Applied Biosystems

同步定量PCR 原理及介紹

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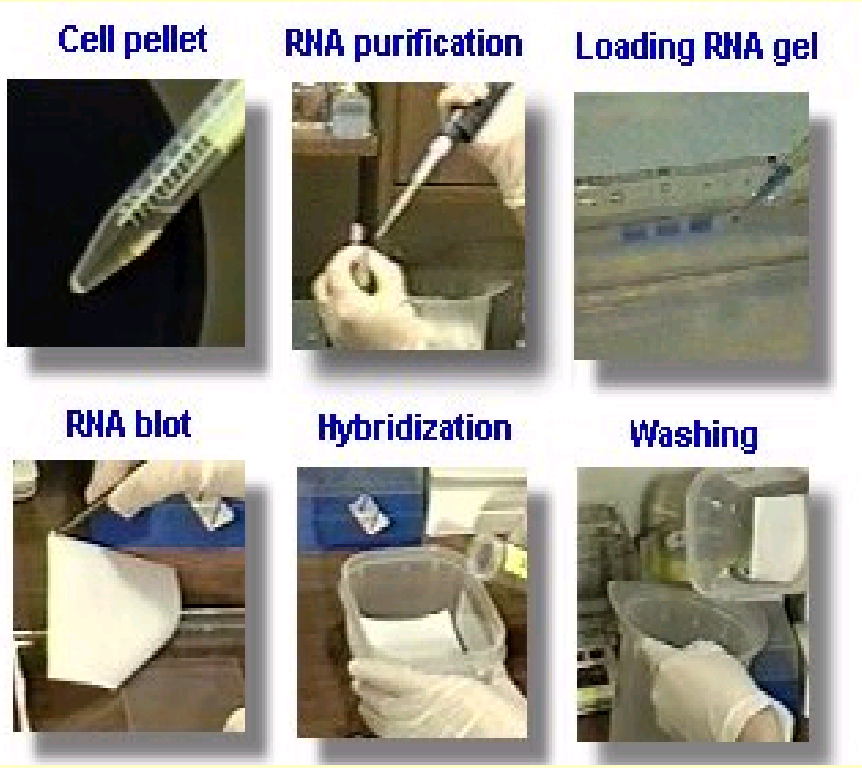
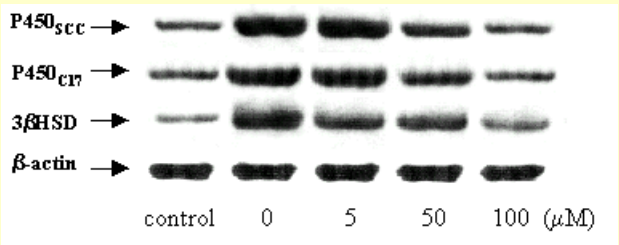
Traditional Quantitative System

- 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

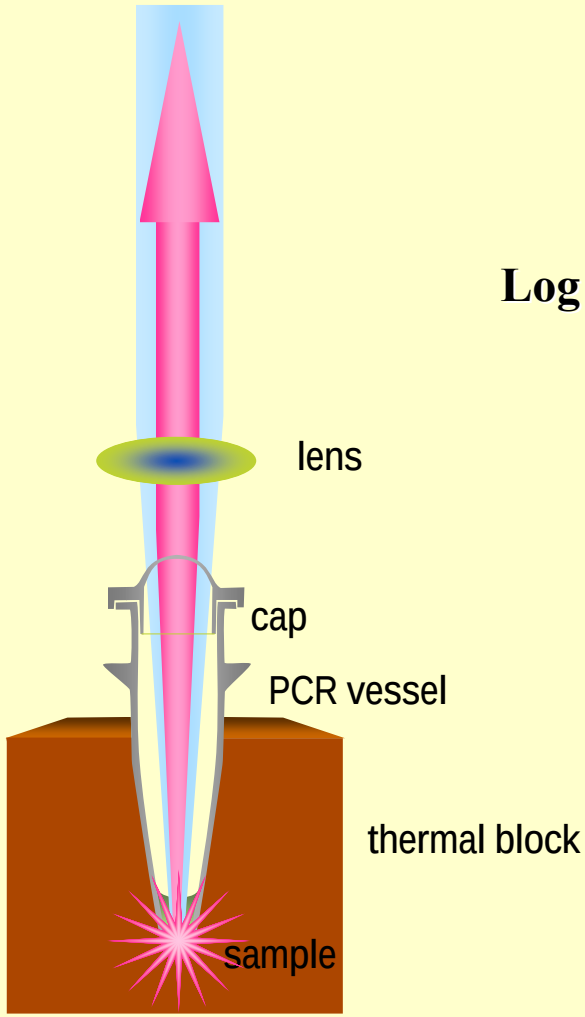
- 檢測基因轉殖食品(**GMO**)
- **癌症基因及免疫基因的定量**
- 心臟血管疾病基因監測
- 病毒定量: HBV, HCV,HPV…
- 病原菌偵測
- **Quantifier Detection**
- **miRNA 基因調控研究**

❖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



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

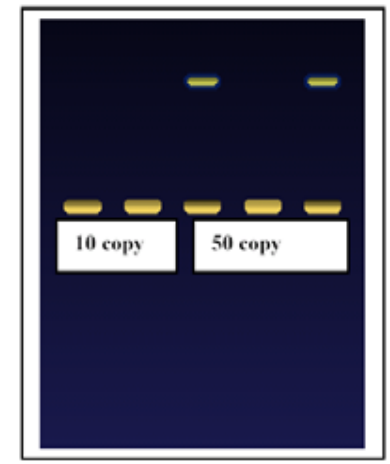
Plateau

Log [DNA]

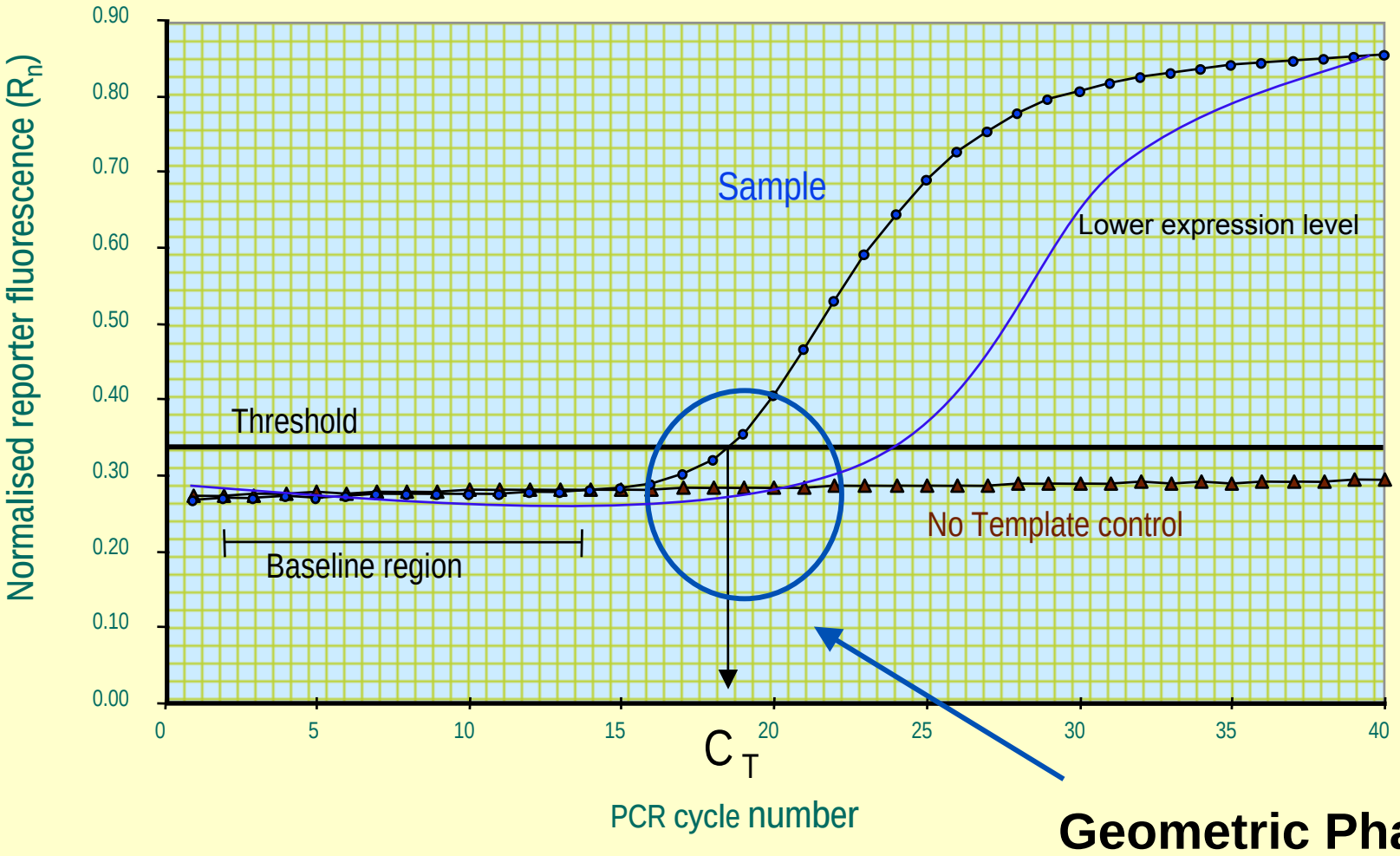
Geometric

$Y=X2^n$

Cycle #



Amplification plot features



$Y = N_0 2^n$，C_T 與起始濃度之對數值成反比

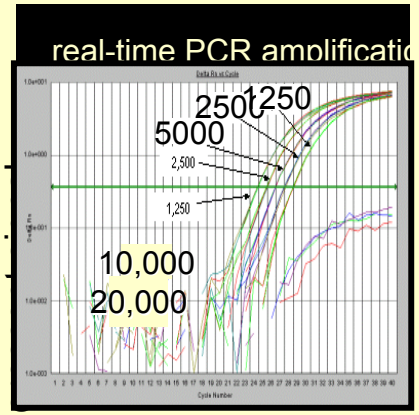
High ThroughPut Nucleotide Quantitation System

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

Pipette Samples

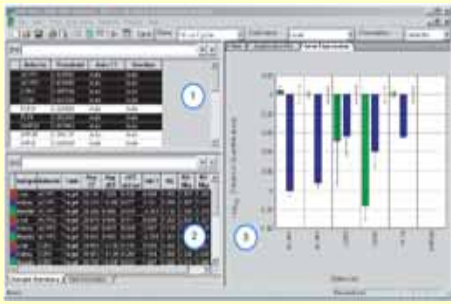
StepOne / 7300 / 7500 / 7500fast / 7900

Read Results



Standard curve

Relative quantitation



ABI Real Time PCR 的發展史

Applied Biosystems

Total Solutions for you!

ABI 發明 TaqMan Chemistry & Real-Time PCR 的理論

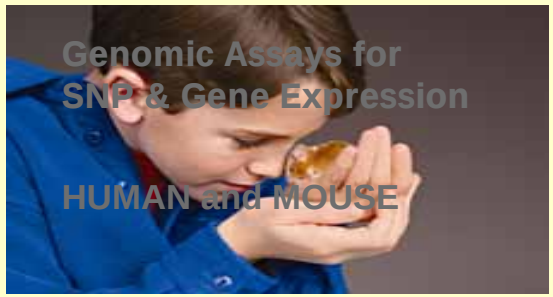
1997

ABI 獨創 相對定量 & ΔCt 的觀念

1998

ABI 特有的定量實驗 保證 PCR efficiency達 100%

2000



2001

2004

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型

ABI 7000

2001 上市超人氣機種
2002 獲選最佳儀器

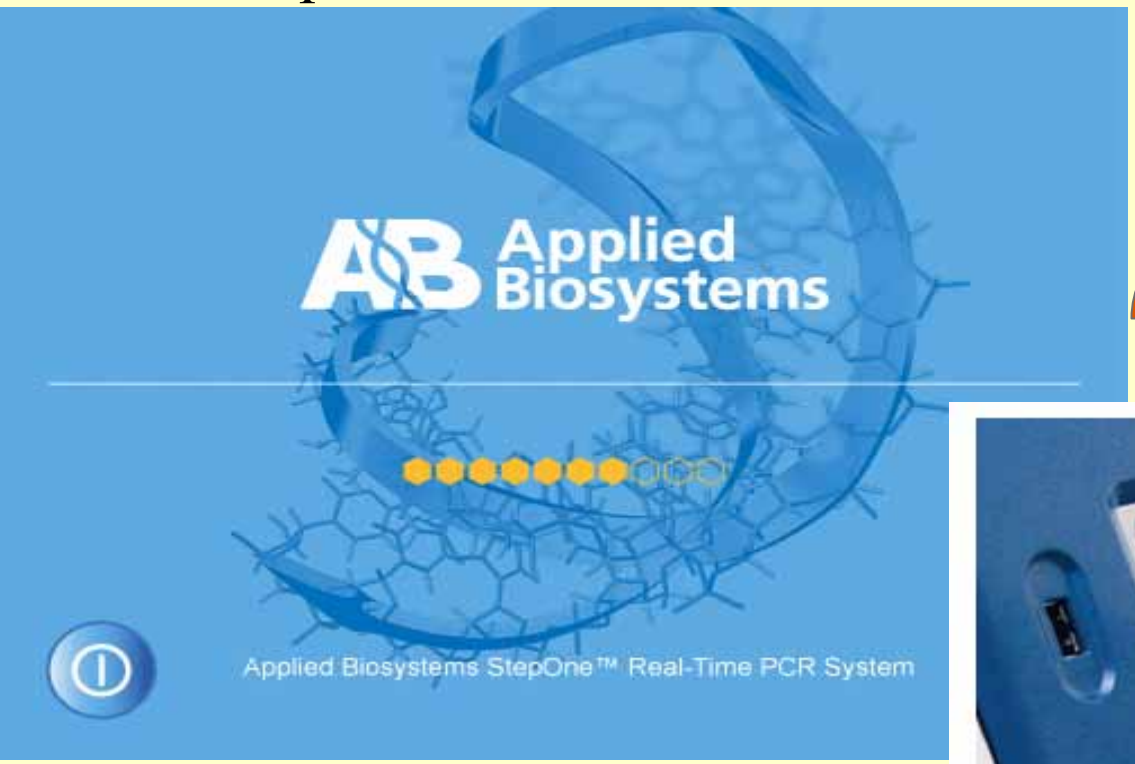
ABI 7500
第三代全新機種

StepOne
最新機種

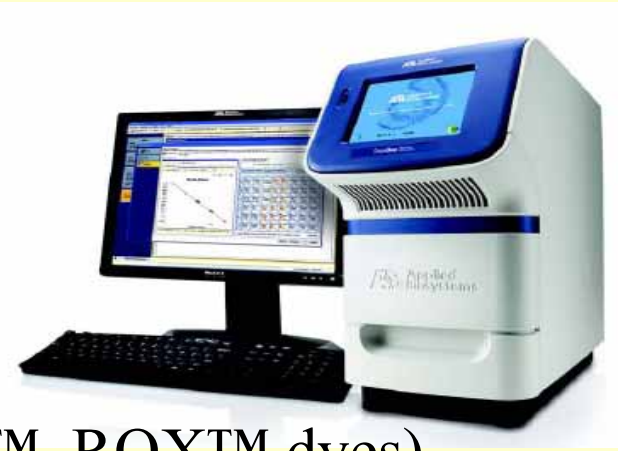
簡易三步驟!!

Basic Operation

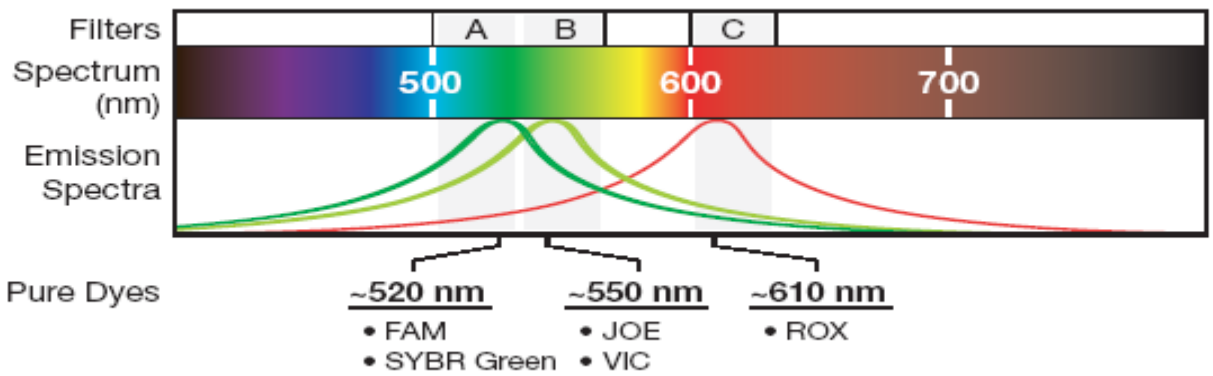
- Soft power button on the LCD touchscreen



The **StepOne™** Real-Time PCR System Introduction

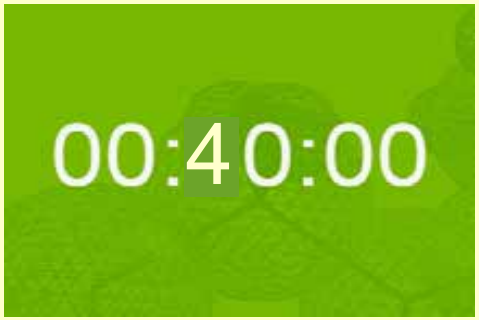


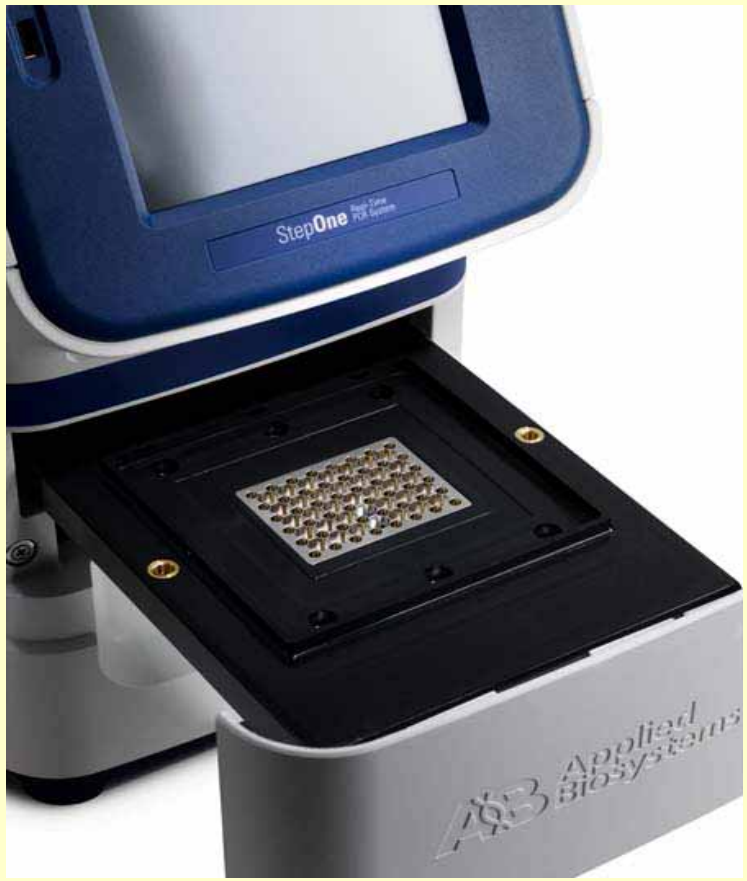
- 48 well 0.1 ml tube
- Single LED excitation
- 3 color System (FAM™/ SYBR® Green, VIC®/JOE™, ROX™ dyes)
- Fast and Standard ramping in the same block (1-100%)
- Standalone or co-located
- Remote monitoring, LAN



StepOne™ Real-Time PCR System: The Basics

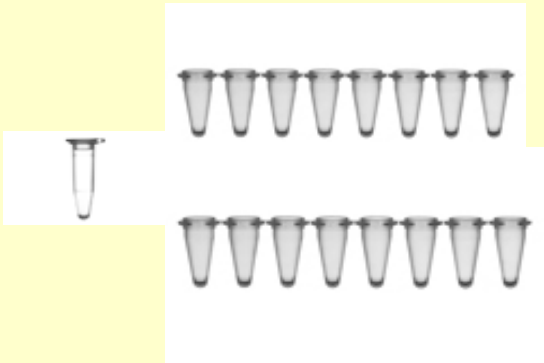
- One block, Two speeds
  - –Fast cycling: 40 cycles in under 40 minutes
  - –Standard cycling: 40 cycles in under 2 hours
  - – 48-wells
  - –10-30µl reaction volume





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

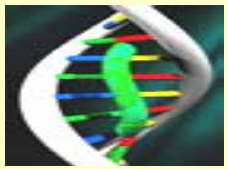
- 兩種Sample ramp rate 供選擇---不需更換Block
  - Standard mode ：40 cycles 2hr
  - Fast mode ：40 cycles 40min

- 全能多功，可滿足所有實驗應用需求

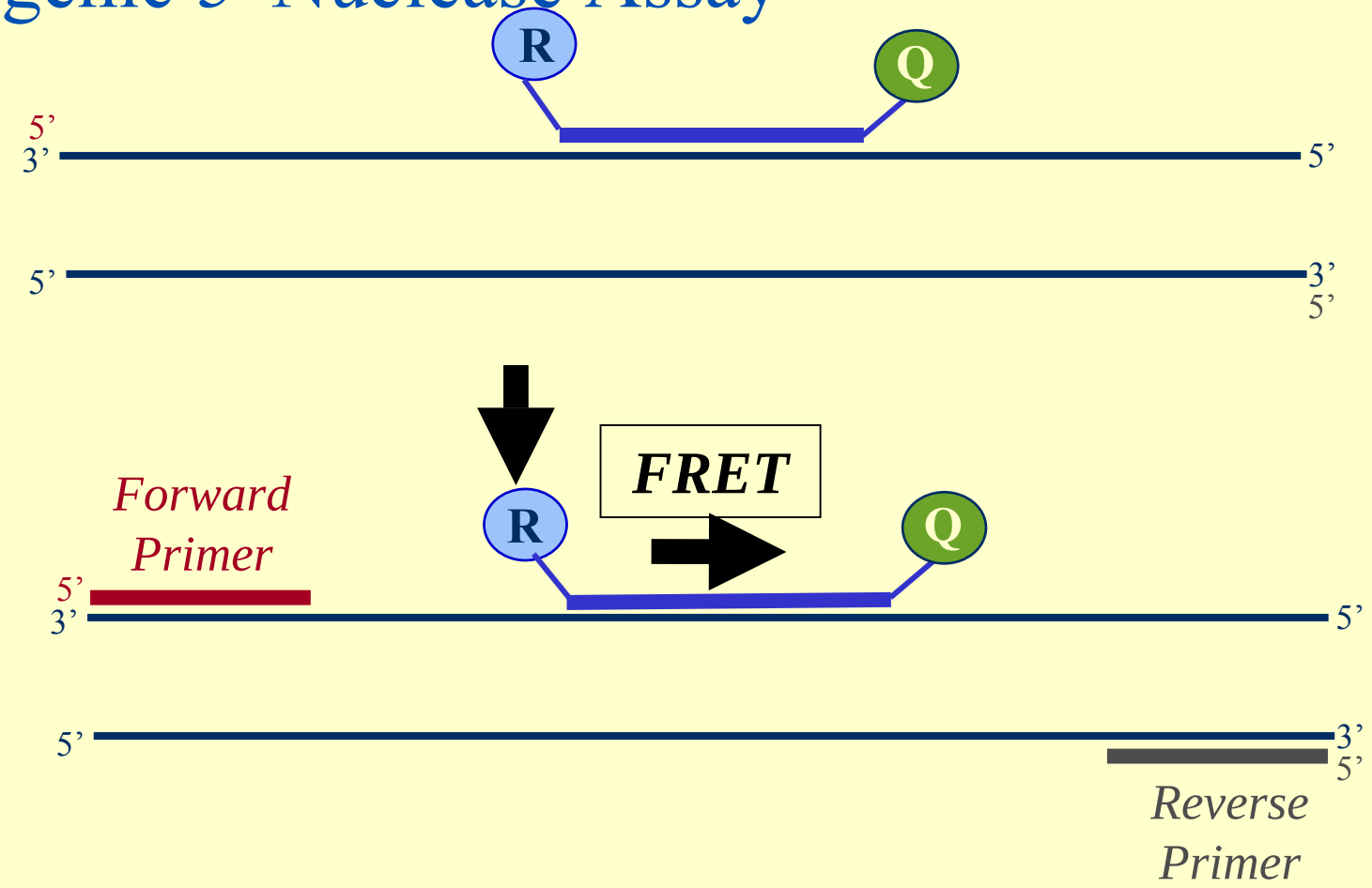
- 人性化操作介面：機器為觸控式螢幕、電腦軟體圖形化介面操作簡單

- 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	SYBR® Green 1 Dye
		
Specificity	• Primer binding • Probe hybridization • PCR conditions	• Primer binding - • PCR conditions
Flexibility	• Multiple Targets per tube • SNP Detection • +/- Application	- - -
Optimization	• Universal guidelines	• Universal guidelines • Check Primer dimer formation

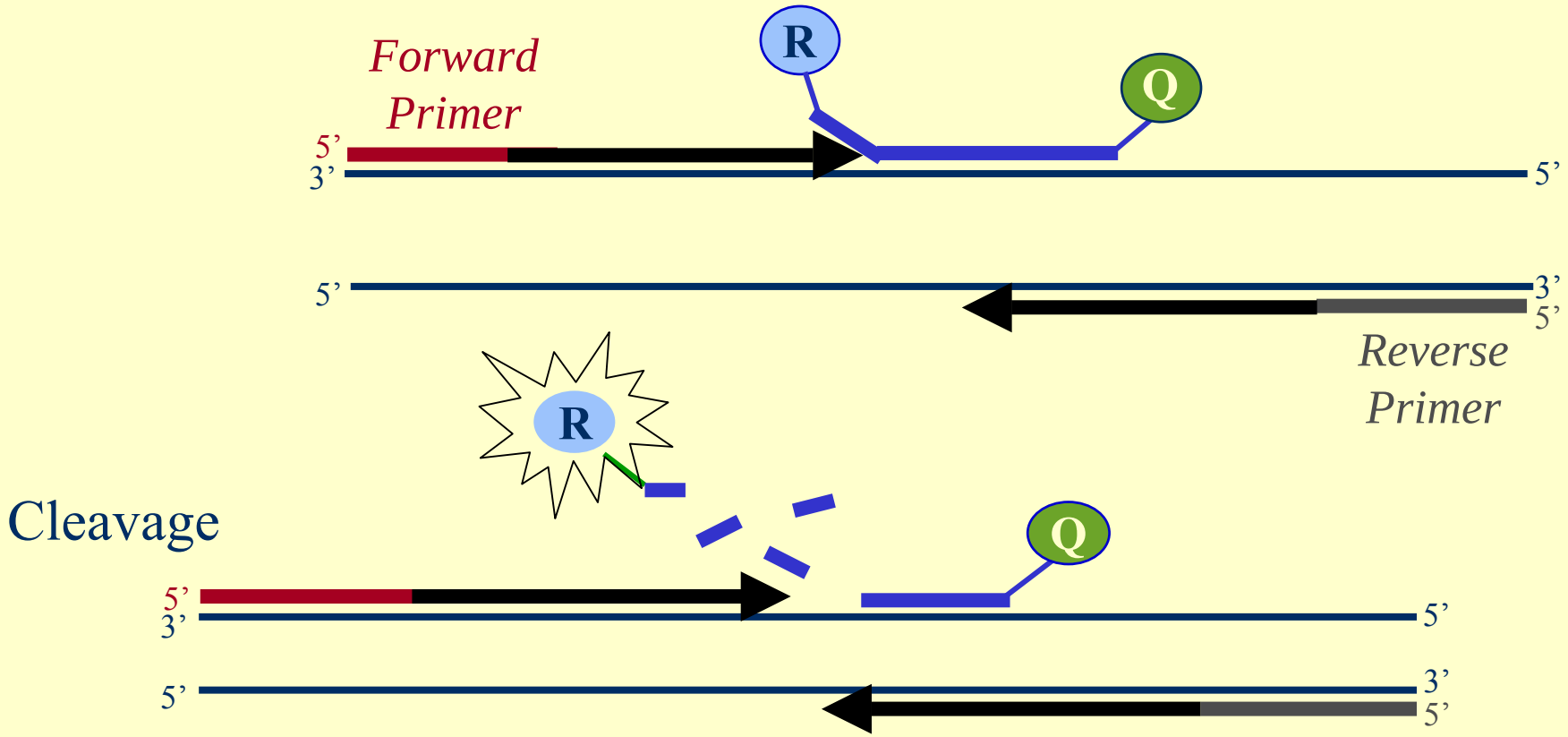
Fluorogenic 5' Nuclease Assay



***FRET= Fluorescence Resonance Energy Transfer**

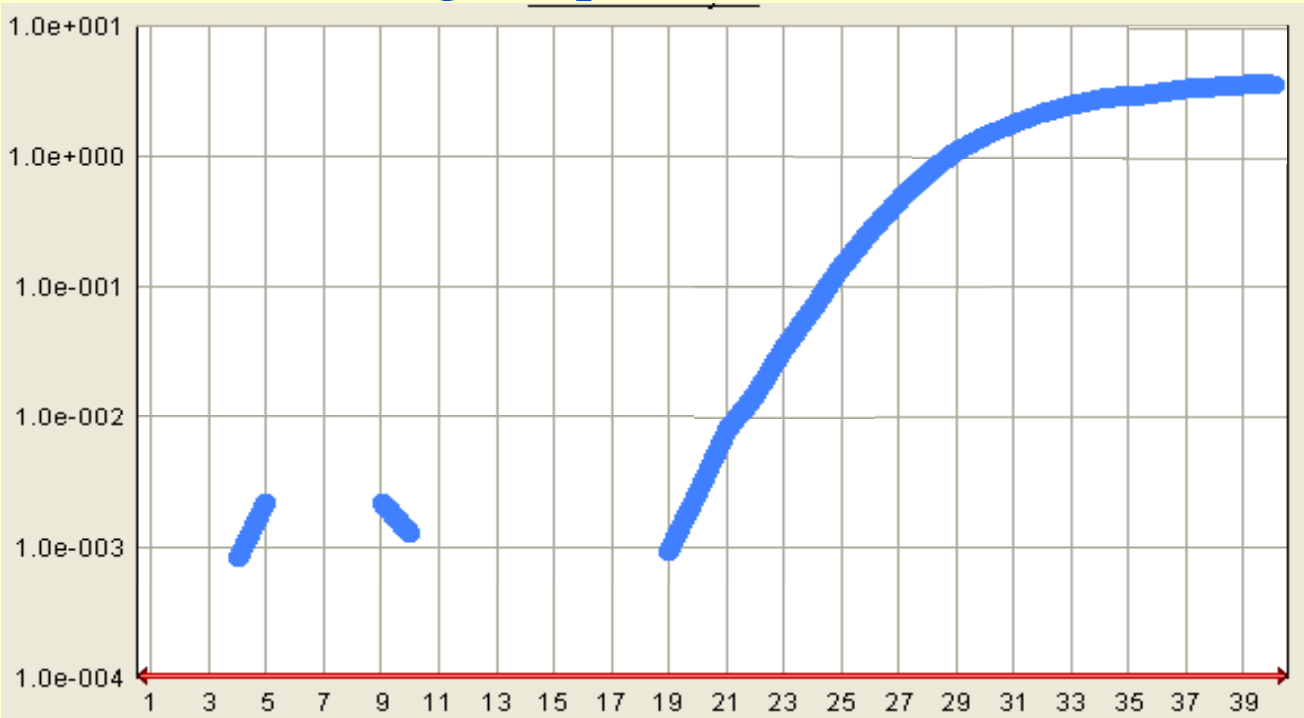
Fluorogenic 5' Nuclease Assay

Displacement during Polymerization



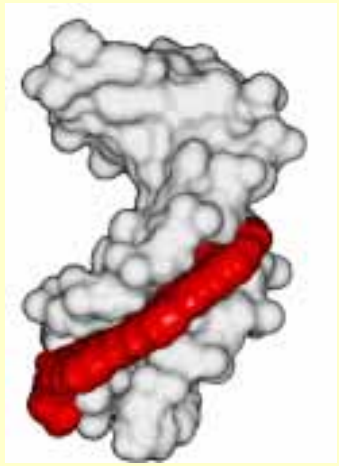
PCR Reaction Monitored in Real-Time

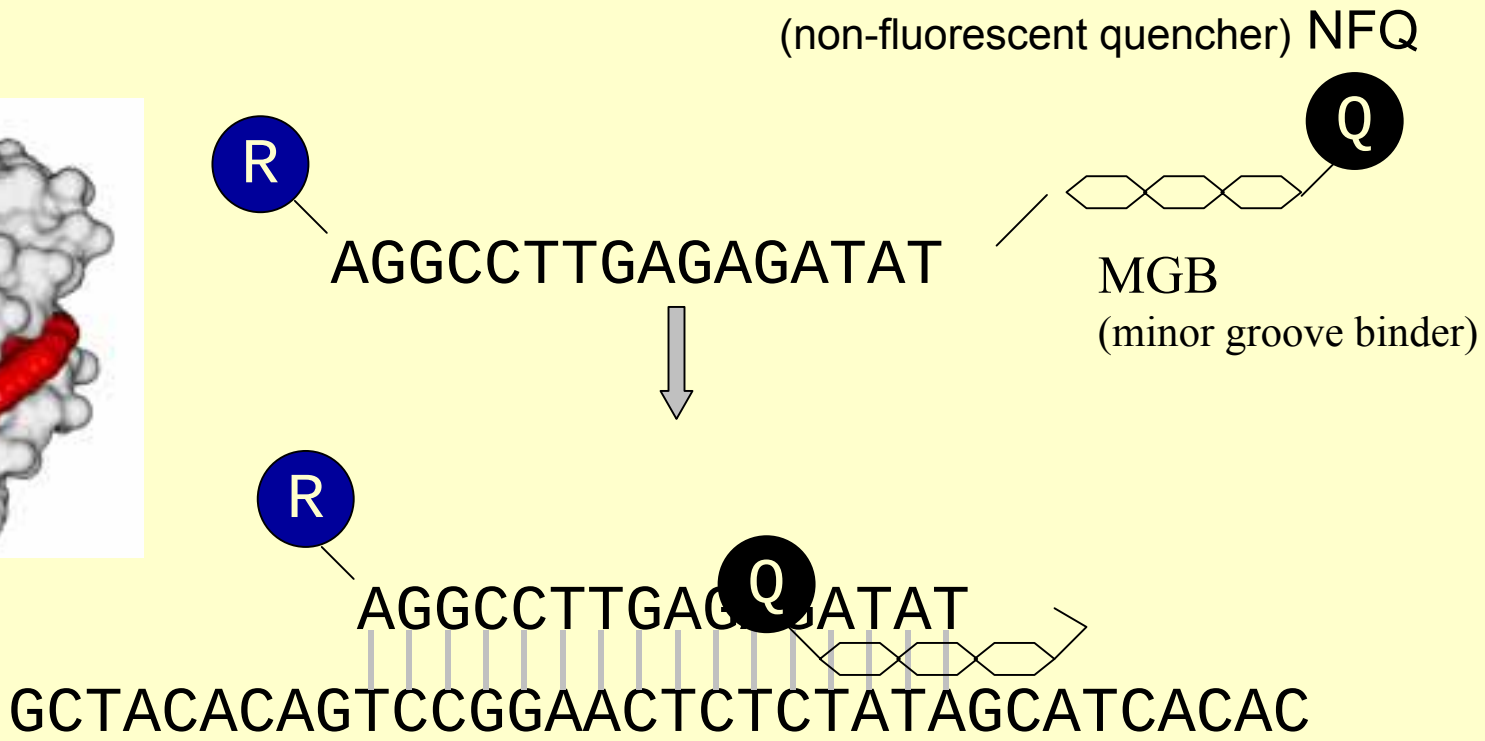
Generating Amplification Curves



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

next-generation chemistry: TaqMan® MGB Probes



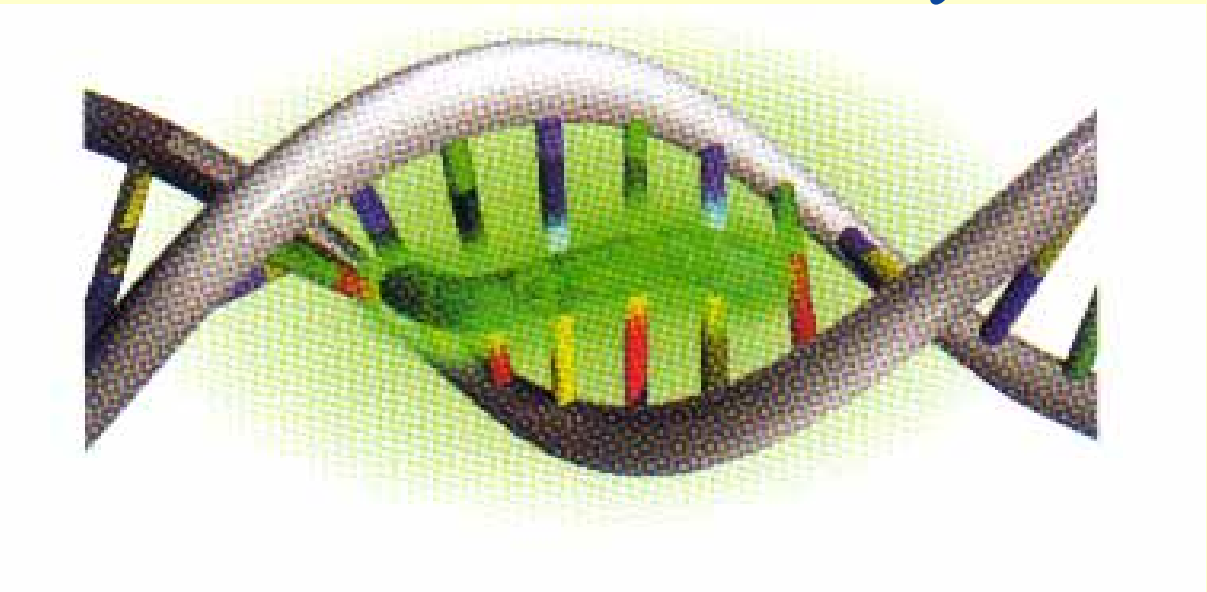


- **All probes will be short (13-20-mers)**
- **High resolution, Low Background**

TaqMan chemistry的優點

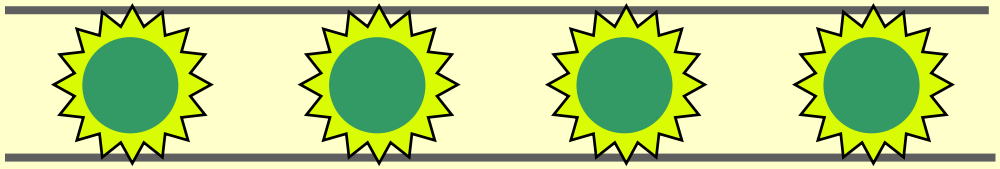
- Noiseless data專一性高
  due to the second level of specificity provided by the probe
- Multiplex compatible可在一反應中進行兩種基因之定量
  each probe can be differently coloured and thereby mixed with others
- Signal proportional to product螢光強度與反應產物呈正比
  signal related to amount of amplified product (not mass/length)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

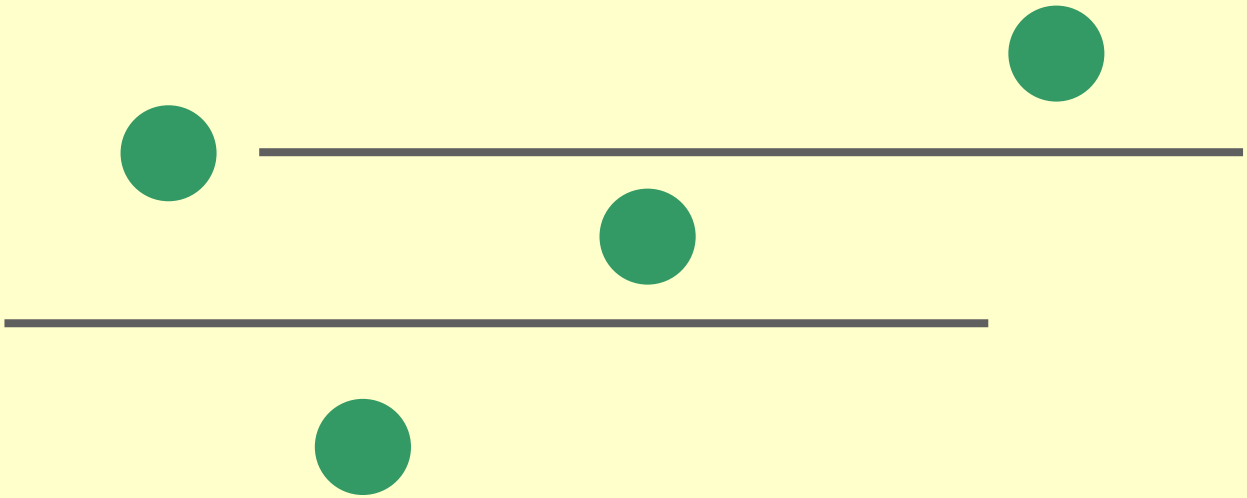


Double Stranded DNA:

Dye will fluoresce when bound to minor grove

Denaturation:

No Signal



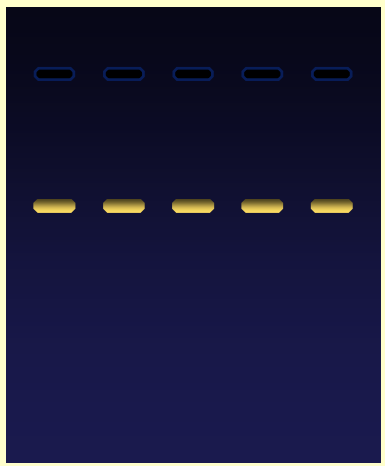
- Chemistry reports the accumulation of dsDNA in PCR
  - Need to verify target specific amplification by other means

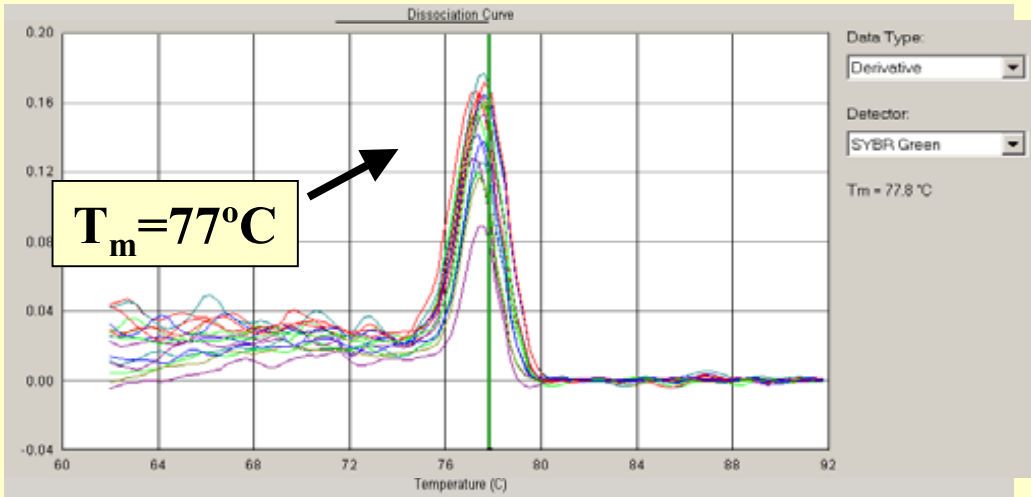
Specificity check

SYBR® Green 1 dye

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

178bp



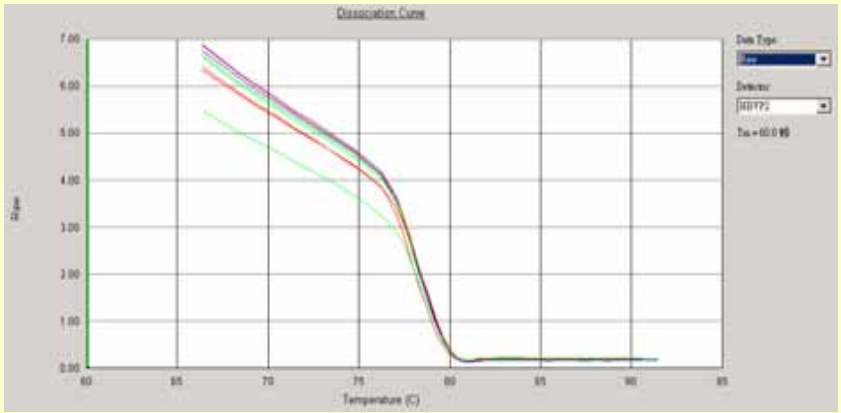


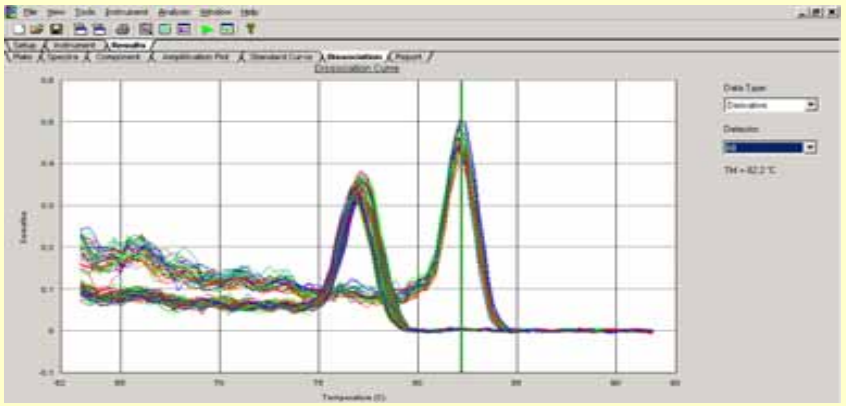
- Gel electrophoresis to determine PCR product size

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

SYBR Green I Chemistry 的特點

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



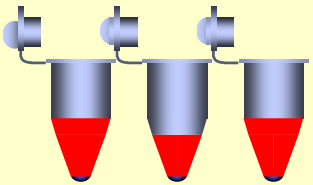


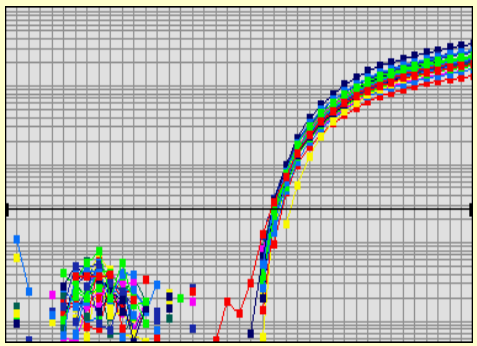
Compatible with dissociation curve analysis

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

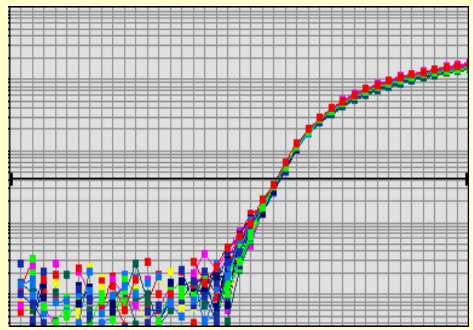
完整的試劑組合_master mix

- All Chemlink Hot start (5~ 15 min)
- Carry over protection, UNG
- Real Master Mix, all in one
- 4 ^{0}C 保存, 室溫操作
- ROX 體積校正





ROX normalize

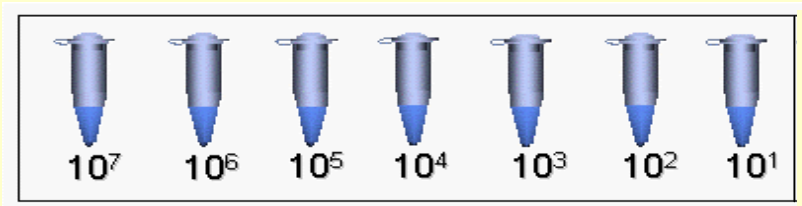


All in One Features 麻雀雖小五臟俱全!!
一套軟體可以符合全方位的應用

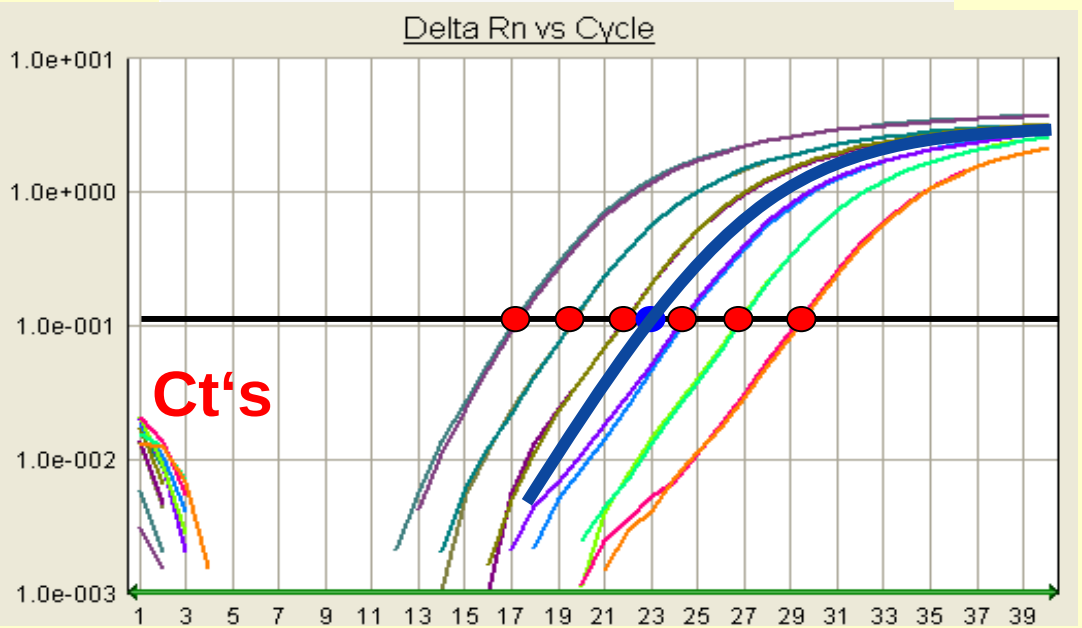
- 絕對定量Absolute Quantification - Standard curve
- 相對定量Realtive Quantification – △△Ct study
- 相對定量Realtive Quantification - Relative standard curve
- Melting Curve Analysis
- SNP Genotyping Assay
- Presence/Absence

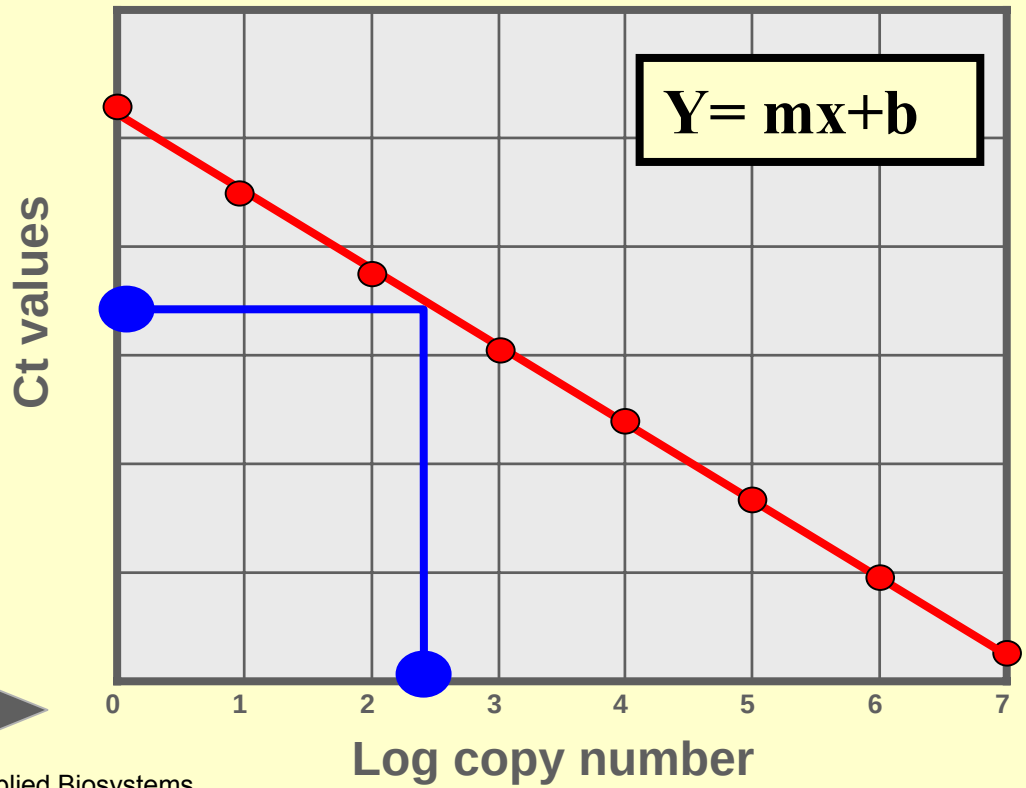
絕對定量(Absolute Quantitation)

-主要應用於病毒量及病原菌偵測-



The well contains 400 target copies



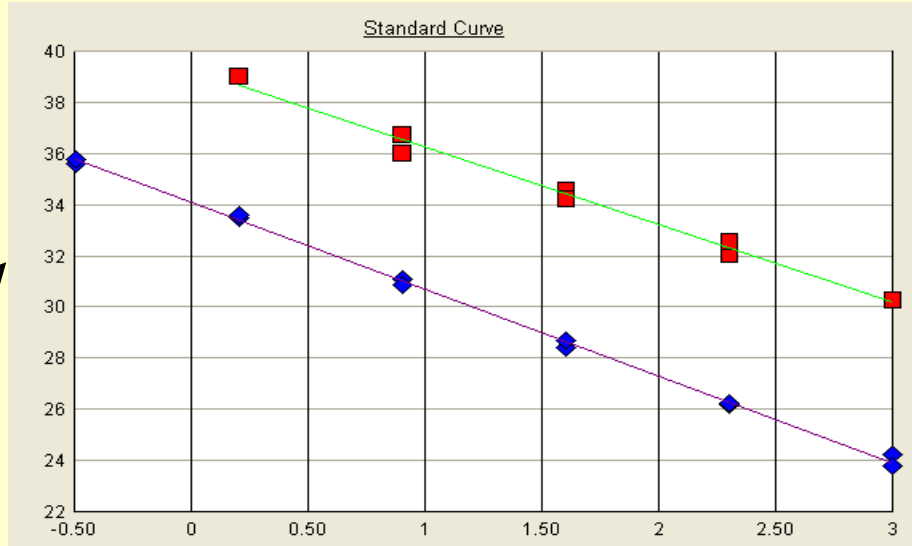


相對定量

Relative Quantification Analysis Methods

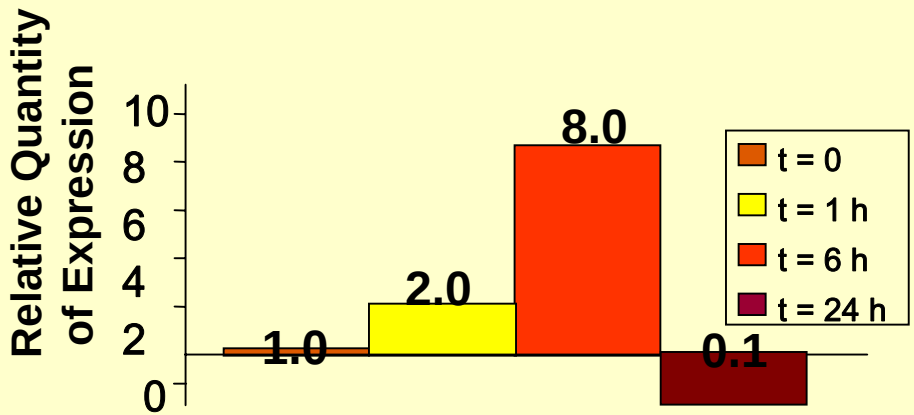
Standard Curve

	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

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

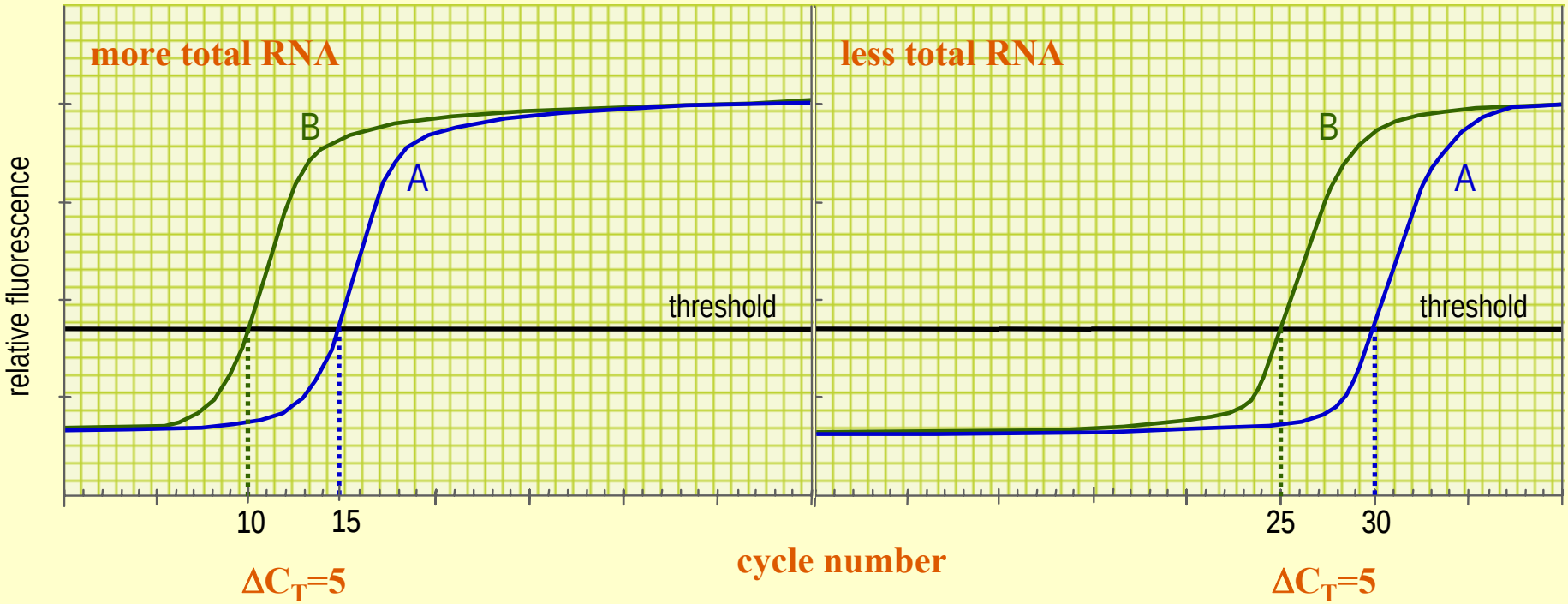


Relative Quantitation theory 相 對 定 量

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

△△Ct Definition

$$\triangle\triangle Ct = \triangle Ct\ (Unknown) - \triangle Ct\ (Control)$$

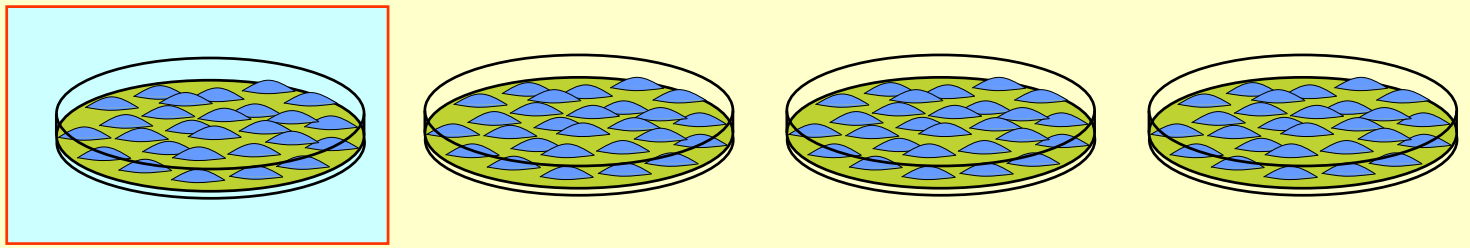
Xrel = $(1+E)^{-\triangle\triangle Ct}$, If Efficiency = ~100 %,

Relative Quantity = $2^{-\triangle\triangle Ct}$

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

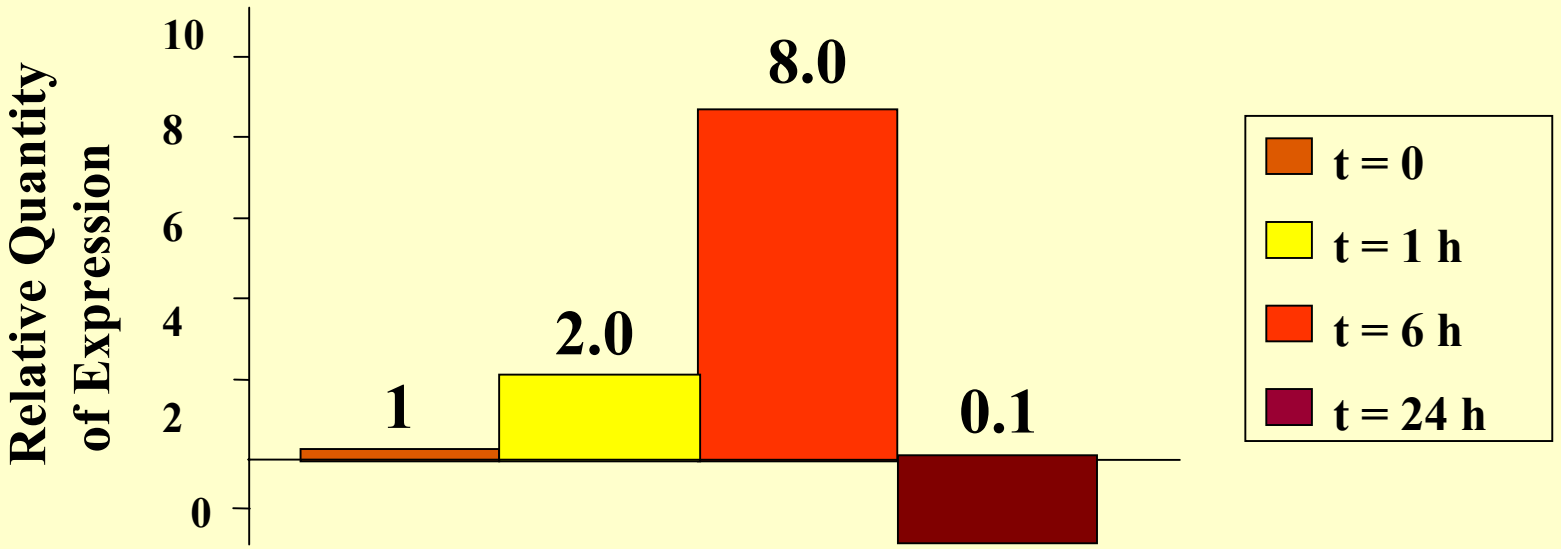
[GOI] [GOI] [GOI] [GOI]

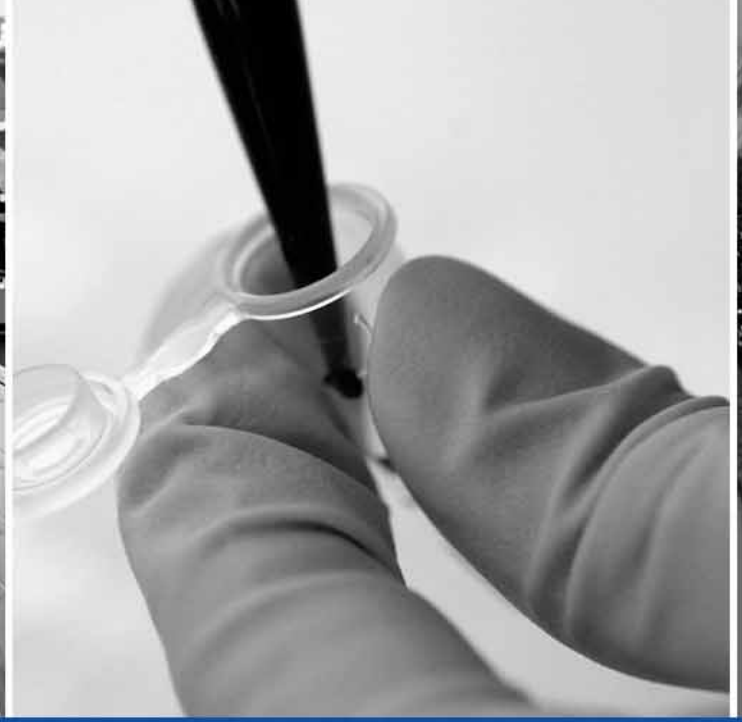
[ENDO] [ENDO] [ENDO] [ENDO]

Control for sample variability

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

	IL-2	18S	DC_t	DDC_t	2^{-DDCt}
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



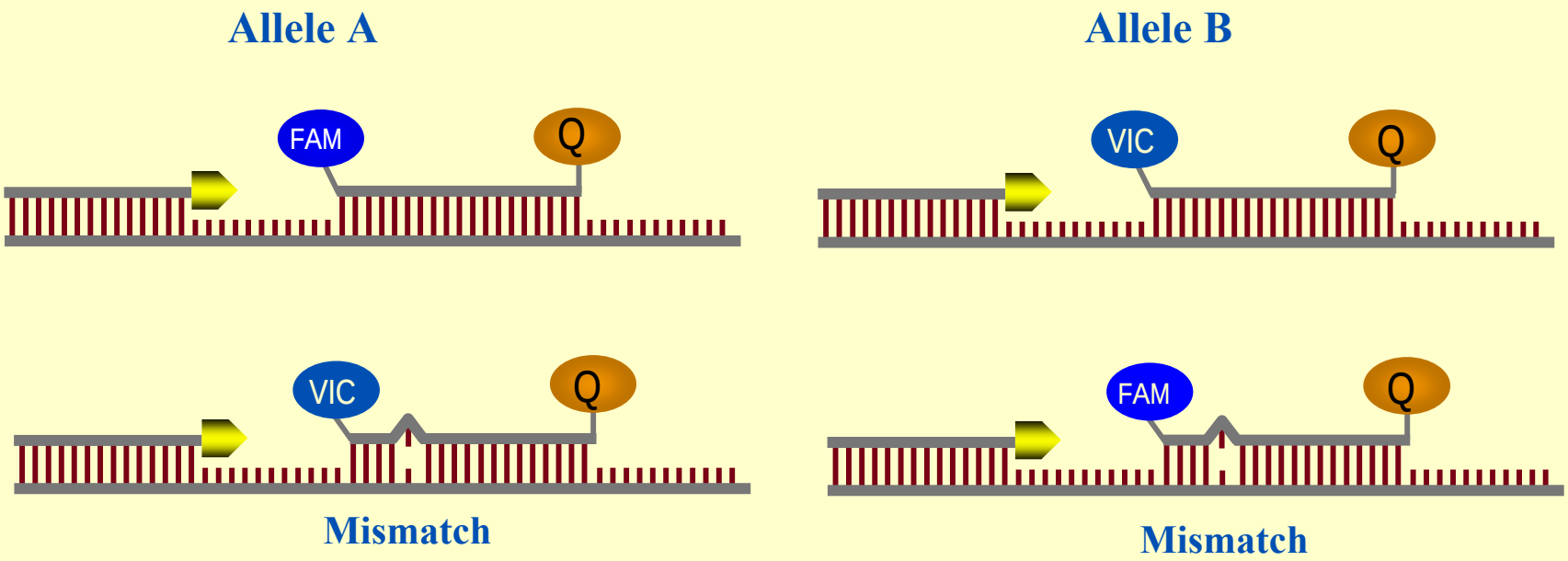


Applied Biosystems

The Application of SNP Genotyping on Real Time PCR System

Genotyping—competing probes

discriminating alleles and single nucleotide polymorphisms

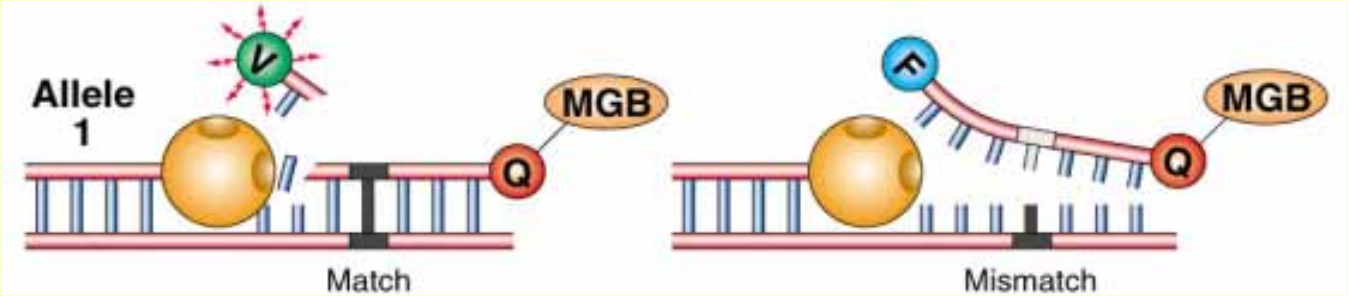


FAM = homozygous allele A

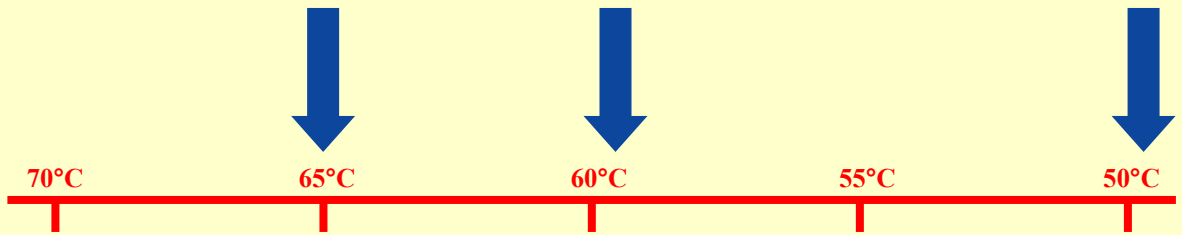
VIC = homozygous allele B

FAM & VIC = heterozygous (A & B)

Delta Tm (ΔT_m) or Mismatch Probe Stabilization



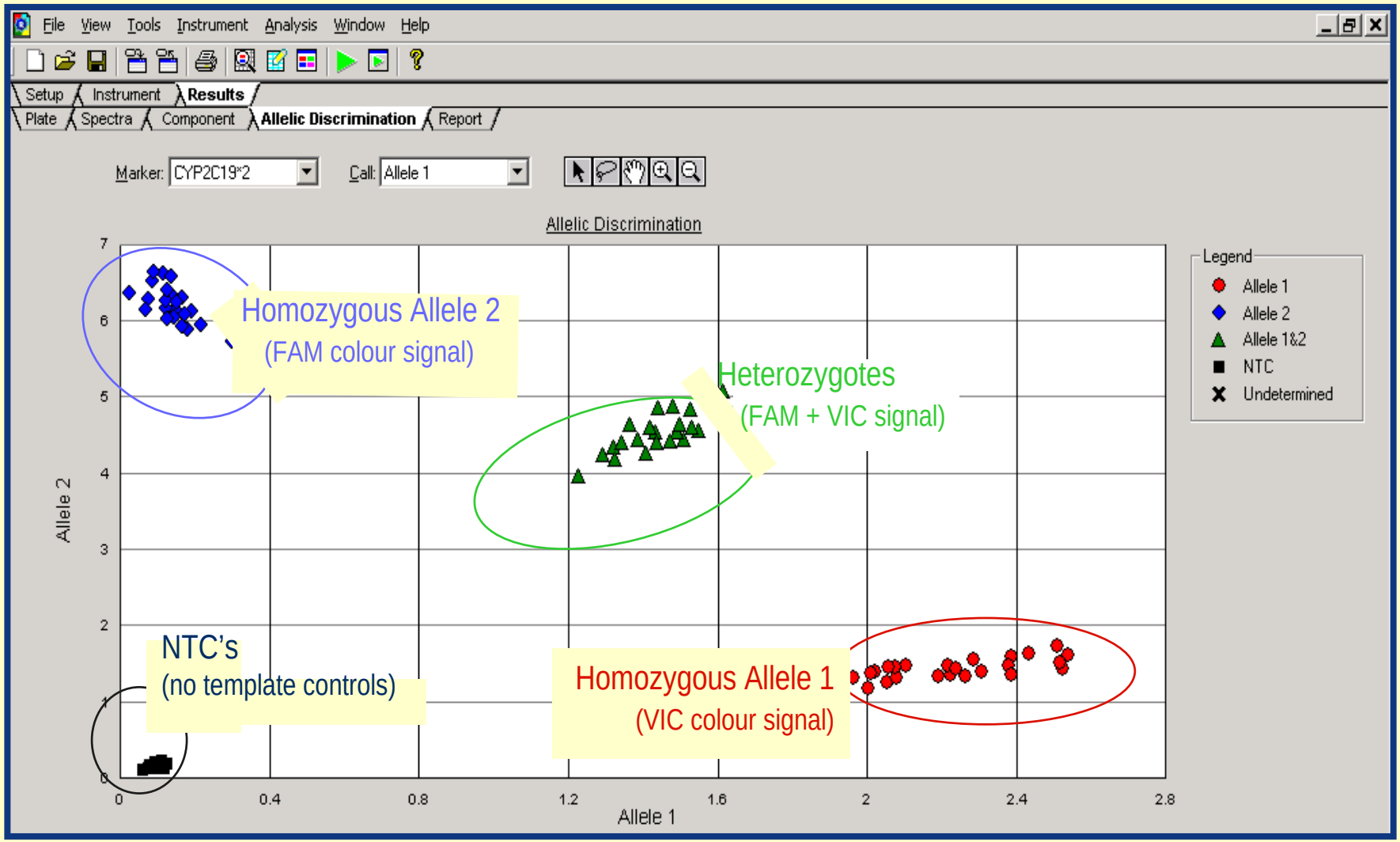
MGB T_m=65 T_m=49 (ΔTm = 17°C)

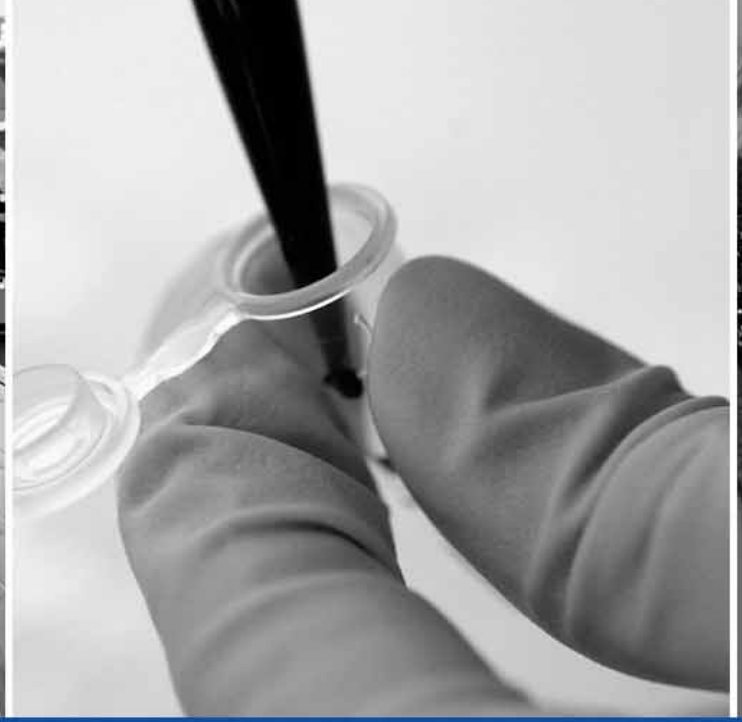


ΔT_m = T_m of perfectly matched probe - T_m of mismatched

Discrimination of alleles is achieved by using an annealing/ extension temperature within the ΔT_m window

allelic discrimination (SNP) data





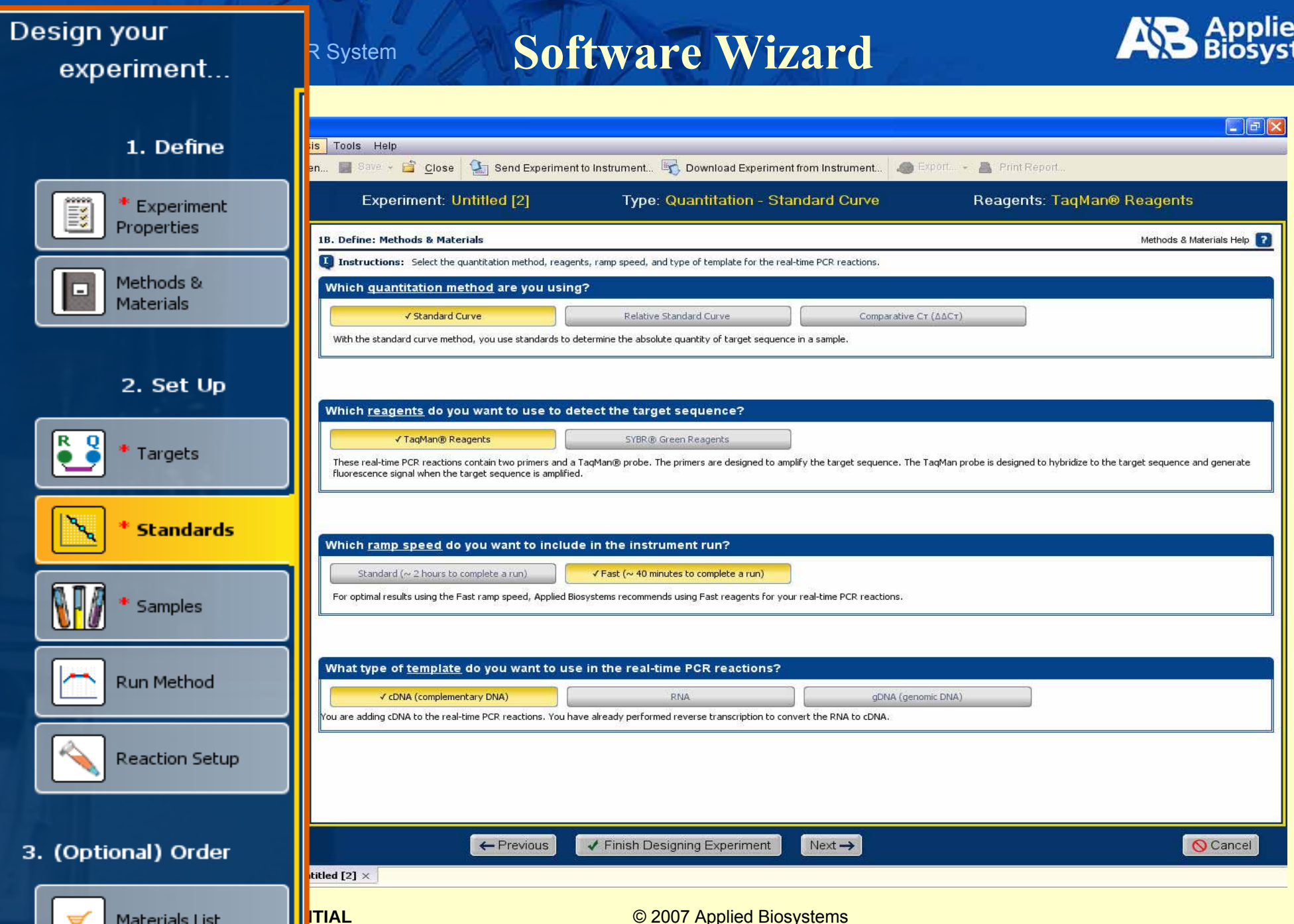
Applied Biosystems

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

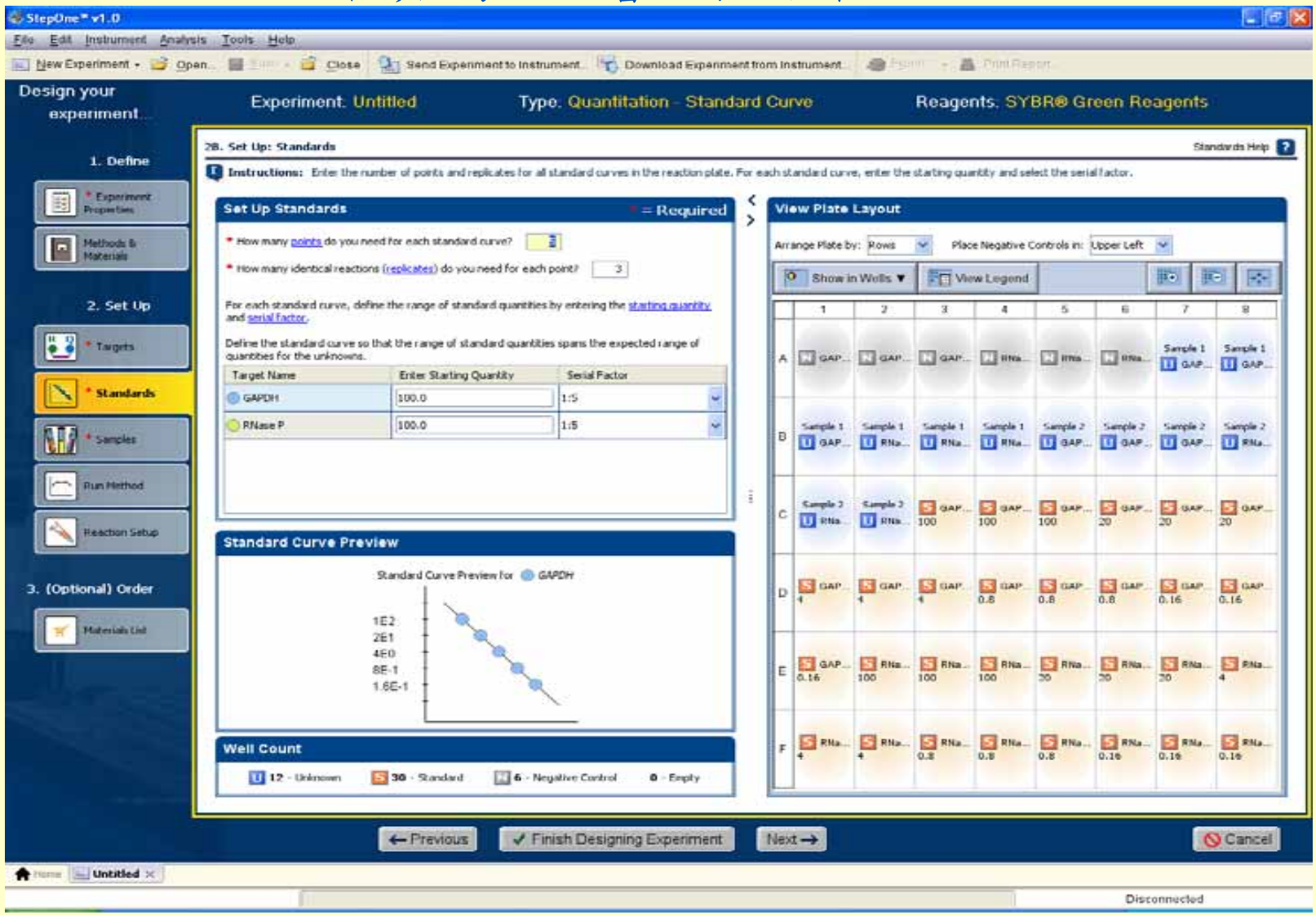
- 絕對定量Absolute Quantification - Standard curve
- 相對定量Realtive Quantification – △△Ct study
- 相對定量Realtive Quantification - Relative standard curve
- Melting Curve Analysis
- SNP Genotyping Assay
- Presence/Absence

Software Wizard

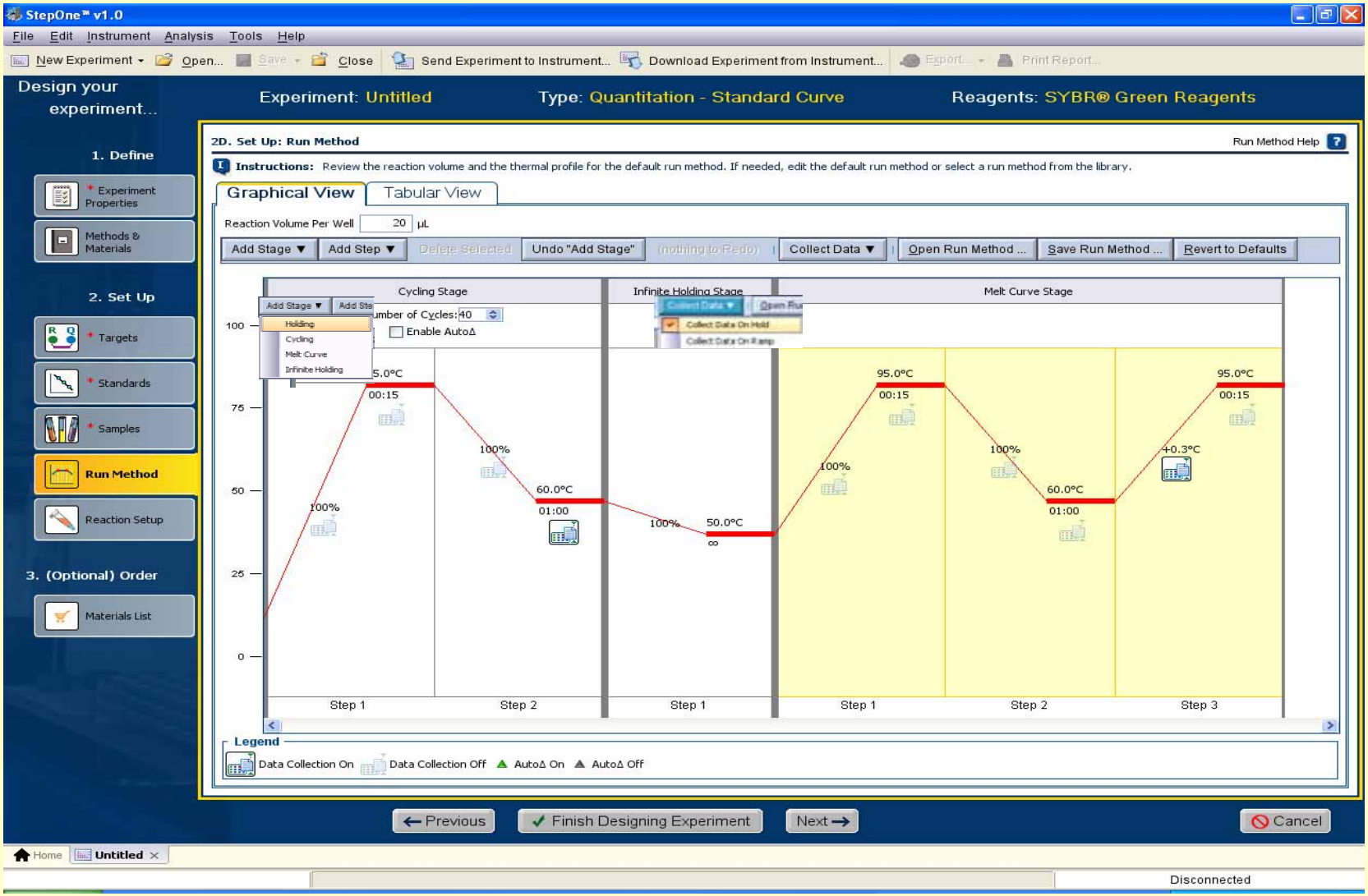


Automatic plate layout in the Design wizard

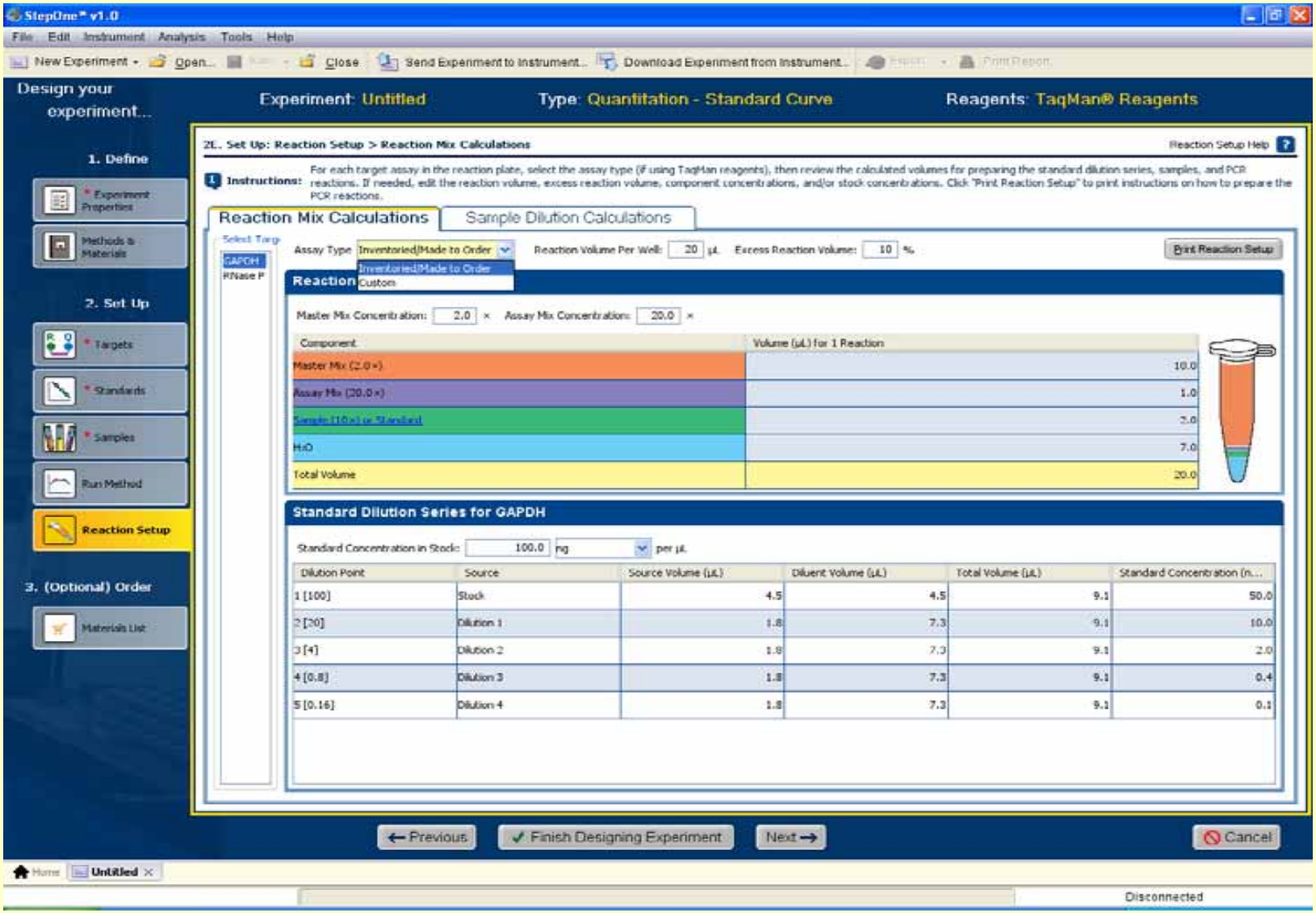
Real Time PCR 很難嗎??照著做就好囉!!



Run Module – 圖形化介面，操作簡單易懂



Reaction Protocol Setup

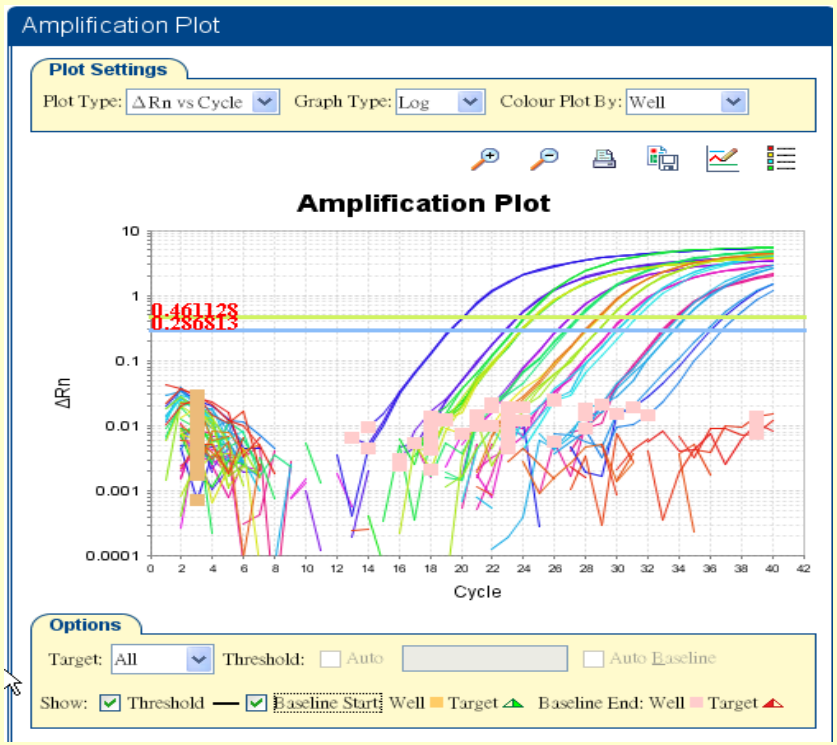


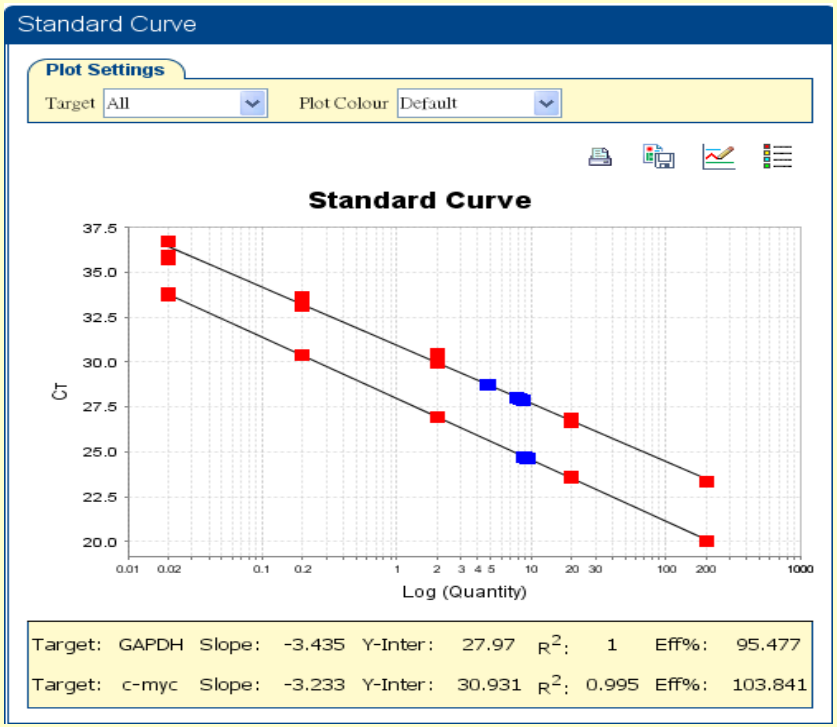
Additional New Features in the Software

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



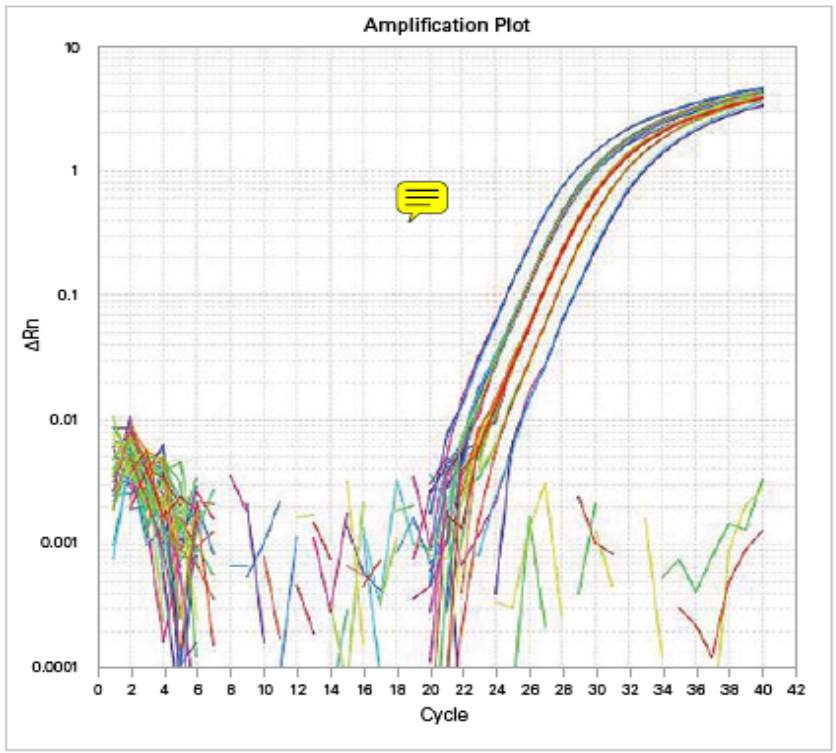


Genotyping Assay

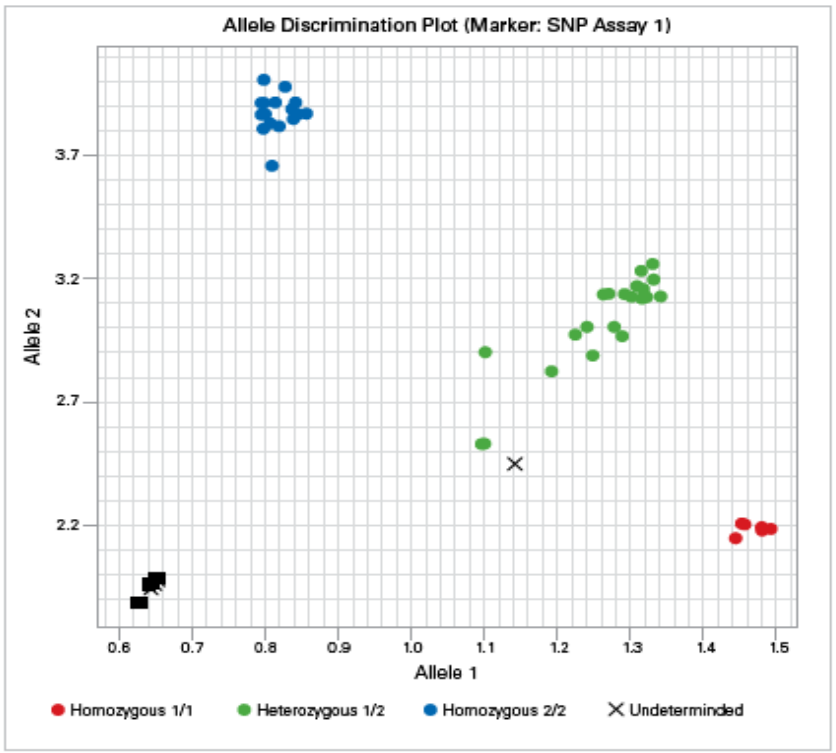
(Real-Time data collected in the same file as pre- and post-read)

Amplification plot 幫你做簡易troubleshooting

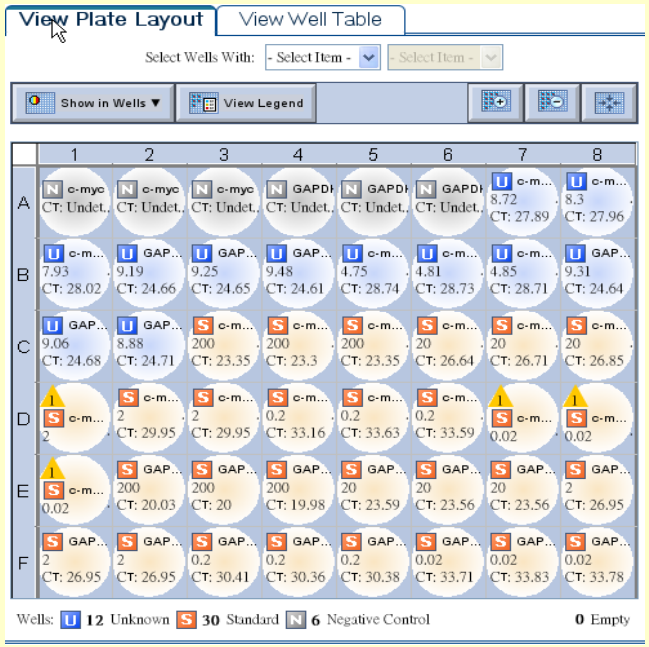
Amplification Plot

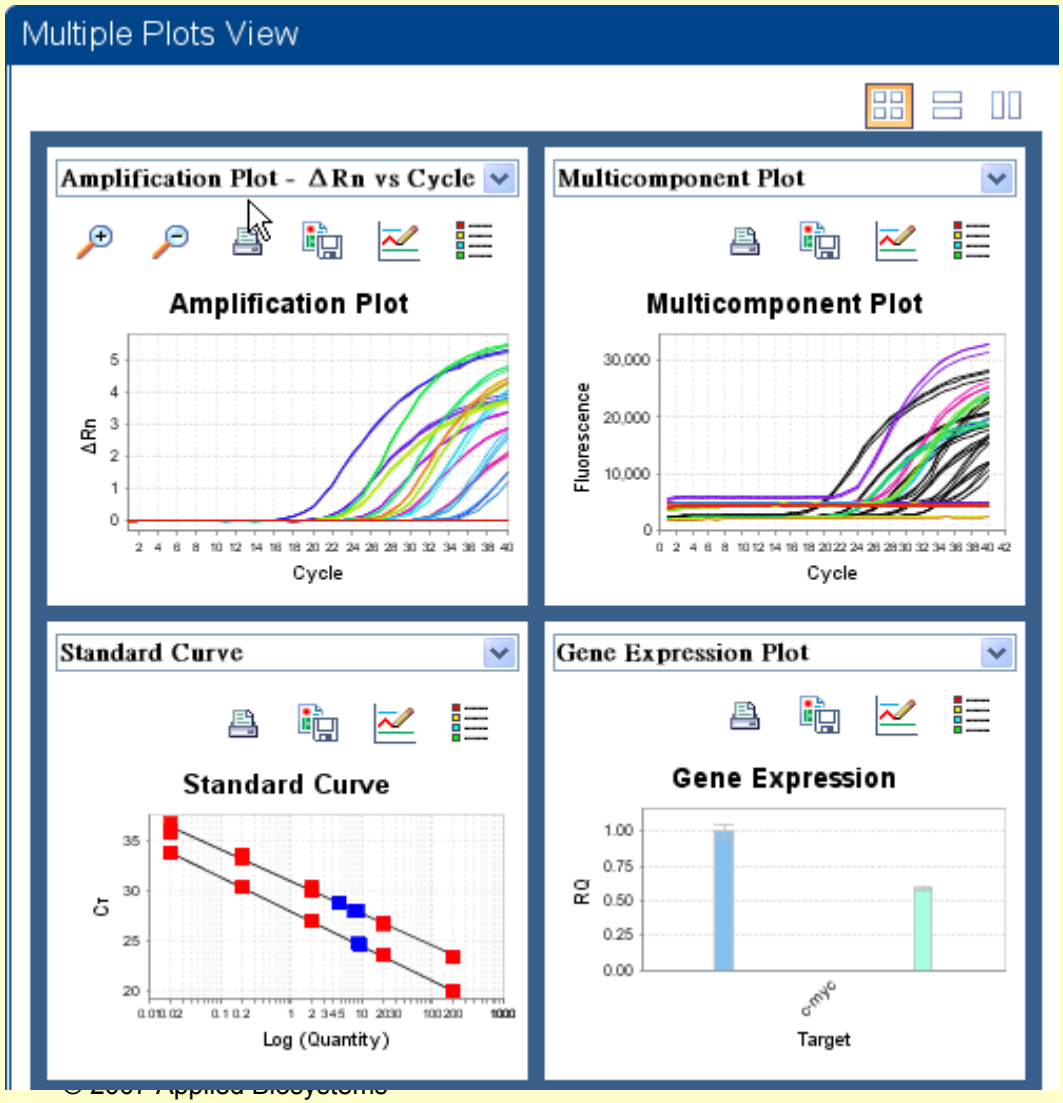


Gene Expression Analysis



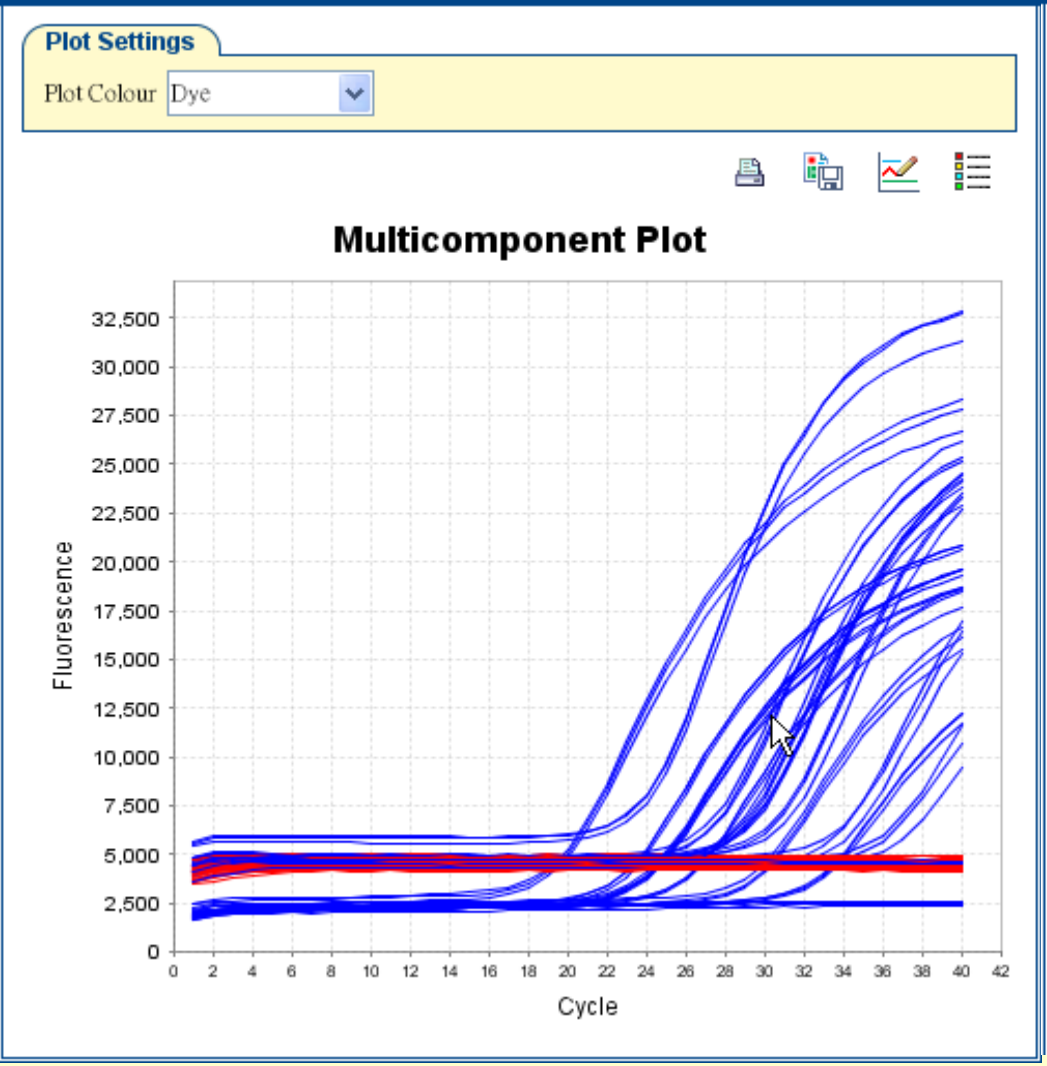
Result: Export to Excel, PowerPoint, or save as JPEG



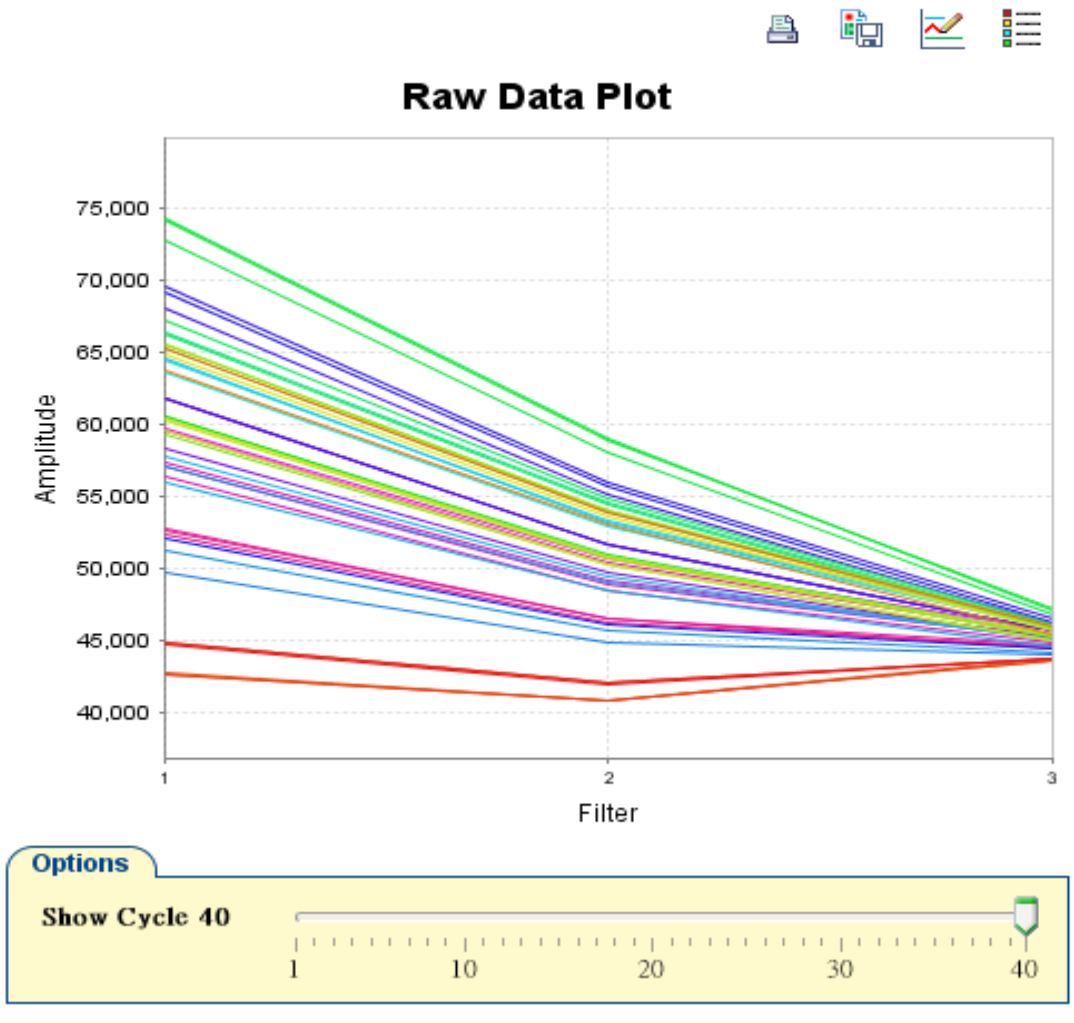


Multicomponent Plot and Raw Spectra

Multicomponent Plot



Raw Data Plot



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

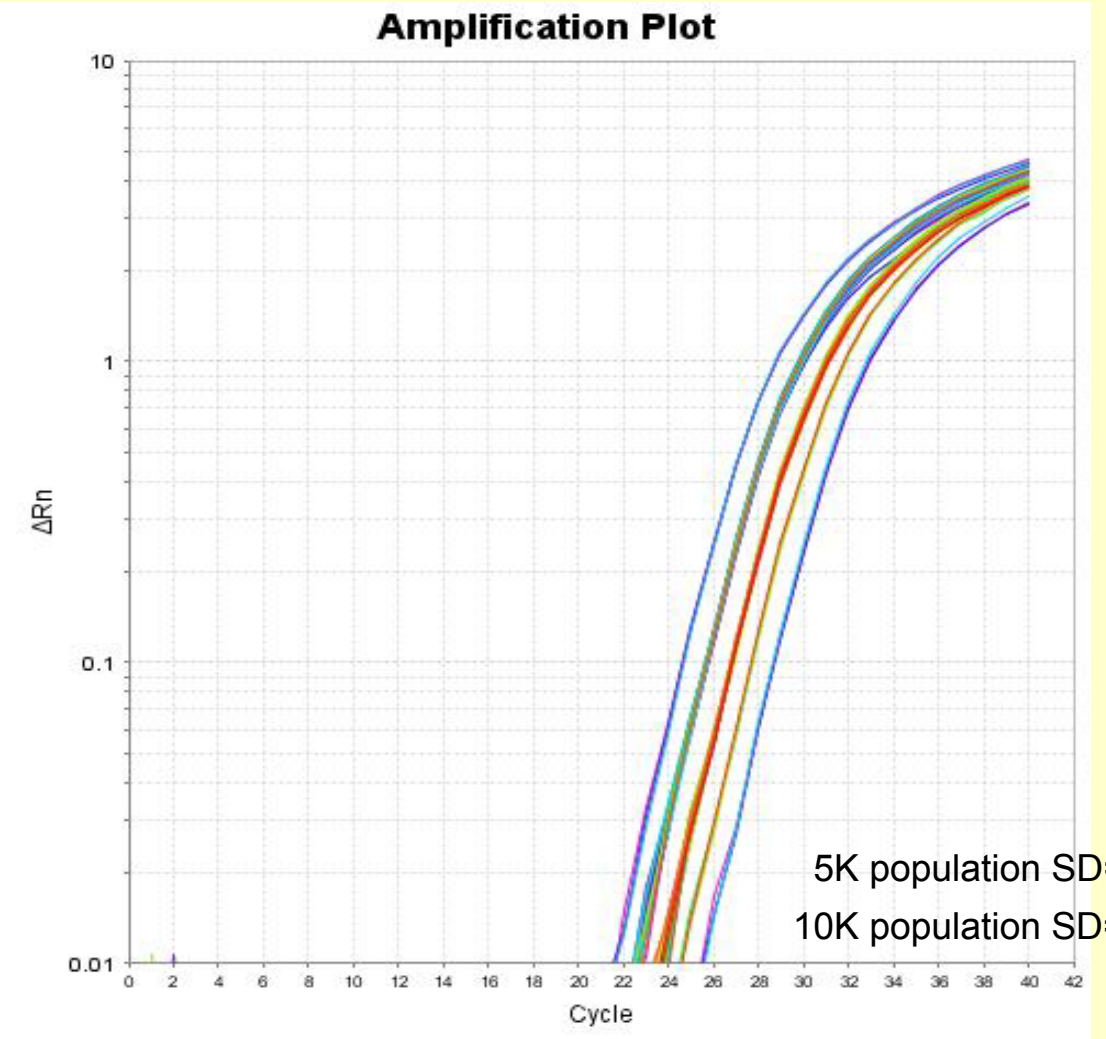
Can the StepOne System be used as a conventional thermal cycler ?

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

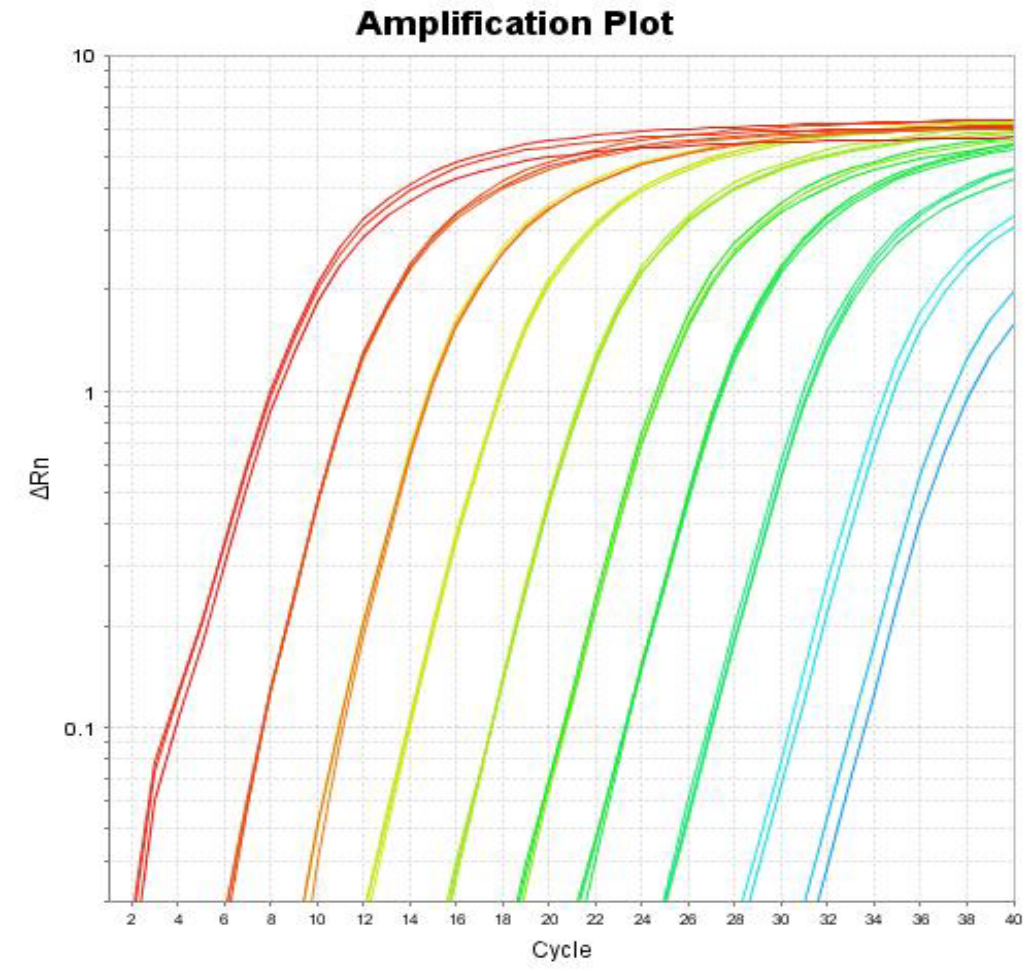
Performance specifications customers have come to expect with AB instruments

- 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

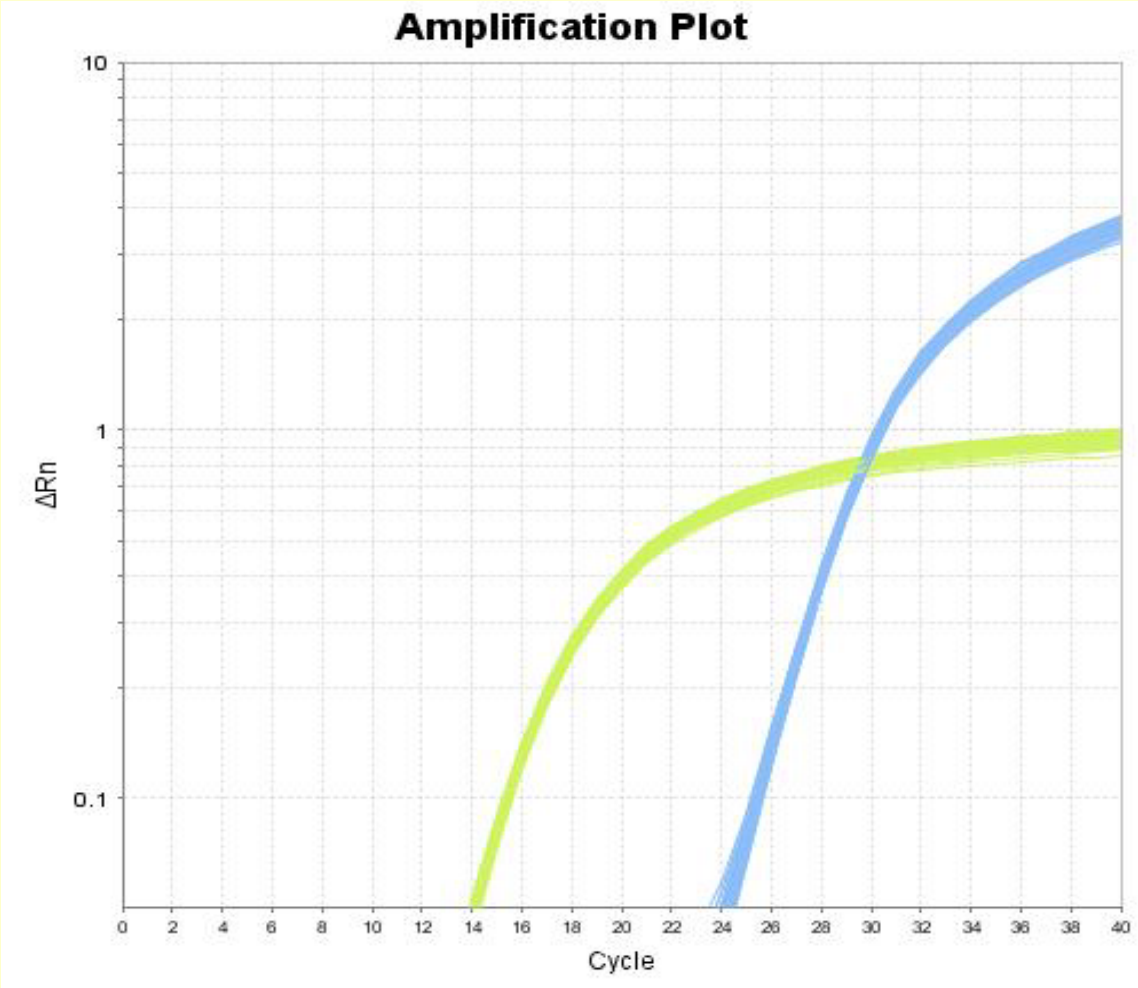


9-Log Dynamic Range



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而耽擱時間的困惱
  - 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

Applied Biosystems' Integrated Solution on Genomic Assays

Assay Development

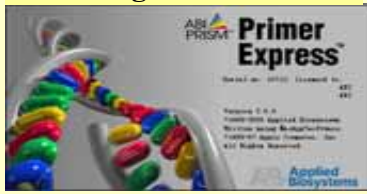
TaqMan Gene Expression Assays

TaqMan SNP Genotyping Assays

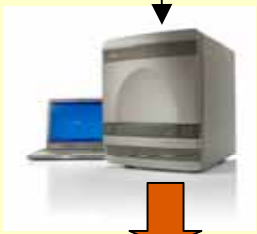
Custom TaqMan GX & SNP Assays

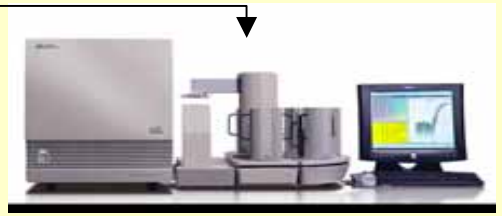
miRNA assays

Custom Primers/Probe Design Software



Universal PCR Condition on Applied Biosystems' Real Time PCR System

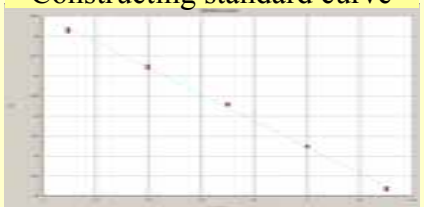




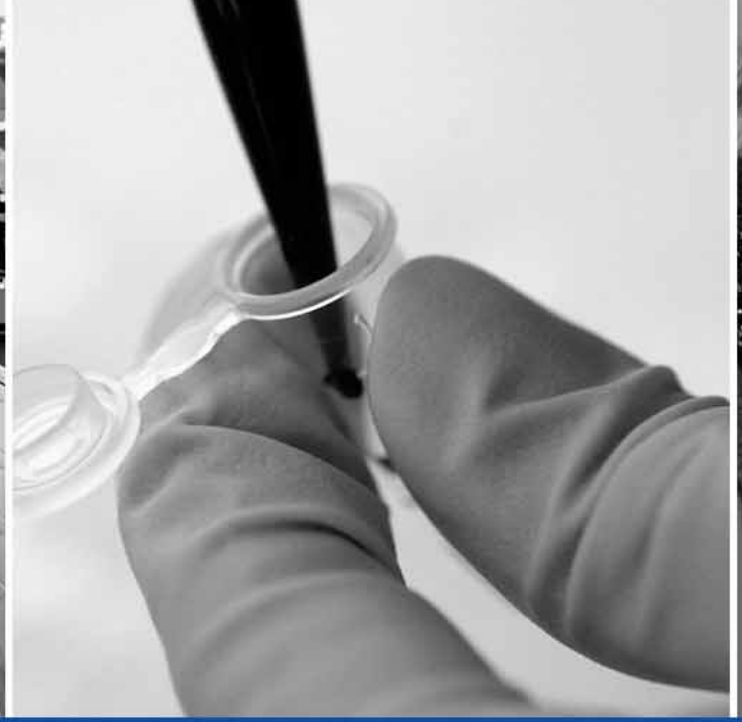
Automatic Data Analysis

Auto-clustering in SNP Genotyping

Gene Expression: automatic Constructing standard curve



Automatic relative quantitation Data analysis



Applied Biosystems

如何選擇Real-Time PCR 試劑耗材

Two-Step

RT (High Capacity cDNA Archive Kit" P/N 4322171)

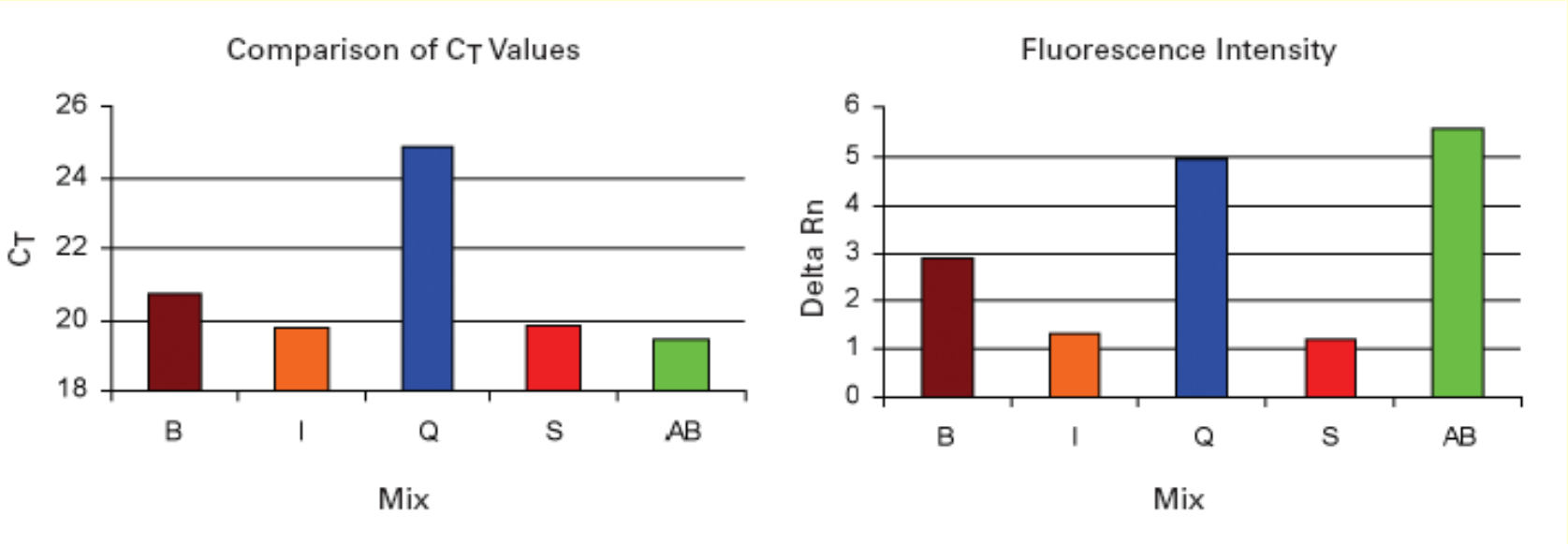
PCR: cDNA template or gDNA

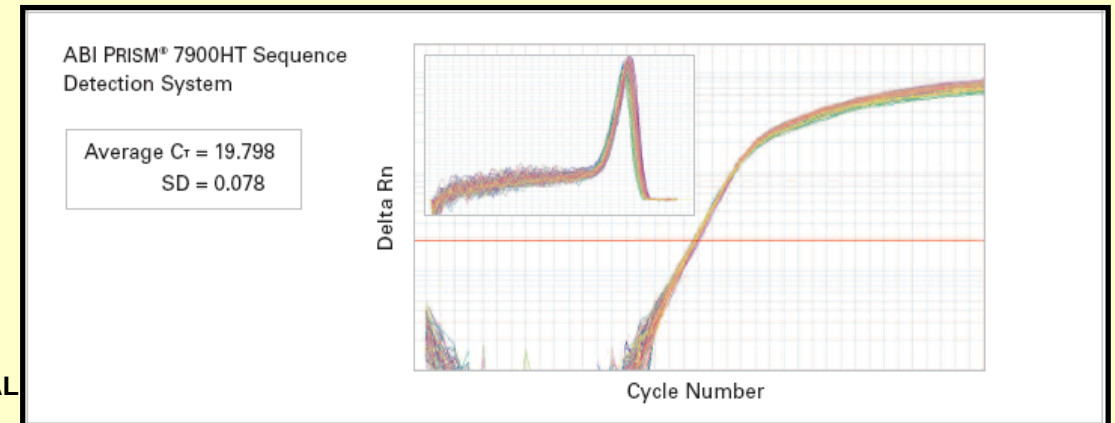
	選擇Probe	選擇Reagent
TaqMan Probe Systems	**20 x probe/ primer mix** 由AB網站選購或代客設計 **(TaqMan Gene Expression Assay)** **(Custom TaqMan Gene Expression Assay)** **Or** **Primer Express software design** **(300 nM primer + 200 nM probe)**	**+ P/N 4304437 (200 Rx)** **2x TaqMan Universal PCR Master Mix**
SYBR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

One-step RT-PCR: RNA template

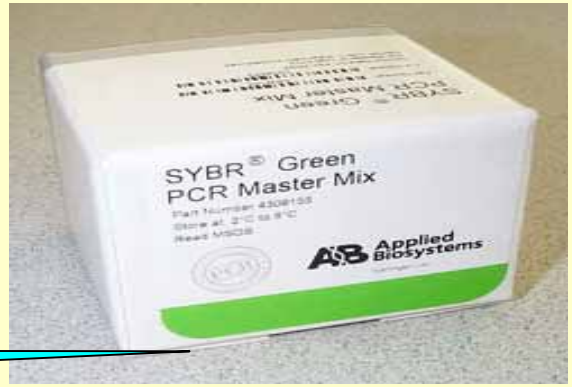
	選擇Probe	選擇Reagent
TaqMan Probe Systems	**20 x probe/ primer mix** 由AB網站選購或代客設計 (TaqMan Gene Expression Assay) (Custom TaqMan Gene Expression Assay) **Or** **Primer Express software design** (300 nM primer + 200 nM probe)	**P/N 4309169 (200 Rx)** **TaqMan One-Step RT-PCR Master Mix Reagents Kit** **Or** **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 *"Power* SYBR® Green Master Mix"

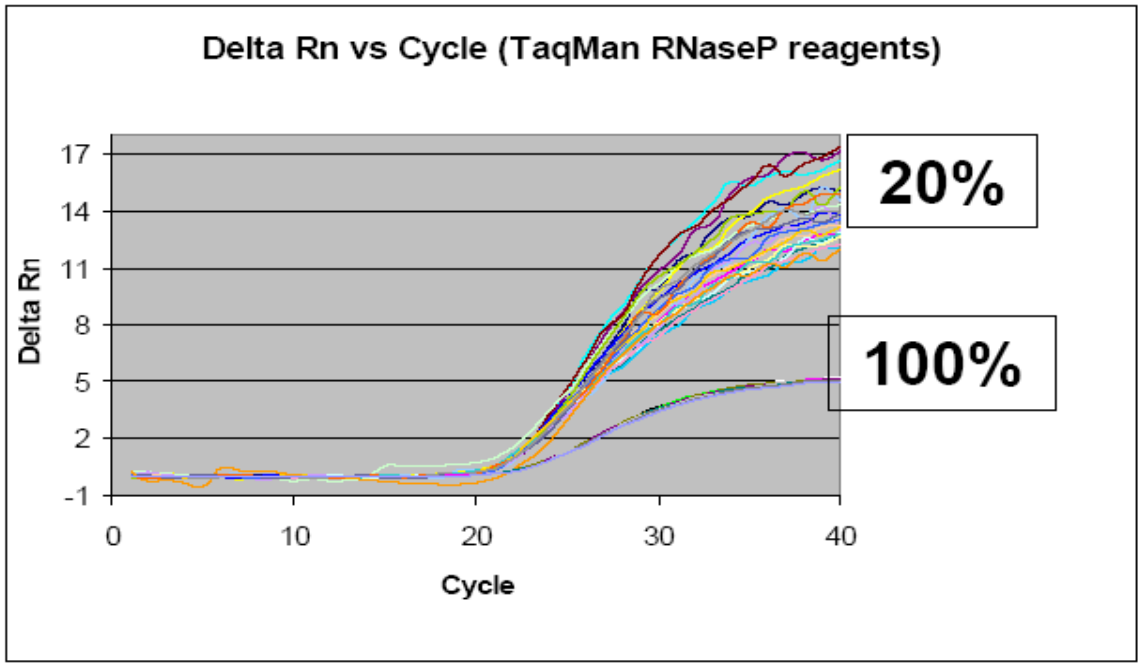




完整的試劑組合



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.

如何製備Real Time PCR的反應

Reverse Transcription : High Capacity cDNA archive Kit

Component
10x Reverse Transscription 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

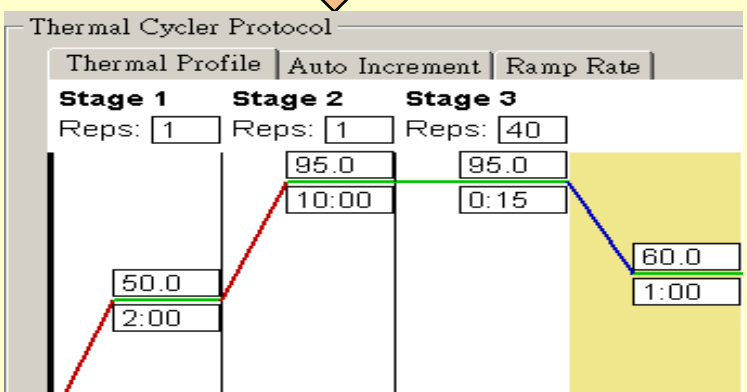
Real Time PCR:

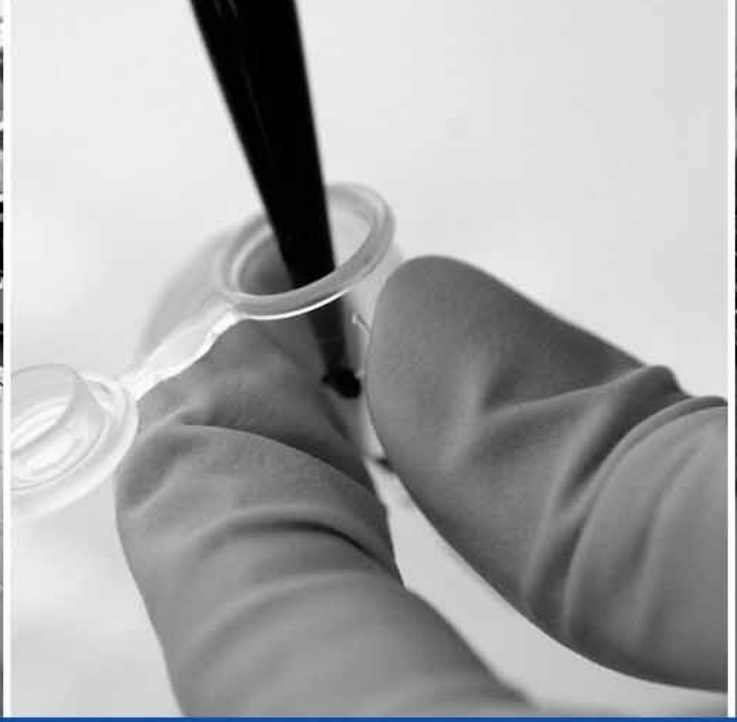
TaqMan Chemistry

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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Applied Biosystems, Taiwan

Accelerate the Pace of Discovery, Enable Sciences for Life

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