iQ5 & MYQ™ Real-Time PCR

UNIVERSITY OF CALIFORNIA
UC RIVERSIDE
Institute for Integrative Genome Biology
Why Real Time PCR?

- More accurate than RT PCR
- Publications are requiring real time PCR data rather than RT PCRs
- Ease and speed of quantification
- Can do many technical replicates without exhausting cDNA template
Real-Time qPCR provides information for **relative** or **absolute** measurements of starting material.

- Gene Expression Studies
- Chromatin Immunoprecipitation (ChIP)
- Microarray Validation
- Transgene Analysis/GMO Testing
- Viral/Bacterial Load Studies
- Allelic Discrimination/SNP
Limitations of Conventional PCR

Amplification is exponential initially

In theory, the amount of DNA produced at every cycle should double,

Product = Template \times 2^n 
(n = \# of cycles)

But the exponential phase becomes linear as reactants become rate-limiting
RT PCR is an End-Point Analysis

96 identical reactions will have very different final amounts of fluorescence at endpoint due to differences in kinetics and pipetting.
Quantitative PCR

Through the use of fluorescent dyes that detect PCR products, real-time PCR measures the reaction directly during amplification.
iQ5™ Utilizes Filter Wheels

- Light Source (Simple halogen bulb)
- Excitation Filter
- Emission Filter
- Detector
Emission Filter Windows

- iQ5 detects up to 5 dyes colors
- MYQ detects SYBR Green only
Detection of quantitative Data

Theoretical vs. Observed Detector

Log Target DNA vs. Cycle #
Intercalation Dyes: SYBR Green I

Displays strong fluorescence in the presence of dsDNA
Cleavage-based assay: TaqMan™

Add iQ Supermix, Hybridization Probe and sample

Denaturation

Annealing
Cleavage-based assay: TaqMan™

Extension Step
Threshold Cycle ($C_T$)

- Assigned point at which the fluorescence rises appreciably above background
- The $C_T$ can be placed anywhere in the exponential (log-linear) phase
Results are More Accurate in the Log Phase

96 identical reactions will have nearly identical $C_T$ values when measured at the linear phase of amplification.
Threshold Cycle ($C_T$)

- At the CT, the fluorescence correlates well with the starting copy number
- BioRad claims a dynamic range of 9 orders of magnitude
- Each 10x dilution equals 3.3 cycles

\[
2^n = 10 \text{ fold} \\
n(\ln 2) = \ln 10 \\
n = \frac{\ln 10}{\ln 2} \\
n = 3.32
\]
1) To perform absolute quantification
2) To examine the efficiency of the qPCR reaction (This can influence the results of your experiment significantly)
C<sub>T</sub> Values Determine the Copy Number

Standard Dilution Series can be used to...

...determine the Copy Number or Concentration of an Unknown
Example of Good Efficiency

- Efficiency >90%
- Correlation Coefficient >0.9
Example of Unreasonably High Efficiency

How do you get an efficiency of >200%?
Real-time PCR Efficiencies

Efficiency = $10^{-\frac{1}{\text{slope}}}$

% Efficiency = $10^{-\frac{1}{\text{slope}}} - 1 \times 100$

- **Primer-dimer!**
- Pipette calibration
- Dilution Errors

Target Range

- 110%
- 90%

- Secondary structures
- Pipette calibration
- Dilution errors
Optimization of RT-qPCR: Annealing Temperature

Primer Dimers Raise the Apparent Efficiency

A = Template + primers  B = Template only (NO primers)

data from Walling lab

Melt curve
What Are Primer Dimers?
Primer Concentration Affects qPCR Efficiency

Optimization of RT-qPCR: Primer Concentration

Primer Concentration Affects qPCR Efficiency

Optimization of RT-qPCR: Primer Concentration

data from Walling lab
Primer Concentration

Primer-dimers can be influenced by template concentration

Fluorescence vs Temperature  $dF/dT$ vs Temperature

- **10,000 copies template**
  - $dF/dT$ vs Temperature graph showing a peak at a certain temperature.
  - Temperature (Celsius) range from 50 to 100.

- **2,000 copies**
  - $dF/dT$ vs Temperature graph showing a different peak.
  - Temperature (Celsius) range from 50 to 100.

- **400 copies**
  - $dF/dT$ vs Temperature graph showing an intermediate profile with both dimers and amplicon peaks.
  - Temperature (Celsius) range from 50 to 100.

- **No template control**
  - $dF/dT$ vs Temperature graph showing dimers only.
  - Temperature (Celsius) range from 50 to 100.
Genomic DNA Contamination Can Affect PCR Efficiency

Optimization of RT-qPCR: Genomic DNA Contamination

- DNAse treatment will reduce contamination
- Also design primers across intron/exon boundaries

data from Walling lab
Melt Curve Analysis

Analysis at the end of the qPCR run to determine the melting temperature ($T_m$) of PCR products

Plot rate of change of fluorescence vs. temperature
Why Do Melt Curve?

• Identification of non-specific products

• Mutation detection/allelic discrimination
Primer Dimers Raise the Apparent Efficiency

Optimization of RT-qPCR: Annealing Temperature

A = Template + primers   B = Template only (NO primers)

data from Walling lab
iQ5 Features for Reaction Optimization

• Continuous Data Collection
• Thermal Gradient
• Melt Curve
Continuous Data Collection

- iCycler iQ features a continuous data collection that is designed to focus analytical scrutiny on subsets of the data gathered
  - Optimize annealing/extension time
  - Chemistry kinetics
Optimizing PCR Reactions: 2-Step PCR vs 3-Step

In some cases, can eliminate extension step since all amplification has already occurred during annealing.
iQ5/MYQ Thermal Gradient

- Used for annealing/extension temperature optimization for PCR reaction specificity and efficiency
- Up to 24°C gradient range programmable across block.
iQ5/MYQ Thermal Gradient

Look for specific product formation without primer dimers

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Dilution series of primer [ ]

Temperature Gradient (annealing temperature)
How do I get started?
RNA preps (Qiagen)

cDNA prep (1μg starting RNA) using 2-step kits
- Ambien Retroscript for RT
- In Vitrogen Superscript III
- BioRad iScript RT

Dilute cDNA template (~1:10)

qPCR (BioRad SYBRGreen SuperMix)

Data analysis
### Reaction Set-Up

<table>
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<tr>
<th>Component</th>
<th>Volume/Concentration</th>
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<tr>
<td>SYBRGreen SuperMix (Biorad)</td>
<td>12.5ul of 2x</td>
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<tr>
<td>Primer forward (10uM)</td>
<td>0.5ul (200nM) (can use 100 to 500nM)</td>
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<tr>
<td>Primer reverse (10uM)</td>
<td>0.5ul</td>
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<tr>
<td>Water</td>
<td>9.5ul</td>
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<tr>
<td></td>
<td>23ul</td>
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<tr>
<td>Template</td>
<td>2ul (typically 1:10 dilution cDNA)</td>
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</table>

**Total volume**: 25ul

Set-up can be at room temperature if using the BioRad SuperMix other hot-start Taq polymerase.
**Plate Set-Up**

Set-up can be at room temperature if using the BioRad SuperMix.
A. Optimize conditions:
1) Annealing temperature
2) Primer concentration
3) Annealing/extension time
4) Sample prep protocol

B. Test efficiency of primers:
1) Primer-dimer
2) Secondary structure

C. Set up SYBR Green I experiments:
1) Standards and unknowns
Template DNA

**Genomic DNA**
- Cut with restriction enzyme that does not cut within amplified region
- Boil DNA stock for 10 min and then onto ice

**Plasmid DNA**
- If it doesn’t work, linearize plasmid with restriction enzyme that does not cut within amplified region

**cDNA**
- Treat total RNA with RNase-free DNase prior to reverse transcription
- Try to design primers at exon boundaries to avoid genomic DNA amplification
- Use enzyme that has RNaseH activity to digest away RNA from RNA:DNA hybrid after making cDNA (kits are designed with this feature)
Primer Design

There is nothing special about qPCR primers, but there are some considerations:

- Optimal Amplicon length 80-300 bp (can be longer)
- When designing primers, check for
  - predicted secondary structure
  - predicted primer-dimer formation
  - can also check for predicted hairpins in the amplicon
- Free tools at
  - IDT website (Google IDT)
  - mfold website
    http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi
  - Premier Biosoft
    http://www.premierbiosoft.com/index.html
- When designing multiple primers to be run on the same plate try to design primers with similar $T_m$
Amplicon Secondary Structure

Reverse primer A

Primer efficiency = 66.3%
Amplicon Secondary Structure

Primer efficiency = 95.8 %

Reverse Primer B

Primer efficiency = 95.8 %
Getting Consistent Results

- Do not underestimate the importance of using:
  - Dedicated set of pipettors for real-time PCR
  - Screwcap tubes
  - Aerosol-barrier filter tips
  - Hot-start Taq polymerase (antibody mediated)
  - PCR-grade water
  - Pipet carefully!
Getting Consistent Results

- Wear gloves
- Create master mixes to average out error
- Mix very well by vortexing (at least 5 secs)
- No-template control to check for contamination
- Prepare reactions in replicate – ideally triplicate
- Seal the tape well – press firmly
- Occasionally inspect reaction module for salt or dirt buildup
- Centrifuge to eliminate bubbles (bottom of well)
Data Analysis

- Relative Quantification
- Absolute Quantification
- Allelic Discrimination
Calculating Relative Expression

### Pfaffl method
*Efficiencies are normalized*

<table>
<thead>
<tr>
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<th>Reference Primers (C_T)</th>
<th>GOI Primers (C_T)</th>
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<tr>
<td><strong>Tissue #1:</strong></td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td><strong>Tissue #2:</strong></td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>(From Standard curve)</td>
<td>Efficiency:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>90% = 1.9</td>
<td>100% = 2</td>
</tr>
<tr>
<td>Delta C_T:</td>
<td>20-21 = -1</td>
<td>24-22 = 2</td>
</tr>
</tbody>
</table>

Fold induction in tissue #1 relative to tissue #2:

\[
\frac{2^{\frac{\text{deltaCt}_{\text{target}}}{\text{deltaCt}_{\text{reference}}}}}{2^{\frac{\text{deltaCt}_{\text{reference}}}{\text{deltaCt}_{\text{target}}}}}
\]

\[
= \frac{2^{\frac{24-22}{20-21}}}{2^{\frac{20-21}{24-22}}}
\]

\[
= \frac{4}{0.53}
\]

\[
= 7.5
\]

All samples should be in triplicate with at least two biological replicates.
## Calculating Relative Expression

**Pfaffl method:**
*(efficiencies are normalized)*

<table>
<thead>
<tr>
<th></th>
<th>Reference Primers ($C_T$)</th>
<th>GOI Primers ($C_T$)</th>
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<tr>
<td>Mutant or treated:</td>
<td>21</td>
<td>22</td>
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<tr>
<td>Wild type or untreated:</td>
<td>20</td>
<td>24</td>
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</table>

(From Standard curve)

<table>
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<tr>
<th></th>
<th>Efficiency:</th>
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<td>90% = 1.9</td>
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<tr>
<td>Wild type or untreated:</td>
<td>20-21 = -1</td>
<td>24-22 = 2</td>
<td></td>
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</table>

Fold induction in treated relative to untreated:

\[
\frac{2^{\Delta C_T_{\text{target}}} \cdot 10^{-\frac{\Delta C_T_{\text{reference}}}{2^{\Delta C_T_{\text{target}}}}}}{1.9}
\]

\[
\frac{2^{24-22} \cdot 10^{-\frac{20-21}{2^{24-22}}}}{1.9}
\]

\[
\frac{4}{0.53} = 7.5
\]

All samples should be in triplicate with at least two biological replicates.
## Displaying Results

Can export results to Excel within the iQ5 software

<table>
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<th>Well</th>
<th>Fluor</th>
<th>Type</th>
<th>Identifier</th>
<th>Replicate #</th>
<th>Threshold Cycle (Ct)</th>
<th>Ct Mean</th>
<th>Ct Std. Dev</th>
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<td></td>
</tr>
</tbody>
</table>
Displaying Results

- Comparison of chemical vs non-treated samples
- Histogram generated in Excel

BioRad iQ5
Data Analysis

FINIS