iQ5 & MYiQ Real-Time PCR

CFX-96 and CFX Connect
Real-Time PCR

CFX96/CFX Connect

iQ5/MYQ
What is Quantitative PCR?

The monitoring of PCR reactions in real time for the estimation of relative or absolute gene expression
Quantitative PCR

Through the use of fluorescent dyes (SYBR Green, fluorescent probes) that detect PCR products, real-time PCR measures the reaction directly during amplification.
Why Use Quantitative PCR?

More accurate than gel-based reverse transcriptase 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 PCR: Applications

Real-Time qPCR provides information for **relative** or **absolute** measurements of starting material.

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

Amplification is exponential initially

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

Product = Template quantity \times 2^n

\( n = \# \) of cycles

But the exponential phase becomes linear as reactants become rate-limiting
96 identical reactions will have very different final amounts of fluorescence at endpoint due to differences in kinetics and pipetting.

Gel-based RT PCR is an End-Point Analysis

Relative fluorescence (DNA amount)

qPCR reactions

Gel-based RT PCR to measure gene expression
How do real-time PCR instruments work?
Lamp Based-Optical System

Biorad iQ5 instrument

Optical unit

Thermocycler base
Optics Utilize Filter Wheels

- Detector
- Emission Filter
- Microplate well
- Excitation Filter
- Light Source (Simple halogen bulb)
- Biorad iQ5 instrument

UC RIVERSIDE Institute for Integrative Genome Biology
- Some (iQ5, ABI) instruments detect up to 5 dyes colors
- Simpler instruments (MYQ) detect SYBR Green only
Fast Thermal Cycling Instruments

- Some instruments have thermocyclers that can ramp very rapidly to save time
- These instruments have more advanced blocks
- Examples include Biorad CFX96, ABI 7500 and others

<table>
<thead>
<tr>
<th>Feature</th>
<th>Specification</th>
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</thead>
<tbody>
<tr>
<td>Max ramp rate</td>
<td>5°C/sec</td>
</tr>
<tr>
<td>Average ramp rate</td>
<td>3.3°C/sec</td>
</tr>
<tr>
<td>Temp Accuracy</td>
<td>± 0.2°C</td>
</tr>
<tr>
<td>Temp Uniformity</td>
<td>± 0.4°C in 10 sec</td>
</tr>
<tr>
<td>Temp Range</td>
<td>0-100°C</td>
</tr>
</tbody>
</table>

Biorad CFX96 instrument
Fast Block Design

Honeycomb architecture produces more uniform heating and cooling

Bio-Rad CFX96 block

6 independent zones of thermal control
LED Optical Technology

- CFX96 uses a scanning shuttle
  - 6 filtered LEDs for excitation
  - 6 filtered photodiodes for detection
  - LEDs fire sequentially
- Multiplex up to 5 targets
- Fixed optical path length for all wells
- Reduce cross talk between wells

Once detector/filter for each color detected

Fixed optical path length
LED Optical Technology

- LEDs are long lasting
- Factory calibrated.
  - Does not require periodic calibration.
- No need for Passive Reference (Rox/Fluoroscein)

- LED instrument always acquires data from all wells in all channels (CFX)
- Laser Homing of shuttle at every scan

Shuttle moves in zig-zag pattern
Fast Scanning on CFX

- Flexibility of 3 data acquisition scan modes
- Shorten run times using the SYBR/FAM Only fast scan mode

<table>
<thead>
<tr>
<th>Mode</th>
<th>Channel(s)</th>
<th>Scan Time (sec)</th>
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</thead>
<tbody>
<tr>
<td>All Channels</td>
<td>1-5</td>
<td>12</td>
</tr>
<tr>
<td>SYBR/FAM Only</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>FRET (Fluorescence resonance energy transfer)</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

FRET useful for examining protein-protein interactions
**Multiple Filter Sets for Multiplex Assays**

<table>
<thead>
<tr>
<th>Channel</th>
<th>Excitation (nm)</th>
<th>Detection (nm)</th>
<th>Calibrated Fluorophores</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>450-490</td>
<td>515-530</td>
<td>FAM™, SYBR Green I™</td>
</tr>
<tr>
<td>2</td>
<td>515-535</td>
<td>560-580</td>
<td>VIC®, HEX™, TET™, Cal Gold 540™</td>
</tr>
<tr>
<td>3</td>
<td>560-590</td>
<td>610-650</td>
<td>ROX™, TEXAS RED®, Cal Red 610™</td>
</tr>
<tr>
<td>4</td>
<td>620-650</td>
<td>675-690</td>
<td>CY5, Quasar 670™</td>
</tr>
<tr>
<td>5</td>
<td>672-684</td>
<td>705-730</td>
<td>Quasar 705™</td>
</tr>
<tr>
<td>6</td>
<td>450-490</td>
<td>560-580</td>
<td>Accommodates FRET Chemistry</td>
</tr>
</tbody>
</table>
Excitation Wavelengths

The LED-filter combinations permit excitation of multiple specific dyes.
Detection Wavelengths

The detection filters can detect fluorescence from multiple dyes.
Practical Differences (iQ5/MYiQ vs CFX96)

- CFX96 requires low profile plate (Biorad MLL 9601)

- All instruments can use SYBRGreen Supermix or other qPCR reagents with fluoroscein. However,
  - CFX96 does not require any normalization dye
  - CFX96 can use SooAdvanced reagents (about 45min run)
  - even using SYBRGreen Supermix will take <1.5hrs for run vs 2.5 hrs for iQ5/MYiQ

- CFX96 and iQ software are sharable
  - CFX96 software will be somewhat familiar to iQ users and offers a few additional features
Quantitative PCR workflow

Purified total RNA

→

Convert to 1st strand cDNA

cDNA template, primers, Taq polymerase, dNTPs, SYBR Green

→

Determine relative gene expression (ddCt method)

<table>
<thead>
<tr>
<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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</thead>
<tbody>
<tr>
<td>A01</td>
<td>SYBR</td>
<td>Unkn</td>
<td>1A_DMSO30_Ub</td>
<td>1</td>
<td>21.65</td>
<td>22.19</td>
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<tr>
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<td>SYBR</td>
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<td>1A_DMSO30_Ub</td>
<td>1</td>
<td>22.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A04</td>
<td>SYBR</td>
<td>Unkn</td>
<td>1B_DMSO30_BL</td>
<td>2</td>
<td>30.56</td>
<td>30.42</td>
<td>0.147</td>
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<td>A05</td>
<td>SYBR</td>
<td>Unkn</td>
<td>1B_DMSO30_BL</td>
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<tr>
<td>A07</td>
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<td>Unkn</td>
<td>2A_Pru30_Ub</td>
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<td>21.64</td>
<td>21.83</td>
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<td>2A_Pru30_Ub</td>
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<td>SYBR</td>
<td>Unkn</td>
<td>3A_BL30_Ub</td>
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<td>21.5</td>
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<td>3A_BL30_Ub</td>
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<td>12</td>
<td>29.96</td>
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</tbody>
</table>
Detection Methods
Intercalation Dyes: SYBR Green I

SYBR displays strong fluorescence in the presence of dsDNA

Ethidium bromide is 25x more fluorescent when intercalated into dsDNA
SYBR Green 125x more fluorescent when intercalated into dsDNA
Hybridization Probes

Currently, hybridization probe strategies fall into three main categories

- **Cleavage-based assay**
  - TaqMan™ Assays
  - Locked nucleic acids (LNA)

- **Displaceable probe assays**
  - Molecular Beacons
  - Dual-oligo FRET probes

- **Probes incorporated directly into the primers**
  - Amplifluor & Scorpions
Cleavage-based assay: TaqMan™

Add iQ Supermix, Hybridization Probe and sample

Denaturation

Annealing
Cleavage-based assay: TaqMan™

Extension Step

Released signal is proportional to quantity of starting mRNA in the cDNA population
Threshold and Standard Curves for Primer Efficiency
Cycle Threshold ($C_T$)

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

![Graph showing the cycle threshold](image)

$C_T$ value = 24.5 cycles

High expression = low $C_T$
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

$C_T$ values nearly identical
Cycle Threshold (C_T)

- At the C_T, the fluorescence correlates well with the starting copy number.
- Instruments have a dynamic range of up to 9 orders of magnitude.
- Each 10x dilution of template equals 3.3 cycles. Thus qPCR is relatively insensitive to the concentration of the target gene(s).

Assuming exact doubling of amplicon at each cycle then

\[ 2^n = 10\text{-fold} \]
\[ n = 3.32 \]

If PCR products double at each cycle (100% efficient), it will take 3.32 cycles to reach a 10-fold difference.
Standard Dilution Series

1) To perform absolute quantification
2) To examine the efficiency of the qPCR reaction (This can influence the results of your experiment significantly; -3.32 slope is 100% efficiency)

Efficiency = $10^{(-1/slope)}$

% Efficiency = $10^{(-1/slope)} - 1 \times 100$

= 1.85

= 85%
Standard Efficiency Curves

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%?
Unreasonably Low Efficiency

Secondary Structure in the template cDNA

Forward Primer

Reverse Primer

Primer efficiency = 66.3%
Efficiency is Now Good

Primer efficiency = 95.8%

Primers designed to avoid secondary structure

Forward Primer

Reverse Primer

110
Real-time PCR Efficiencies

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

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

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

Target Range

- Secondary structures
- Pipette calibration
- Dilution errors
Primer Dimers Raise the Apparent Efficiency

Optimization of RT-qPCR: Annealing Temperature

Primer Concentration

70°C

55°C

Primer Conc. 200 nM

A = Template + primers  B = Primers only (No template)
What Are Primer Dimers?

These double strand products are detected by SYBR Green.
Primer Concentration Affects qPCR Efficiency

Optimization of RT-qPCR: Primer Concentration

1: 100 nM
2: 200 nM
Primer Concentration

Primer-dimers can be influenced by template concentration

10,000 copies template

2,000 copies

400 copies

Amplicon

Dimers

No template control

Dimers only
Genomic DNA Contamination Can Affect PCR Efficiency

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

$T_m$ of amplicon
Why Do Melt Curve?

- Identification of non-specific products
- Mutation detection/allelic discrimination
Optimizing Real time qPCR
Features for Reaction Optimization

- Continuous Data Collection (iQ5/MYiQ)
- Thermal Gradient
- Melt Curve
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

$T_m$ of amplicon
Continuous Data Collection

- iCycler iQ features continuous data collection that is designed to focus analytical scrutiny on subsets of the data gathered
  - Optimize annealing/extension time
  - Chemistry kinetics
In some cases, can eliminate extension step since all amplification has already occurred during annealing.
Thermal Gradient Optimization

Look for specific product formation without primer dimers

Dilution series of primer [ ]

Temperature Gradient (annealing temperature)
iQ5/MYQ/CFX Thermal Gradient

- Used for annealing/extension temperature optimization for PCR reaction specificity and efficiency
- Up to 24°C gradient range programmable across block
How do I get started?
Examples of Reagents and Plates

**Disposables**
- Low profile plates (CFX96) BioRad MLL9601 (Box of 25)
- Regular plates (iQ5) Biorad 2239441 (Box of 25)
- Microseal B Film Biorad MSB1001 (Pkg of 100)

**Reagents**
- iQ SYBR Green Supermix 500 x 50μl reaction (CFX and iQ5)
- iScript RT Supermix (1 step cDNA kit)
- SoAdvanced SYBRGreen Supermix 500 x 20μl reaction (CFX96 only; fast cycle reagent for <1 hr experiment)
Total RNA preps

cDNA prep (1μg starting RNA) using 2-step kits
- Ambien Retroscript for RT
- In Vitrogen Superscript III
- BioRad iScript RT (new one-step kit)
- Reagents can be home made as well

Dilute cDNA template (~1:10)

qPCR (2x SYBR Green Mix)

Data analysis
### Reaction Set-Up

**Master mix**

- **qPCR reaction (per reaction)**
  - SYBRGreen SuperMix (Biorad) 12.5ul of 2x
  - Primer forward (10uM) 0.25ul (100nM) (can use 100 to 500nM)
  - Primer reverse (10uM) 0.25ul
  - Water 10ul
  - 23ul

- **Template** 2ul (typically 1:10 dilution cDNA)

- **Total volume** 25ul

- **Set-up can be at room temperature if using a hot-start Taq polymerase**
- **Can use smaller reaction volumes (15 to 20ul cost less)**
Plate Set-Up

Set-up can be at room temperature if using hot-start Taq

Master mixes for each primer set

<table>
<thead>
<tr>
<th>Condition 1</th>
<th>Condition 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference gene</td>
<td>GOI 1</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
</tr>
<tr>
<td>G</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>1</td>
</tr>
</tbody>
</table>
qPCR Optimization

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
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 $T_m$
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
Getting Consistent Results

Do not underestimate the importance of using:

- Dedicated set of pipettors for real-time PCR
- Screwcap tubes
- Aerosol-barrier filter tips
- PCR-grade water
- Pipet carefully!
Obtaining 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 optical sealer tape
• Inspect block for salt or dirt buildup
• Centrifuge to eliminate bubbles (bottom of well)
Data Analysis

• Relative Quantification
• Absolute Quantification
• Allelic Discrimination
Some References

Pfaffl method for relative gene quantification

MIQE guidelines for publication of qPCR results

Reference gene selection and normalization

QPCR method and reviews
Principle of Relative Gene Expression

Example based on a Northern blot

Gene expression is normalized compared to a reference gene

Corrected fold increase = $\frac{10}{2} = 5$

Ratio target gene in experimental/control = fold change in target gene
fold change in reference gene
Principle of Relative Gene Expression

Normalized gene expression by qPCR (Pfaffl equation)

Example based on a Northern blot

\[
\text{ratio} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target (control-treated)}}}{(E_{\text{ref}})^{\Delta Ct \text{ ref (control-treated)}}}
\]

E = qPCR efficiency

Calculating Relative Gene Expression

Pfaffl method: (Pfaffl, 2001; Nucleic Acid Research)

Fold induction = \[
\frac{\text{Efficiency}_{\text{target}}^{\text{deltaCt}_{\text{target}}}}{\text{Efficiency}_{\text{reference}}^{\text{deltaCt}_{\text{reference}}}}
\]

Efficiency = \[10^{-1/\text{slope}}\]

Reference genes include Ubiquitin, actin, GAPDH, rDNA, and others
Calculating Relative Expression

**Pfaffl method**
*(Efficiencies are normalized)*

<table>
<thead>
<tr>
<th></th>
<th>Reference Primers (C&lt;sub&gt;T&lt;/sub&gt;)</th>
<th>GOI Primers (C&lt;sub&gt;T&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue #1:</td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Tissue #2:</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>

(From Standard curve)

- Efficiency: 90% = 1.9
- 100% = 2
- Delta C<sub>T</sub>: 20-21 = -1
- 24-22 = 2

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

\[
\frac{2^{\Delta C_{T_{\text{target}}}}} {2^{\Delta C_{T_{\text{reference}}}}} = \frac{2^{(24-22)}} {2^{(20-21)}} = \frac{4} {0.53} = 7.5
\]

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

Pfaffl method
*(efficiencies are normalized)*

<table>
<thead>
<tr>
<th>Mutant or treated:</th>
<th>Reference Primers (C&lt;sub&gt;T&lt;/sub&gt;)</th>
<th>GOI Primers (C&lt;sub&gt;T&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21</td>
<td>22</td>
</tr>
<tr>
<td>Wild type or untreated:</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>

(From Standard curve)

| Efficiency: | 90% = 1.9 | 100% = 2 |
| Delta C<sub>T</sub>: | 20-21 = -1 | 24-22 = 2 |

Fold induction in treated relative to untreated

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

\[
\frac{2^{24-22}}{2^{20-21}} = \frac{4}{0.53} = 7.5
\]

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

**Can export results to Excel within the iQ5 software**

<table>
<thead>
<tr>
<th>Well</th>
<th>Fluor</th>
<th>Type</th>
<th>Identifier</th>
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## Displaying Results

### All software permits export of results to Excel

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Displaying Results

Semi-quantitative RT-PCR

- Comparison of chemical vs non-treated samples
- Histogram generated in Excel
Reference Genes (Vandesompele Method)

- There are no true “House keeping” genes
- This method uses more than 1 reference gene (3 is recommended) and takes the geometric mean to normalize fold expression
- Using a single reference gene can lead to erroneous normalization up to 3.0-fold and 6.4-fold in 25% and 10% of the cases, respectively, with sporadic values above 20-fold
- geNorm site: http://medgen.ugent.be/~jvdesomp/genorm/
- geNorm is a popular algorithm to determine the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel
- The CFX96 will provide Target Stability Values (M) to help assess reference genes

CONTROVERSY OVER MEASLES-MUMPS-RUBELLA (MMR) vaccine erupted in 1998 when it was suggested that the measles virus (MV) component of the vaccine was responsible for autistic enterocolitis, a new form of autism spectrum disorder (ASD) characterized by the presence of ileo-colonic lymphonodular hyperplasia, chronic inflammatory colonic disease, and loss of acquired cognitive skills after a period of normal development.


Fear has reduced MMR vaccinations in the US
Poor Technique with Serious Consequences

Non-specific products have correct melt temp for measles product, but not confirmed by sequence

Reagents and Plates

Disposables
- Low profile plates (CFX96) BioRad MLL9601 (Box of 25) - $76
- Regular plates (iQ5) Biorad 2239441 (Box of 25) - $76
- Microseal B Film BIORAD MSB1001 (Pkg of 100) - $109.80

Reagents
- iQ SYBR Green Supermix 500 x 50 μl reaction (CFX and iQ5) - $568
- iScript RT Supermix (1 step cDNA kit) - $387
- SsoAdvanced SYBR Supermix 1000 x 20 μl reaction (CFX fast cycle reagent for <1 hr experiment) - $430