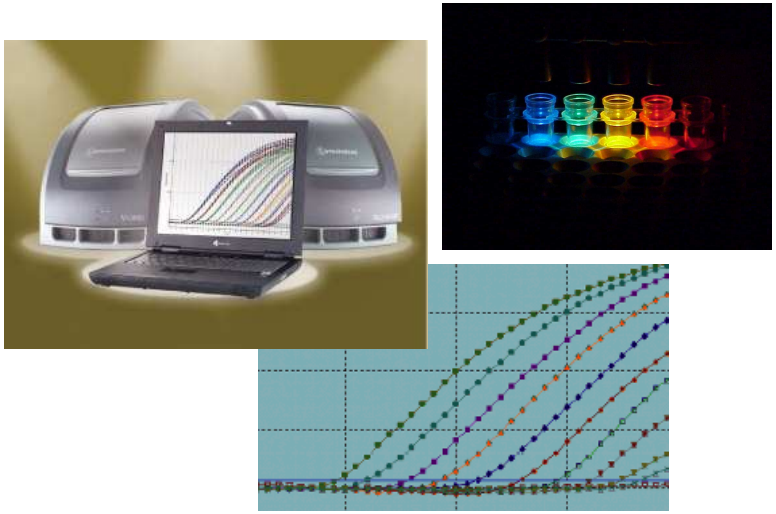
Importance of Experimental Design in QPCR

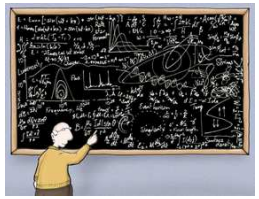
Review of good practice in QPCR for meaningful and robust results



Dr. Steffen Müller
Sr. Field Application Scientist

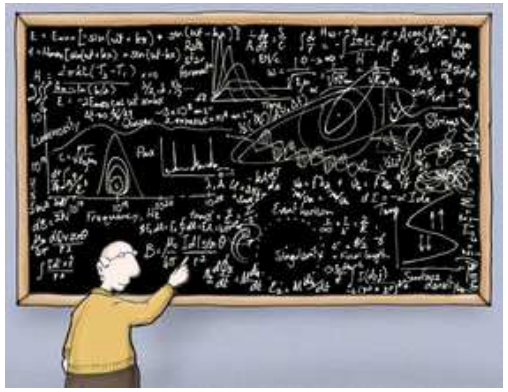
Importance of Experimental Design in QPCR

- **Introduction – Source of Variance**
- **Sampling and Sample Preparation**
- **Quality of Template**
- **Inhibition**

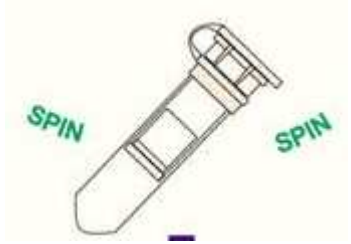


Experimental Design

Understanding experimental variance



Experimental Design

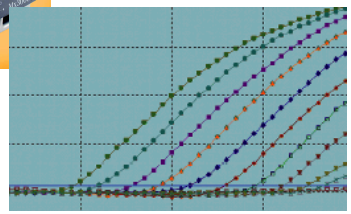


Sample preparation and purification

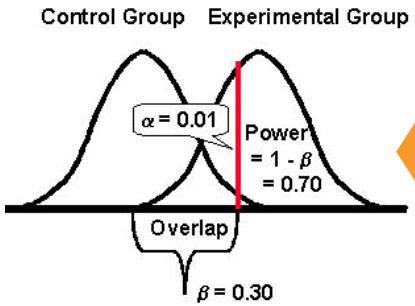
Total RNA

cDNA

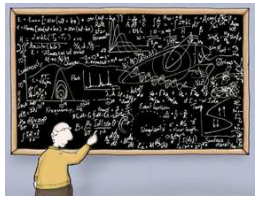
Reverse Transcription



Real time QPCR



Post-run Analysis



Experimental Design

Understanding experimental variance

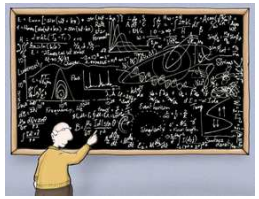
Sources of variance in QPCR experiments:

- Biology of experimental system
  Use of biological replicates

- Technical variance
  Use of technical replicates

  → Pipetting error
  Avoid pipetting small volumes and use of calibrated pipettes
  → Varying template quality and/or quantity
  Use of sample QC and proper normalization
  → Run-to-run variabilty
  Use of inter-run calibrating samples

Number of biological replicates should be determined in a pilot experiment.
Usually 3 technical replicates are sufficient.



Experimental Design

Understanding experimental variance

Sampling and DNA/RNA extraction

- Sample complexity
- Efficiency of extraction procedures
- Quality of template preparation (Inhibitors, RNA or DNA quality)

Nucleic acid quantification and QC

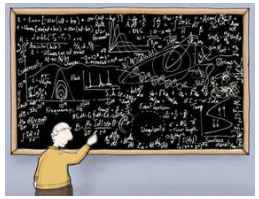
- There is no gold standard method
- Different methods will generate different results
- Any type of QPCR quantification assumes similar template qualities

Reverse Transcription

- Known to be the major source of variability
- RT efficiency is dependent on initial RNA quantity as well as individual level of a certain mRNA
- Type of enzyme and priming method will yield different results

QPCR

- Assay robustness affects results
- Variance of chosen normalizer
- Plasticware and reagents



Experimental Design

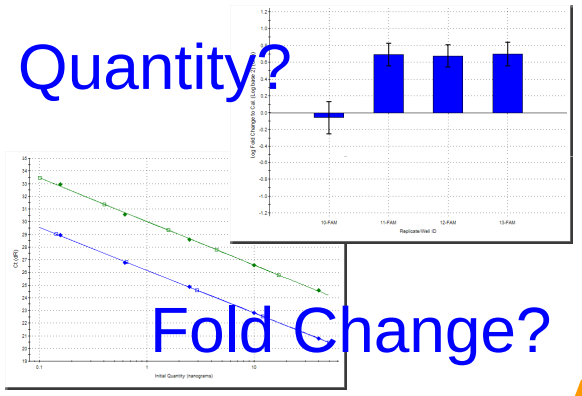
Application specific variance

Linear dynamic range of assay

Quantification should be performed in the range where there is a linear dependency of Ct and log(quantity)

Efficiency of sample and standard amplification

Differences in sample and standard amplification efficiency lead to over- or underestimation of sample quantity

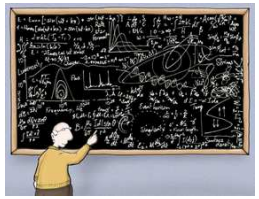


Variance of chosen normalizer

Reference genes should have similar expression levels as the GOI due to RT properties

Quality of samples

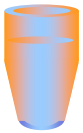
Low quality can have a negative effect on results
If not taken care of



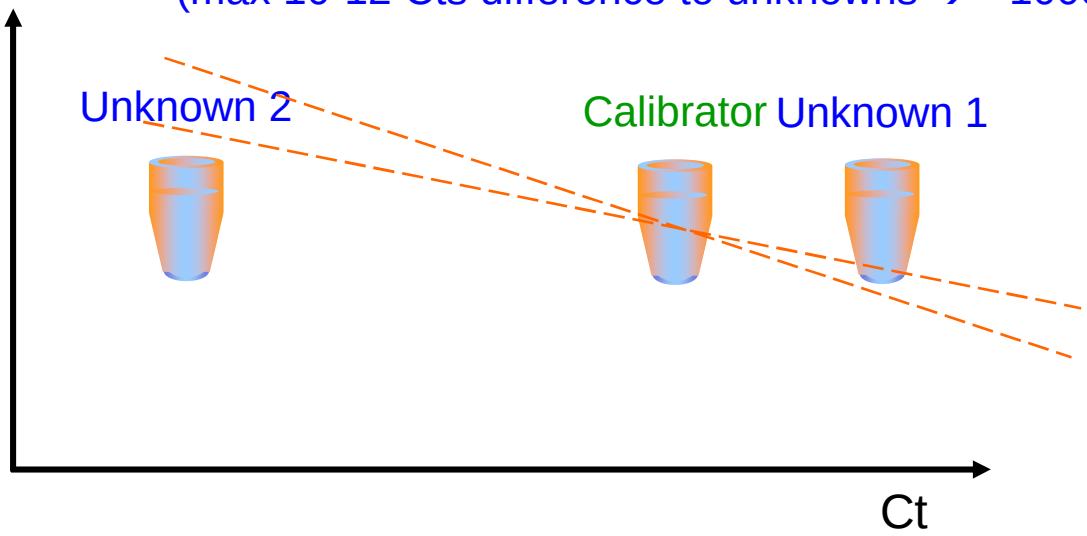
Experimental Design
Application specific variance

What are the limitations of comparative quantification?

Calibrator

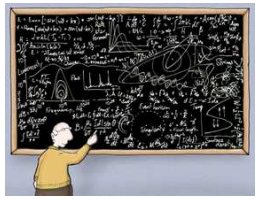


- Needs to be positive for GOI and reference
  → Consider exogenous Calibrators (eg. QPCR reference RNA)
- Should have a Ct value in the range of the unkowns
  (max 10-12 Cts difference to unknowns → ~1000-5000 fold change)



The further away an unknown is from the calibrator the less accurate the FC will be

Sample in the center of the data population best possible Calibrator (reduced variance)

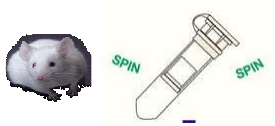


Experimental Design

Application specific variance

Melt curve analysis is influenced by many variables:

- Salt concentration of the reaction mix
  (Spink and Chaires, Biochemistry 38, 1999; Zipper et al., NAR 32, 2004)
- Other compounds present in the reaction mix
  (Spink and Chaires, Biochemistry 38, 1999)
- Concentration and type of double strand binding dye
  (Gudnason et al., NAR 35, 2007; Rasmussen et al., BMC Bioinformatics 8, 2007)
- Ramp rate of the thermal system
  (Gundry et al., Clin. Chem. 49, 2003)
- Data sampling rate and algorithms used in data analysis
- **Sequence of the DNA**
  (Li et al., BioTechniques 35, 2003; Giglio et al., NAR 31, 2003; Zipper et al., NAR 32, 2004; Rasmussen et al., BMC Bioinformatics 8, 2007)



Sample Preparation

Influence of Sampling and Sample Preparation

Sample preparation influences QPCR results

Quality of template

- Quantification assumes comparable quality
- Low quality can lead to failure of detection

Amount of Inhibitors

- Inhibitors can lead to delayed or failure of detection

Amount of co-purified salts

- Affects primer and probe binding affinity

Sample preparation affects QPCR assay performance resulting in lower assay sensitivity if not optimized!

Why Quality Matters

Sample/template quality

RNA quality control (Quality of template):

- RNA degrades naturally due to enzymatic or autocatalytic mechanisms: → Any 5' or 3' biased design might fail or produce misleading results
  → Wrong priming strategy in the RT step can produce misleading results
- Knowing RNA quality allows to accommodate the amplicon design and set expectations avoiding wrong interpretation of results
- All quantifications rely on comparable template quality to be meaningful

Why Quality Matters

Sample/template quality

Quality of assay

QPCR assay validation/optimization (Quality of results):

- The resolution of SYBR Green meltcurves is limited
- T_m depends on dye/template ratio
- Verifying the size of PCR products is a recommended validation procedure: → Resolution of slab gels limited!

Robust and meaningful results

Experimental workflow

Extraction from $5x10^6$ HEK cells using **Absolutely RNA® mini**

RNA extraction

Nucleic acid quantification and QC

Quantification of 1 µl sample on Nanodrop
QC on **Bioanalyzer**: **RNA 6000 nano kit**

RNA degradation @ 70℃

RT from 1 µg of total RNA using **AffinityScript™**

Reverse Transcription

QPCR 5‘ and 3‘ assays

Mx3005P®

QPCR Assay validation

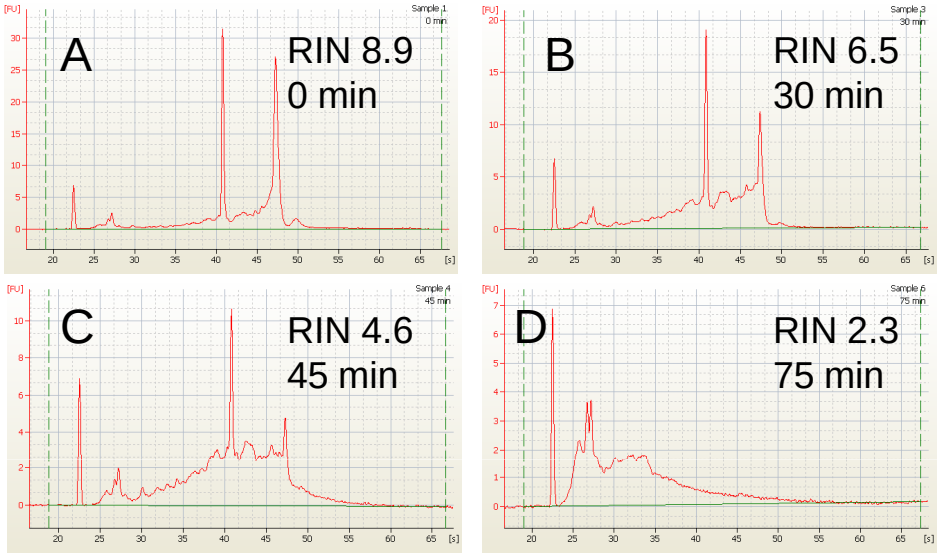
Brilliant® II SYBR® Green

Analysis of QPCR products on **Bioanalyzer: DNA 1000 kit**

Quality and Impact on Gene Expression Results

Effect of RNA quality on gene expression results:

RNA was extracted from HEK293 cells and thermally degraded
All RNAs were tested on the Agilent Bioanalyzer



Assay design:

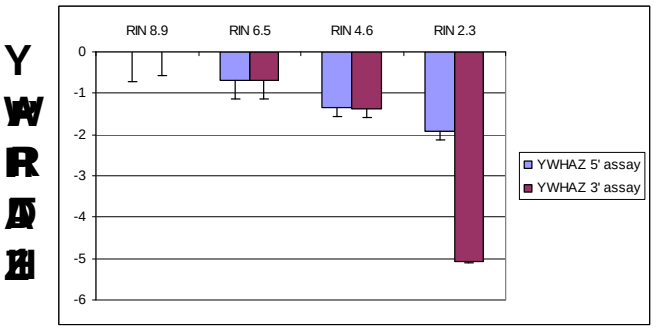
GAPDH

HPRT1

YWHAZ

intron 2-3: 23.6 kb

Results:

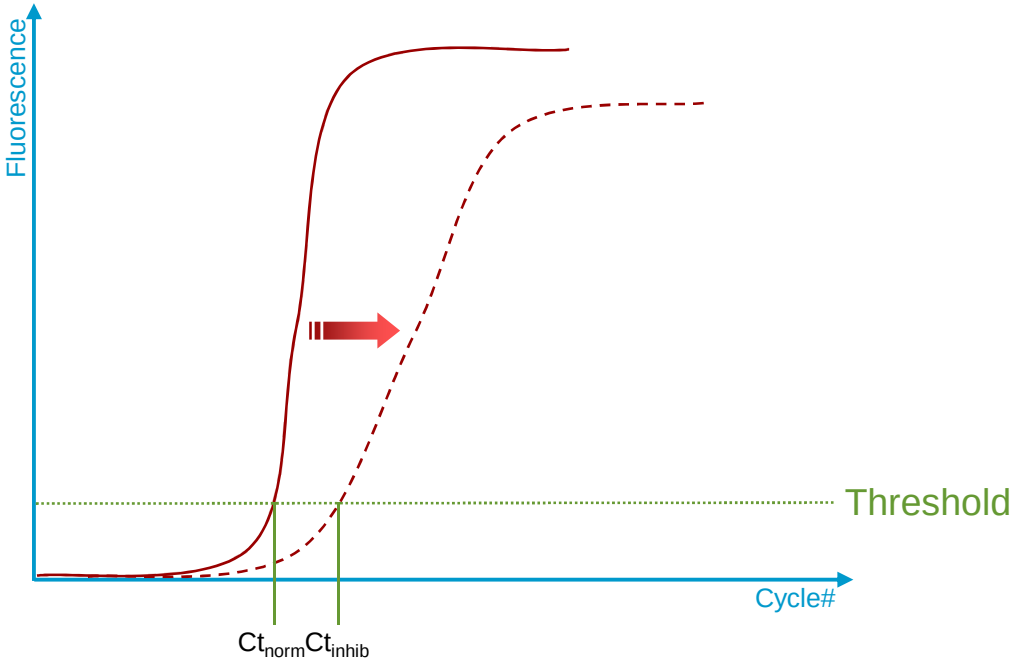


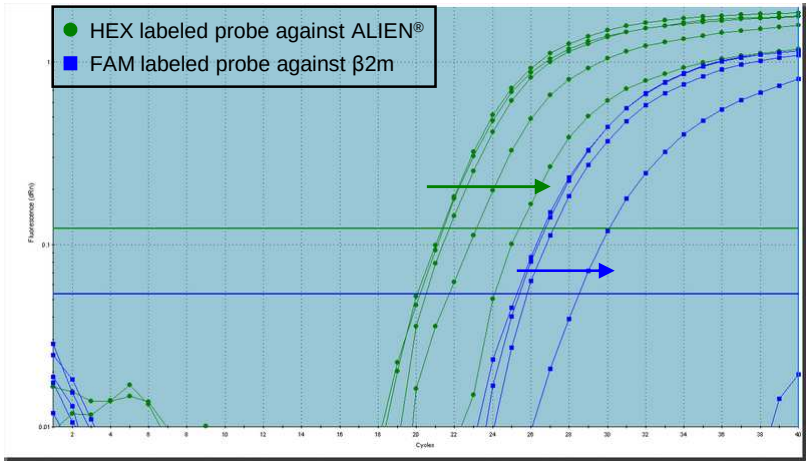
Why DNA Quality matters

DNA degradation in preserved biological tissue, forensic samples or samples commonly used in pathogen detection can negatively impact assay performance and produce misleading results

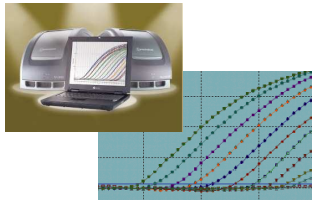
Sample Quality - Inhibition

- Various compounds can act as inhibitors: eg. phenols, polysaccharides, cell debris, EDTA, lipids, high amounts of rRNA + tRNA or gDNA, components of RT reactions,
- Inhibition affects the kinetics of the PCR reaction resulting in later Ct values and reduced amplification efficiency





- Inhibited samples either need to be excluded from quantification or if possible rerun at higher dilutions
- Internal positive controls (eg. ALIEN® Inhibitor Alert) are a good tool to monitor the varying degrees of inhibition in each sample



Real-time PCR

Assay Optimization

DNA/RNA extraction

Optimization of sample extraction for yield, quality and low amounts of inhibitors

Nucleic acid quantification and QC

Reverse Transcription

Choosing the right enzyme and priming method to increase yield

QPCR assay validation and optimization

Optimize primer and probe concentration for increased specificity and efficiency

QPCR

QPCR uses small amplicons. Shorten thermoprofile timings for increased speed

Summary

- It is important to understand sources of experimental variance
- If variability exists try to minimize this by adjusting your experimental design
- All types of quantification assume similar template quality
- Assay validation and optimization are not optional but crucial to obtain robust and meaningful results
- Make sure the analysis parameters are meaningful and make sense in relation to the data obtained in the experiment
- With the drive to detect ever smaller quantities or fold changes it is highly important to address experimental variability at all stages
- Finally we have to accept that in the worst case the overall variability may prevent us from achieving a certain sensitivity of our results

Importance of Experimental Design in QPCR

Thanks for your attention!