Optimization, Validation and Troubleshooting Single and Multiplex QPCR Assays

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Importance of Experimental Design and Assay Validation

QPCR is the optimal method for identifying and verifying changes at the RNA or DNA level

- High sensitivity and specificity of good assay
- Can use limiting amounts of material to generate data

Quality results start with a good assay design and a sound strategy for characterizing biological events

How data is reduced from Ct to reported value can confound experimental results.
QPCR...just put in the nucleic acids and go, right?

• Performance can be good right out of the “box”
• Less emphasis on assay validation/optimization
• Does the assay fit your experimental needs?
• Will the results lead to accurate data interpretation?

• How much work is required to get the assay to a point where I can run my valuable samples and get reliable data?
• will I have the informational leverage to make the right decisions?
The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments

**BACKGROUND:** Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader’s ability to evaluate critically the quality of the results presented or to repeat the experiments.

**SUMMARY:** Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.

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The fluorescence-based quantitative real-time PCR
QPCR Assay Design Considerations

• Initial optimization efforts should identify good control or standard RNA or DNA that you can rely upon throughout data generation

• Generate a range of acceptable QPCR performance data

• Controls should dictate what data is good or bad

• Practice setting up assay, its not a trivial task

• Always try and start with good RNA or DNA of known concentration and purity (Gel, BioA, OD260/280/230)

  If RNA/DNA damaged, make amplicon as small as possible, ~100bp
RNA QC in Routine Gene Expression Workflow

Cells / Culture

RNA isolation

Total RNA

RNA QC via Agilent 2100 bioanalyzer

RIN

Software feature RIN as independent and standardized measure of sample quality

RIN above threshold, ie RIN>8

Continue with downstream Experiment (Microarray, real-time PCR, etc.)

Start again with sample isolation
RIN has become the Gold Standard for RNA quality

- RNA of ≥8 is adequate for QPCR and microarray
- The use of an invariant endogenous transcript for normalization mitigates the effect of RNA quality on quantitation
Cycle of QPCR Assay Development

1. Design Primers
2. Assay Standard curve With negative controls
3. Prime Specificity?
   - No
   - Yes
4. Good %Efficiency?
   - Yes
   - No
5. Quantitative range adequate?
   - Yes
   - No
6. Primer optimization Matrix; Exon cDNA Specific?
   - Yes
   - No
7. Normalization strategy Stability?
   - Yes
   - No
8. Analyze precious samples
QPCR... A Race to Quality
A Sprint and a Marathon... Assay Quality depends upon first few cycles

Primer Dimer

Specific Amplicon

Melt
Anneal primers
Taq

Melt
Anneal
Extend
Detect

Compromised Detection

Accurate Quantification

gDNA, Alternative Sequences, Matrix Effects
Assay Controls and Standards
Leverage for objective data interpretation

• Easy to justify good vs bad data; failed PCR, matrix, quality
• Positive control material should reflect intended sample complexity and matrix, and depends upon assay type
• Numerous examples exist when samples did not perform the same as a plasmid in buffer (gene expression)
  • Spike plasmid or other positive into cDNA background
  • Purified pooled reference RNA, ie Stratagene Universal Reference RNA (human, rat or mouse URR)
  • Armored RNA (phage, resistant to Rnases), ivt cRNA template
  • Documented SNP containing DNA, ie Coriell, CDC (genotyping)
• Negative controls provide reference of assay bounds
  • Zero template is not often zero signal/noCt in QPCR
  • Include NTC (Primers alone) as well as NoRt (RNA prior to RT)
QPCR Standard Curve

Tool for QPCR Validation, your first test

1:5 Serial dilution of cDNA,

7-10 pts in triplicate

~300nM each primer

Expect:

Good linear fit ($R^2 > 0.98$)

High efficiency ($E = 90 - 110\%$)

Stratagene Brilliant II SYBR MM
**Negative vs Positive QPCR Controls**

**Desired “NoCt” call for negative controls**

- NoRT tests for DNA, pseudogenes: $Ct \leq$ test sample, throw out
- NTC test for contaminated MM, primer dimer, spread out, prepare separately
QPCR Standard Curve
Tool for QPCR Validation

- What is the resolution of the assay?
- Can I detect replicates with precision?
- What is the quantitation cut-off at the high and low end of “expression”?
- Does the final assay range have a good %E?

Eff=105.6%
$R^2=0.97$
QPCR Standard Curve Tool for QPCR Validation

- Determine acceptable range of Cts for input into Rel Quant calculations in downstream sample analysis, ie 11-30 Ct range

\[ Eff = 105.0\% \]
\[ R^2 = 1.0 \]
Optimizing for Assay Sensitivity and Specificity

• To determine that the reactions are specific, turn to the melting curve of a DNA binding dye assay

• Generally, specific primers that are not distracted with creating non-specific products yield an Efficiency ~100%

• More sensitive and better resolution
DNA Binding Dye - i.e. SYBR-Green, EVA-Green

- DNA + free dye (weak fluorescence)

- Binds dsDNA (increased fluorescence)

- PCR Amplification
  - Δ Temperature
QPCR Assay Controls
Negative QPCR Controls

Unknown
Good!

NoRT  NTC
Negative vs Positive QPCR Controls

Template Contamination

NTC

Unknown
Negative vs Positive QPCR Controls
One Peak?

No Template Control (NTC)

Standard Curve Template
Negative vs Positive QPCR Controls
One Peak or Two?

Dissociation Curve

- Primer Dimer
- Unknown
- NTC
Negative vs Positive QPCR Controls Or Three?

- Will the results lead to accurate data interpretation?
Beyond Melting Curve Analysis: Agilent BioAnalyzer™

- Why? SYBR Green is a non-saturating dye
- Melting temperatures of products can be misleading based upon length, sequence, and structure
- Gel analysis may not yield needed resolution
- BioAnalyzer analysis will give accurate high resolution information about what is being made in QPCR tube
BioAnalyzer Electrophoresis System

The “Chip”
- Microfluidic capillaries filled with sizing gel and nucleic acid (NA) specific dye
- Samples placed in wells
- Migrate out of well, dye labeled NAs separate on size

The BioA “Box”
- 16 independent electrical probes apply current sequentially across samples
- Fluorescently excited NAs pass detection window
- Background subtracted fluor detected

The Data
- SW algorithm measures each peak
- Calculates quantity and size
BioAnalyzer
Concordant results with SYBR Melt

GAPDH 5' assay: Expected size 118 bp
oligo-dT random
Size: 110 bp

HPRT1 3' assay: Expected size 114 bp
oligo-dT random
Size: 102 bp

Agilent Technologies
BioAnalyzer
Higher resolution analysis of products

NTC 21 + 51 bp
Size: 120 bp
My Standard Doesn’t fit my Need

• **Low sensitivity, Low Efficiency, Short range**
  - Change annealing times and temperatures to favor the lower Tm primer
    - Longer time and lower temp
  - Add more of both primers if assay is specific
  - Re-assay at a greater resolution, 2 fold dilutions

• **Poor Precision, High Efficiency (>100%)**
  - Investigate different primer design
  - Re-assay primers at different use concentrations
    - Not always both primers that are “promiscuous”
      - Limit the bad primer and salvage the pair
**Primer Optimization Matrix**

**Individuated Ideal [Primer]**

<table>
<thead>
<tr>
<th>Mid-range [RNA] plate + NTC</th>
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<td>Vary forward and reverse concentrations in rxn</td>
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<table>
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<th>Forward [Primer]</th>
<th>75 nM</th>
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**Goal:**
- Select Forward and Reverse ratio that yields lowest Ct
- Identify minimum, optimal concentrations
- Reaction with no non-specific products
- Future Multiplex QPCR should be easier to set up
Mitigating Data Effects of non-Specific Signal and Poor Std Performance

Dilute primers to optimal concentrations

DNAse1 treat/clean up RNA using column

• Agilent Absolutely RNA micro column…on column tx

Develop cDNA specific amplicon, span exons

Avoid quantitating at the low end of the assay where the incidence of +NTC may be higher

Track data and understand day to day variation

Redesign primers, order 2 sets, shift 1-3nt from original site
Which Chemistry?

• No real consistent difference in assay quality parameters such as sensitivity
• DNA binding dyes, ie SYBR Green, EVA-Green are reliable and provide analysis of specificity
• Primer non-specificity requires optimization/design around
• Probes allow multiplex QPCR, and add additional layer of detection specificity
• But can present their own issues with troubleshooting chemistry

• Ultimate decision can be economic/convenience
  – Have transient need to screen lots of genes > DNA binding dye
  – Have persistent desire to assay fewer genes, lots of samples > multiplex probes

• Quality QPCR data is driven by the reliability and specificity of priming; if you don’t make it you will not detect it
Plan to optimize using SYBR chemistry

• Constrain the Tms of primers to approx. 60°C
• Optimize on the basis of efficiency and specificity
  – primer concentration-matrix

Choose an amplicon that contains sequence that is compatible with probe based chemistry

Order just primers, work out conditions, run samples until Sybr chemistry limits utility of PCR or multiplex is desired

• Order probe and confirm measuring range of assay.
Subtle differences in efficiency between RNAs may exist, and is part of the expected variability within an assay.

- Due to differences in RNA quality, priming, RT reaction robustness, inhibitors.
- Goal is to reduce these variables by using high quality RNA, employing controls, and testing steps

Very likely there will be differences in efficiency between PCR assays

- Goal is to minimize differences through optimization
- Adjust calculations to reflect true efficiency
- Apply a normalization factor from non-experimental gene or assay
Experimental Design
QPCR Normalizer

Function:

• Correction factor for amount of sample represented in every reaction. (technical error)
  – Control for Rt, QPCR, Nucleic Acid quality
  – Biological changes in sample

Properties:

• No Trending and low variation across experiment
• Ideal if Ct similar to that of your GOI
• Must be validated to show is a good choice
One normalizer does not fit all....

Gene regulation is species and experiment-specific
“Normalization is just GAPDH or β-actin – right?”

β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels

Decreased β-actin mRNA expression in hyperglycemic focal cerebral ischemia in the rat

The Housekeeping Genes GAPDH and Cyclophilin Are Regulated

Differential expression of GAPDH and β-actin in growing collateral arteries

Peptidylprolyl Isomerase A (PPIA) as a Preferential Internal Control Over GAPDH and β-Actin: Quantitative RNA Analyses

J. Dairy Sci. 86:3423–3429

Why normalize?

Remove sources of variation

• Technical error
  – Physical – user, pipettes, instrument
  – Chemical – sample quality, inhibitors, RT efficiency, QPCR efficiency
• Biological – covered by biological reps

Greater precision in gene expression measurements
Normalization of Gene Quantitation

Ideally normalize for:

RNA Prep:
- Sample size error
- Sample quality
- RNA prep quality

RT Step:
- RNA quant error
- Pipetting error
- RT inefficiency

QPCR Step:
- Pipetting error
- QPCR inhibition
Normalization to Total RNA in QPCR...we all do it

Improvement on sampling size

Does not capture variation post-RNA extraction step

Requires RNA quantitation

Total RNA may not reflect mRNA fraction (1 – 5% of total RNA)
Normalization to Total RNA in QPCR...we all do it

Eg: # of cells, mass of tissue, ugs RNA

Many assumptions:

• Cellularity is the same across tissues
• Sample extraction uniformity
• RNA quality consistency
• RT efficiencies across tissues
• QPCR efficiencies
• RNA per cell, mRNA per RNA
Assumptions about total RNA. Normalization to mass RNA not always safe

T. Schmittgen, JBBM., 46, 2000
Normalization to Reference genes

aka “house-keeping genes”
aka “normalizer genes”
aka “endogenous controls”

• Best normalization choice should come from the same biology, and be there from the start of the experiment, ie in the cell
Reference genes
Currently the best methodology

Pros:
- Considers all sources of technical error:
  - RNA prep
  - RNA quant
  - RT step
  - QPCR step

Cons:
- Requires RNA quant
- Requires assay for each Norm
- Requires stable genes
- Assumes equal RT for GOI and normalizer
- Requires validation
Reference genes

How do I use reference genes?

• Pick candidate reference genes
  • Literature review for your model, what has been validated as a good candidate normalizer?
  • Review articles on normalizer targets
  • Array data, if part of experiment

• Design and validate QPCR assays
  – Ensure similar QPCR efficiencies for both
• Quantify in real samples with treatments etc
• Assess expression stability

• Take the most stable → normalize to these!!
Reference genes
Assessing stability

Treatment A

Candidate reference gene assays

Treatment B

Manual assessment

Software assessment
Assessing stability

Software Assisted Normalization Selection and Normalization Factor Calculation:

- **geNORM** (Vandesompele et al, Genome Biol 2002, 3: 34.1-34.11)
  - Uses Relative Quantity or Quantity
- **BestKeeper** (Pfaffl et al, Biotechnol Lett 2004, 509-515)
  - Uses Ct value
- **MultiD Software package** (http://www.gene-quantification.de/genex.html)
  - Has statistical package as well as geNORM fxn, sophisticate, $
Normalizer Target Software

Research

Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes
Jo Vandesompele, Katrien De Preter, Filip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe and Frank Speleman

Address: Center for Medical Genetics, Ghent University Hospital 1K5, De Pintelaan 185, B-9000 Ghent, Belgium.
Reference genes
Other methods of Normalization

• Global Pattern Recognition (Akilesh et al., Genome Research, 2003)
• Equivalence test (Haller et al., Analytical Biochemistry, 2004)
• ANOVA test (Brunner et al., BMC Plant Biology, 2004)
• Normfinder (Andersen et al., Cancer Research, 2004)
• Szabo et al., Genome Biology, 2004
• Abruzzo et al., Biotechniques, 2005
Reference genes

Summary

Housekeeping genes control for most sources of variability

**BUT**

Are tissue & experiment specific

Must be validated

May require > 1 gene for robust data

Best advice is to assay a few Housekeeping genes and determine if the inclusion of the normalizer drastically changes the data
Exogenous controls

What: “Spiked-in” sequences at known concentration
  • RNA or DNA oligo templates

When: No stable normalizer available

When: detection/ titering applications, eg: virus or bacteria
DNA spikes

Sample RNA extraction and quantification

DNA spike preparation

Linear Plasmid

RT rxn

normalization of target Ct to spike Ct

QPCR rxn

Agilent Technologies
RNA spikes

Sample RNA extraction and quantification

RT rxn

Alien Poly A+ mRNA

RNA spike preparation

normalization of target Ct to spike Ct

QPCR rxn
Alien™ spikes

Artificial DNA or polyA+ RNA template

No homology to any sequence submitted to GenBank

Primers provided

Spike control can be used as:

• Inhibition indicator due to matrix effects
• Internal positive control
• Normalizing Ct for QPCR data analysis
Alien™ spikes act like housekeeping gene in inhibition assay

- Alien and B-Actin QPCR to model effect of inhibition of RT by addition of EDTA
- Alien can be used to detect inhibition as well as contribute a normalizing factor
Genomic DNA Normalizers

What: Normalize to genomic equivalents in sample from gDNA

When: if RNA quant not possible, eg LCM or embryo samples, small sample size

Validated single copy gDNA target
Requires both RNA and DNA to be captured and stabilized in solution
Genomic DNA

Work flow

RNA & DNA stabilization

RNA + DNA prep

RT QPCR

QPCR

QPCR – gDNA and mRNA targets

Normalized Gene of Interest QPCR
Genomic DNA Normalizers

Extraction method must recover RNA and gDNA

**Agilent SideStep Lysis & Stabilization kit**

Do not have to quantify RNA

gDNA specific primers detect validated single copy gDNA sequence

cDNA/intron spanning specific primers essential to detect GOI
Agilent SideStep™
QPCR Reagent

Direct to QPCR nucleic acid analysis from small amounts of material
• Fast, stabilization of RNA and DNA, direct to QPCR
• No Loss, enhanced assay sensitivity

Offers the opportunity to normalize to genomic DNA targets in lysate
• Good normalization strategy for gene expression or copy number analyses
• Stratagene designed/validated single copy primers
QPCR with SideStep Cell Lysate

- Two-fold serial dilutions of SideStep HeLa cell lysate
- DNA-specific primer set was used in SYBR Green QPCR
- RNA-specific B2M TaqMan primers and probes were used for 1 step QRT-PCR

%E (gDNA) = 101
%E (β2M) = 97

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<th>RT-PCR (Ct)</th>
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Strategies for Normalization

Summary

Normalization is system-dependent

Validated reference genes are the gold standard for gene expression

Other methods compromise but may be necessary in some contexts

New methods under development may simplify normalization in the future
Conclusion

• Positive and negative controls determine your bounds of performance. Use them.
• If you get good Standard curve performance, move on.
• Priming determines quality of assay…start with SYBR.
• Minimize primer/probe concentrations in singleplex format to reduce the probability of oligo duplex formation.
• Confirm that Ct values, efficiencies and LOD do not change dramatically when shifting to a multiplex format.
• Limit primers of dominant PCR if necessary.
• Increasing reagent concentration will allow easier optimization of multiplex QPCR reactions.
Thanks!

- Quality, publishable data requires optimization of assay

- Agilent provides products and support to help you succeed in QPCR

QPCR technical support:

Email: QPCR@Agilent.com
800-894-1304, La Jolla, CA