



同步定量PCR 原理及介紹

高慈娟 Catherine

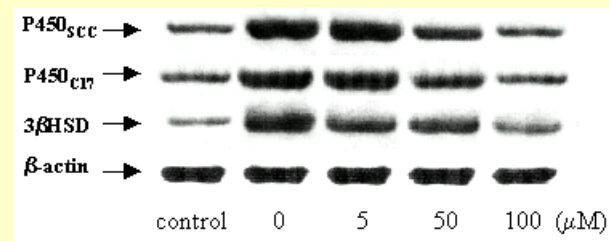
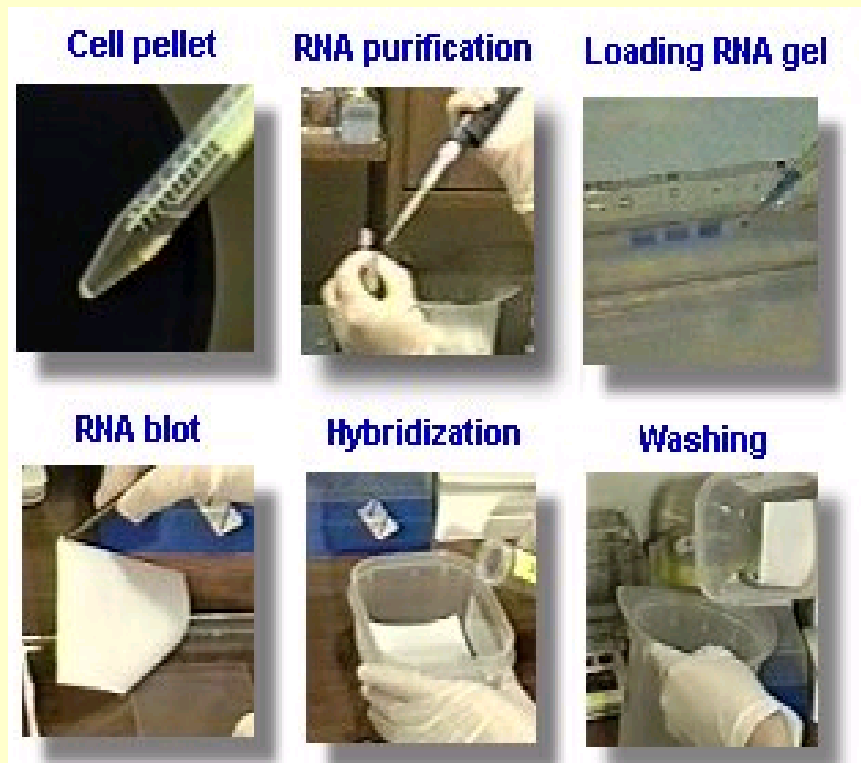
產品應用專員 Technical Product Specialist

美商應用生命系統公司

- ❖ Northern Blots Analysis
- ❖ Competitive PCR
- ❖ RT-PCR followed by South blots

Disadvantages

- Labor intensive, many manual steps
- Semi-quantitative
- Low Resolution
- Limited dynamic
- Low throughput



同步定量PCR之應用



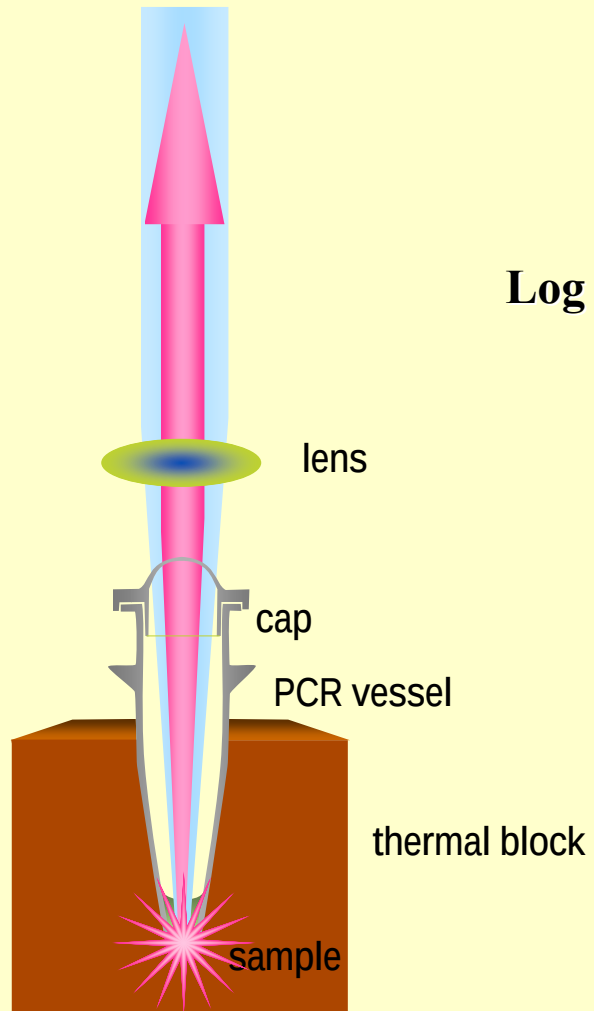
❖ 基因定量

- 檢測基因轉殖食品(**GMO**)
- 癌症基因及免疫基因的定量
- 心臟血管疾病基因監測
- 病毒定量: HBV, HCV, HPV...
- 病原菌偵測
- Quantifier Detection
- **miRNA 基因調控研究**

❖ 定性研究

- 基因型研究
- **-- SNP與疾病關聯性**

Real-time PCR
is “*sequence detection*”
in a closed-tube



Log [DNA]

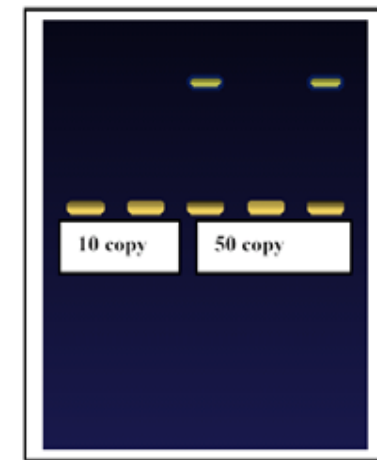
$$Y = X(1+e)^n$$

Plateau

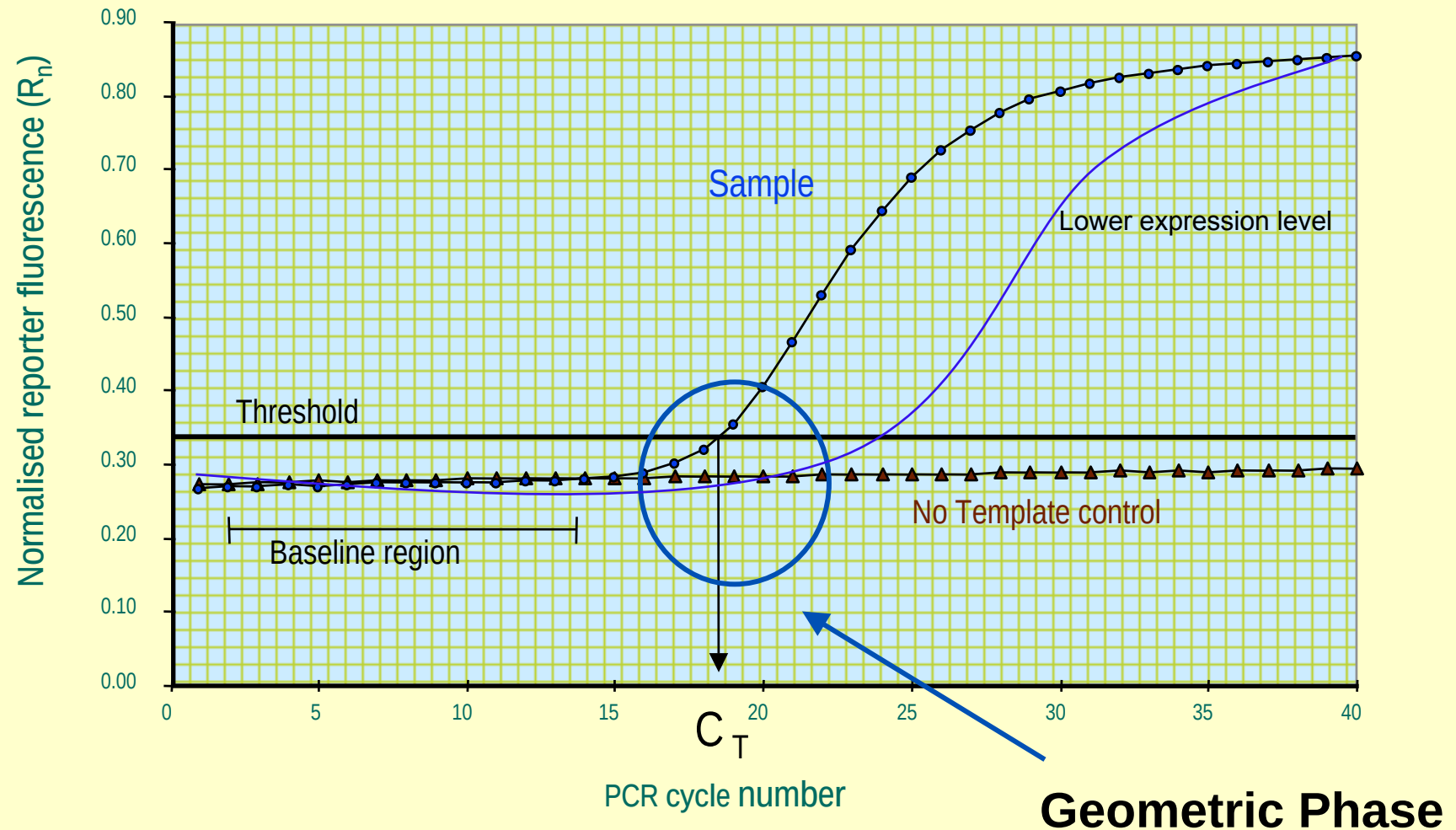
Geometric

$$Y = X2^n$$

Cycle #



Amplification plot features

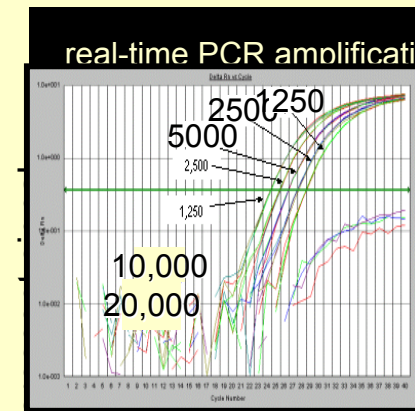
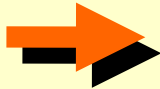


$Y = N_0 2^n$, C_T 與起始濃度之對數值成反比

- 2 小時PCR反應後即可分析 96 PCR Amplification Curves獲得定量結果

StepOne / 7300 / 7500 / 7500fast / 7900

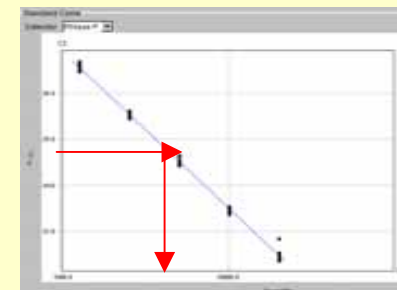
Pipette
Samples



PCR cycles →



Standard curve



Relative quantitation



ABI 發明
TaqMan Chemistry
& Real-Time PCR
的理論

↓ 1997

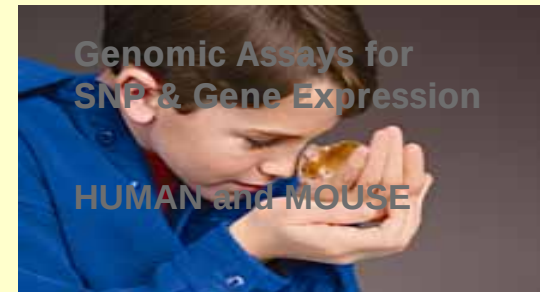
ABI 獨創
相對定量 &
 ΔC_t 的觀念

↓ 1998

ABI 特有的定量實驗
保證 PCR efficiency 達
100%

↓ 2000

Total Solutions for you!



未來趨勢
High Throughput
Multi Channels
Speed

2007



ABI 7700

全世界第一台
定量PCR



ABI 5700

1998 單一波長
組裝機種



ABI 7900HT

2000 目前最高樣
品處理量的機種



ABI 7000

2001 上市超人
氣機種
2002 獲選最佳
儀器

↑ 2001

2004



ABI 7500
第三代全新機種



StepOne
最新機種

↑

簡易三步驟!!

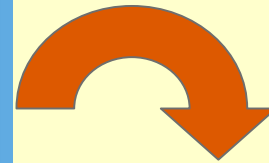


Basic Operation

- Soft power button on the LCD touchscreen

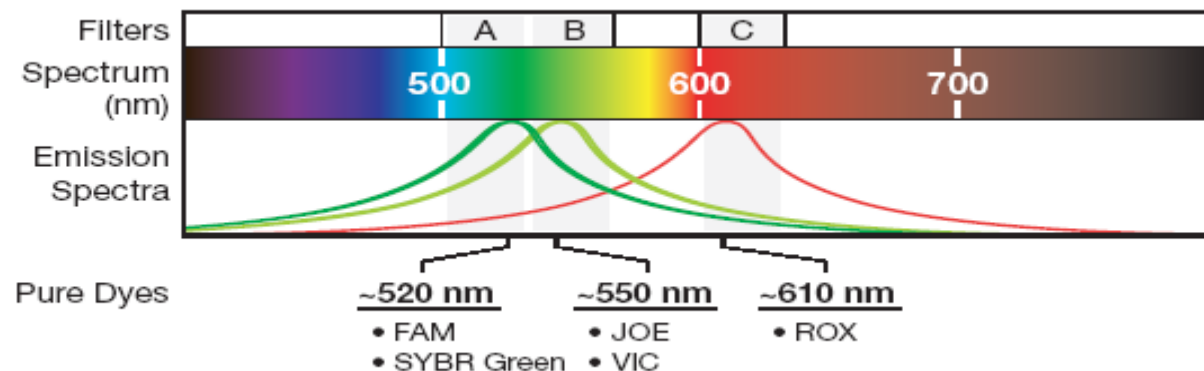
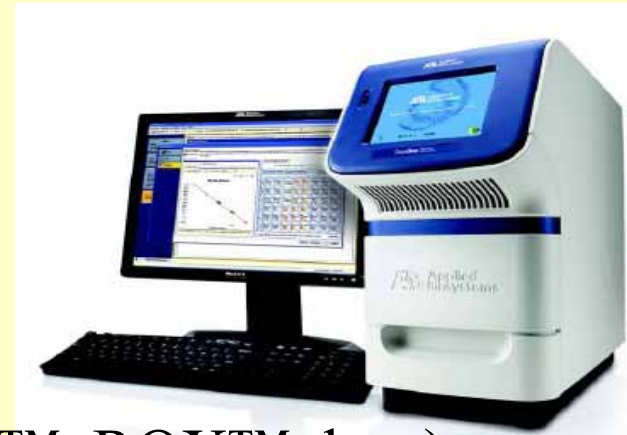
AB Applied Biosystems

Applied Biosystems StepOne™ Real-Time PCR System



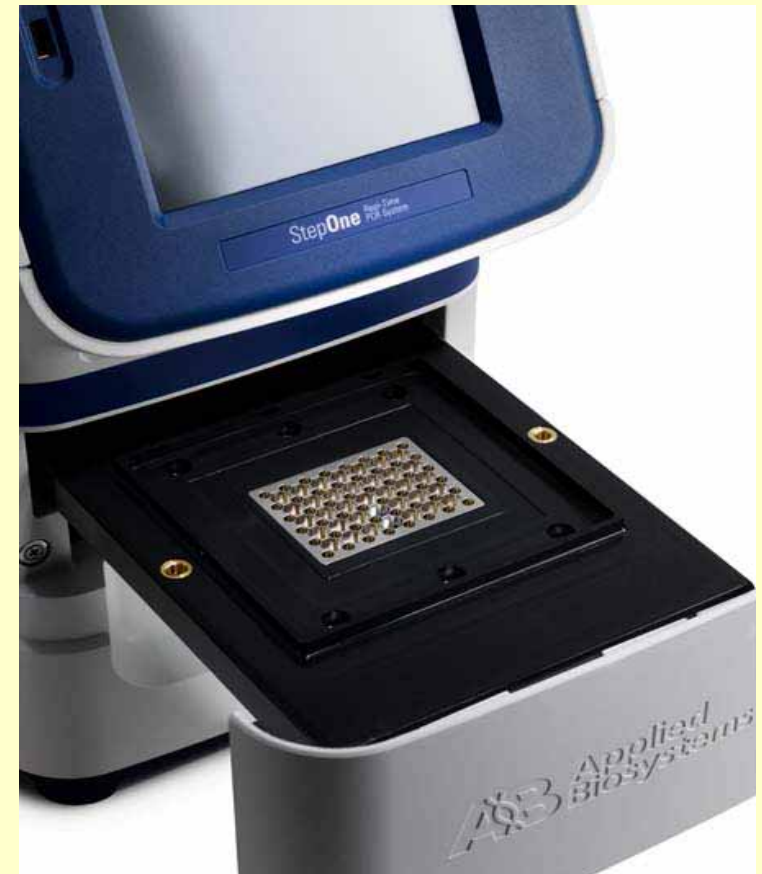
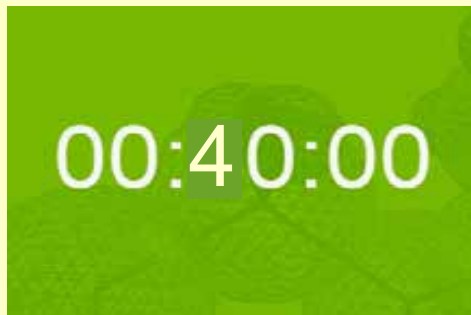
The StepOne™ Real-Time PCR System Introduction

- 48 well 0.1 ml tube
- Single LED excitation
- 3 color System (FAM™/ SYBR® Green, VIC®/JOE™, ROX™ dyes)
- Fast and Standard ramping in the same block (1-100%)
- Standalone or co-located
- Remote monitoring, LAN



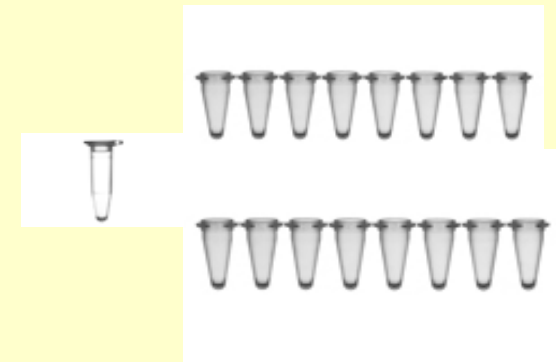
StepOne™ Real-Time PCR System: The Basics

- One block, Two speeds
 - **Fast cycling**: 40 cycles in under 40 minutes
 - Standard cycling: 40 cycles in under 2 hours
 - 48-wells
 - 10-30µl reaction volume



StepOne™ Real-Time PCR System: The Basics

- Supported consumables:
 - 48-well plates with optical adhesive cover
 - 48-well plates with caps
 - Fast tubes
 - Fast 8-tube strips



Networkability: Remote Monitoring, E-mail Notification



or

LAN



Summary

- 全機一體成型，可有效避免光學路徑偏移
- 操作方便性: 48 well plate or Single tube
 - 精確、穩定的PCR控溫系統: Peltier technology
- 兩種Sample ramp rate 供選擇---不需更換Block
 - Standard mode : 40 cycles 2hr
 - Fast mode : 40 cycles 40min
- 全能多功，可滿足所有實驗應用需求
- 人性化操作介面：機器為觸控式螢幕、電腦軟體圖形化介面操作簡單
- 多種搭配組合，可依客戶實際需求達到全面客製化

Chemistry Comparison

TaqMan® Probe



Specificity

- Primer binding
- Probe hybridization
- PCR conditions

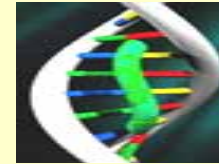
Flexibility

- Multiple Targets per tube
- SNP Detection
- +/- Application

Optimization

- Universal guidelines

SYBR® Green 1 Dye



- Primer binding
-
- PCR conditions

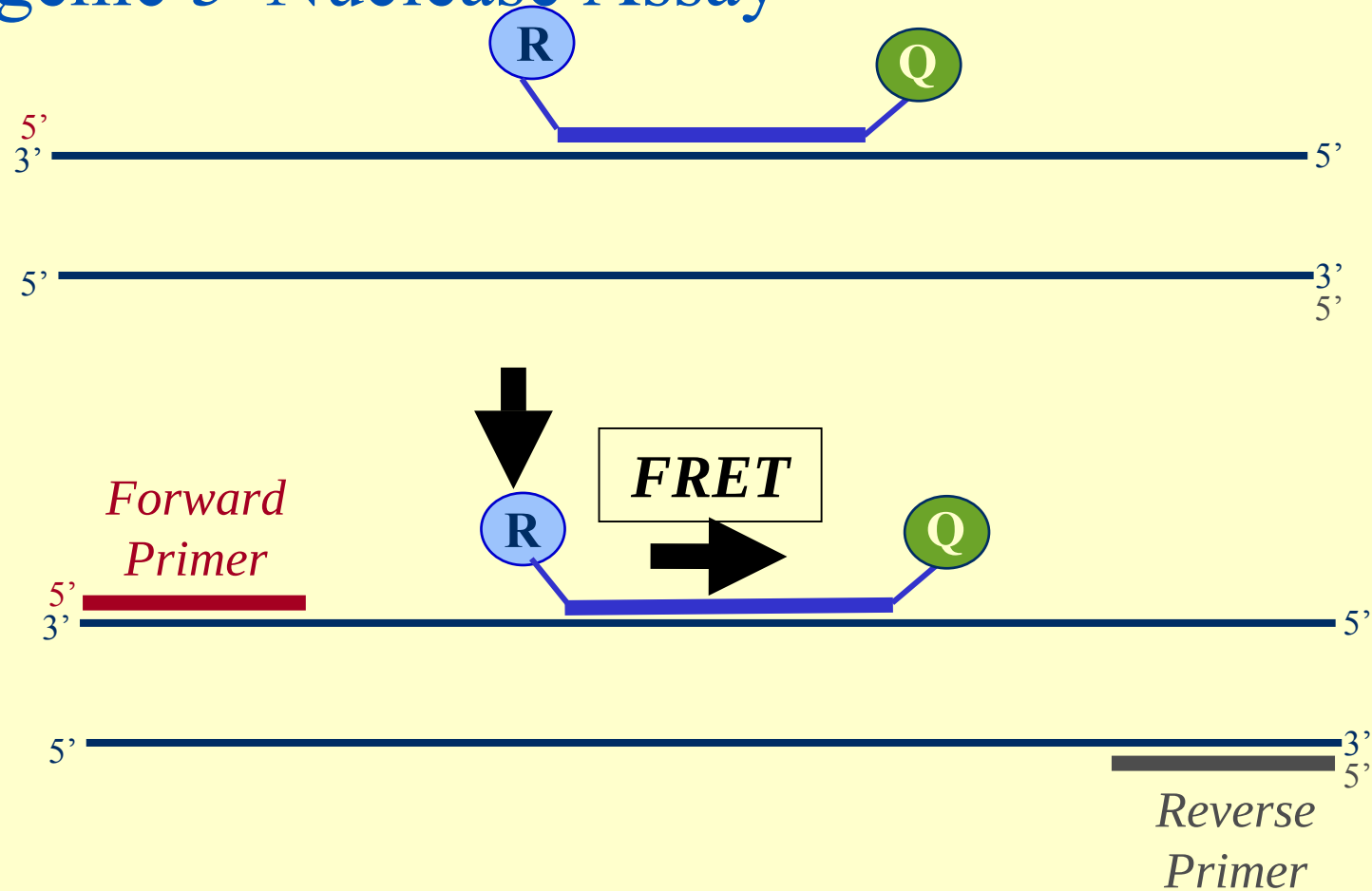
-

-

-

- Universal guidelines
- Check Primer dimer formation

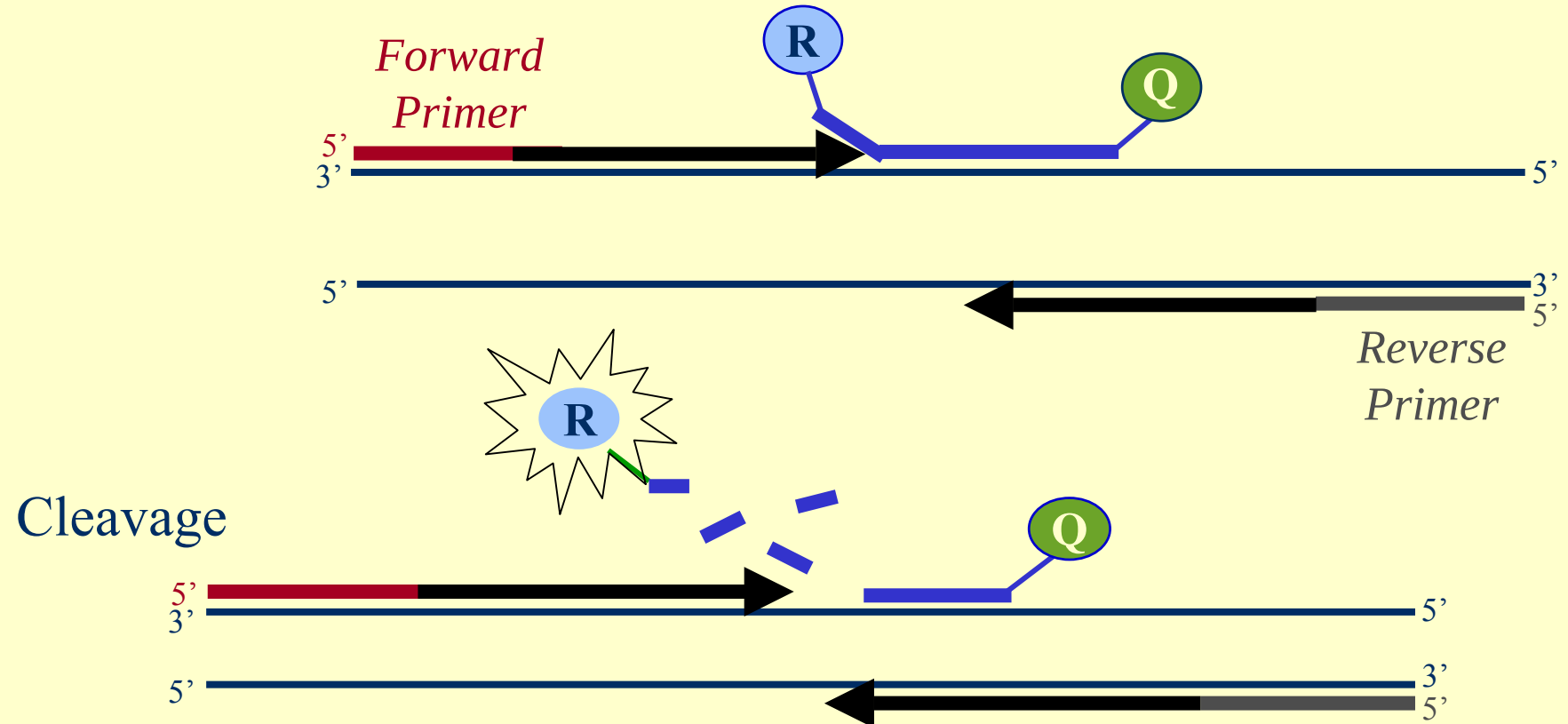
Fluorogenic 5' Nuclease Assay



***FRET= Fluorescence Resonance Energy Transfer**

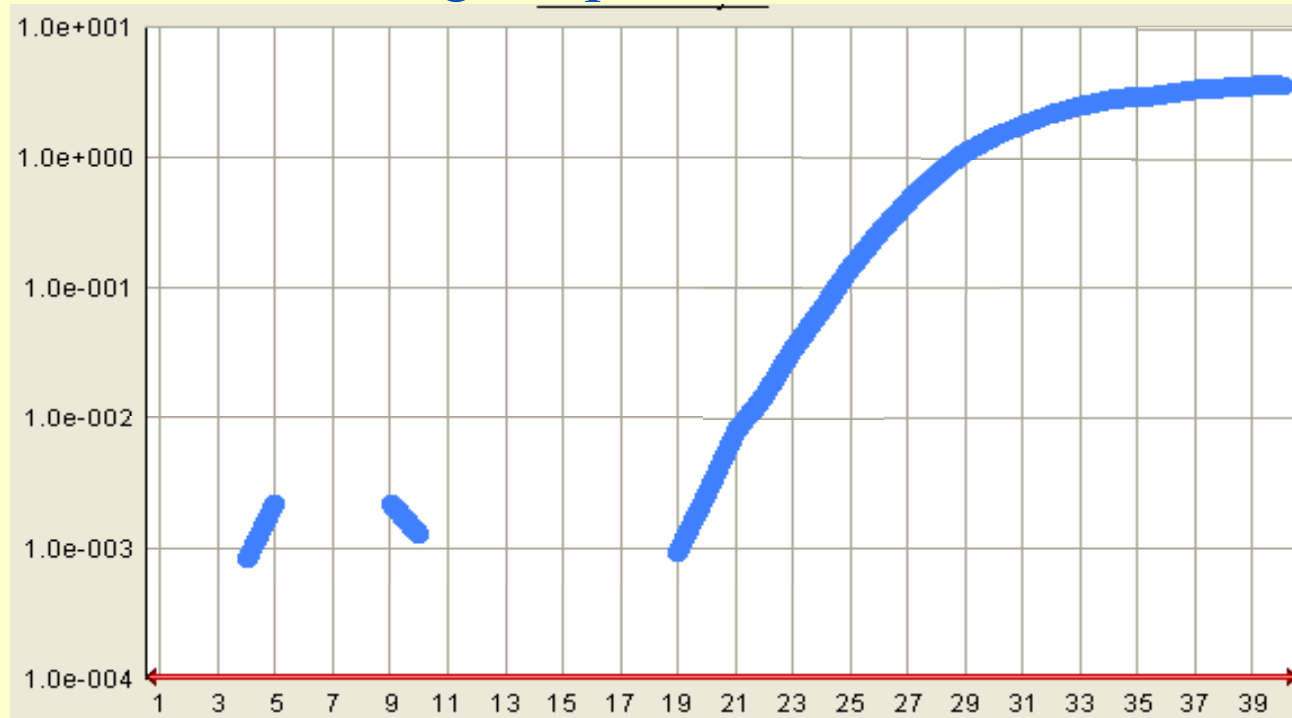
Fluorogenic 5' Nuclease Assay

Displacement during Polymerization



PCR Reaction Monitored in Real-Time

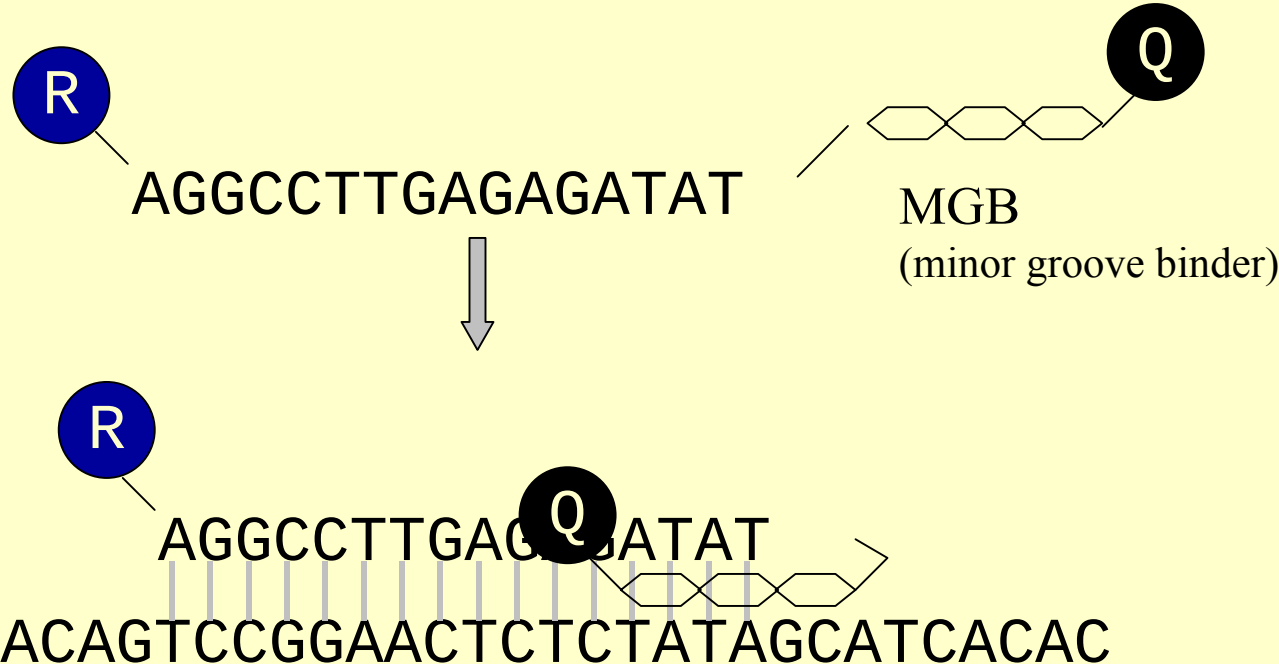
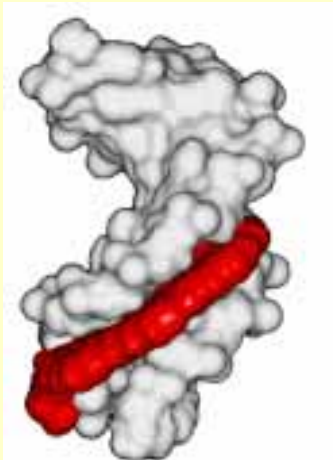
Generating Amplification Curves



- **Fluorescent signal is proportional to the amount of PCR product amplifying in the tube.**

next-generation chemistry: TaqMan® MGB Probes

(non-fluorescent quencher) NFQ

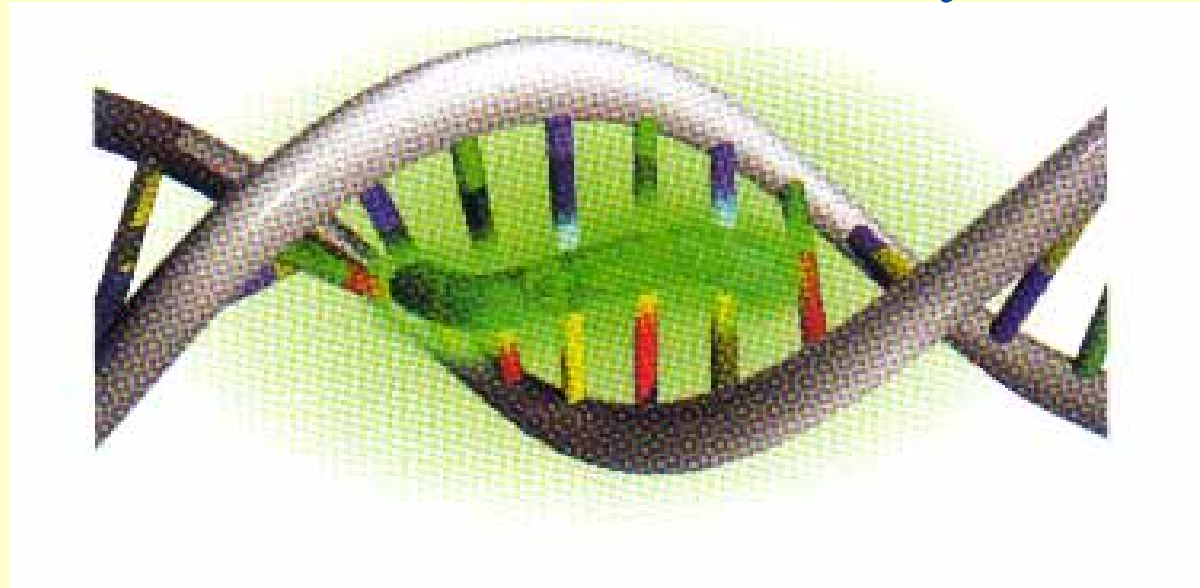


✱ All probes will be short (13-20-mers)

✱ High resolution, Low Background

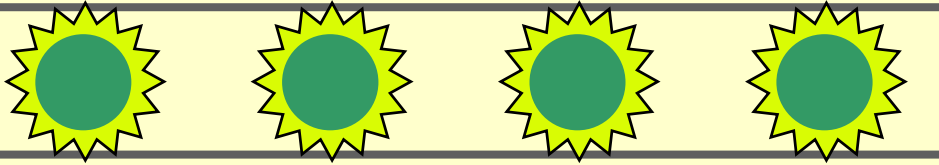
- Noiseless data 專一性高
due to the second level of specificity provided by the probe
- Multiplex compatible 可在一反應中進行兩種基因之定量
each probe can be differently coloured and thereby mixed with others
- Signal proportional to product 螢光強度與反應產物呈正比
signal related to amount of amplified product (not mass/length)
- Optimized assays available
Assays on Demand (AoD), Assays by Design (AbD), PDAR

Signal Generation with SYBR® Green 1 dye



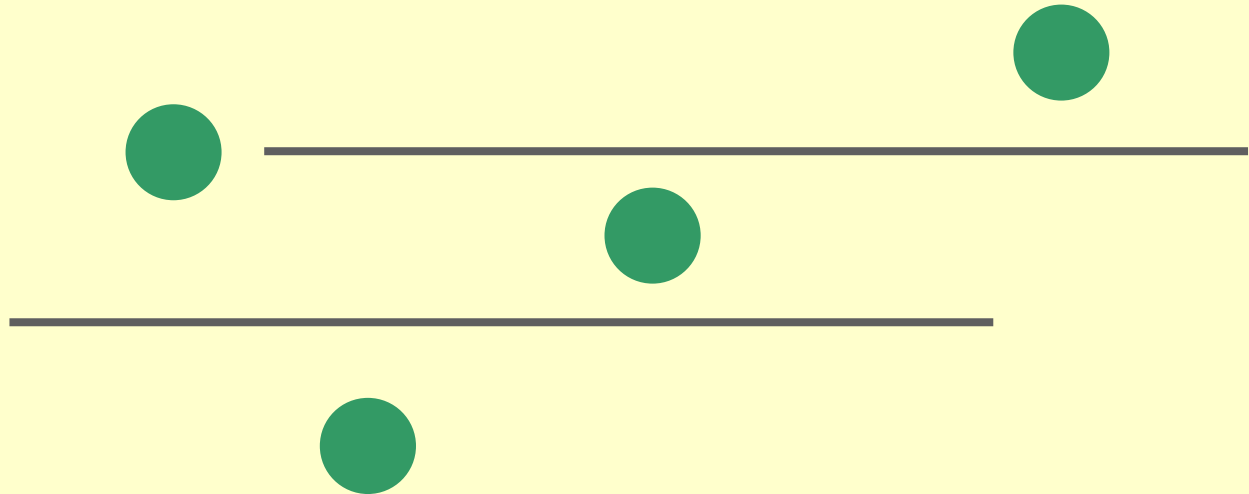
- SYBR® Green 1 dye binds to the minor groove of ds DNA
- Detects specific and unspecific products

SYBR® Green I Dye



Double Stranded DNA:
Dye will fluoresce when bound to minor groove

Denaturation:
No Signal



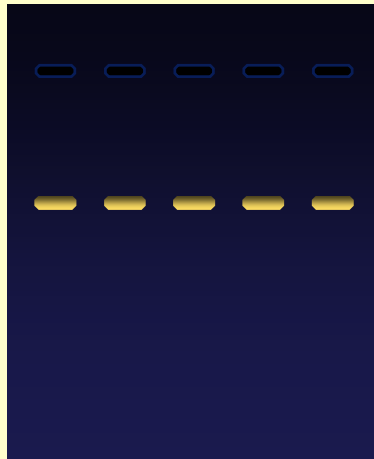
- Chemistry reports the accumulation of dsDNA in PCR
 - Need to verify target specific amplification by other means

Specificity check

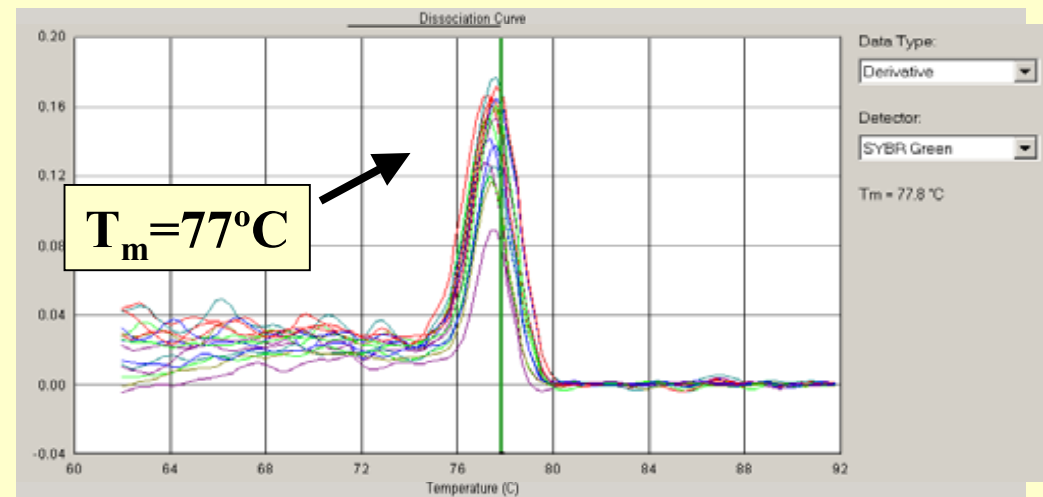
SYBR® Green 1 dye

- How ? Gel or Real-Time PCR System *add Melting Curve stage*
- When ? Post PCR Analysis

178bp

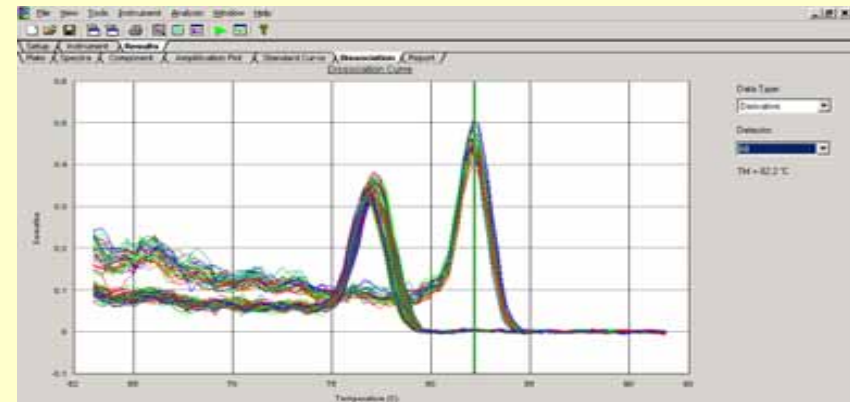
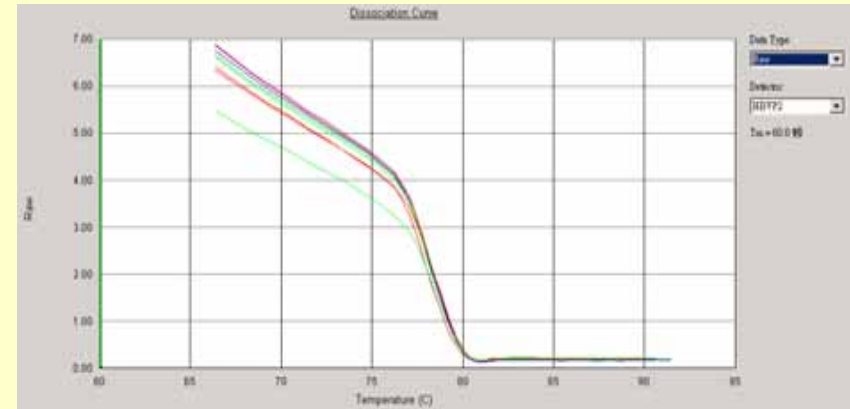


- Gel electrophoresis to determine PCR product size



- Melt Analysis to determine PCR product Tm (performed on the Real-Time PCR instrument).

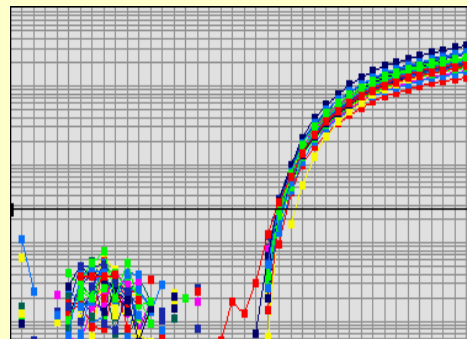
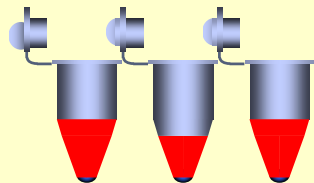
- ◎ 可省下Probe的費用
- ◎ 理想的篩檢的工具
- ◎ Primer Dimer 也會產生訊號
- ◎ 必須要做PCR解離曲線
- ◎ 無法做 multiplex PCR



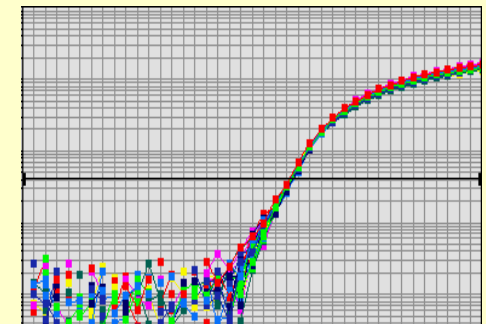
Compatible with dissociation curve analysis

利用dissociation curve assay確定PCR反應產生幾種產物

- All Chemlink Hot start (5~ 15 min)
- Carry over protection, UNG
- Real Master Mix, all in one
- 4 °C 保存, 室溫操作
- ROX 體積校正



ROX normalize

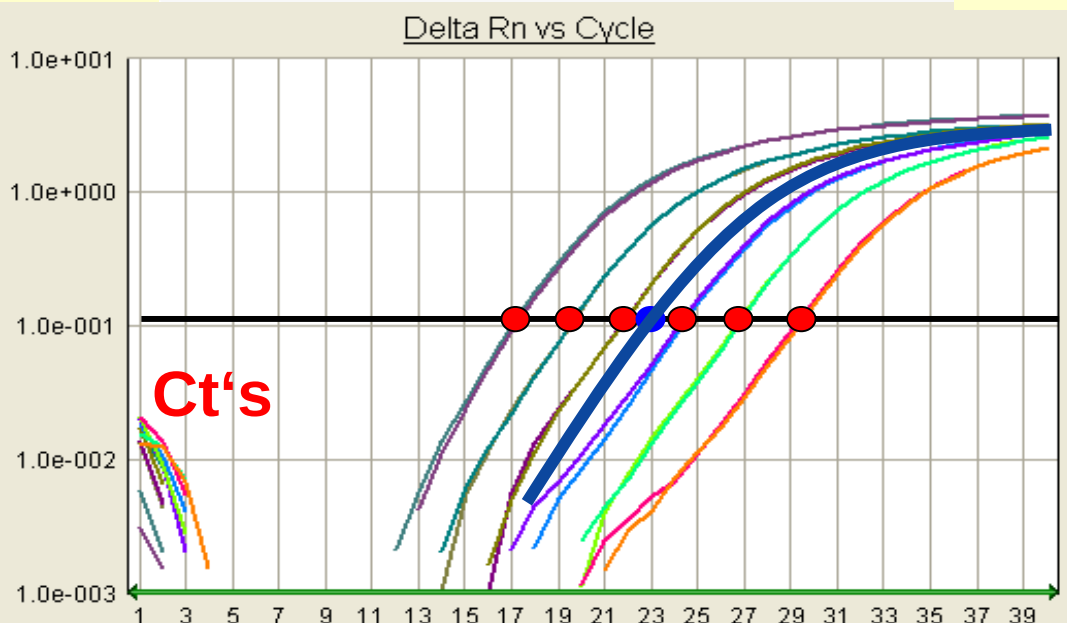
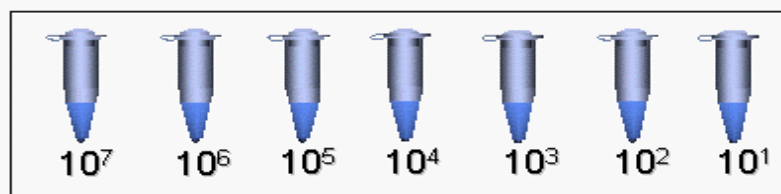


All in One Features 麻雀雖小五臟俱全!! 一套軟體可以符合全方位的應用

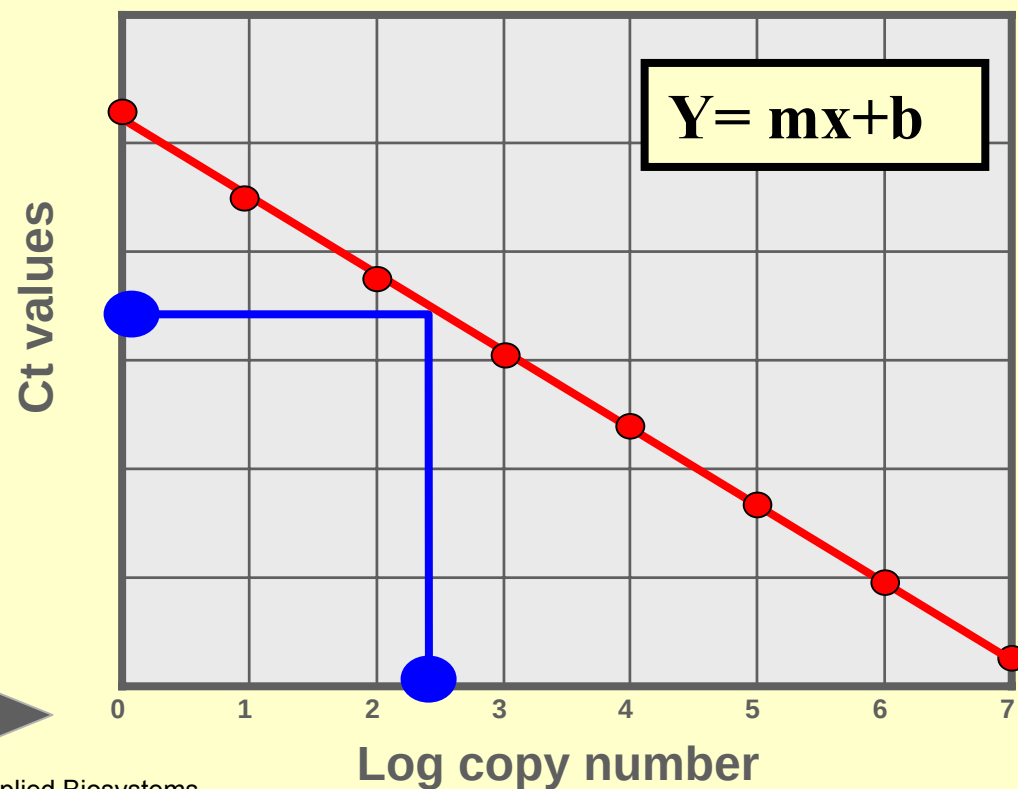
- 絕對定量Absolute Quantification - Standard curve
- 相對定量Relative Quantification – $\Delta\Delta$ Ct study
- 相對定量Relative Quantification - Relative standard curve
- Melting Curve Analysis
- SNP Genotyping Assay
- Presence/Absence

絕對定量 (Absolute Quantitation)

-主要應用於病毒量及病原菌偵測-



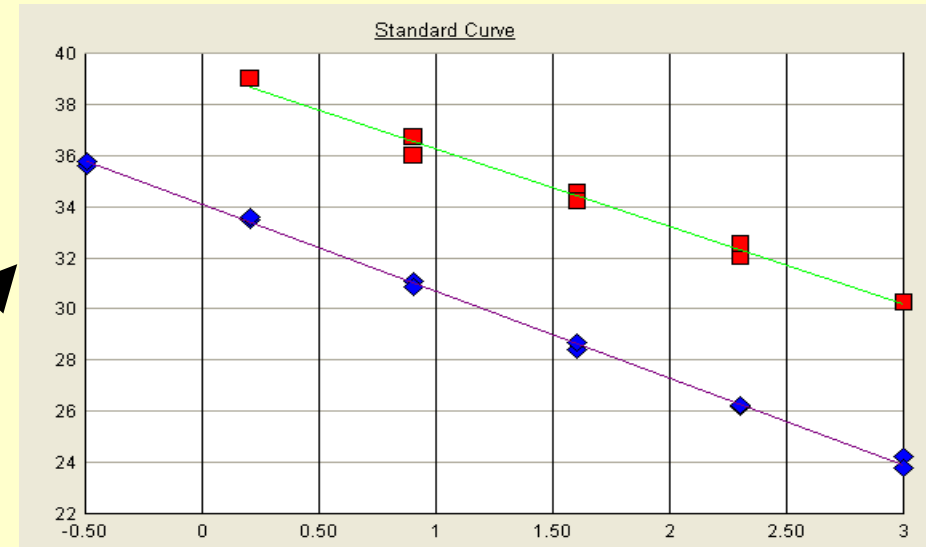
The well contains 400 target copies



Relative Quantification Analysis Methods

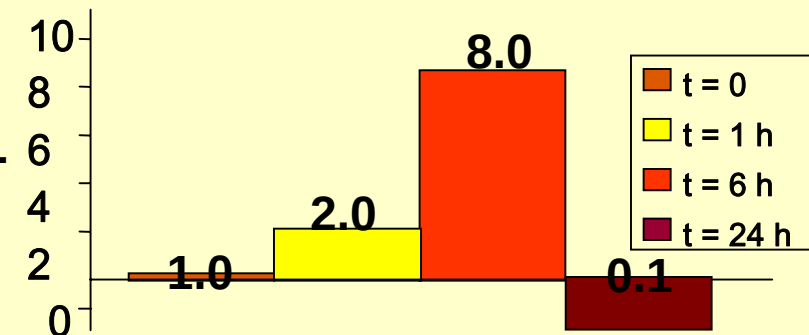
Standard Curve

	Gene A	Gene B
Heart	25.2	18.1
Liver	30.2	17.5
Lung	21.4	18.2
Brain	25.3	17.1

Comparative C_t

$$2^{-(\Delta\Delta C_t)}$$

Relative Quantity of Expression

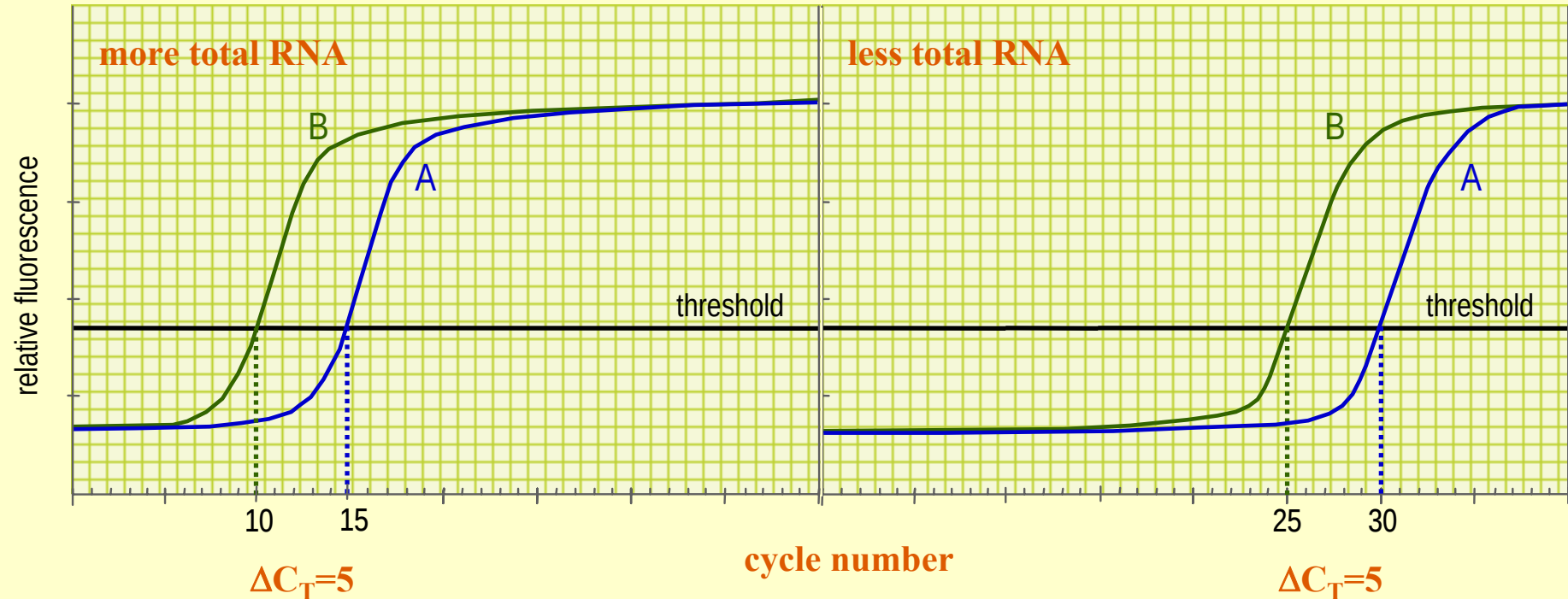


Relative Quantitation theory 相對定量

the ΔC_T

a measure of the ratio of one target to another

ΔC_T is a constant value



When the PCR efficiency of both systems is the same
the ΔC_T remains constant

Converting ΔC_T into a ratio

$$\frac{\text{A (target gene)}}{\text{B (endogenous gene)}} = 2^{-\Delta C_T}$$

e.g. if the ΔC_T between mRNA species A & B = 5 cycles
(i.e. it took 5 more cycles to see amplification of A)

then there is: $2^{-5} = 1/2^5 = 1/32$ as much A as B

$$\Delta\Delta C_t = \Delta C_t (\text{Unknown}) - \Delta C_t (\text{Control})$$

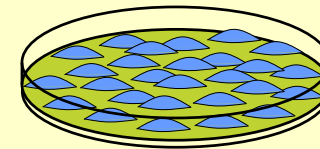
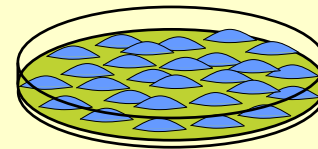
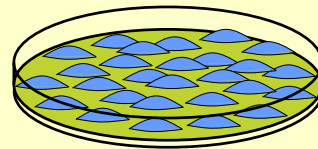
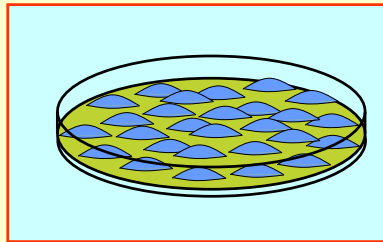
$$X_{\text{rel}} = (1+E)^{-\Delta\Delta C_t} \quad , \quad \text{If Efficiency} = \sim 100 \%,$$

$$\text{Relative Quantity} = 2^{-\Delta\Delta C_t}$$

Relative Quantification

Calibrator

The sample used as the basis for comparative results



time

t = 0

t = 12

t = 24

t = 48

Convert purified total RNA to cDNA

[GOI]

[ENDO]

[GOI]

[ENDO]

[GOI]

[ENDO]

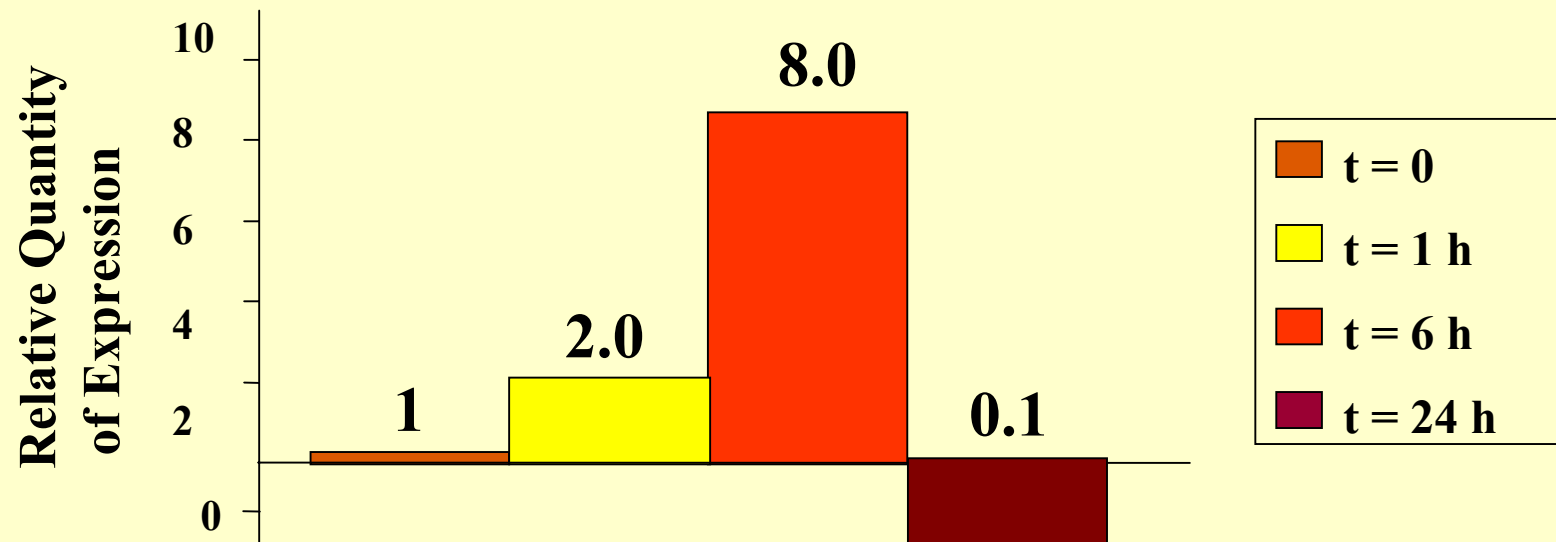
[GOI]

[ENDO]

Control for sample variability

$\Delta\Delta C_t$ Calculations (Comparative C_t)

	IL-2	18S	DC_t	DDC_t	2^{-DDC_t}
T=0 (calibrator)	24	9	15	0	1.0
T=12hr	24	10	14	-1	2.0
T=24hr	23	11	12	-3	8.0
T=48hr	28	10	18	3	0.1

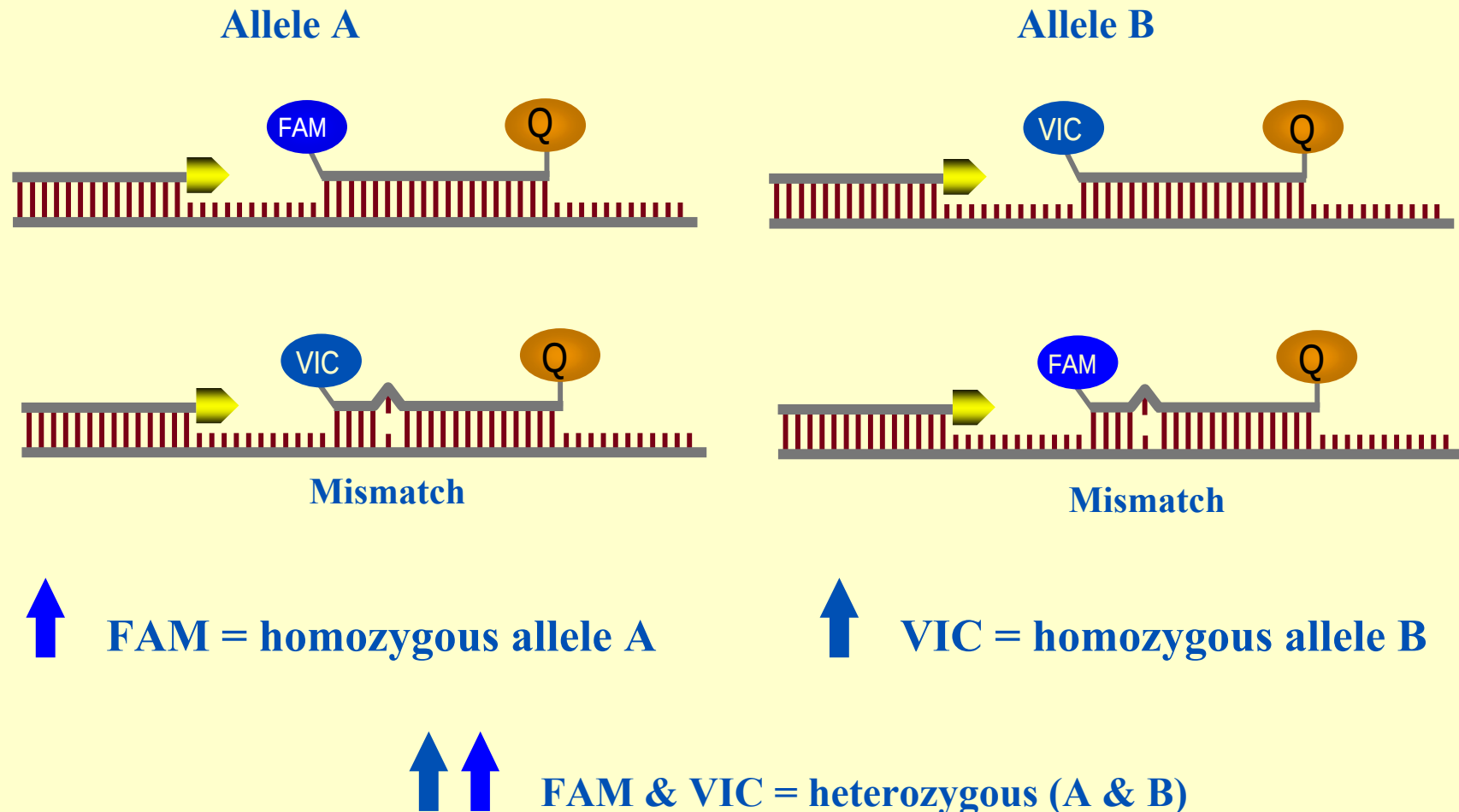


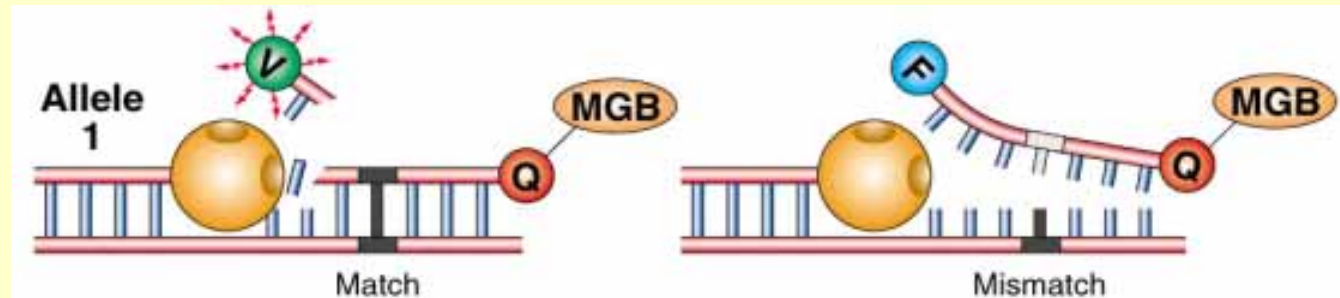


The Application of SNP Genotyping on Real Time PCR System

Genotyping—competing probes

discriminating alleles and single nucleotide polymorphisms

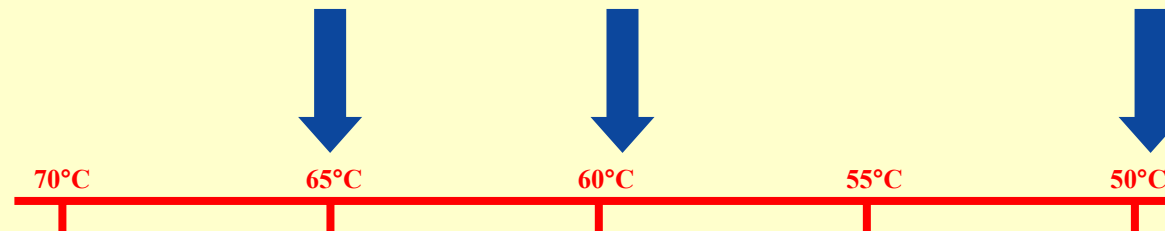




MGB

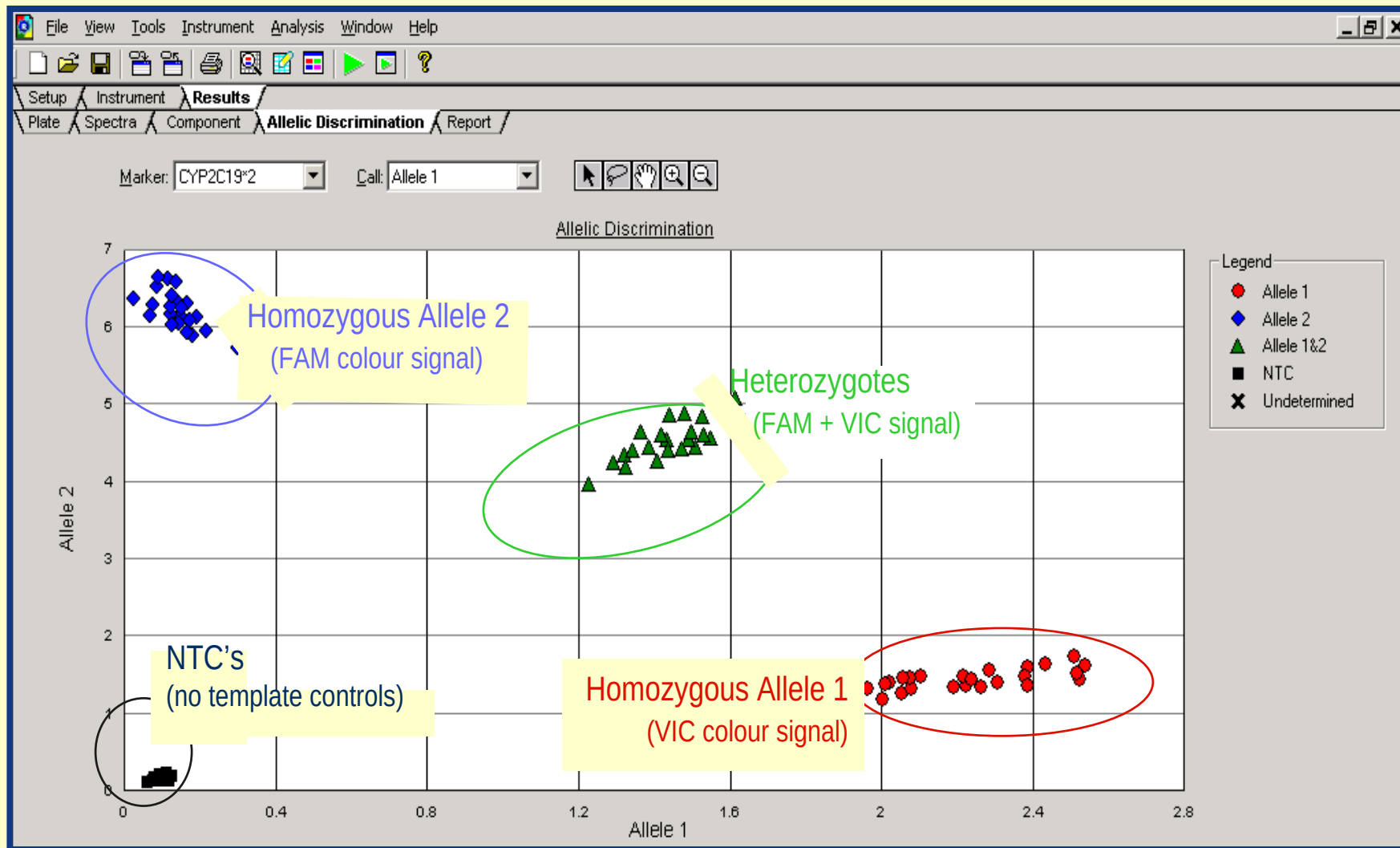
T_m=65

T_m=49 (ΔT_m = 17°C)



$\Delta T_m = T_m$ of perfectly matched probe - T_m of mismatched
Discrimination of alleles is achieved by using an annealing/
extension temperature within the ΔT_m window

allelic discrimination (SNP) data





StepOne Software Introduction

All in One Features 麻雀雖小五臟俱全!! 一套軟體可以符合全方位的應用

- 絕對定量Absolute Quantification - Standard curve
- 相對定量Relative Quantification – $\Delta\Delta$ Ct study
- 相對定量Relative Quantification - Relative standard curve
- Melting Curve Analysis
- SNP Genotyping Assay
- Presence/Absence

Design your
experiment...

1. Define



* Experiment
Properties



Methods &
Materials

2. Set Up



* Targets



* Standards



* Samples



Run Method



Reaction Setup

3. (Optional) Order



Materials List

R System

Software Wizard

AB Applied
Biosystems

is

Tools

Help

en... Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export... Print Report...

Experiment: **Untitled [2]** Type: **Quantitation - Standard Curve** Reagents: **TaqMan® Reagents**

1B. Define: Methods & Materials

Methods & Materials Help ?

I

Instructions:

Select the quantitation method, reagents, ramp speed, and type of template for the real-time PCR reactions.

Which quantitation method are you using?

✓ Standard Curve

Relative Standard Curve

Comparative Ct ($\Delta\Delta C_T$)

With the standard curve method, you use standards to determine the absolute quantity of target sequence in a sample.

Which reagents do you want to use to detect the target sequence?

✓ TaqMan® Reagents

SYBR® Green Reagents

These real-time PCR reactions contain two primers and a TaqMan® probe. The primers are designed to amplify the target sequence. The TaqMan probe is designed to hybridize to the target sequence and generate fluorescence signal when the target sequence is amplified.

Which ramp speed do you want to include in the instrument run?

Standard (~ 2 hours to complete a run)

✓ Fast (~ 40 minutes to complete a run)

For optimal results using the Fast ramp speed, Applied Biosystems recommends using Fast reagents for your real-time PCR reactions.

What type of template do you want to use in the real-time PCR reactions?

✓ cDNA (complementary DNA)

RNA

gDNA (genomic DNA)

You are adding cDNA to the real-time PCR reactions. You have already performed reverse transcription to convert the RNA to cDNA.

← Previous

✓ Finish Designing Experiment

Next →

Cancel

itled [2] x

ITIAL

© 2007 Applied Biosystems

Automatic plate layout in the Design wizard

Real Time PCR 很難嗎??照著做就好囉!!

StepOne™ v1.0

File Edit Instrument Analysis Tools Help

New Experiment Open Save Close Send Experiment to Instrument Download Experiment from Instrument Print Print Report

Design your experiment

1. Define

- Experiment Properties
- Methods & Materials

2. Set Up

- Targets
- Standards**
- Samples
- Run Method
- Reaction Setup

3. (Optional) Order

- Materials List

Experiment: **Untitled** Type: **Quantitation - Standard Curve** Reagents: **SYBR® Green Reagents**

26. Set Up: Standards Standards Help ?

Instructions: Enter the number of points and replicates for all standard curves in the reaction plate. For each standard curve, enter the starting quantity and select the serial factor.

Set Up Standards = Required

- How many **points** do you need for each standard curve?
- How many identical reactions (**replicates**) do you need for each point?

For each standard curve, define the range of standard quantities by entering the **starting quantity** and **serial factor**.

Define the standard curve so that the range of standard quantities spans the expected range of quantities for the unknowns.

Target Name	Enter Starting Quantity	Serial Factor
GAPDH	100.0	1:5
RNase P	100.0	1:5

Standard Curve Preview

Standard Curve Preview for GAPDH

Well Count

12 - Unknown 36 - Standard 6 - Negative Control 0 - Empty

View Plate Layout

Arrange Plate by: Rows Place Negative Controls in: Upper Left

Show in Wells View Legend

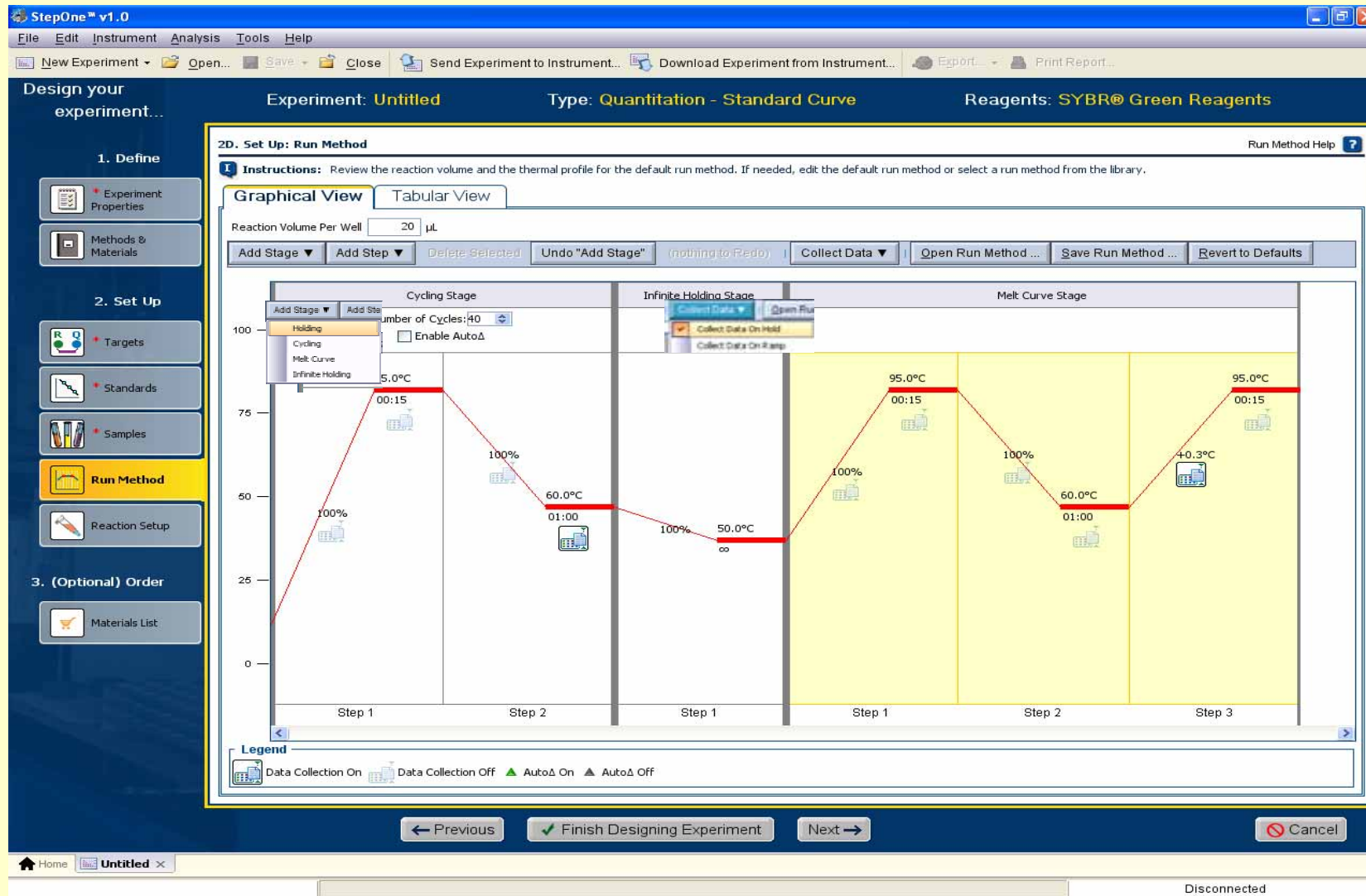
	1	2	3	4	5	6	7	8
A	GAP...	GAP...	GAP...	RNA...	RNA...	RNA...	Sample 1	Sample 1
B	Sample 1	Sample 1	Sample 1	Sample 1	Sample 2	Sample 2	Sample 2	Sample 2
C	Sample 2	Sample 2	GAP...	GAP...	GAP...	GAP...	GAP...	GAP...
D	GAP...	GAP...	GAP...	GAP...	GAP...	GAP...	GAP...	GAP...
E	GAP...	RNA...	RNA...	RNA...	RNA...	RNA...	RNA...	RNA...
F	RNA...	RNA...	RNA...	RNA...	RNA...	RNA...	RNA...	RNA...

← Previous Finish Designing Experiment Next → Cancel

Home Untitled x

Disconnected

Run Module – 圖形化介面，操作簡單易懂



Reaction Protocol Setup

StepOne v1.0

File Edit Instrument Analysis Tools Help

New Experiment Open Run Close Send Experiment to Instrument Download Experiment from Instrument Experiment Print Report

Design your experiment... Experiment: **Untitled** Type: **Quantitation - Standard Curve** Reagents: **TaqMan® Reagents**

1. Define

- Experiment Properties
- Methods & Materials

2. Set Up

- Targets
- Standards
- Samples
- Run Method
- Reaction Setup**

3. (Optional) Order

- Materials List

2E. Set Up: Reaction Setup > Reaction Mix Calculations

Instructions: For each target assay in the reaction plate, select the assay type (if using TaqMan reagents), then review the calculated volumes for preparing the standard dilution series, samples, and PCR reactions. If needed, edit the reaction volume, excess reaction volume, component concentrations, and/or stock concentrations. Click "Print Reaction Setup" to print instructions on how to prepare the PCR reactions.

Reaction Mix Calculations Sample Dilution Calculations

Select Target: **GAPDH** **RNAse P**

Assay Type: **Inventoried/Made to Order** Reaction Volume Per Well: **20** μ L Excess Reaction Volume: **10** % **Print Reaction Setup**

Reaction: **Custom**

Master Mix Concentration: **2.0** $\times$ Assay Mix Concentration: **20.0** $\times$

Component	Volume (μ L) for 1 Reaction
Master Mix (2.0 $\times$)	10.0
Assay Mix (20.0 $\times$)	1.0
Sample (10.0 $\times$ Standard)	2.0
H ₂ O	7.0
Total Volume	20.0

Standard Dilution Series for GAPDH

Standard Concentration in Stock: **100.0** ng per μ L

Dilution Point	Source	Source Volume (μ L)	Diluent Volume (μ L)	Total Volume (μ L)	Standard Concentration (nM)
1 [100]	Stock	4.5	4.5	9.1	50.0
2 [20]	Dilution 1	1.8	7.3	9.1	10.0
3 [4]	Dilution 2	1.8	7.3	9.1	2.0
4 [0.8]	Dilution 3	1.8	7.3	9.1	0.4
5 [0.16]	Dilution 4	1.8	7.3	9.1	0.1

← Previous Finish Designing Experiment Next → Cancel

Home Untitled x

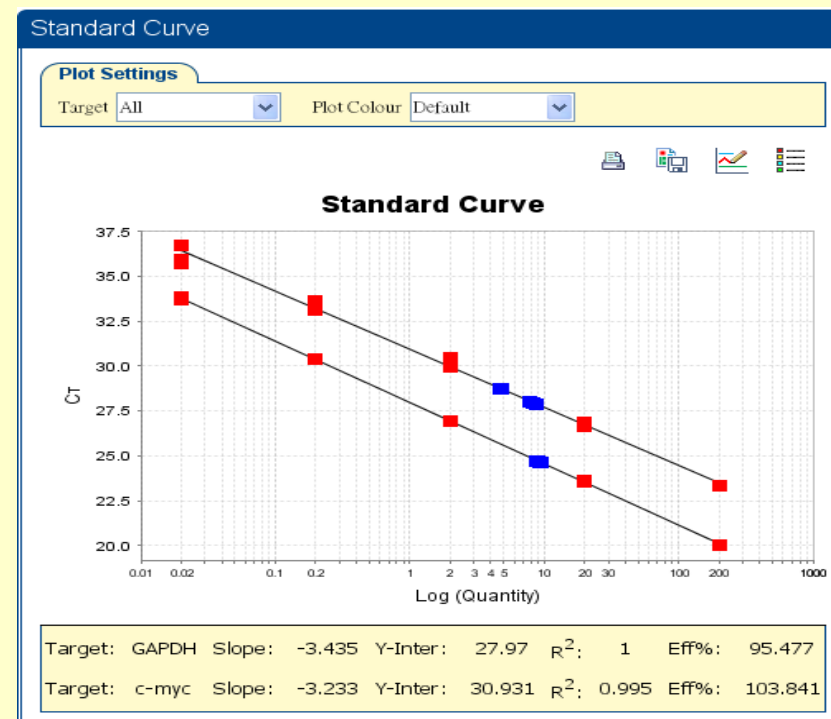
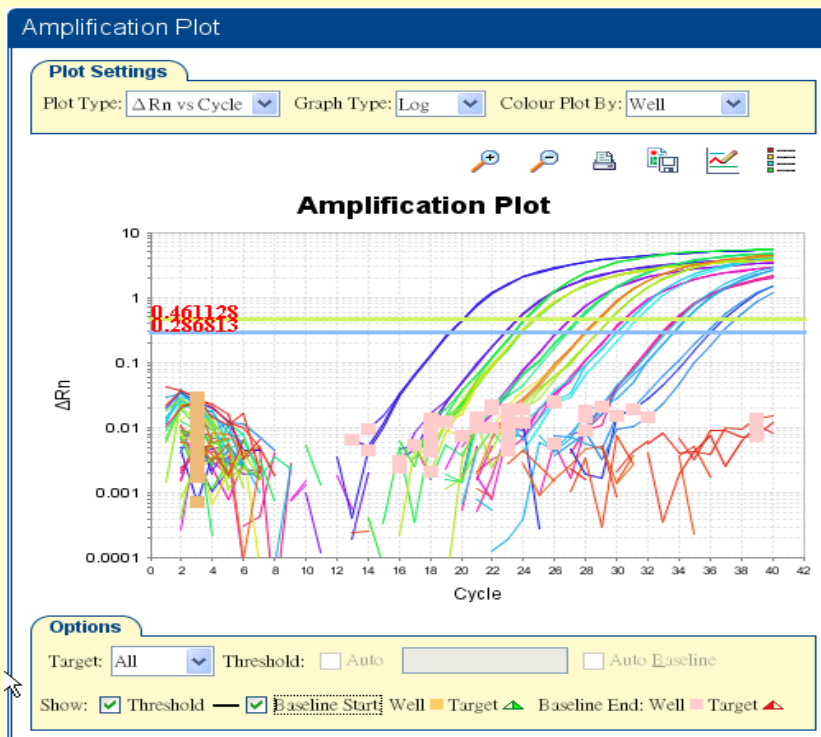
Disconnected

Additional New Features in the Software

Absolute & Relative Standard Curve

Relative Quantification can read Absolute Quantification Data

不用擔心設定錯誤造成分析上的困擾



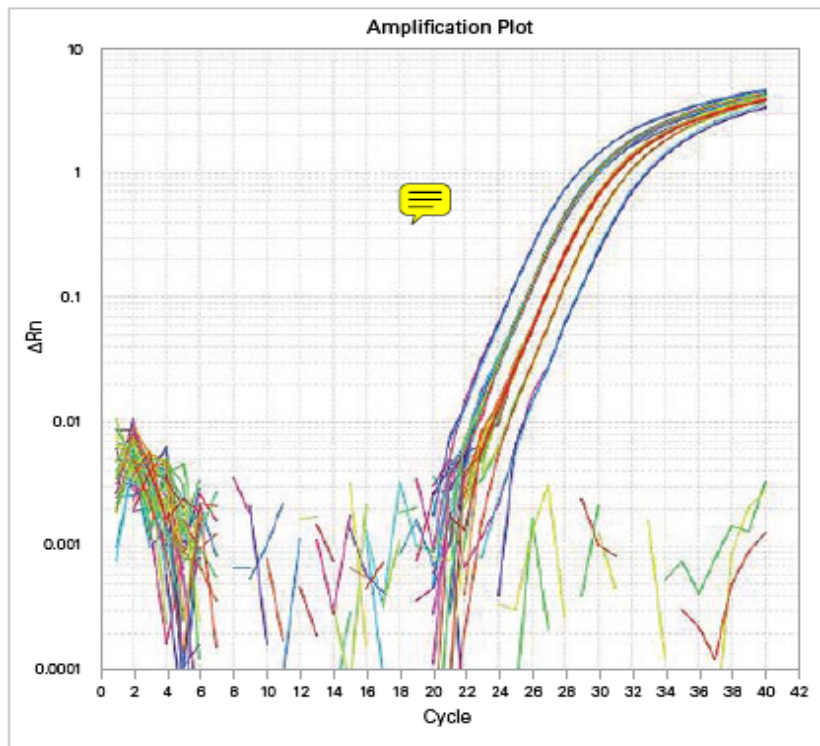
Genotyping Assay

(Real-Time data collected in the same file as pre- and post-read)
Amplification plot 幫你做簡易troubleshooting

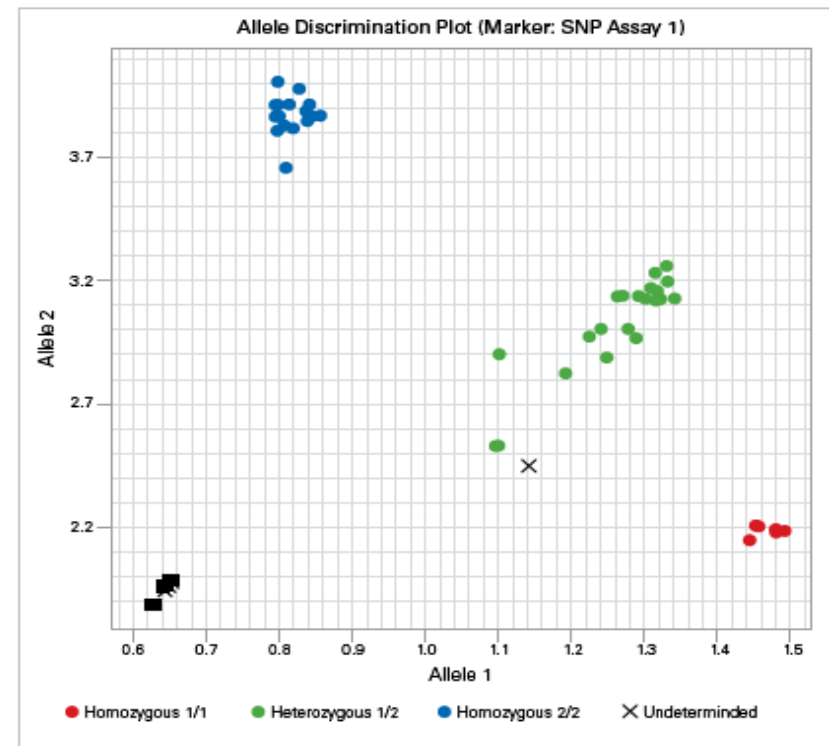
What do you want to include in the instrument run?

Include Stages: ☒ Pre-PCR Read ☒ Amplification ☒ Post-PCR Read

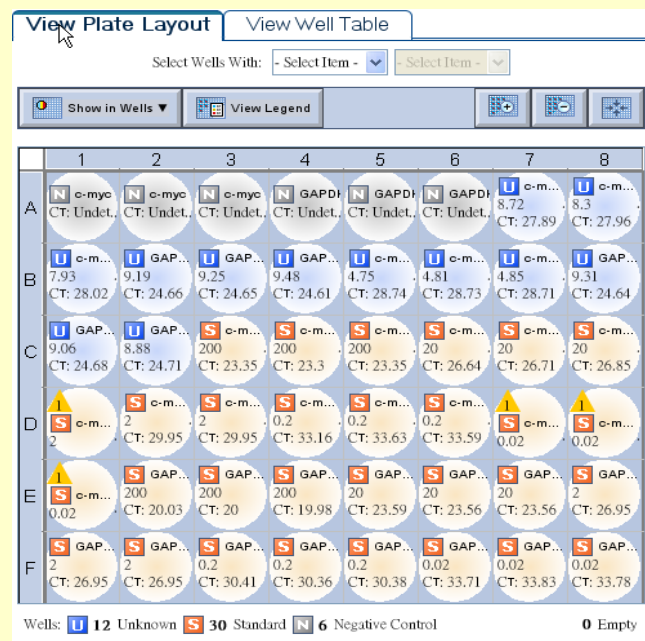
Amplification Plot



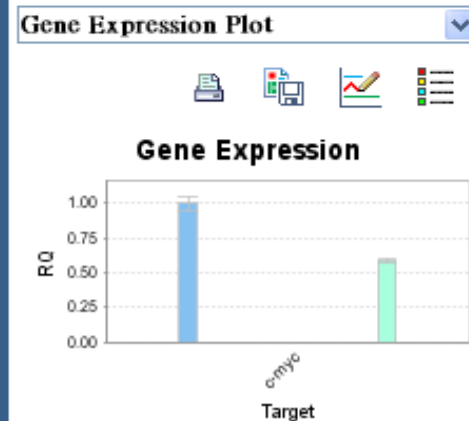
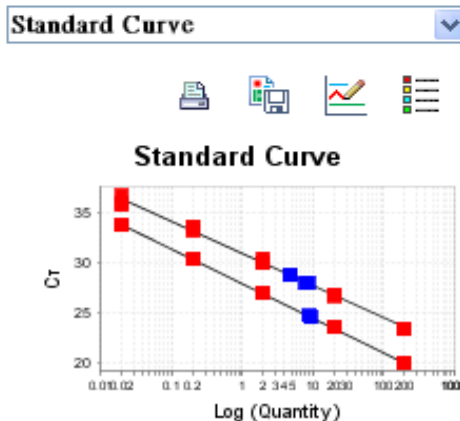
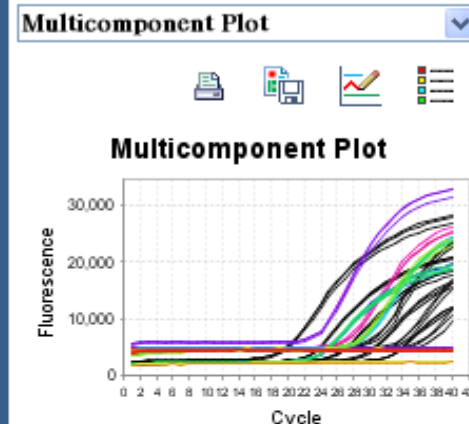
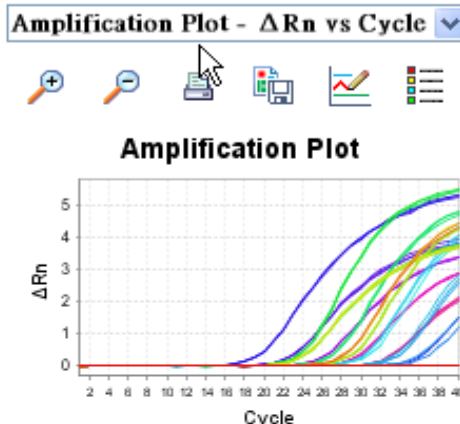
Gene Expression Analysis



Result: Export to Excel, PowerPoint, or save as JPEG

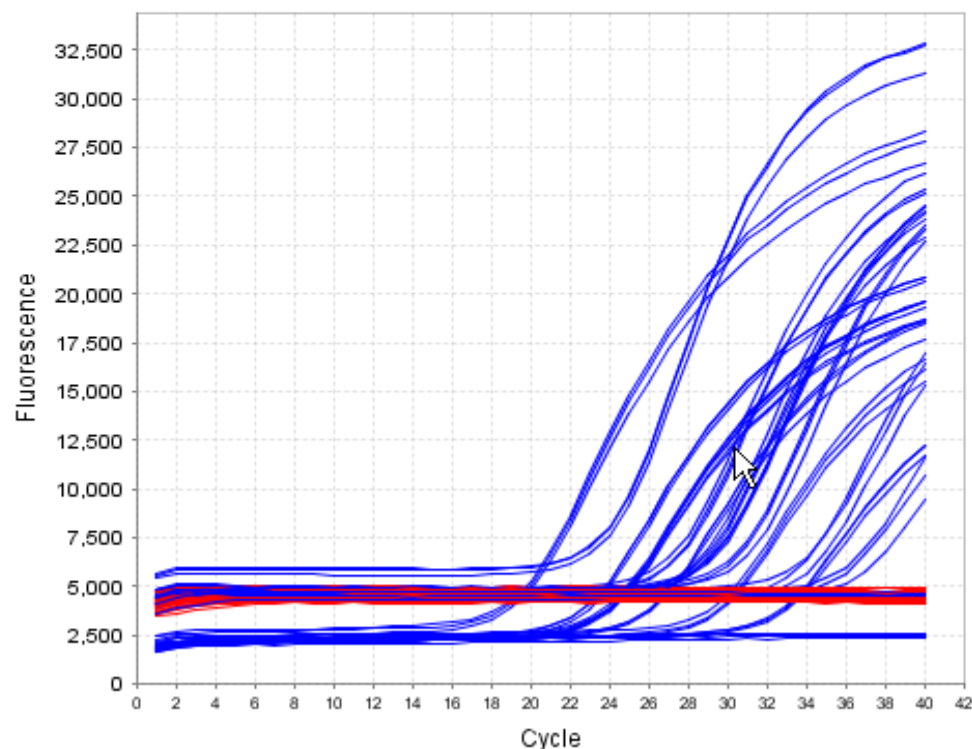


Multiple Plots View

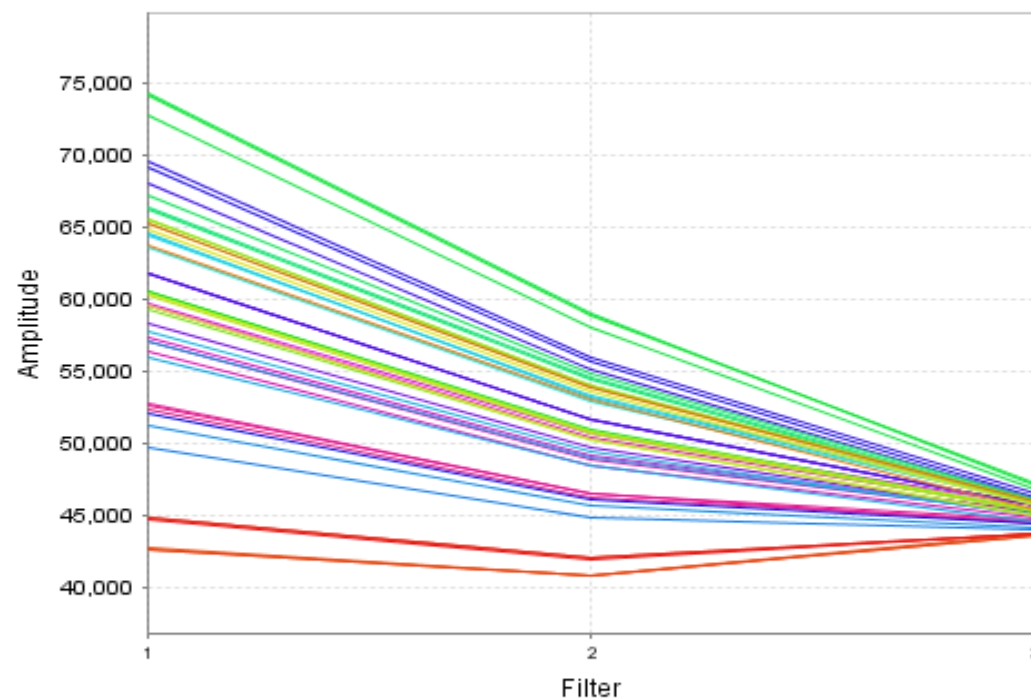


Multicomponent Plot and Raw Spectra

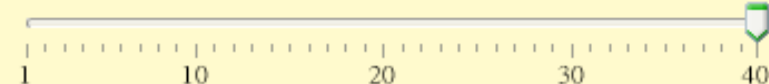
Multicomponent Plot

Plot SettingsPlot Colour Dye **Multicomponent Plot**

Raw Data Plot

**Raw Data Plot****Options**

Show Cycle 40



StepOne 操作軟體主要特點



- 超優標準配備，不需額外添購其他套裝軟體
- 內建軟體操作精靈 – 淺顯易懂
- 貼心軟體設計 - 擁有防呆功能
- ❖ 自動化reaction condition setup – 不必再擔心任何人為疏失
- ❖ 所有data都存入同一檔案 – 不必擔心因設定錯誤所造成實驗failed
- ❖ 分析結果只需一個按鈕，可直接export to Excel , Power Point 或 JPEG 檔案
- Remote Monitoring, E-mail Notification – 無需耗時等待 data

Can the StepOne System be used as a conventional thermal cycler ?

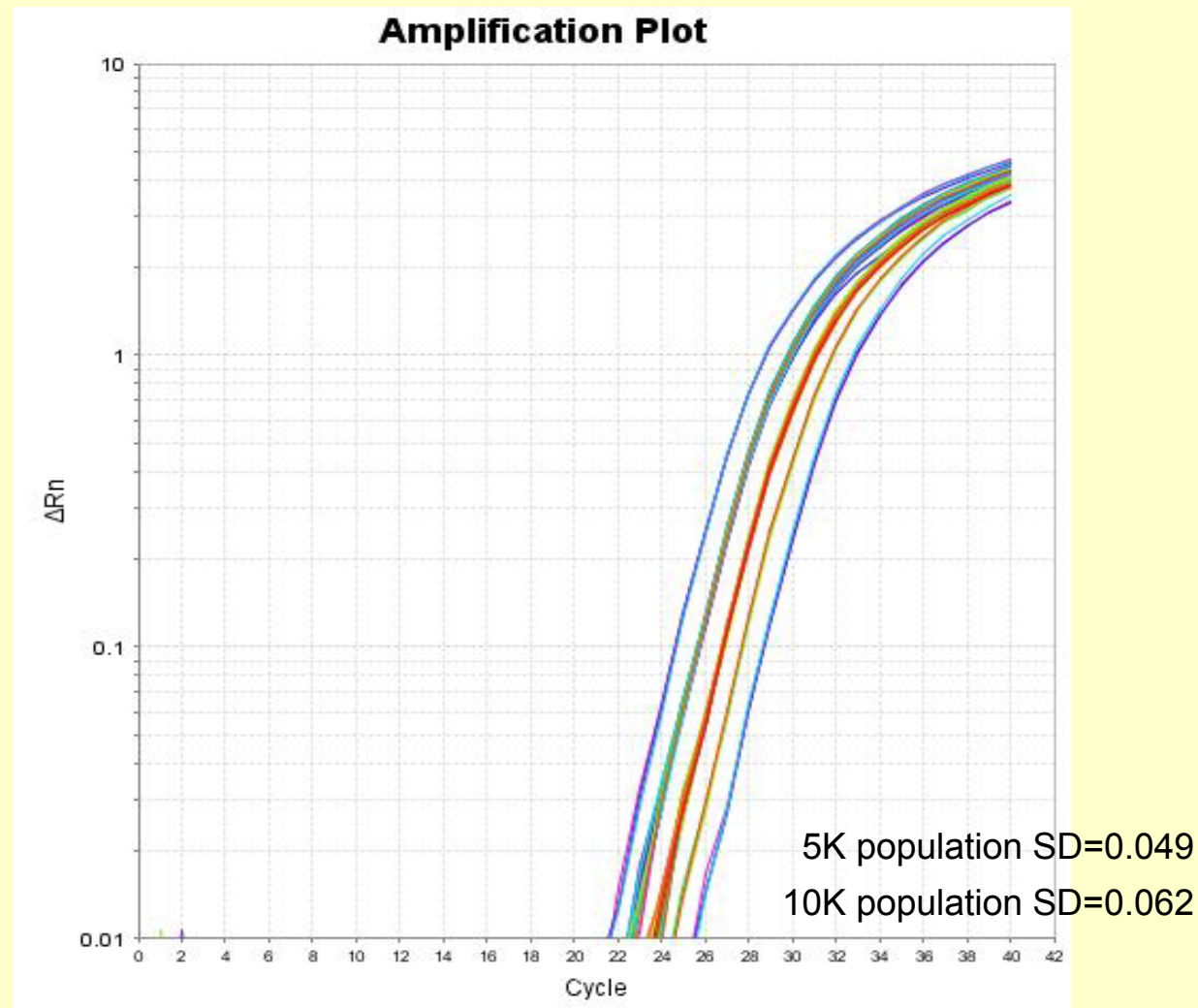
You can create a thermal cycler protocol on the touchscreen and use the StepOne System as a thermal cycler.



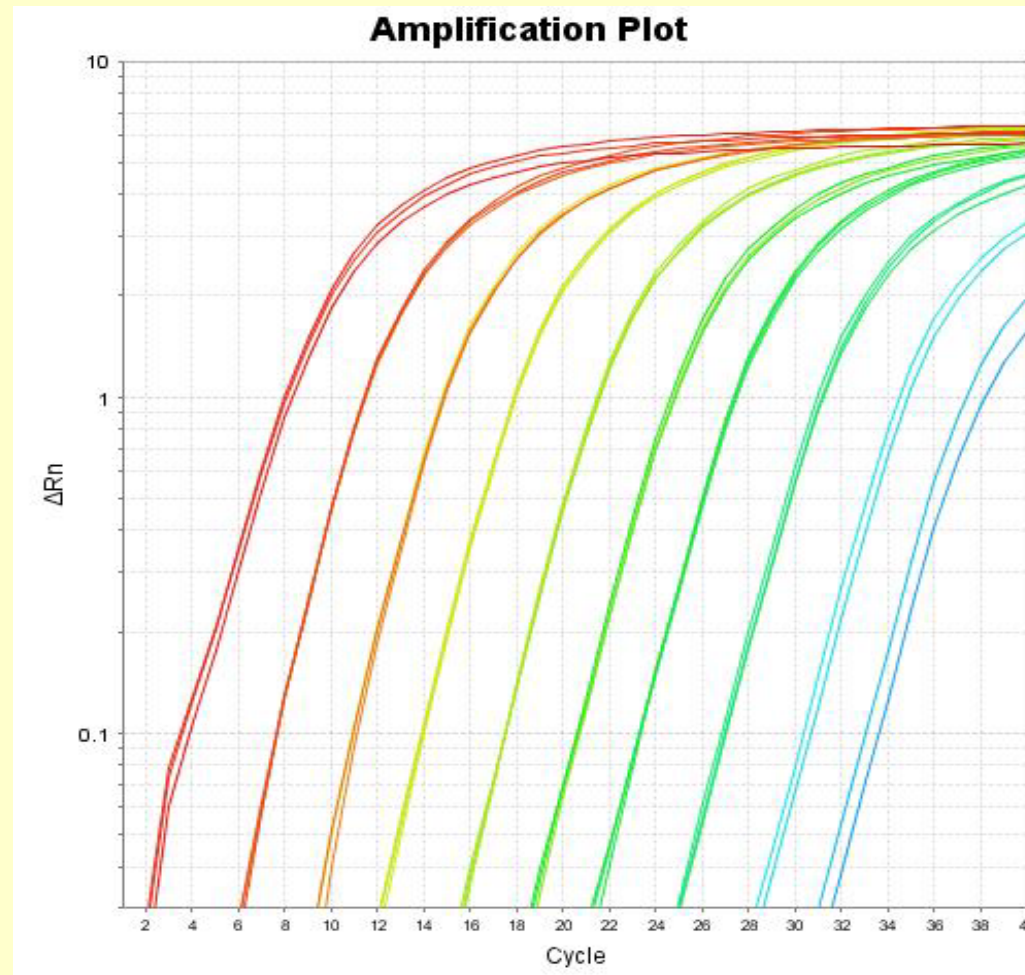
Performance specifications customers have come to expect with AB instruments

- High Precision : 2- fold discrimination with 99.7% confidence
- High Sensitivity : 10 copies per 30 ul
- Broad Dynamic Range : 9 logs
- Dual Reporter and Single Reporter Uniformity

RNase P Instrument Verification Plate

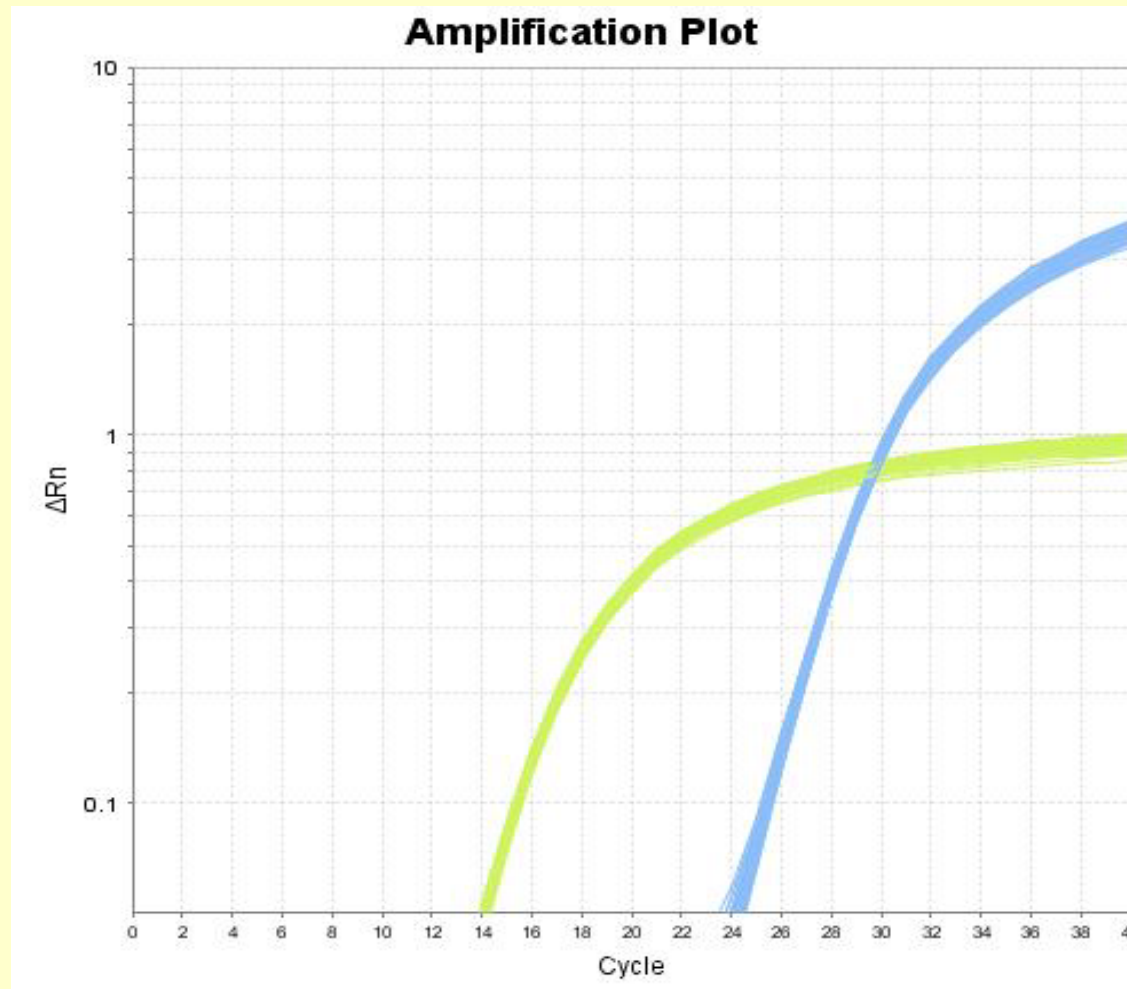


9-Log Dynamic Range



Dilution series of gDNA using 18S TaqMan probes and primers

Dual Reporter in Fast Mode



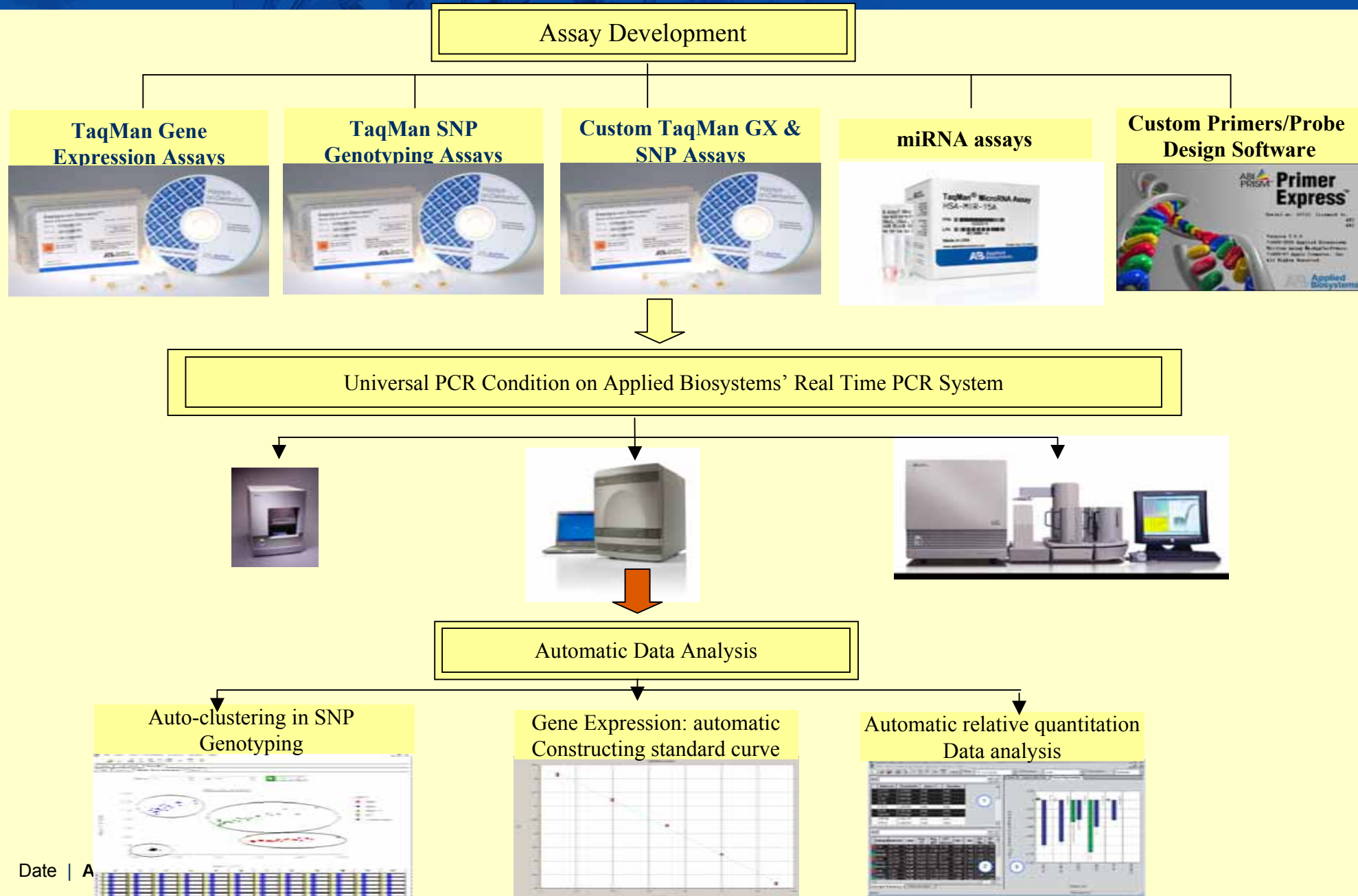
18S SD=0.088

TGFβ SD=0.080

Multiplexed 18S VIC-labeled and TGFβ FAM-labeled TaqMan® probes and primers

Summary

- 輕鬆擁有百萬級以下配備齊全的首選機器!!
- 操作方便性: 48 well plate or Single tube
 - 精確、穩定的PCR控溫系統: Peltier technology
 - No Gradient !! 準確度及再現性高 !!
- 兩種Sample ramp rate 供選擇---不需更換Block
 - Standard mode : 40 cycles 2hr
 - Fast mode : 40 cycles 40min
- 多重模式操作機器：減少為等待data而耽擱時間的困擾
 - 可遠端控制機器(download data、monitor experiment process)
- 人性化操作介面：機器為觸控式螢幕、電腦軟體圖形化介面操作簡單
- 一機多用：除了可當Real-Time PCR，亦可當一般PCR使用
- 獨家驗收規格





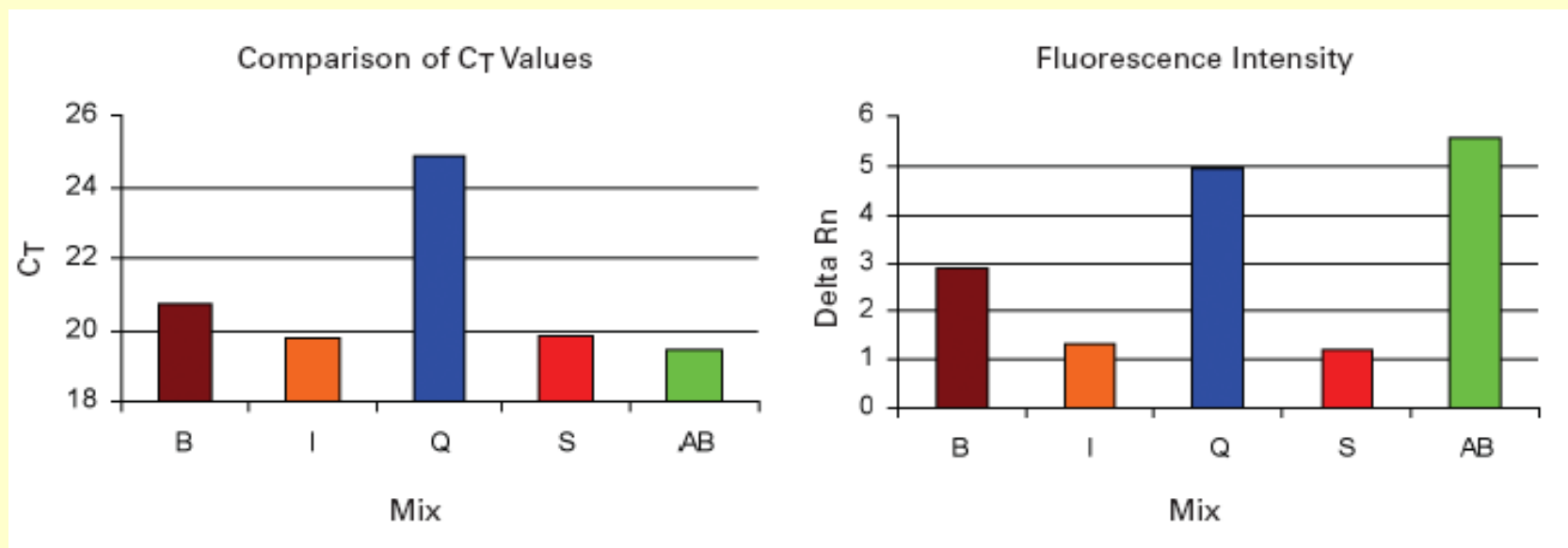
如何選擇Real-Time PCR 試劑耗材

RT (High Capacity cDNA Archive Kit” P/N 4322171)**PCR: cDNA template or gDNA**

	選擇Probe	選擇Reagent
TaqMan Probe Systems	20 x probe/ primer mix 由AB網站選購或代客設計 (TaqMan Gene Expression Assay) (Custom TaqMan Gene Expression Assay) Or Primer Express software design (300 nM primer + 200 nM probe)	P/N 4304437 (200 Rx) + 2x TaqMan Universal PCR Master Mix
SYBR Green I Systems	Primer Express software design 或自行設計 (optimized primer conc.)	P/N 4309155 (200 Rx) + 2x SYBR Green I PCR Master Mix

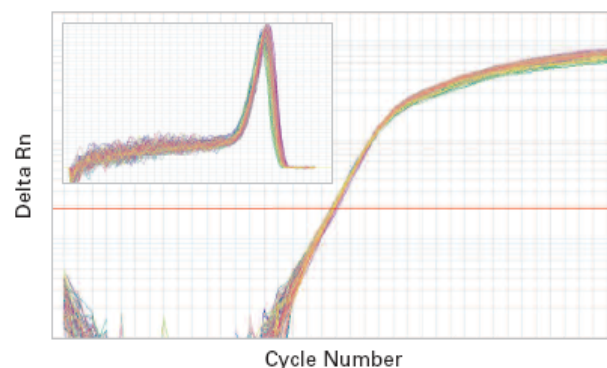
	選擇Probe	選擇Reagent
TaqMan Probe Systems	20 x probe/ primer mix 由AB網站選購或代客設計 (TaqMan Gene Expression Assay) (Custom TaqMan Gene Expression Assay) Or Primer Express software design (300 nM primer + 200 nM probe)	P/N 4309169 (200 Rx) TaqMan One-Step RT-PCR Master Mix Reagents Kit Or N808-0236 (200 Rx) TaqMan EZ RT PCR core Reagents
SYBR Green I Systems	Primer Express software design 或自行設計的primer (optimized primer conc.)	P/N 4310179 SYBR Green RT-PCR Reagent (P/N 4309155+N808-0234)

Performance of “PowerSYBR® Green Master Mix”



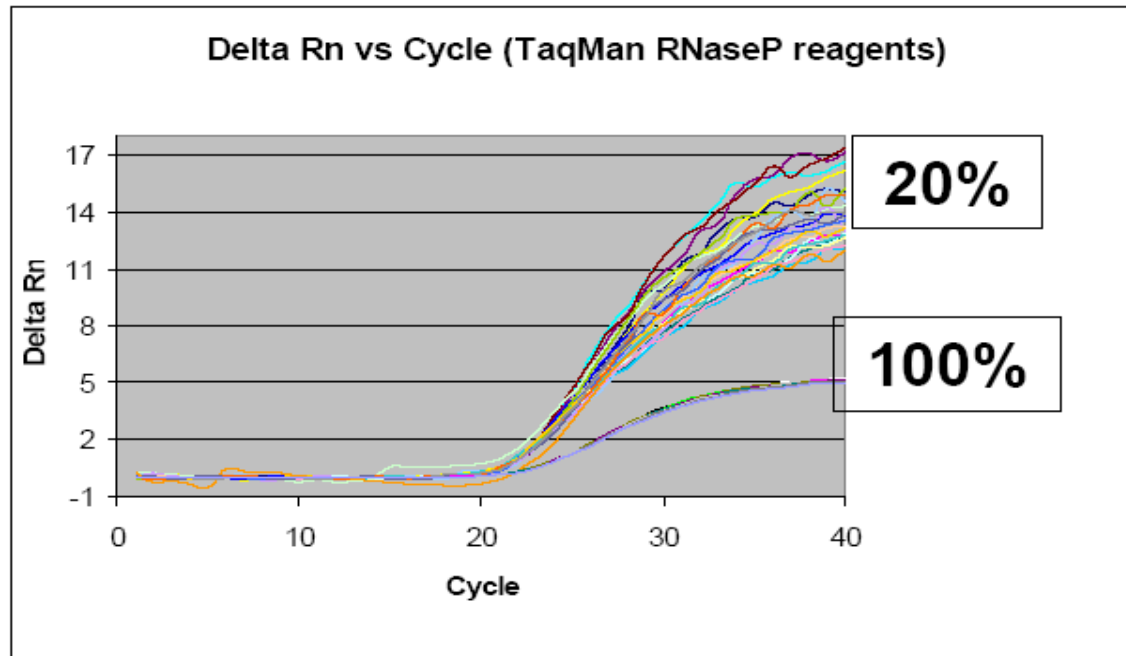
ABI PRISM® 7900HT Sequence
Detection System

Average C_T = 19.798
SD = 0.078



完整的試劑組合

All include ROX as the internal passive reference



With only 20% of the Passive Reference Dye I, amplification becomes noisy with broad C_T spread.

At 100% of the Passive Reference Dye I, C_T replicates are tight and precise.

Reverse Transcription : High Capacity cDNA archive Kit

Component
10x Reverse Transcription Buffer
25x dNTP
10x Random Primer
RT Enzyme 50U/uL
RNA (0.1~10 ug)
H2O



Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C

Real Time PCR:

TaqMan Chemistry

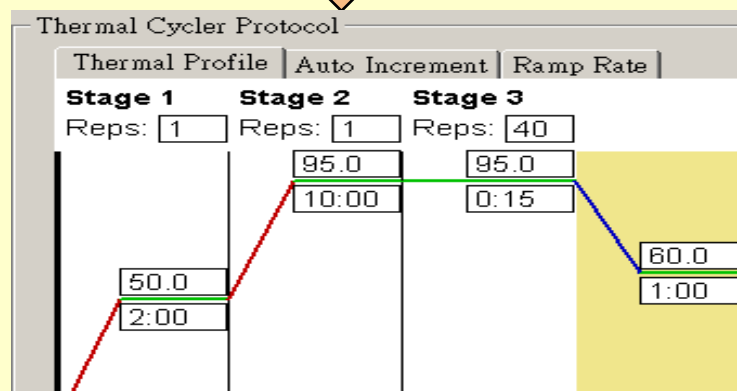
2x TaqMan Master Mix	1x	12.5 ul
20x Probe/primer Assay Mix		1.25 ul
Water		NA
cDNA	10-100 ng	5-10 ul

25 ul

SYBR Chemistry

2x SYBR Master Mix	1x	12.5 ul
F Primer	optimized	NA
R Primer	optimized	NA
Water		NA
cDNA	10-100 ng	5-10 ul

25 ul





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技術服務E-mail: twsupport@appliedbiosystems.com

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