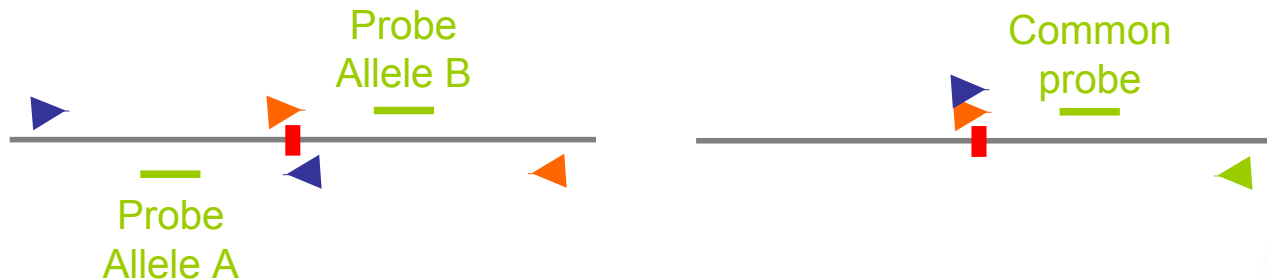


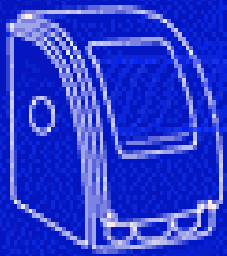
Application specific Design

Allelic Discrimination/SNP Detection

⇒ Allele specific PCR using either SYBR or probe

- Position the SNP at the 3' end of one of the primers
 - Incorporation of LNA bases at SNP position improves discrimination ability
- You have to make sure you don't get amplification from the other allele: Usually a mismatch at the 3' end is not enough:
 - introduce a second mismatch 3-4 bases from the 3' end of the allele specific primer

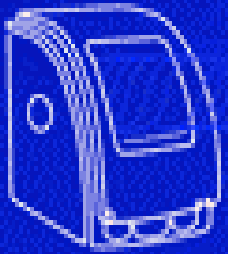




Quantification Methods

Prerequisites

- ➔ Standards and samples amplify with similar efficiency
 - ➔ Mimic sample complexity in standard by using carrier DNA, keeping the overall DNA concentration constant
 - ➔ Use of positive cDNA dilution series in comparative quantification (eg. pool of cDNA aliquots)
- ➔ Apply efficiency correction when observing different amplification efficiencies
- ➔ Quantification is performed in the linear working range of the assays
- ➔ How do I want to quantify:
 - ➔ **Absolute Quantification:**
Is your standard accurately quantified (eg. copy number)?
 - ➔ **Relative Quantification**
 - Is your control positive for your target sequence?
 - Is the Ct for your control in the range of your unknowns (7-10 Cts)?



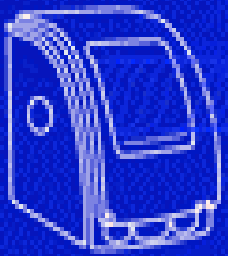
Quantification Methods

Absolute Quantification – Efficiency Correction

If you observe efficiency differences between your external standard and your sample, apply efficiency correction to the quantity as determined by the standard:

$$\text{Real Quantity}_{\text{Sample}} = \text{Quantity}_{\text{vsStd}} \times \left(\frac{E_{\text{Sample}}}{E_{\text{Std}}} \right)^{Ct_{\text{Sample}}}$$

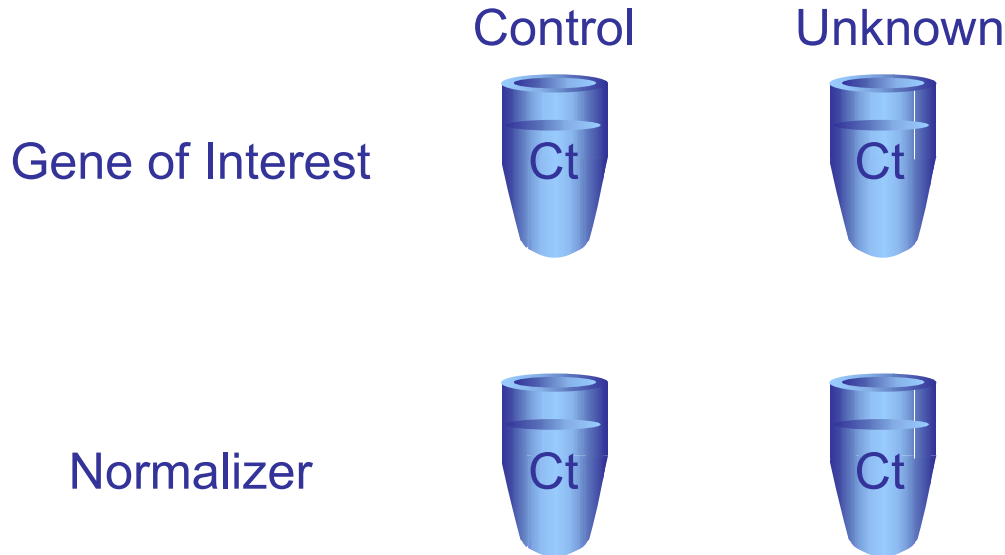
Requires a second relative standard curve from a positive sample to assess amplification efficiency in the sample



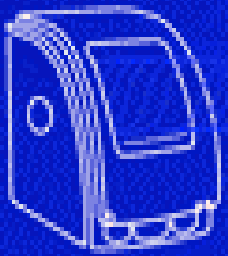
Quantification Methods

Comparative Quantification

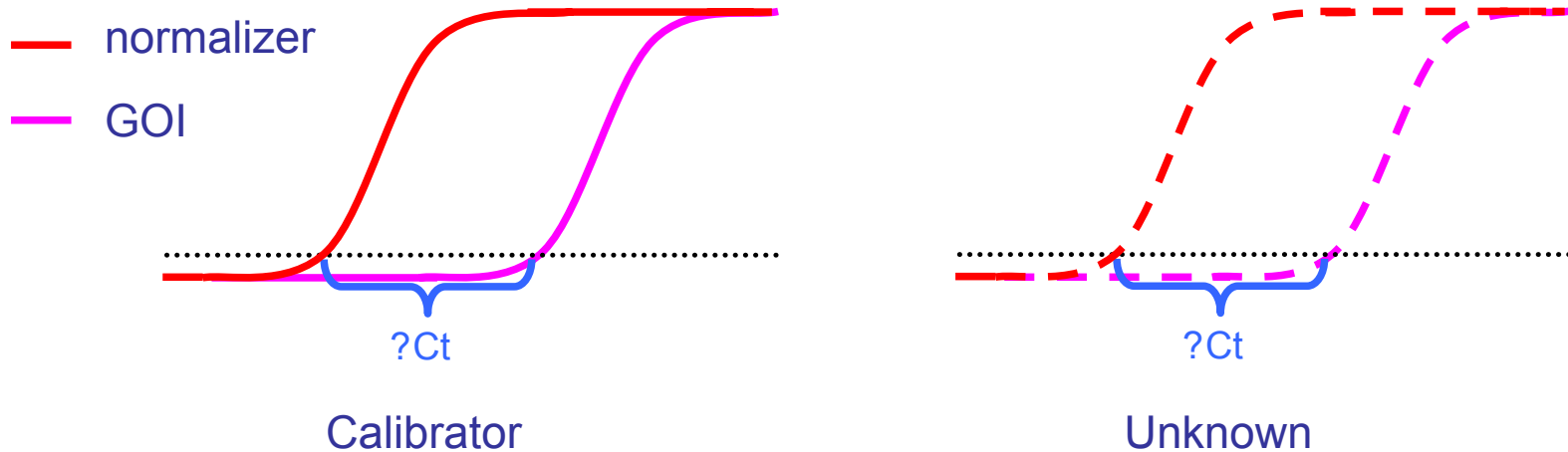
Given two samples: What is the difference in gene expression?



QPCR Human
Reference
Total RNA



Comparative Quantification The $\Delta\Delta Ct$ Method

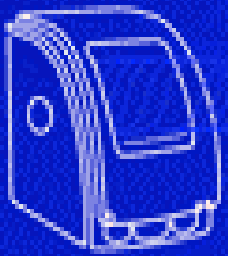


Relative quantity to calibrator = $2^{-\Delta\Delta Ct}$

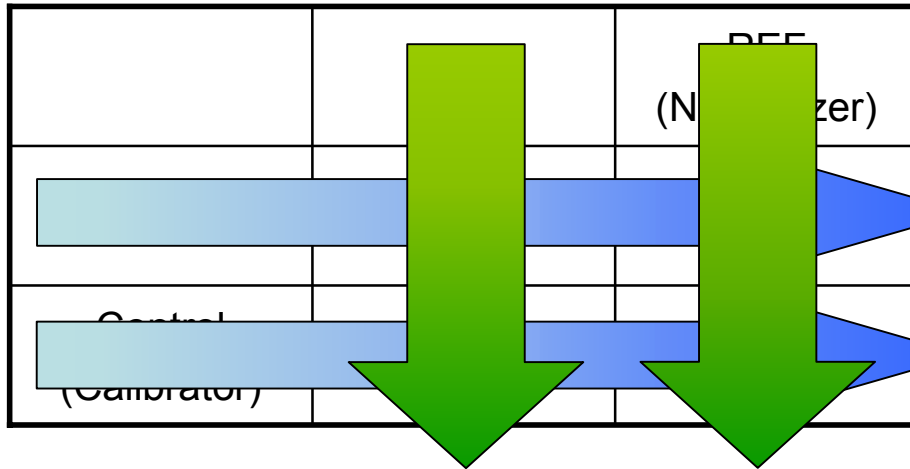
Where $\Delta\Delta Ct = Ct(\text{calibrator}) - Ct(\text{unknown})$

Assumes $E_{GOI} = E_{REF} = 100\%$

*Applied Biosystems. User Bulletin #2 Relative Quantitation of Gene Expression. Dec. 11, 1997



Comparative Quantification The Modified ?? Ct Method



The standard ΔCt :

$$\Delta Ct_U = Ct_{REF_U} - Ct_{GOI_U}$$

$$\Delta Ct_C = Ct_{REF_C} - Ct_{GOI_C}$$

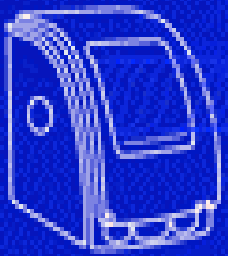
$2^{-\Delta Ct}$

The efficiency corrected ΔCt :

$$\Delta Ct_{GOI} = Ct_{GOI_C} - Ct_{GOI_U}$$

$$\Delta Ct_{REF} = Ct_{REF_C} - Ct_{REF_U}$$

$$Rel.Quant. = \frac{(1 + E)_{GOI} (Ct_{control} - Ct_{sample})}{(1 + E)_{Norm} (Ct_{control} - Ct_{sample})}$$

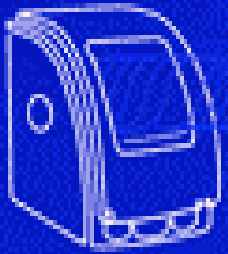


Importance of Efficiency Correction

GOI ΔCt \ GOI Eff.	70	80	90	100
1	1.7	1.8	1.9	2
2	2.9	3.2	3.6	4
3	4.9	5.8	6.9	8
4	8.4	10.5	13.0	16
5	14.2	18.9	24.8	32
6	24.1	34.0	47.0	64
7	41.0	61.2	89.4	128
8	69.8	110.2	169.8	256
9	118.6	198.4	322.7	512
10	201.6	357.0	613.1	1024
12	582.6	1156.8	2213.3	4096
15	2862.4	6746.6	15181.1	32768

Fold changes assuming normalizer Cts are identical

Without Efficiency correction you will overestimate the real fold change!



Data Analysis

Statistical Data Analysis

- As biologists we try to draw general conclusions from limited amounts of data

Are results using our sample dataset transferable to the whole population?

Statistics allows to measure that applicability depending eg. on replicate number and variability.

- **Combination of biological and technical variability can make it challenging to identify differences between experimental groups**

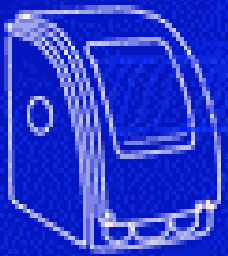
Statistics enables comparison of variant groups and provides a metric that addresses the confidence of two or more groups being different.

- **Which QPCR data can I use in statistical data analysis?**

Most commonly used statistical tests need normal distribution of the data:

Most of the QPCR data might not be normally distributed

Log transformation can restore a normal distribution



Data Analysis

Statistical Data Analysis

Which tests to use?

Use with log normal data:

eg. \log_2 transformed gene expression changes

- Parametric (normal distribution)

- Analysis of Variance - (ANOVA) for Class differences

- works for more than 2 experimental conditions/groups (eg. multiple dosages, time course...)

- tells you if there is a change between the different groups/conditions

- t-tests for member differences

- works for 2 experimental conditions/groups

- tells you if there is a difference between two conditions

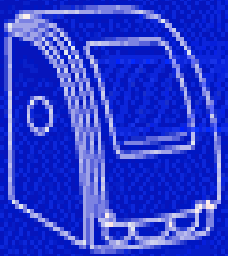
Use with non normal data:

eg. patient samples

- Non-parametric (Non-normal distribution)

- Mann-Whitney or Wilcoxon Rank

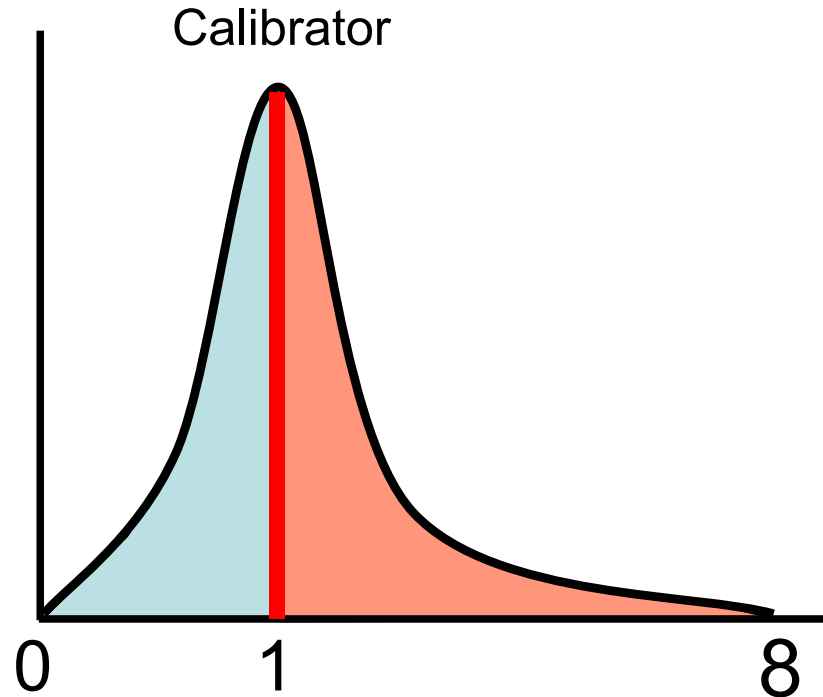
- Kruskal Wallis



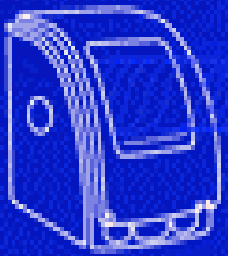
Data Analysis

Statistical Data Analysis

Restoration of Normal Distribution



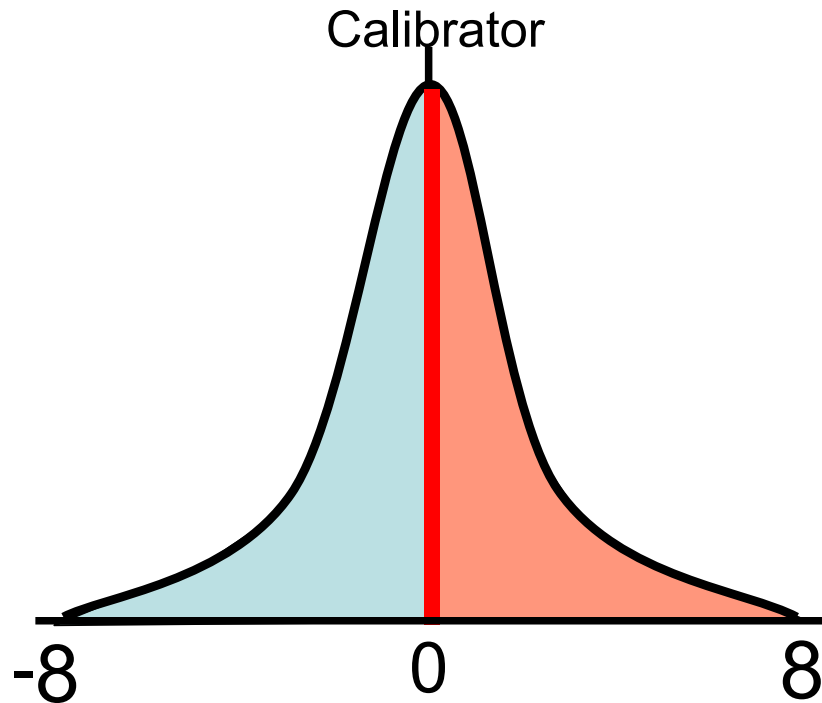
Relative quantity is expected to be positively skewed do to differing limits in positive and negative direction



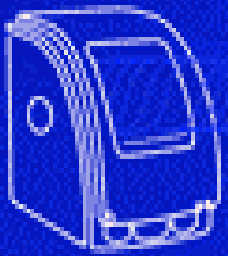
Data Analysis

Statistical Data Analysis

Restoration of Normal Distribution

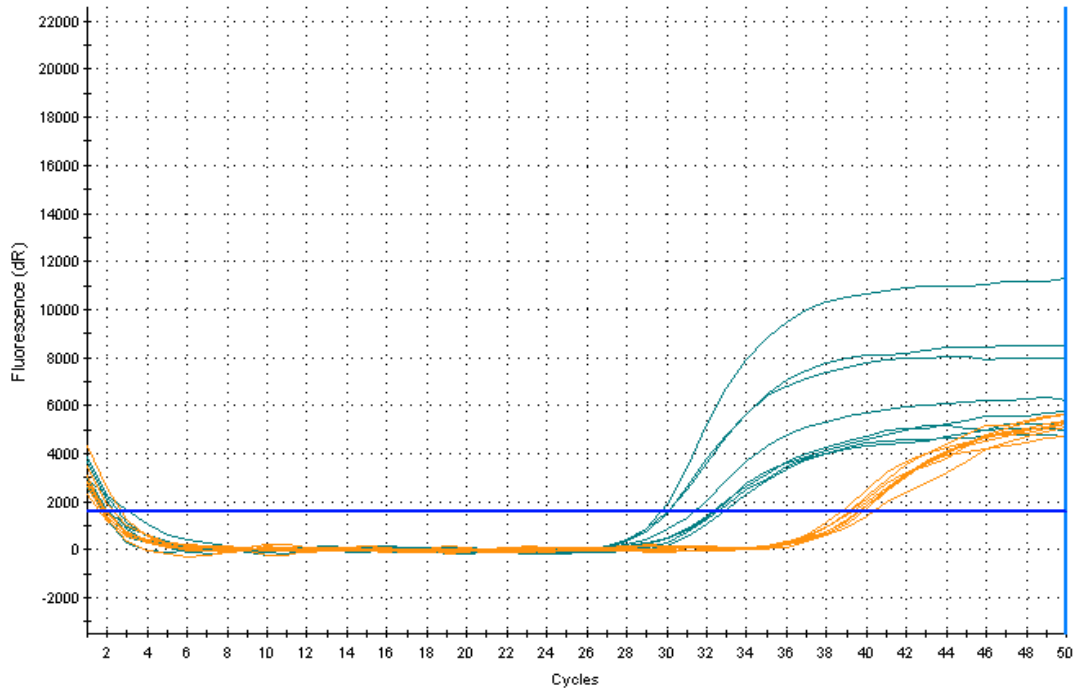


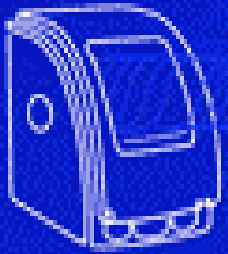
Log fold change is expected to have more normal distribution



Basic Protocol for Troubleshooting

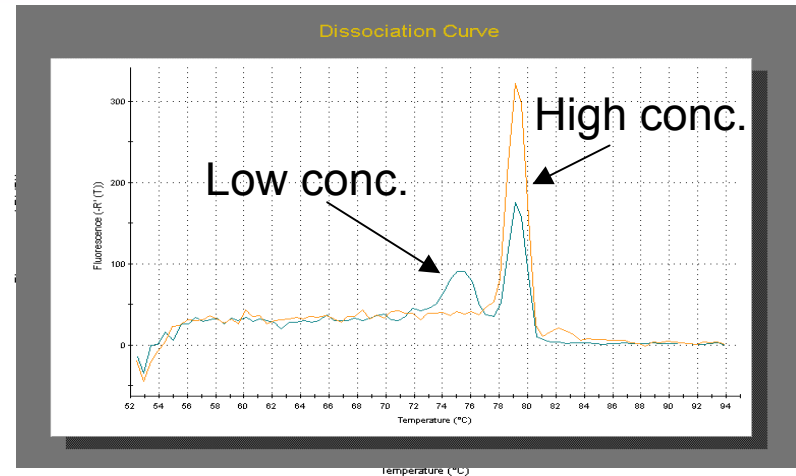
Look at the baseline corrected view (dR).
Amplification plots still look perfect?
Tilted plots hint at baseline correction problem!

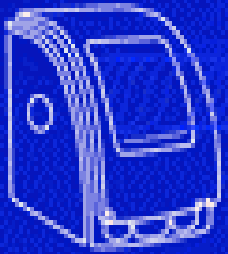




Troubleshooting - Primer Dimers

- ➔ Detection of primer dimers is only possible with SYBR melt curves. They tend to have a T_m between 72-78°C depending on sequence
- ➔ Primer dimers tend to occur in low concentrated samples or NTCs at late Cts
- ➔ In SYBR they contribute to the overall signal and may make accurate quantification impossible.
- ➔ Using a 4th plateau at higher temperature or moving to a probe based chemistry:
→ allows specific detection BUT doesn't remove competition resulting in higher variability and loss of sensitivity
- ➔ Try to get rid of them by primer titration, decreasing annealing time, or redesign





Troubleshooting - Low Signal

➤ Low Signal is often caused by:

low signal yield of dye

- excitation not at absorption maximum
- emission is not measured at maximum
- dye has low quantum yield (eg. TAMRA)

low labeling efficiency of probe

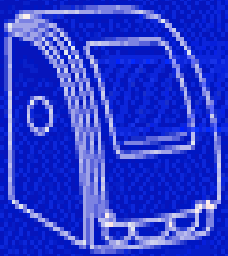
only a small percentage of probe is labeled (eg. ROX)

inefficient probe cleavage

eg. probe too far from primer on the same strand

inefficient probe or primer binding

probe/primer binds in a region of high secondary structure



Efficiency out of the recommended range

Properties of a good standard curve:

➔ high efficiency (80% < 85% < 90% - 105% < 110% < 115%)

good R^2 (>0.98)

low replicate variability for individual standards ($SD_{rep}/mean_{rep} * 100 = \%CV < 1\%$)

Low efficiencies:

➔ Inhibition of amplification

➔ Primer and/or probe don't bind efficiently

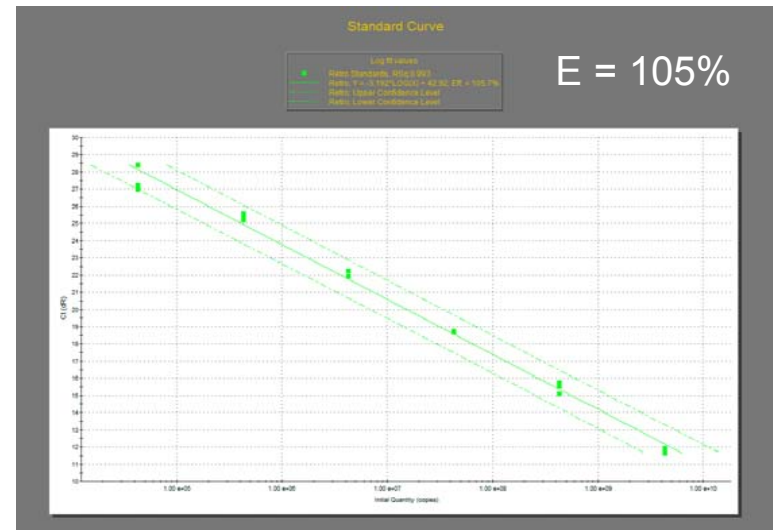
➔ High variability/loss of linearity at high concentrations

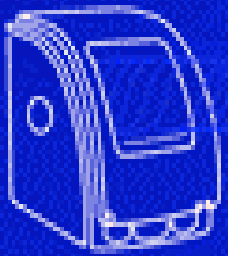
High efficiencies:

➔ High variability/loss of linearity at low concentrations

➔ Amplification/detection of more than one product

➔ Template independent probe degradation

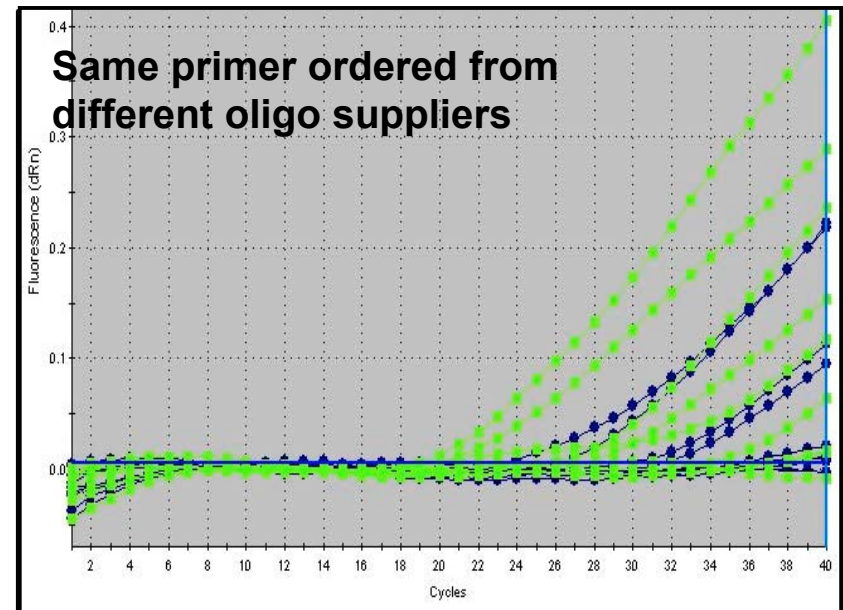




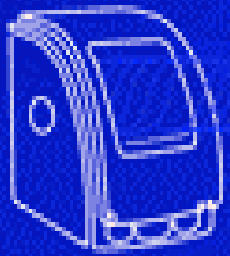
Troubleshooting - Probes and Primers

Varying primer and probe quality may lead to unexpected results

- ➔ You can't expect to get the same results with new lots of already tested primers and probes
- ➔ It is beneficial to quality control every new lot of primers and probes:
 - Test your primers and probes with a standard curve
 - Quality control new probe lots with quantitative plate reads and DNase I digest



pDNA with copy numbers from 10^7 to 10^1
Stoverock, v. Samson, Hannover (Germany)



Application specific Design, Data Analysis and Troubleshooting

Thanks for your attention!

Educational material can be found at
<http://www.stratagene.com/fasttrack/>