Let our MyiQ on line!

Bio-Rad Real-Time PCR Training Course

Pedro Lam 林華峰
Bio-Rad Laboratories 台灣分公司
Jan, 2008
Today Outline

• Part I: What is the Real-Time PCR?

• Part II: Software Training: Operation and Data Analyze

• Part III: Open Discussion
Monitor the amplification reaction as it occurs.
Real Time PCR 原理

- Plateau 螢光強度
- Log phase 切線斜率
- Threshold 閾值

$Y = N_0 2^n$
C_T 值 v.s. 濃度

- 1 cycle = 2 fold difference
- 3.32 cycles ≅ 10 fold difference
- Assumes 100% efficiency

\[ Y = N_0 \ 2^n \quad \rightarrow \quad Y = N_0 \ (1+E)^n \]
Threshold Cycle, $C_T$, can be used to generate standard curves.

$r$ is a measure of how well the actual data fit to the standard curve.

$$r = \frac{\text{explained variation}}{\text{total variation}}$$

The slope of the standard curve can be directly correlated to the efficiency of the reactions:

$$\text{Efficiency} (\eta) = \left[10^{-\frac{1}{\text{slope}}} \right] - 1$$
The MyiQ Detection System

**PCR System**
- Gradient, easy to optimized
- 6 peltiers, High uniformity and long life
- Black block, no need special optical tube, lower cost
- 15~100 ul, 10 ul demonstrated

**CCD Camera**
- 12 bit
- 350,000 pixel
- 96 well 同步收光, no time delay

**Lamp**
- >20000 hrs
- 400 – 700 nm
- 更換光源, 無需重新校正
Detection Chemistries

- Non-specific dsDNA binding dyes
  - SYBR™ Green I
  - Ethidium Bromide

- Specific Hybridization Probes
  - Cleavage probes (TaqMan®)
  - Molecular beacons
  - dual-oligo FRET pairs
  - Others: Scorpions™ / Amplifluor™ / LUX™
The dsDNA Binding Dyes
SYBR Green I

Extension

Extension Continued
Apply Excitation
Wavelength
- Easy to start qPCR Exp.
- Non-specific binding: Primer dimer
- Melting Curve Analysis
- No need to adjust using Bio-Rad
**Extension Step**

1. Strand Displacement

2. Cleavage

3. Polymerization Complete

4. Detection
• More specific than SYBR Green I
• More high cost than SYBR green I
• Data collected in extension stage
How to choose the right Consumable

• **Quantitative PCR Reagent**
  
  iQ SYBR Green Supermix (100 / 500 / 1000 / 2000)
  
  iQ Supermix (100 / 500 / 1000)
  
  iQ multiplex powermix (50 / 200)

• **One-Step Quantitative PCR**
  
  iScript One-Step RT-PCR Kit with SYBR Green (50 / 200)
  
  iScript One-Step RT-PCR Kit for Probes (50 / 200)

• **Disposable Consumables**
  
  223-9441  iQ 96-Well PCR Plates, 25
  
  MSB-1001  Microseal ‘B’ Adhesive Seals, 100
  
  TCS-0803  Optical Flat 8-Cap Strips, for 0.2 ml, ultraclear, 120
  
  TBS-0201  0.2 ml 8-Tube Strips Without Caps, natural, 125

• **All reagents are 2x stock and ready to use**
  
  • All 3 mins Hot Start (vs 10 or 20 mins Hot Start)
  
  • All reagent get 1yr Exp. Day in –20 and ½ yr in 4 °C
Real-time PCR Sample Preparation

**SYBR Green Chemistry**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ SYBR Green Supermix</td>
<td>25 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Primer 1</td>
<td>x µl</td>
<td>100 nM-500 nM</td>
</tr>
<tr>
<td>Primer 2</td>
<td>x µl</td>
<td>100 nM-500 nM</td>
</tr>
<tr>
<td>Sterile water</td>
<td>x µl</td>
<td></td>
</tr>
<tr>
<td>DNA template</td>
<td>x µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50 µl</td>
<td></td>
</tr>
</tbody>
</table>

**Probe Chemistry**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQ Supermix</td>
<td>25 µl</td>
<td>1X</td>
</tr>
<tr>
<td>Primer 1</td>
<td>x µl</td>
<td>100 nM-500 nM</td>
</tr>
<tr>
<td>Primer 2</td>
<td>x µl</td>
<td>100 nM-500 nM</td>
</tr>
<tr>
<td>Probe</td>
<td>x µl</td>
<td></td>
</tr>
<tr>
<td>Sterile water</td>
<td>x µl</td>
<td></td>
</tr>
<tr>
<td>DNA template</td>
<td>x µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50 µl</td>
<td></td>
</tr>
</tbody>
</table>

**Hot Start**

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>Cycle 2</th>
<th>Cycle 3</th>
<th>Cycle 4</th>
<th>Cycle 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1</td>
<td>Step 1</td>
<td>Step 2</td>
<td>Step 1</td>
<td>Step 1</td>
</tr>
<tr>
<td>95.0</td>
<td>95.0</td>
<td>55.0</td>
<td>95.0</td>
<td>55.0</td>
</tr>
<tr>
<td>3:00</td>
<td>0:10</td>
<td>0:30</td>
<td>1:30</td>
<td>1:00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0:10</td>
</tr>
</tbody>
</table>

**PCR**

**Melting Curve**

Life Science Group
Remember...

Real-Time PCR is not ‘cookbook chemistry’ - a real-time instrument will not optimize your experiments for you

However, once you do **optimize your reactions**, you will get **reproducible, accurate results**
Bio-Rad
Gene Expression Gateway

http://bio-rad.com/genomics
Amplification Central

PCR Doctor
Cure your data ailments with this interactive troubleshooting tool for PCR and real-time PCR assays.
- Real-Time PCR Doctor
- Conventional PCR Doctor
- Bio-Rad Technical Support

Tutorials
Learn the key principles of conventional and real-time PCR.
- Real-Time PCR Fundamentals
- Real-Time PCR Chemistry
- Downloadable Presentations

Assay Design
Access design considerations for conventional and real-time PCR assays.
- Primer and PCR Product Design
- Assay Validation and Optimization Overview
- Quantitative PCR Analysis

Top 20 Citations
Recommended articles featuring the most popular amplification-related applications. Learn more.

RNAi Solutions
From design to detection, Bio-Rad offers an extensive set of tools for effective gene silencing and analysis. Learn more.

Tip of the Week
To avoid genomic DNA amplification when using cDNA as the starting template, it is helpful to design primers at splice junctions.
Part II: How to operate the MyiQ?
開機順序

1. PCR 系統開關
2. 光學系統開關
3. 電腦開關
4. 點選桌面 iQ5 圖示，開啓 iQ5 軟體

注意：使用機器前需暖機十分鐘。
檢查是否連線

未連線

已連線

（HOST CONTROL MODE）
How to use the software

• Step 1: Protocol Setup

• Step 2: Plate Setup

• Step 3: Put your sample

• Step 4: Begin Run
Edit the template protocol
Protocol Editing
Gradient Function
Save the protocol *.tmo
Plate Setup

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Repeats</th>
<th>Step</th>
<th>Dwell Time</th>
<th>Setpoint</th>
<th>PCR/ melting data acquisition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>10s</td>
<td>30s</td>
<td>95.0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>10s</td>
<td>10s</td>
<td>95.0</td>
<td></td>
</tr>
</tbody>
</table>

Template button highlighted in the setup window.
Sample Type
Save Plate Setup file *.pts
Run
Begin Run- save *.opd
當實驗完成時...

請務必先關閉 iQ5 操作軟體，再關閉 Real-Time PCR
iQ5 Software Training-Data analysis
Software Overview
Data Analysis
Edit Plate
Ct Value
Detailed Results
Select Samples You Wanted

- **Display Wells...**: Just show the Selected Samples without re-analysis
- **Analysis Wells...**: Select and re-analysis samples

Life Science Group
Ct: Automatic Determination
Three Analysis Modes for Troubleshooting
Melting Curve Analysis
Quantification strategies in real-time PCR

**Absolute Quantification**
- External standard curve
- Major applied in:
  - Viral load
  - Bacterial load
  - Any screening test need “Unit”

**Relative Quantification**
- Normalization by a reference gene
- Major applied in:
  - Investigate physiological changes in gene expression levels
  - Exp: Drug treatment, microarray validation
1. Set up the Sample Type and Concs: Standard or Unknown

2. Determine Ct Value

3. Auto-Calculation

Absolute Quantization

絶對定量
AQ Results
Relative Quantization
相對定量 (ΔΔCt method)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Exp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target gene</strong></td>
<td>Ct SB</td>
<td>Ct SA</td>
</tr>
<tr>
<td><strong>Reference gene</strong></td>
<td>Ct CB</td>
<td>Ct CA</td>
</tr>
</tbody>
</table>

\[ \text{Ratio} = \left( \frac{2}{E} \right)^{-\Delta\Delta C_t} \]

\[ \Delta\Delta C_t = (\Delta C_t A - \Delta C_t B) \]
Normalized Expression

• $\Delta \Delta C_T$ (Livak)
  – Assume 100% efficiency
  – Only one Ref Gene

• Pfaffl Modification
  – Accounts for efficiency differences
  – Only one Ref Gene

• Vandesompele
  – Accounts for efficiency differences
  – Allows multiple reference genes for normalization
Gene Expression Tab

[Image of the Gene Expression Tab interface with highlighted elements]

- Workshop
- Run-Time Central
- Data Analysis
- Calibration
- User Profile

[Table with gene names and data]

- **Gene List**
  - Name: Gapdh, Full Name: Gapdh
  - Name: IL1B, Full Name: IL1B
  - Name: Tubulin, Full Name: Tubulin
  - Name: Actin, Full Name: Actin

- **Gene Expression Options**
  - Condition
  - Log 2
  - Linear

- **Graph Options**
  - Graph Data
    - Relative to control
    - Relative to zero

- **Scaling Options**
  - Highest
  - Lowest
  - Unscaled

- **Graph Show**
  - y-Shelf Line

- **Data Table**
  - Gene Name: Gapdh, Condition Name: Control
  - Gene Name: IL1B, Condition Name: Treatment

- **Normalization**
  - Normalized expression (dCt)
  - Relative quantity (dCt)

- **Data Entry**
  - Columns: A, B
  - Rows: 1-12
  - Values: Various expression levels
Grouping

<p>| | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- Actin
- Myosin
- Spectrin
- Basal
- Spectral
- IL-1β
- IL-6
- Tubulin

Note: The table may require interpretation due to the visual presentation style.
Select Ref. Gene and Control

<table>
<thead>
<tr>
<th></th>
<th>Name</th>
<th>Full Name</th>
<th>Ref</th>
<th>Color</th>
<th>Show Graph</th>
<th>Auto Efficiency</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gapdh</td>
<td>Gapdh</td>
<td>✔</td>
<td>Blue</td>
<td>✔</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>IL1b</td>
<td>IL1b</td>
<td></td>
<td>Violet</td>
<td>✔</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>3</td>
<td>Tubulin</td>
<td>Tubulin</td>
<td></td>
<td>Pink</td>
<td>✔</td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>4</td>
<td>Actin</td>
<td>Actin</td>
<td>✔</td>
<td>Green</td>
<td>✔</td>
<td></td>
<td>100.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Name</th>
<th>Full Name</th>
<th>Ctrl</th>
<th>Color</th>
<th>Show Graph</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 Hr</td>
<td>0 Hr</td>
<td>✔</td>
<td>Pink</td>
<td>✔</td>
</tr>
<tr>
<td>2</td>
<td>1 Hr</td>
<td>1 Hr</td>
<td></td>
<td>Brown</td>
<td>✔</td>
</tr>
<tr>
<td>3</td>
<td>2 Hr</td>
<td>2 Hr</td>
<td></td>
<td>Blue</td>
<td>✔</td>
</tr>
</tbody>
</table>
RQ Results
Multifile Gene Expression - MFGE

- Accomplished through new file type called “Gene Study”

- Created as .gxd file

- .gxd files maintain Sample ID and $C_T$ information only

- Over 50 data files can be imported into a Gene Study
  - Over 5,000 wells of data can be analyzed in a Gene Study using the iQ5 Gene Expression module
  - This is approx. 50 full plates (data files) of single color real-time PCR data or 25 plates of dual-color data, etc…
Detailed Reports

PCR Quantification Detailed Report
PCR Base Line Subtracted Curve Fit Data (Texas) Contains All Available Data

General Data
- Data File Name
- Data File Path
- Collected Data
- Current Date
- Run Date
- User aborted the run
- Active RMEs
- Active Wall Factors
- Background Readings Valid
- RME Valid
- Well Factors Valid
- Plate Setup File Name
- Plate Setup File Path
- Protocol File Name
- Protocol File Path
- Computer name
- Created by app
- Created by user
- Creation Date
- Created in Security Edition
- Last Creation GUID
- Modified by user
- Last modified date
- OS Build and Service Pack

Sample Dynamic Range Data.xlsx
C:\Program Files\Bio-Rad\iQ5\SampleFile

03/14/2007 PM 12:47:57
03/14/2007 PM 12:47:57
No
Yes
Original
Dynamic
Yes
Yes
Yes
Yes

LSGXP.01094735
I05.exe (v2.0.104.60221)
BioRad\admin
03/13/2006 PM 03:50:47
No
71 y-int-36
12
71 y-int-36
12

Life Science Group
General QPCR Working Process
-- Data analysis in iQ5 software

- Amplification plot
  - Reproducibility?  So you need duplication or triplication....
  - Determination Ct Value?  Threshold

- Absolute Q., Relative Q. or SNP
  - ΔCt or ΔΔCt
  - Allelic Discrimination

- PCR efficiency from std. Curve
  - <100%
  - >100%
  - Dynamic range

- Reproducibility
  - Duplication or triplication

- Melting curve analysis
  - Primer dimmer
  - Non specific production

---

Life Science Group

---

BIO-RAD