StepOne Plus

real-time PCR system

即時定量系統

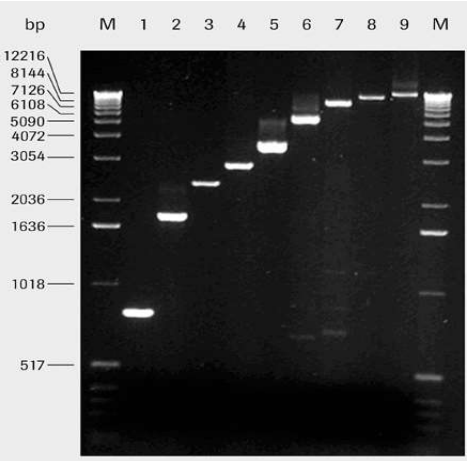
金萬林企業股份有限公司

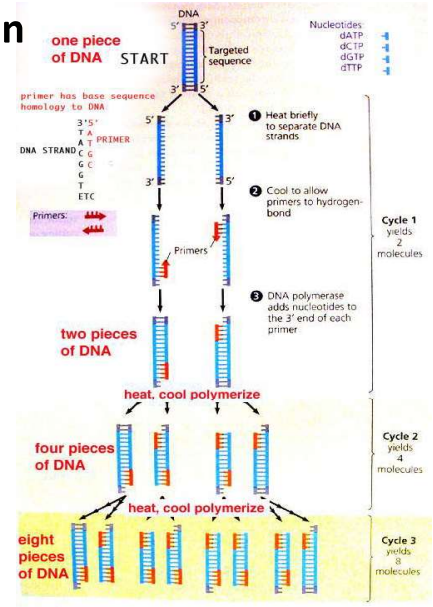
劉儀君 Jessie

Agenda

- Principle of real-time PCR
- Real-time PCR chemistries
- Real-time PCR Quantitation methods and applications
- Standalone operating, PC-controlled operating and data analyzed

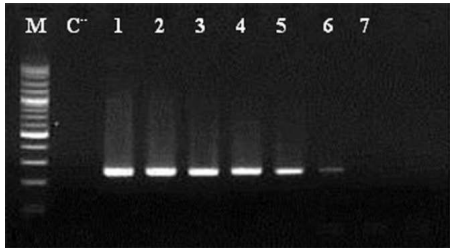
Polymerase Chain Reaction (PCR)





Conventional PCR Quantitative System

- Low resolution
- Limited dynamic
- Semi-quantitative
- Many manual steps
- Labor intensive
- Low throughput

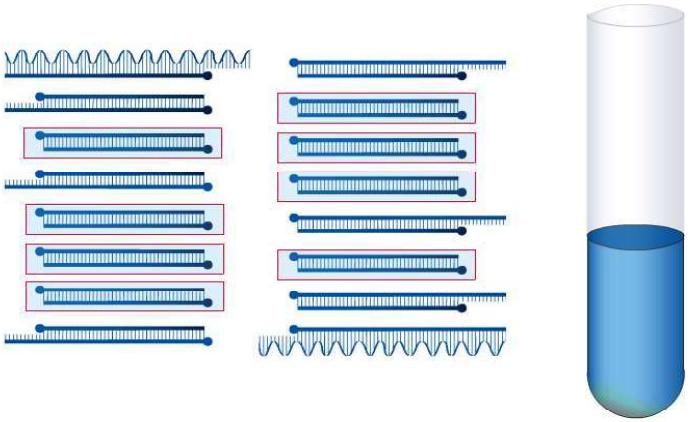


M: Marker
Lane 1: 42 pg
Lane 2: 4.2 pg
Lane 3: 420 fg

C: Control
Lane 4: 42 fg
Lane 5: 4.2 fg
Lane 6: 0.42 fg
Lane 7: 0.042 fg

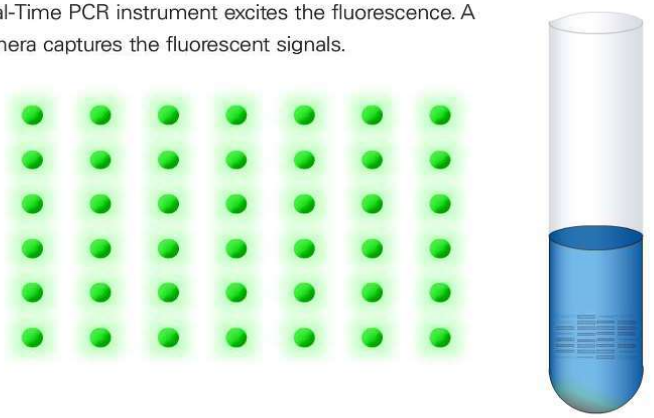
Real-Time PCR System

To perform Real-Time PCR, start with a basic PCR mix.



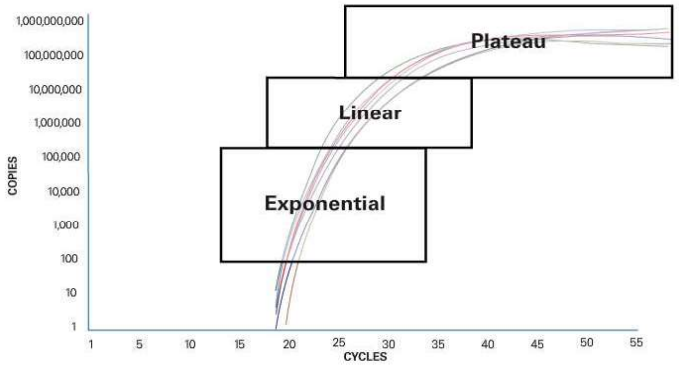
Real-Time PCR System

Add fluorescent labels to the PCR mix. A light source in the Real-Time PCR instrument excites the fluorescence. A camera captures the fluorescent signals.



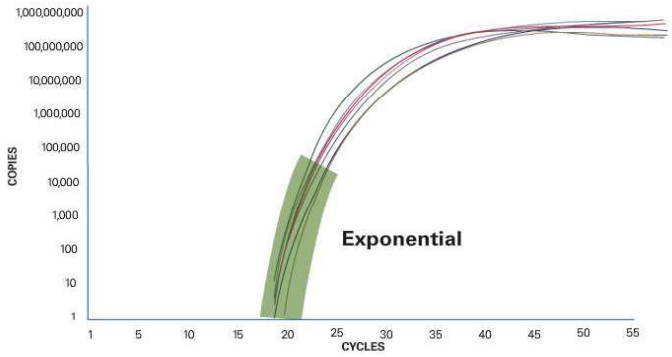
Polymerase Chain Reaction

With Real-Time PCR, there are three amplification stages:
1) Exponential, 2) Linear, and 3) Plateau.



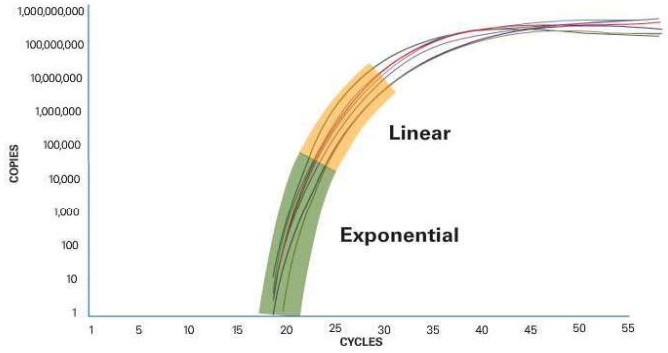
Polymerase Chain Reaction

In the Exponential phase, the reagents are in abundance and the PCR product doubles every cycle.



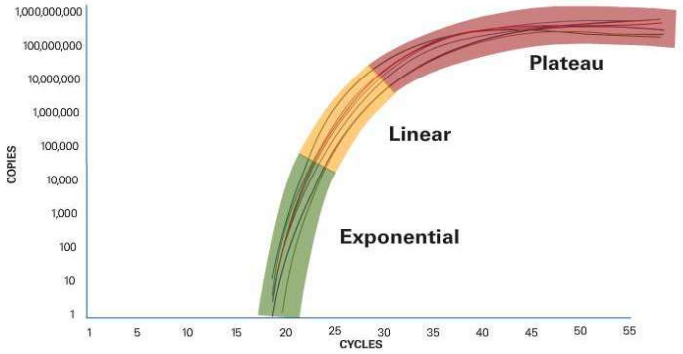
Polymerase Chain Reaction

In the Linear phase, the reagents begin to run out. The PCR reaction slows down.



Polymerase Chain Reaction

In the Plateau phase, the reagents are depleted and the PCR reaction stops.

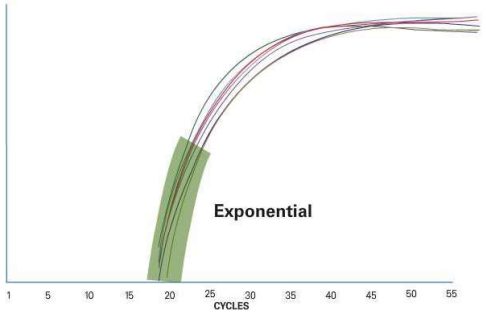


Real-Time PCR System

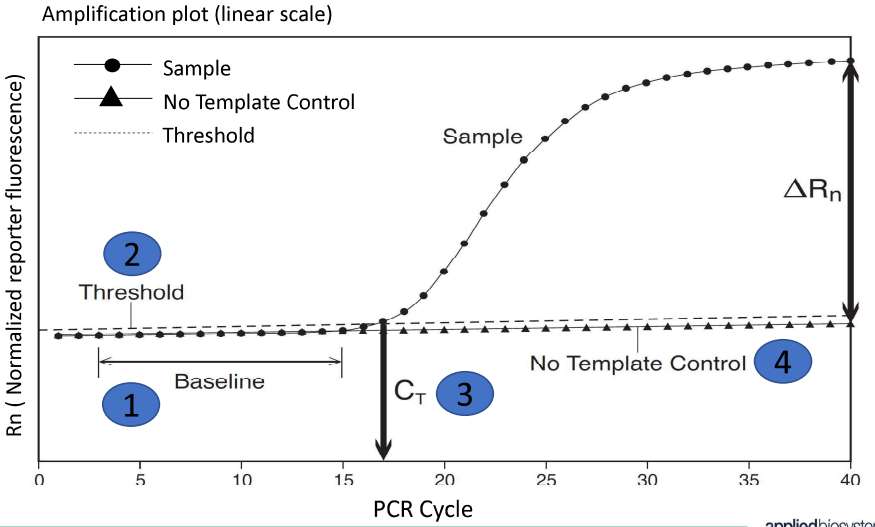
If PCR efficiency is 100% → $Y = X \cdot 2^n$

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

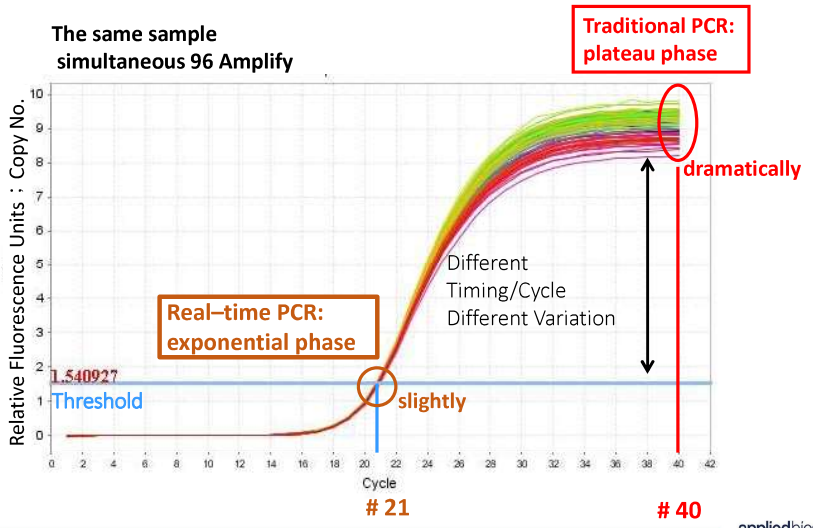
Y: 經n個cycle後, PCR product 之總產量
X: Gene起始濃度(原始表現量)
e: Efficiency
n: PCR cycle number, Ct value



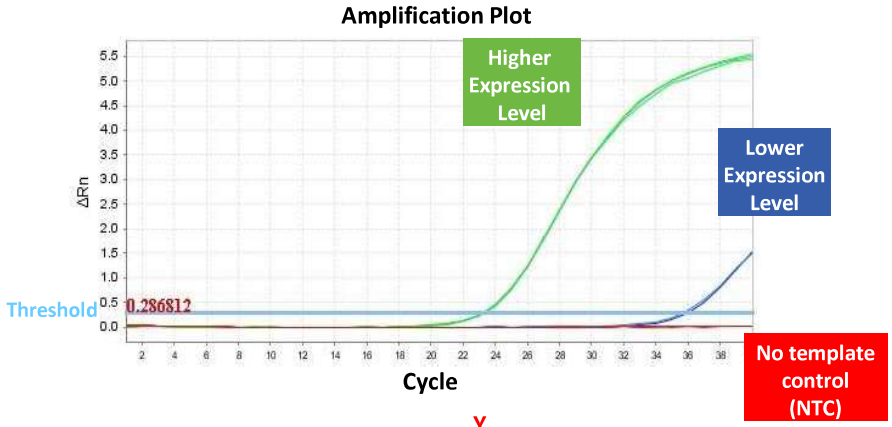
Real-Time PCR System



Real-time PCR signal detection: Exponential phase



Real-Time PCR System



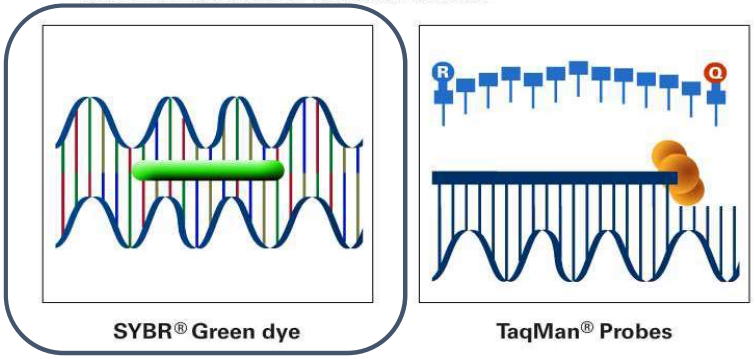
$Y = X \cdot 2^n$ ➡ $X = \frac{Y}{2^n}$

若Y固定,則 n (C_t) 與起始濃度之對數值成反比

Real-time PCR chemistries

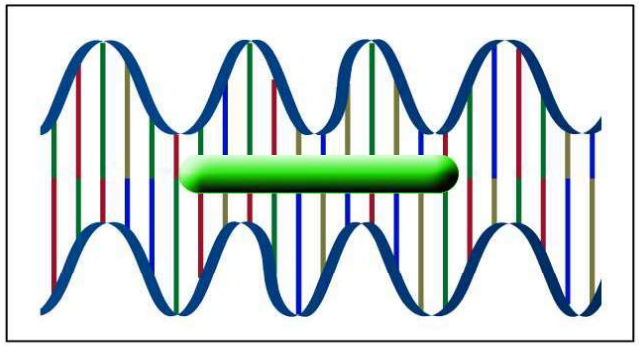
Real-Time PCR Chemistries

Real-Time PCR systems from Applied Biosystems use two main fluorescence detection formats.



Real-Time PCR System – **SYBR® Green System**

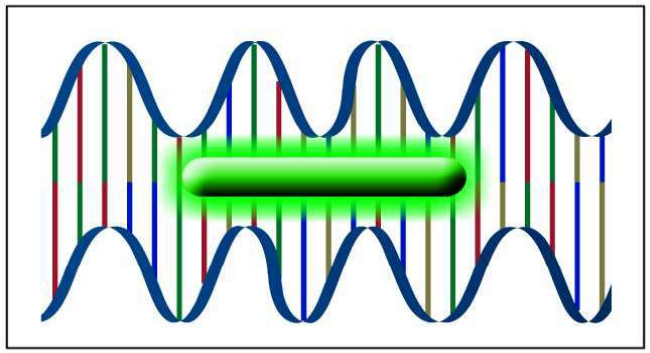
The SYBR® fluorescence format uses a dye called SYBR® Green, which binds non-specifically to double-stranded DNA.



SYBR® Green dye

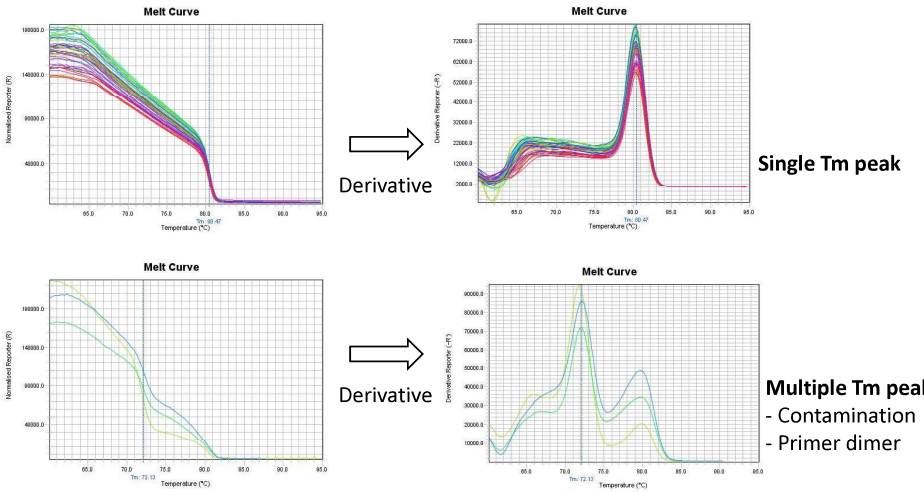
Real-Time PCR System – **SYBR® Green System**

The DNA-dye complex emits green light, which is recorded by the Real-Time PCR instrument.



SYBR® Green dye

Real-Time PCR System – **SYBR® Green System (Melting Curve)**



Real-Time PCR System

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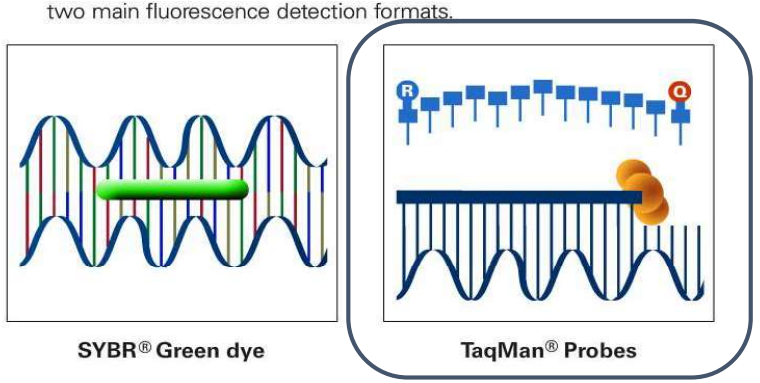


SYBR® Green dye

TaqMan® Probes

Real-Time PCR System – **TaqMan® Probe System**

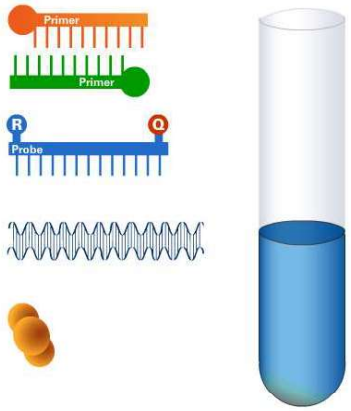
The TaqMan® Probe fluorescence format uses:

Two primers

A probe with a fluorescent Reporter dye and a Quencher

Target DNA

Polymerase

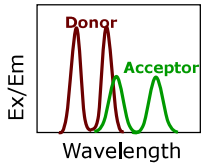


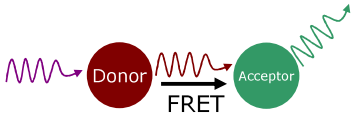
TaqMan® Probes

Real-Time PCR System – **TaqMan® Probe System**

The design of the probe is key. The TaqMan® probe is an oligonucleotide that contains a fluorescent reporter dye bound to the 5′ end and a quencher on the 3′ end.

A probe with a fluorescent Reporter dye and a Quencher



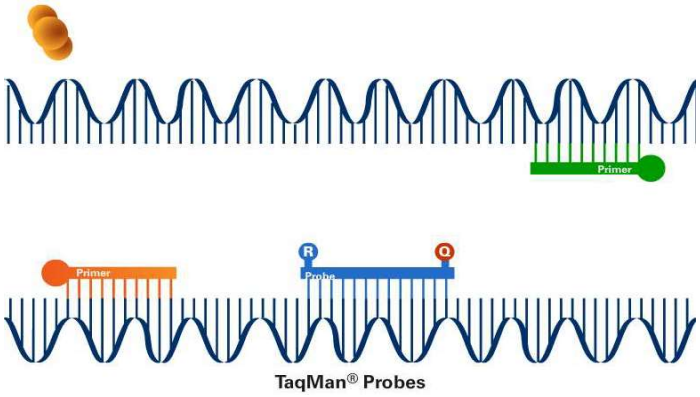


Fluorescence Resonance Energy Transfer (FRET)

TaqMan® Probes

Real-Time PCR System – **TaqMan® Probe System**

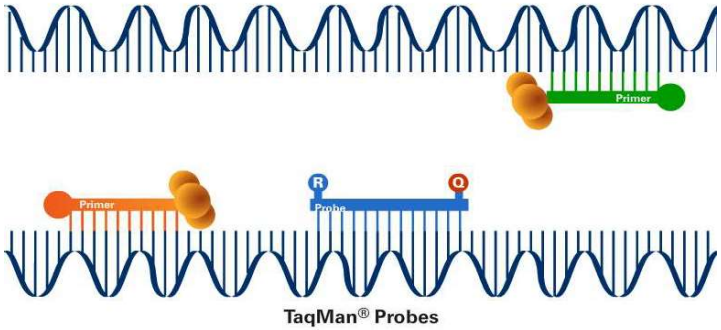
While the dye and quencher are intact, there is no fluorescence.



TaqMan® Probes

Real-Time PCR System – **TaqMan® Probe System**

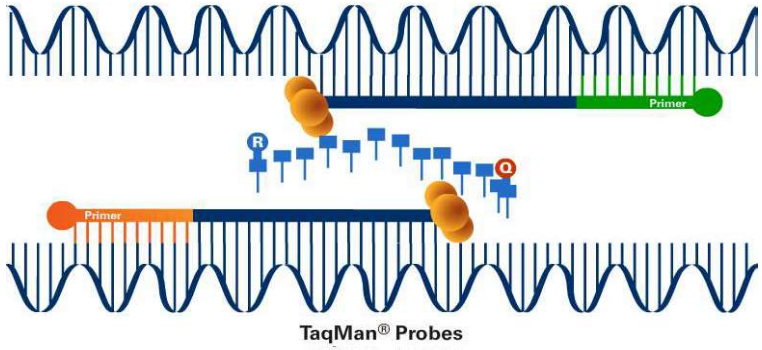
While the dye and quencher are intact, there is no fluorescence.



TaqMan® Probes

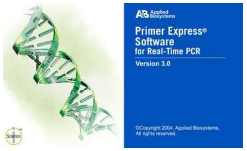
Real-Time PCR System – TaqMan® Probe System

When the polymerase elongates, it is able to cleave the probe, separate the reporter from its quencher, and fluoresce. This fluorescent signal is captured by Real-Time PCR.



Real-Time PCR System (SYBR® Green I vs. TaqMan probe system)

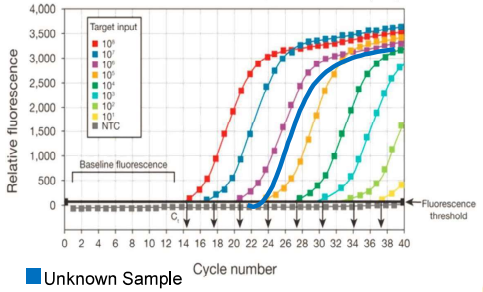
	TaqMan® Probe System	SYBR® Green I Dye System
Specificity	• **Highly specific** • Probe Hybridization	• Less specific
Sensitivity	• Very High	• Very High
Flexibility	• **Multiplex PCR** • SNP detection • +/- application	• **No Probe is required** • **Screening tool**
Optimization	• Universal Guideline • Optimized 20x probe/primer mix • PCR efficiency 100±10%	• Universal Guideline • Need to **optimize PCR program、PCR efficiency** • Need to check primer-dimer information **(Melting curve)**

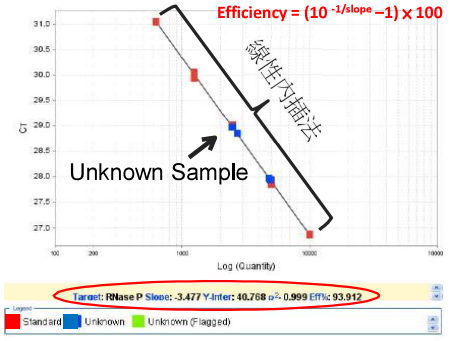


Real-time PCR Quantitation methods and applications

Absolute Quantitation

已知濃度Standard Sample繪製標準曲線
利用內插法求得Unknown Sample之濃度



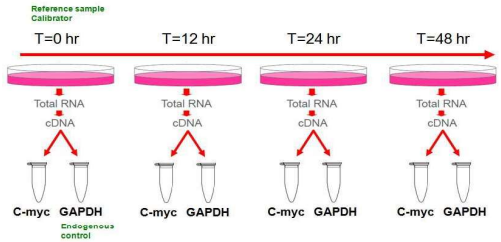


Problem	Possible cause and solution
PCR efficiency is >110%	**Non-specific products.** Use melting curve analysis and gel electrophoresis to identify non specific amplicons. Optimize your primer design to avoid such artifacts or use validated pre-designed primers.
PCR efficiency is <90%	**PCR inhibitors present in a reaction mixture.** Re-purify your template DNA. **PCR conditions are suboptimal.** Verify the primer concentrations. Verify storage conditions of qPCR master mix. **Primer design.** Verify your primer design, use primer design programs or validated pre-designed primers. Avoid designing primers in regions with high DNA secondary structure.

Standard Sample

- When slope = -3.32 Efficiency = 100%
- The optimal efficiency range: 90-110%
- R-value should be ≥0.99

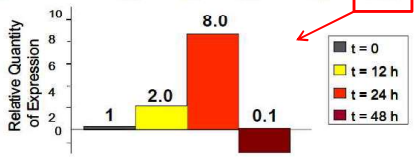
Relative Quantitation (comparative Ct)



- ✓ Target gene: ***c-myc***
- ✓ Reference gene (Endogenous control): ***GAPDH***
- ✓ Calibrator : **T= 0 hr**

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

	c-Myc	GAPDH	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta Ct}$
T=0 (calibrator)	25	10	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



step 1: Normalization to endogenous control

Sample: Ct c-myc – Ct GAPDH = ΔCt sample

Calibrator: Ct c-myc – Ct GAPDH = ΔCt calibrator

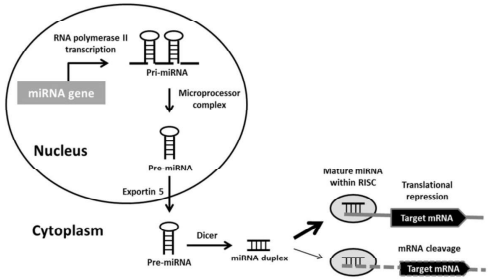
step 2: Normalization to calibrator sample

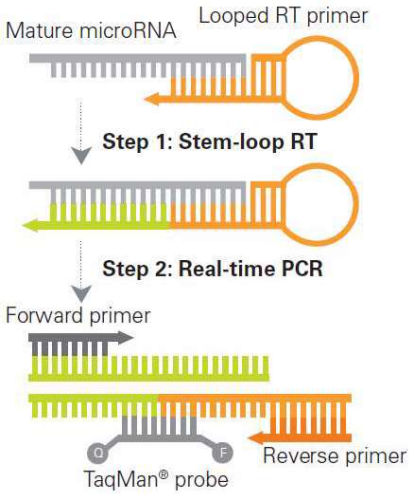
ΔCt Sample – ΔCt Calibrator = ΔΔCt

step 3: use the formula

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

TaqMan microRNA Assays





- Each assay contain:

  miRNA-specific RT primer、miRNA-specific TaqMan Assay
- human, mouse, rat, *Drosophila, C. elegans, and Arabidopsis* etc. available; coverage for miRBase v.21

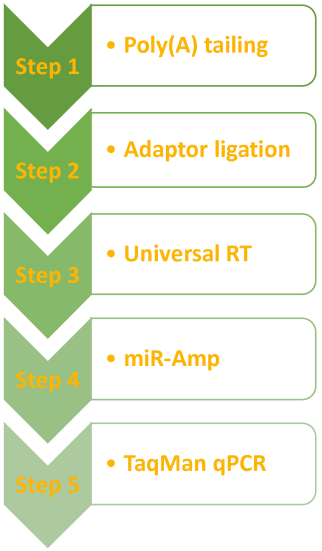
Front Cell Neurosci, Oct 2013 vol.7, article178

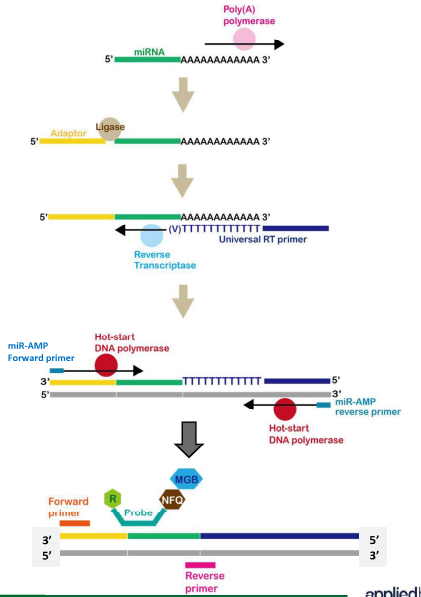
TaqMan Advanced miRNA Assays

- superior sensitivity in tissue, serum, and plasma
- 1 ng of total RNA from tissue or 2 µL of eluant from serum or plasma

- Have multiple miRNA targets from a single amplified sample
- TaqMan Advanced miRNA Assays is compatible with TaqMan advances miRNA cDNA synthesis kit

TaqMan Advanced miRNA Assays: workflow





TaqMan Advanced miRNA Assays (High specificity and sensitivity)

	Synthetic template							
TaqMan Advanced miRNA Assay	Let-7a	Let-7b	Let-7c	Let-7d	Let-7e	Let-7f	Let-7g	Let-7i
Let-7a	100%	0%	0%	0%	4%	2%	0%	0%
Let-7b	0%	100%	3%	0%	0%	0%	0%	0%
Let-7c	1%	2%	100%	0%	0%	0%	0%	0%
Let-7d	0%	0%	0%	100%	0%	0%	0%	0%
Let-7e	0%	0%	0%	0%	100%	0%	0%	0%
Let-7f	1%	0%	0%	0%	0%	100%	0%	0%
Let-7g	0%	0%	0%	0%	0%	0%	100%	4%
Let-7i	0%	1%	0%	0%	0%	0%	0%	100%

miRNA name	miRNA sequence
hsa-let-7a-5p	UGA GGU AGU AGG UUG UAU AGU U
hsa-let-7b-5p	UGA GGU AGU AGG UUG UGU GGU U
hsa-let-7c-5p	UGA GGU AGU AGG UUG UAU GGU U
hsa-let-7d-5p	AGA GGU AGU AGG UUG CAU AGU U
hsa-let-7e-5p	UGA GGU AGG AGG UUG UAU AGU U
hsa-let-7f-5p	UGA GGU AGU AGA UUG UAU AGU U
hsa-let-7g-5p	UGA GGU AGU AGU UUG UAC AGU U
hsa-let-7i-5p	UGA GGU AGU AGU UUG UGC UGU U
	* * * * * *

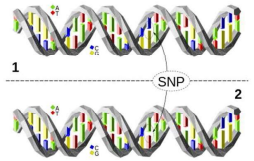
* Represent Differences in nucleotide

Highly homologous members of the let-7 miRNA family

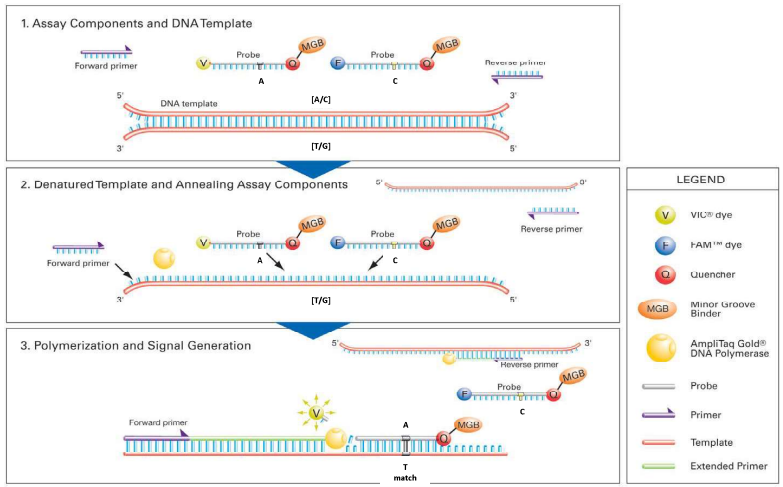
There is minimal or no cross-reactivity.

What are Single Nucleotide Polymorphism (SNP)?

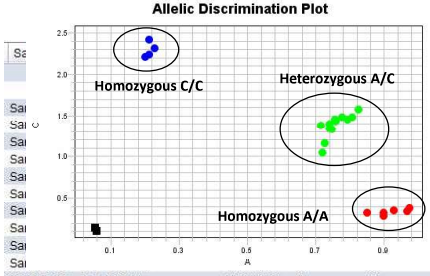
- Diploid organisms – 2 sets of chromosomes
- Each person receives 1 alleles from each parent.
- If both alleles are the same, the person is ***homozygous*** for that gene.
- If the alleles differ, the person is ***heterozygous*** for that gene.
- They occur once in every 300 nucleotides on average.
- The rarer allele having a frequency of at least 1%.



TaqMan Probe System (Single Nucleotide Polymorphism)



Allelic Discrimination Plot (SNP genotyping)



					Allele 1 Dyes	Allele 2 Dyes	Allele 1 Δ...	Allele 2 Δ...	Pass.Ref	Call
Sample 19	C__117114	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.795	1.528	4,026.385	Heterozygous A/C
Sample 2	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.84	0.364	3,737.051	Homozygous A/A
Sample 20	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.888	0.37	4,099.657	Homozygous A/A
Sample 3	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.189	2.251	3,643.654	Homozygous C/C
Sample 4	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.963	0.421	3,826.977	Homozygous A/A
Sample 5	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.705	1.418	3,982.397	Heterozygous A/C
Sample 6	C__117114...	Unknown	A	C	VIC-NFQ-MGB	FAM-NFQ-MGB	0.729	1.386	4,048.287	Heterozygous A/C

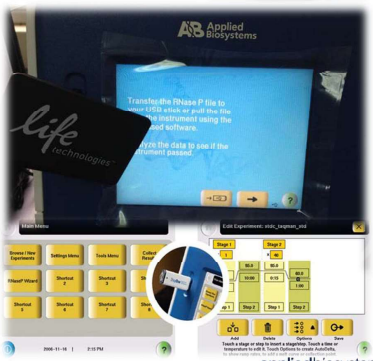
Application of TaqMan Assays and SYBR systems

TaqMan assays	SYBR Green
➢ Gene expression analysis (ex: Genetically Modified Organism, GMO)	➢ Gene expression analysis
➢ MicroRNA and noncoding RNA analysis	
➢ Drug metabolism genotyping ➢ SNP genotyping ➢ Somatic mutation detection	
➢ Pathogen Presence/ Absence	
➢ Protein expression	

The StepOnePlus Real-Time PCR System

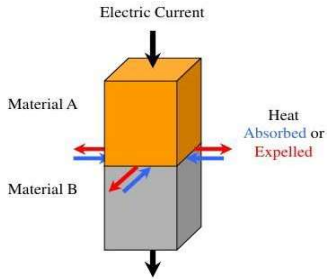
Design and Feature

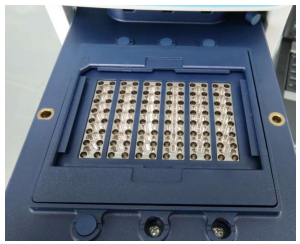
StepOnePlus 96 well



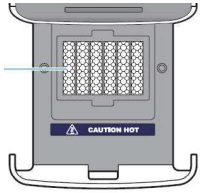
1. PCR +Optical sys. 避免光徑偏移
2. Flash LED 偵測才開啟，耗損少
3. Peltier effect 精確穩定控溫系統
4. USB 存出 (主機會暫存最後一筆檔案)

Block





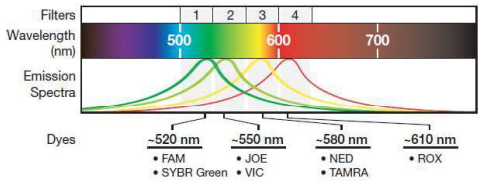
Tech. →	Ramping ←	Volume
1. Peltier effect 不同導體 電流改變 熱能吸放 熱電效應 精確控溫	1. 鎳鉻合金基座 4.6 ℃/sec 2. 樣品升降溫 **Fast : ± 2.2 ℃/sec** Std: ± 1.6 ℃/sec 3. Auto delta Time by cycle Temp by cycle	1. 反應管 **(0.1 mL)** 96 well plate 8-tube strip single tube ***耗材需使用"Optical"** 2. 反應體積 **10-30 µL**



StepOnePlus
instrument VeriFlex™
Sample Blocks

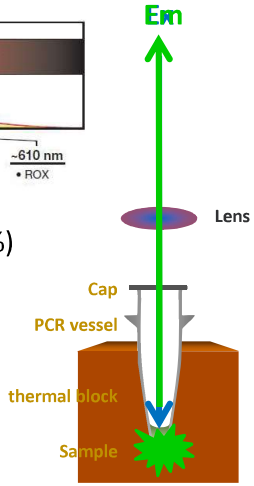
StepOne™ Plus Real-Time PCR System

- 96-well
- 4 color system (TaqMan MGB probe)

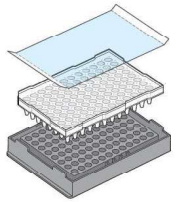


Common Features

- Fast and Standard ramping in the same block (1-100%)
- Single LED excitation
- Supported volumes are 10-30 µL
- Standalone or co-located
- Remote monitoring - LAN



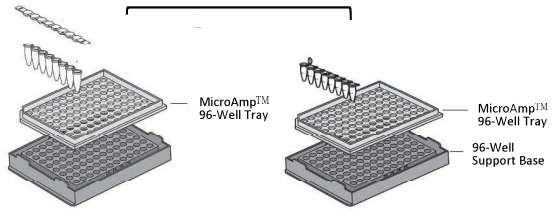
Supported Consumables



more than 48 reactions/run

✓96-well plate + Optical adhesive film

● for ***0.1 mL Optical*** tube strip/single tube
✓管子及蓋子上**請勿標記任何記號**
避免墨色脫落沾到 PCR block增加背景值



less than 48 reactions/run

✓使用八連排或單管必須將其置放在 MicroAmp™ 96-Well Tray
✓對稱放置，避免加熱板上升時， 受力不均，造成機械性的損傷。

上機前/時之注意事項

✓光學封膜/瓶蓋是否平整緊密貼附，且表面無指紋或其他髒汙沾黏。

✓確認已將 reaction mixture完全離心至反應管底部，且無氣泡產生 (Centrifuge the tubes for 2 min at less than 1500 rpm)

Correct	Incorrect
Liquid is at the bottom of the well.	• Not centrifuged with enough force, or • Not centrifuged for enough time

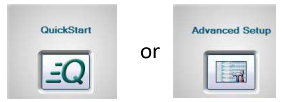
✓上機時，反應管放置位置應以"平行、偶數、對稱"為原則。

✓為了避免卡盤，不建議8連排剪裁後上機使用。

SetUp

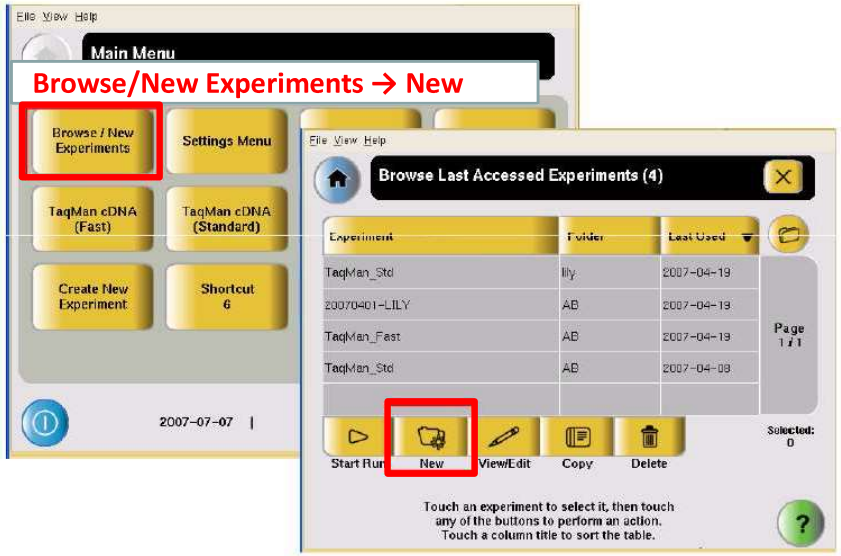
1. PC free

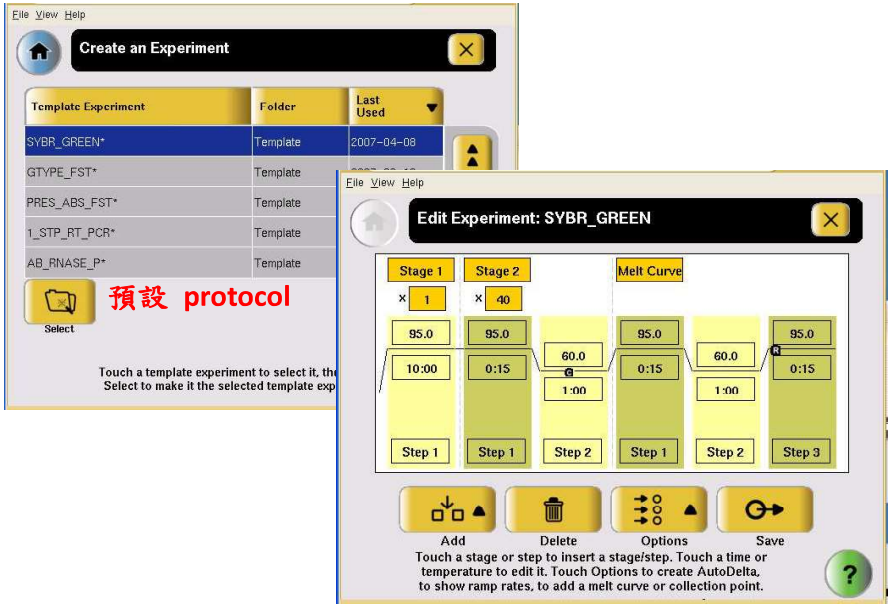
2. PC controlled

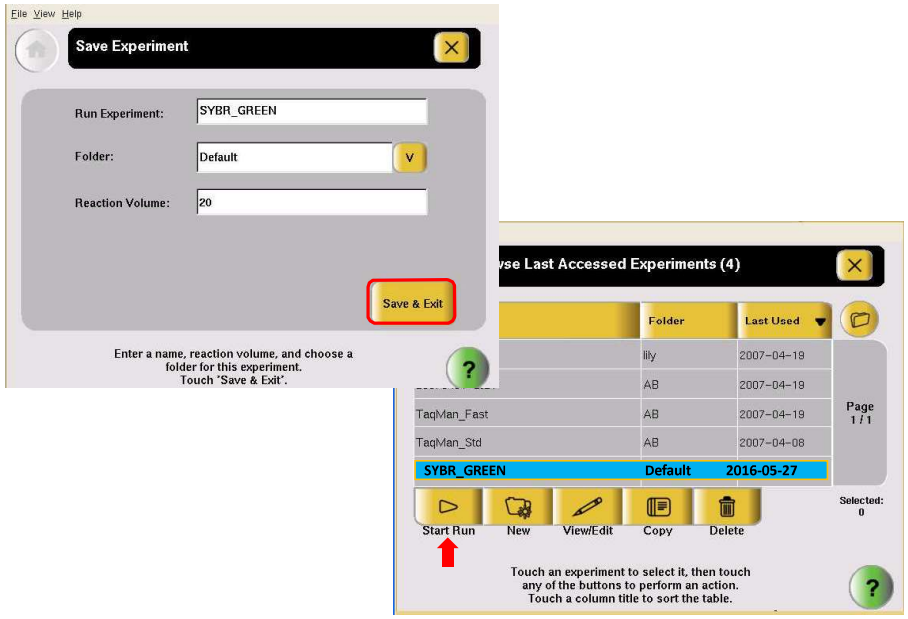


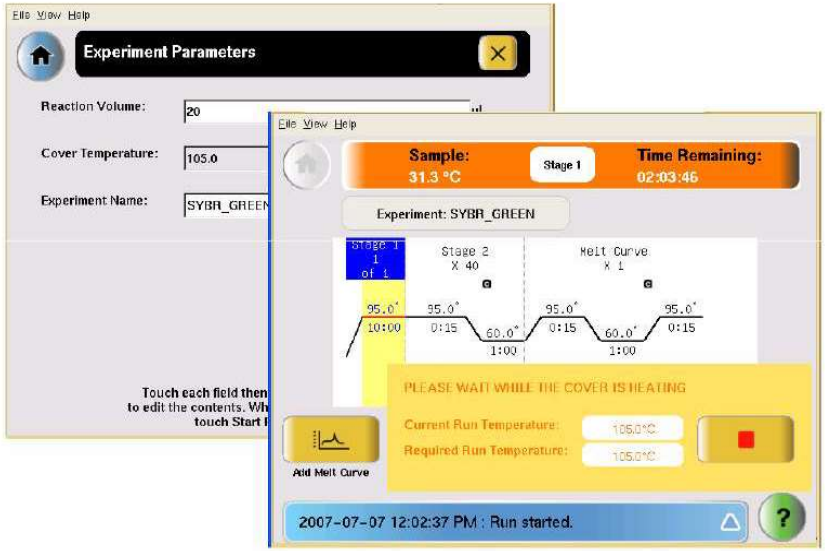
Standalone (PC-free)

1. Start the run from the touch screen.
2. Download the file (**.eds**) to your PC.（主機暫存最後一筆檔案）
3. Analyze your data.





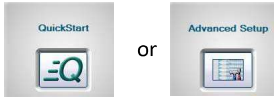




SetUp

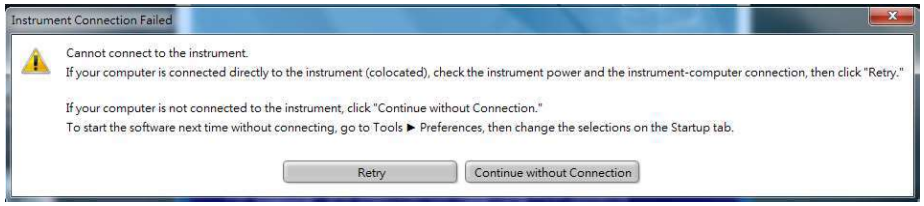
1. PC free

2. PC controlled

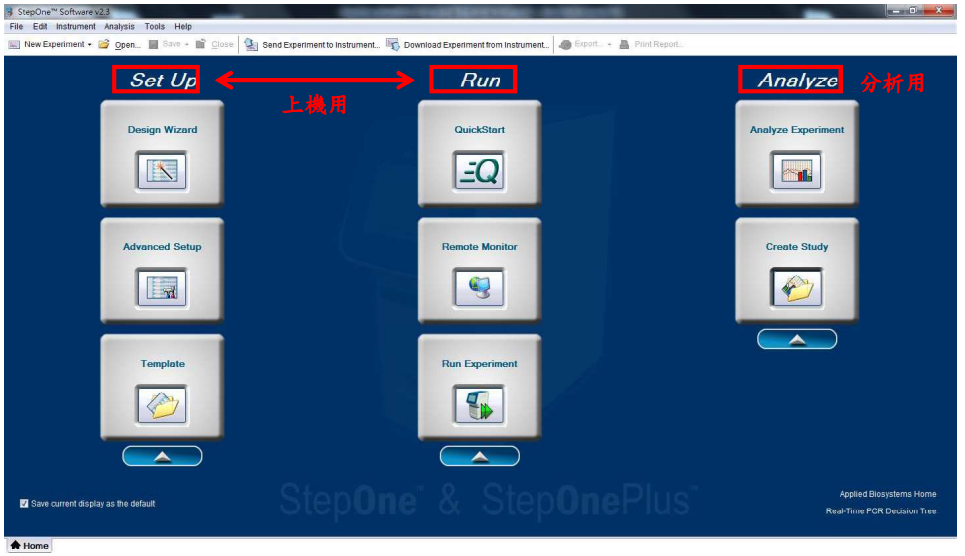


StepOne Software v2.3
軟體操作

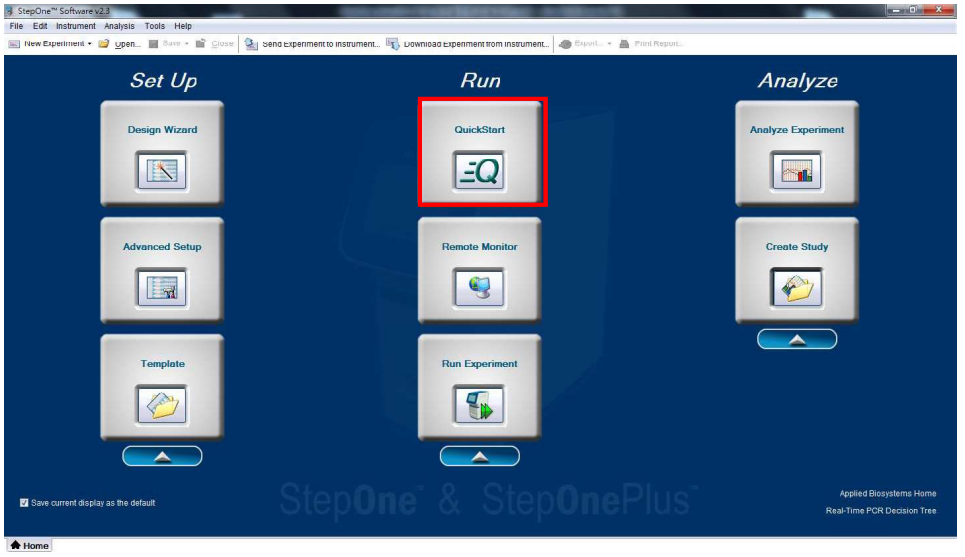
Login the software



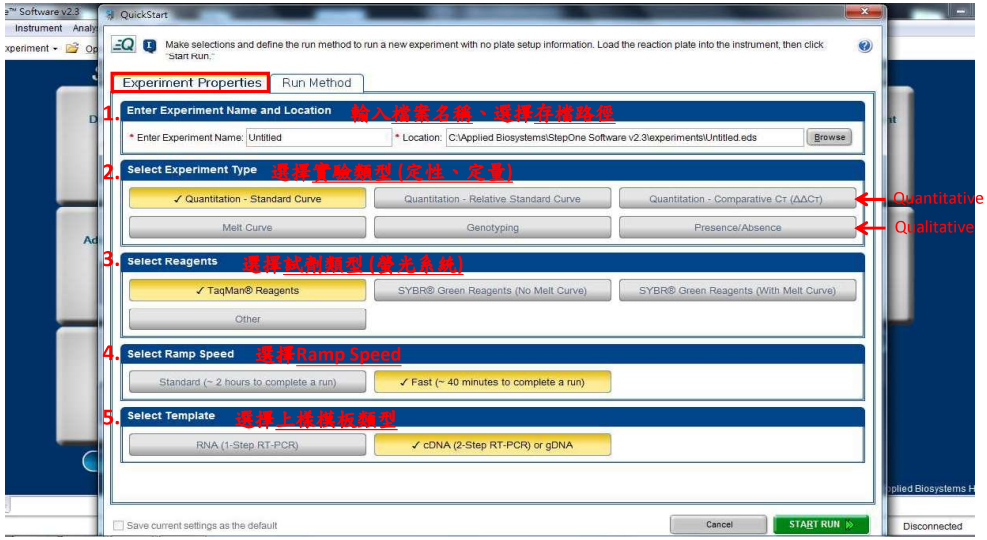
*** Analyze data -> Continue without Connection**



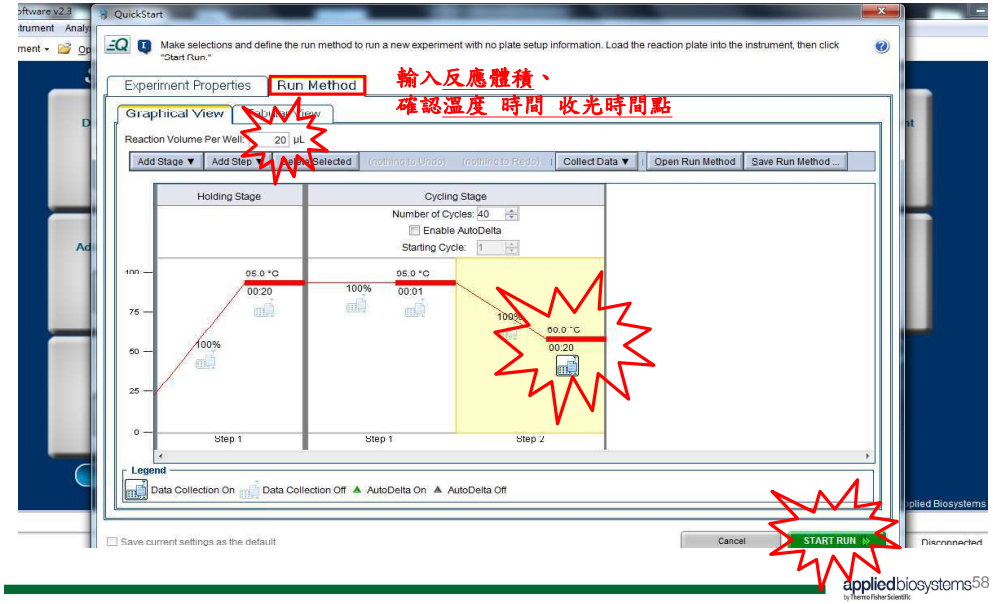
Run the experiment



Run (QuickStart) : Experiment Properties

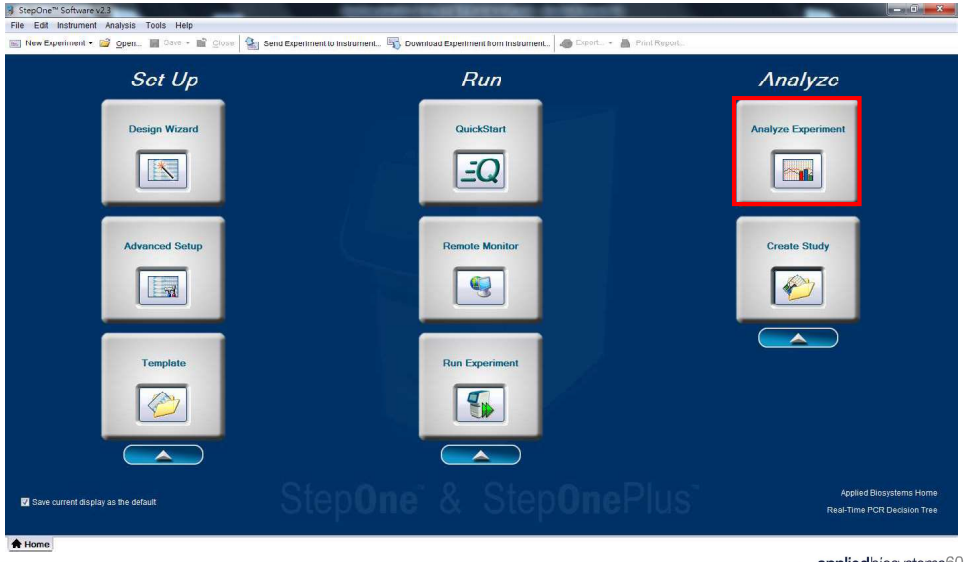


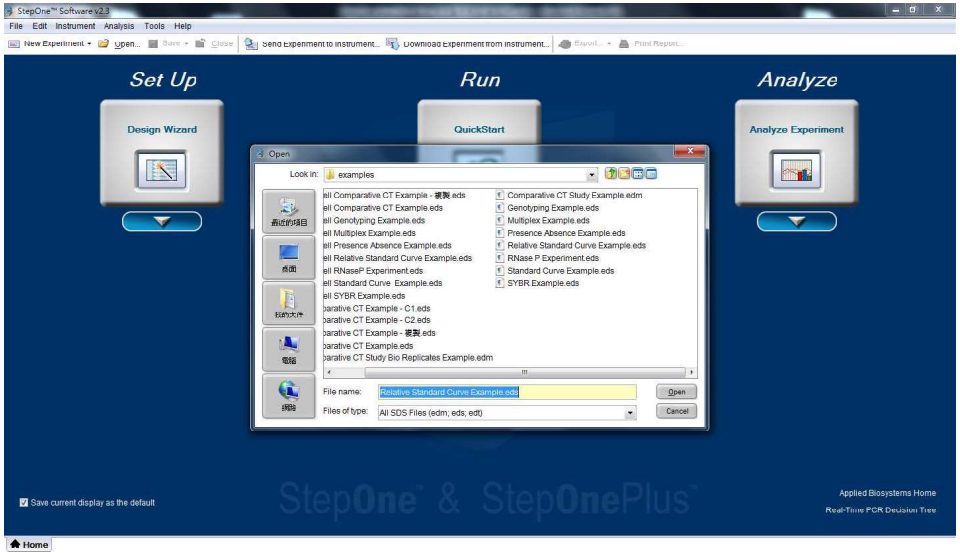
Run (QuickStart) : Run Method



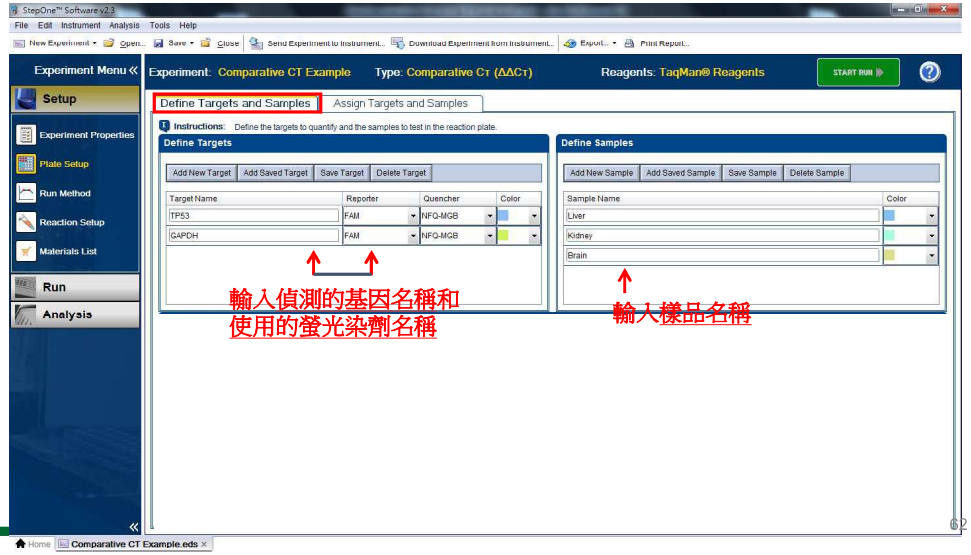
Analyze the data

Analyze: Analyze Experiment



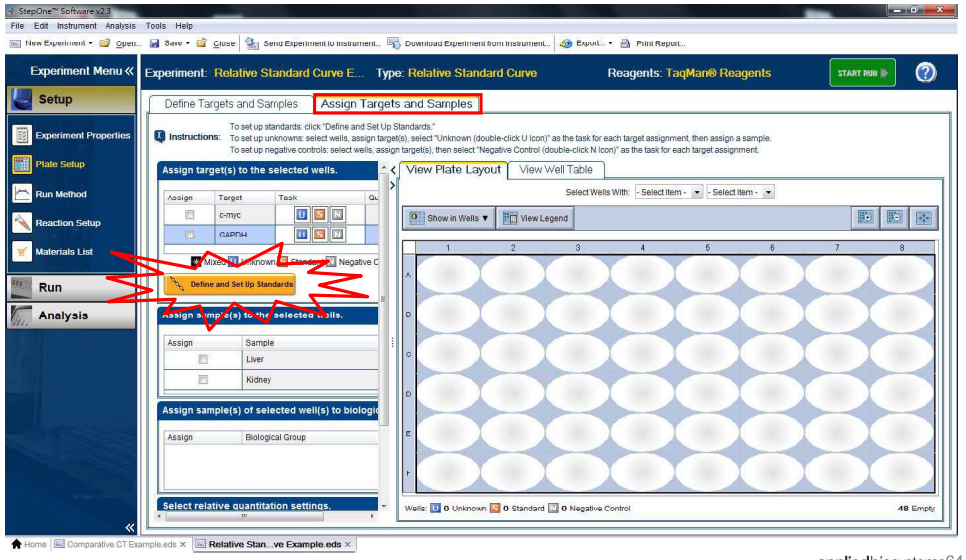


Setup: plate Setup
→ Define Targets and Samples 定義偵測的基因和樣品名稱



Setup: plate Setup
→ Assign Targets and Samples 定義基因和樣品位置、設定 Reference Sample和 Endogenous Control

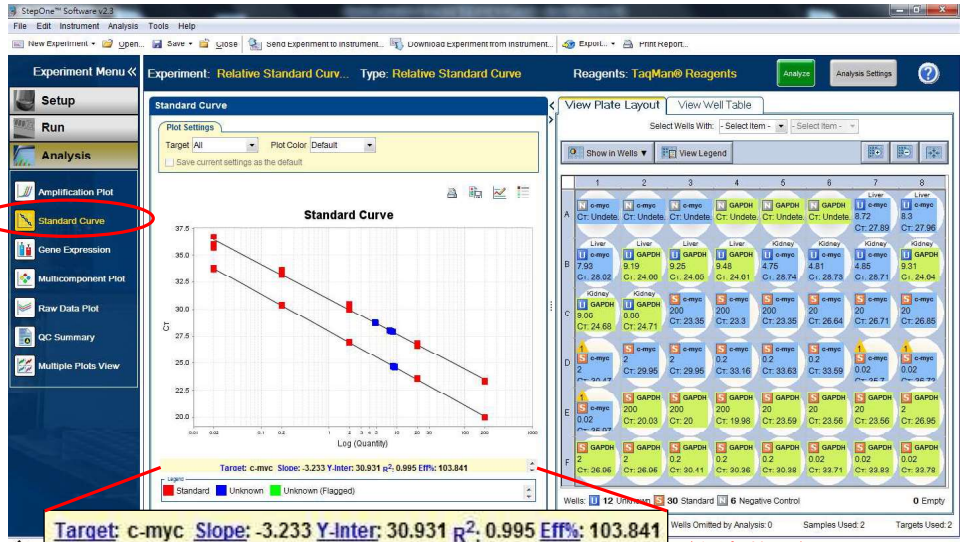
Setup: plate Setup
→ Assign Targets and Samples 定義標準品樣品位置、濃度



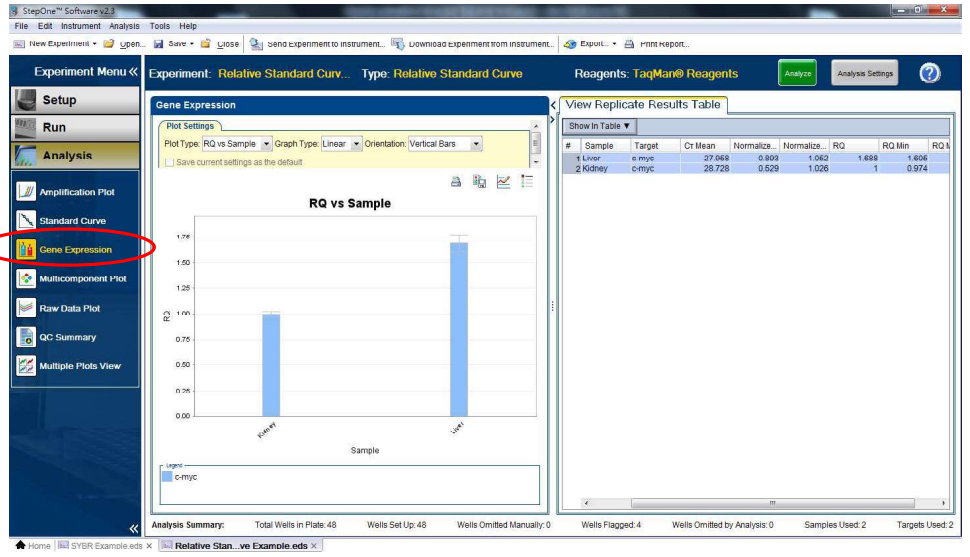
Analysis: Melt curve (SYBR Green only)

Analysis: Amplification plot

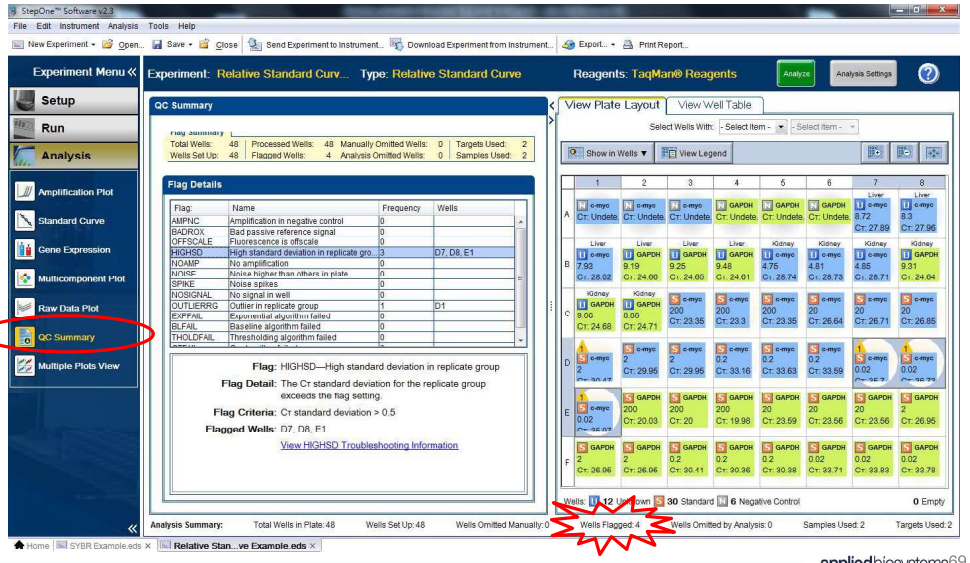
Analysis: Standard curve



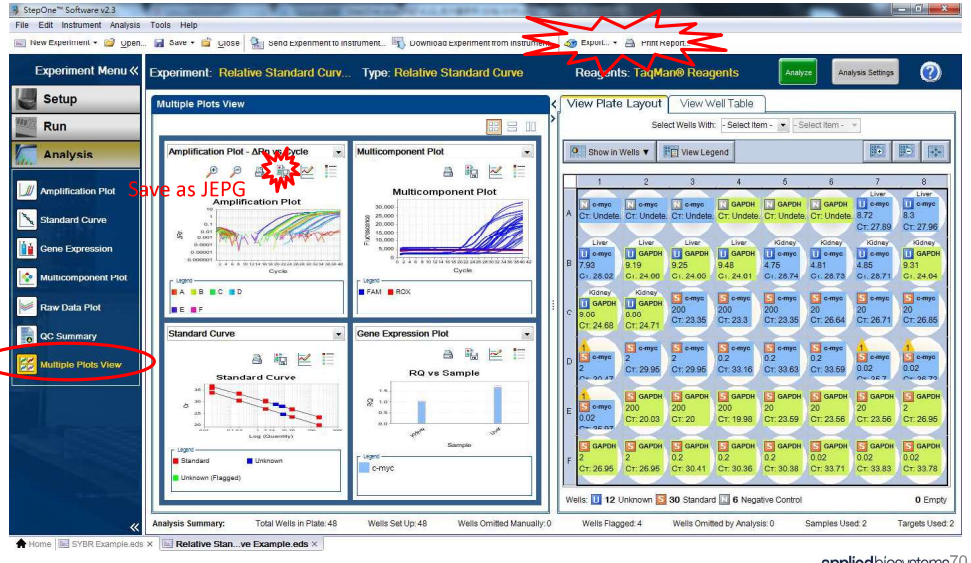
Analysis: Gene Expression



Analysis: QC Summary
→ 協助 troubleshooting



Analysis: Multiple Plots View
→ Export to Excel or save as JEPG



Thank you for your attention!

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Email: jessie@kimforest.com