Importance of experimental design and optimisation in QPCR
Experimental Design
Understanding experimental variance

Experimental Design

Sample preparation and purification

Post-run Analysis

Real time QPCR

Reverse Transcription

Total RNA

cDNA

Agilent Technologies

QPCR Seminars 2012
Make sure your sampling doesn’t introduce a bias

Smiley gene is expressed in 1/10 of cells:

SMILEY present at $10^6$ copies per cell:
QPCR of 10 mixed cells would give a result of $10^5$ copies per cell

SMILEY is present at 10 copies per cell
QPCR of 10 mixed cells would give a result of 1 copy per cell (and be challenging to detect)
Possibly giving a negative result

Tissue complexity may result in UNDERESTIMATION of quantity
Sample Preparation
Influence of Sampling and Sample Preparation

Smiley gene is expressed in 1/10 of cells:

Cut around position 1 –
No SMILEY gene detected

Cut around position 2 –
SMILEY gene detected in 4/8 cells

Cut around position 3 –
SMILEY gene detected in 1/10 cells

POSITIONAL effect of cell type sampled
effects gene quantification results
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!
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.

- **Reduced population of DNA with full length of amplicon:** Underestimation of quantity
- **Small DNA fragments compete with primers:** Unspecific amplification,
- **Competition by abortive amplicons:** Loss of sensitivity or inhibition
- **Alterations of bases:** Reduced affinity of primers and probes

Assay performance and success

QPCR Seminars
Agilent 2100 Bioanalyzer
The Agilent 2100 Bioanalyzer platform

Fast and automated separation, sizing and quantification by miniaturized on-Chip electrophoresis. Comparable but superior to traditional *slab-gel-type analysis*.

Optional available: easy access to desktop flow cytometry

**On a single Platform to perform:**

- Sizing, Quantitation and Purity of *Proteins* (5 - 250 kDa)
- Sizing, Quantitation and Purity of *DNA fragments* (25 – 12000 bp)
- Integrity check, Separation and Quantitation of *RNAs*, Small RNA sizing
- *Cell Fluorescence Assays* with stained cells (Apoptosis, Transfection, Expression, )

- Fast results
- High Reproducibility
- Qualitative and quantitative in one run
- Digital and normalized data
- Easy to use
- Small sample volumes

1. Load sample  
2. Run analysis  
3. Analyze data
Agilent 2200 TapeStation
ScreenTape

- Exploit microscale benefits
- Budget
Customer Genomic DNA samples
0.8% Agarose and Genomic DNA Screentape

All samples were derived from blood and were purified by precipitation (no column-based cleanup). Some of the samples were approx. 10 years old.
ScreenTape system overview

1. **ScreenTape**
   - Place ScreenTape and some tips in the TapeStation.

2. **2200 TapeStation**
   - Place your samples in the TapeStation and press ‘Start’ on the instrument controller software.

3. **TapeStation Analysis Software**
   - View your analysed results in around 1 min per sample.
TapeStation Software RNA file

1. Gel Image
2. Electropherogram
3. Tabulated results:
   - Quantity
   - 28s/18s ratio
   - RIN<sup>e</sup>
4. Easy report setup and preview
5. RNA QC on ScreenTape with RIN<sup>e</sup> result
6. Colour coded RIN<sup>e</sup>
Control for Inhibition

- Inhibition by various substances in your sample can lead to delayed detection or failure of amplification.

- Known inhibitors are eg. lipids, phenol, polysaccharides, guanidiniumHCl

- To test for inhibition the most common way is to have an internal positive control (IPC) at low copy numbers

- **Alien® QRT-PCR Inhibitor Alert** is a Stratagene solution for SYBR green
  - Polyadenylated Alien® transcript as spike-in to your RT reaction
  - Designed to have no homology to known sequences
Reverse transcription is a main source of error in RT-qPCR

The RT reaction is only 30-40% efficient (e.g. for input RNA 1 mg, cDNA ~300-400 ng)

Sample-to-sample efficiency is variable

Therefore optimizing the RT step improves your PCR results

High quality RNA gives the most reproducible and robust results
  ➔ Essential for detection of low abundant transcripts

MMLV based enzymes (AffinityScript™ RT) usually work at higher temperatures
  ➔ Enables full-length cDNA from RNA with high secondary structure
  ➔ RNaseH activity can improve PCR sensitivity from GC rich messages

RT is a non-linear process: Standardize your input amount
  ➔ Use of same amount of RNA (or same number of cells) for all samples

RT reagents are inhibitory to PCR ➔ dilute the reaction
Real-time PCR
Assay Design

Get all necessary sequence information:
Many databases available

Use transcript structure information (exon/intron information):
Enables transcript variant specific design

For species or subtype specific design get as many related sequences as possible
Align sequences to find conserved regions or regions specific to sequence of interest

Detect secondary structure in your sequence:
Use mfold to avoid regions with stable secondary structure
http://frontend.bioinfo.rpi.edu/applications/mfold/
Primers and probes don’t bind with high affinity to regions with secondary structure
Amplicon length affects assay performance:
- In general amplicon length between 70 – 300 bp is recommended.
- Genotyping: Small amplicons 70 – 150 bp are preferred

Amplicon position should reflect template quality:
- Degradation of template can cause failure of amplification.
- RNA Degradation: depending on directionality a 5’ or 3’ biased design might fail to produce a PCR product

Ensure specificity by BLASTing amplicon sequence:
- Especially in Pathogen detection it is obligatory to ensure uniqueness of amplicon design: BLAST search against database.
- Previous alignment with related as well as unrelated sequences allows good initial choice of position.
Assay Design Considerations

Primer Design

- Avoid long primers (> 25 bp) and BLAST primers: Specificity is key to avoid getting false positive results
- Avoid strong GC clamps and degenerated primers: Not more than 2 GC in the last 4 bp

- Design Primers against a region without stable secondary structure: Binding affinity of oligo will be higher
- Aim at a Tm of 60°C and a Tm difference < 2°C: Reduces probability of primer dimers, increases specificity and allows reuse of SYBR primers in a probe based chemistry

- Gene expression: At least one of the primers should overlap an Exon-Exon junction: Avoids amplification from genomic DNA
Assay Design Considerations
Probe Design

Probes

**Avoid long probes to ensure specific binding:**
Probes should be in the range of 17-30 bp (17 – 25 bp in genotyping)

**Design probes against a region without stable secondary structure:** Ensures high affinity probe binding

Probes

**Probes should be close to primer on same strand:**
- 4 – 15 bp distance between 3‘ end of primer and 5‘ end of probe.

**Tm of probe should be higher than primers:**
- 5 – 10°C higher to ensure probe binding before primer binding

Probes

**Probes are obligatory in diagnostic pathogen detection:**
Avoids false positives due to detection of unspecific amplification
Real-time PCR
Assay Optimization

Optimizing your assay can help you to

Increase specificity: Get rid of unspecific amplification
eg. primer dimers

Increase sensitivity: Get earlier Ct values, detect lower
concentrations

Increase reproducibility: Low replicate variability,
high amplification efficiency

Assay optimization will improve assay robustness
and minimize assay variability

QPCR Seminars
2012
Real-time PCR
Assay Optimization

Why optimize forward and reverse primer concentrations?

• It is difficult to design a primer pair with identical $T_m$. Even with theoretical identical $T_m$: In real life differences exist.

• Changing annealing temperature affects all oligos in the reaction at the same time and only optimizes specificity.
• $T_m$ (affinity to template) of primers depends on concentration: perform a primer matrix test to identify optimal concentration

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• choose concentrations that results in the lowest Cq and still gives signal
**Assay Optimization**

Primer titration 50 nM – 200 nM
duplicates for pos. Control & NTC

**Aims:**
- low Cq values → sensitivity
- no unspecific amplification or primer dimers → specificity
- Low inter-replicate variability
- high efficiency of Amplification → separate run
Real-time PCR
Assay Optimization

Optimal concentration is the lowest concentration that results in

• the lowest Cq
• Minimal variation between replicates
• and adequate fluorescence
### Effect of efficiency on copy no.

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Real-time PCR
Multiplexing - Optimization

Validation of multiplex

Acceptable limits of variance
• Efficiencies within 5%
• ΔCq less than 1 Cycle difference
Assay Design Considerations
Multiplexing – Success Starts at Design Stage

• All assays compete for the reagents in the reaction
  → Similar amplification efficiencies are key for successful multiplexing

• Design considerations for multiplexing:
  • All amplicons should be within ±5 bp and have similar GC content (±2-3%)
  • Maximum Tm difference for multiplex primer sets ±1°C
  • Tm of probes should be within 1°C
  • Use non-fluorescent quenchers to reduce background (BHQ, DarkQuencher)
  • Use software that is able to validate multiplex designs
Real-time PCR
Multiplexing - Optimization

• Optimize all individual assays to achieve optimal performance:

• Get every single assay to its peak performance
  → Use primer matrix titration and titration of probe

• Determine limiting primer concentration for the most abundant target

• More Targets need more reagents
  → Increase Polymerase, dNTP and Mg$^{2+}$ as they will be limiting a lot earlier
  or use a mastermix format like Brilliant® QPCR Multiplex Mastermix

• Sometimes individual reagent optimization is necessary to avoid compromising sensitivity. (Brilliant® QPCR Core Reagents)
Real-time PCR
Multiplexing - Optimization

Optimize all individual assays to achieve optimal performance:

- Assemble those assays that have similar efficiencies into a multiplex
  - Validate all assays as single and multiplex on the same plate

Probe titration
Ensuring Quality of Results
Assay Validation

Validating an assay generates valuable assay performance data:

- The specificity of your primers and probes
  Melting curves, appropriate controls

- The working range and sensitivity of your assay
  Standard curves

- The reproducibility of your experiments
  Replicates, Statistics

Assay validation makes it easy to avoid or understand unexpected results in future experiments.
Real-time PCR
Assay Validation - Controls

Controls enable you to understand unexpected results and are necessary components of assay validation:

No Template Control (NTC):
Template contamination, primer dimers, probe degradation

No Reverse Transcriptase Control (no RT):
Amplification from genomic DNA

Negative Sample:
Non-specific amplification (non-specific primer/probe binding)

Positive Controls:
Necessary component of assay validation, control for inhibition

Only controls will tell you which data is good or bad!
Real-time PCR
Assay Validation - Specificity

Electrophoresis
- Size information
- Primer dimers
- Non-specific products

SYBR Dissociation Curve
- Tₘ information
- Amplicon heterogeneity
- Primer dimers
- Non-specific products

Sequencing
- Only necessary in specific cases
- Subtle amplicon variations
Dissociation Curve

Temperature (°C)

Fluorescence (-Rn(T))

Template 86.5°C

Primer dimer 79°C
Real-time PCR
Assay Validation - Specificity

Benefit from the superior resolution of the Bioanalyzer:

- **Validation of amplicon size**
  - oligo-dT random: Size 110 bp
  - oligo-dT random: Size 121 bp

- **Validation of unclear results**
  - NTC: Size 21 + 51 bp
A standard curve enables you to identify the linear working range and the efficiency of your assay.

Perform a serial dilution series over a range of concentrations that reflects your experimental samples with a minimum of 4 orders of magnitude (better 6-8) with 6-8 individual standards.

Use replicates for your standard curve enables outlier detection and statistics.

Properties of a good standard curve:

- high efficiency (80% < 85% < 90% - 105% < 110% < 115%)
- good R² (> 0.98)
- low replicate variability for individual standards
Standard Curve

Serial dilution of pre-determined known quantities

Plot of the log of the initial template quantity

- Use: Absolute concentration determination of unknowns

If slope = -3.323
Then efficiency = 100%
Eff. = 10^{-1/slope} - 1
Summary

- It is important to understand sources of experimental variance
- If variability exists try to minimize this by adjusting your experimental design
- DNA/RNA quality can have a dramatic effect on QPCR results
- For successful QPCR it is advisable to optimize sample preparation methods to achieve highest template quality possible
- Assay validation and optimization are crucial to minimise variation and obtain robust and meaningful results
- 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 and optimisation in QPCR

Thanks for your attention!