

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

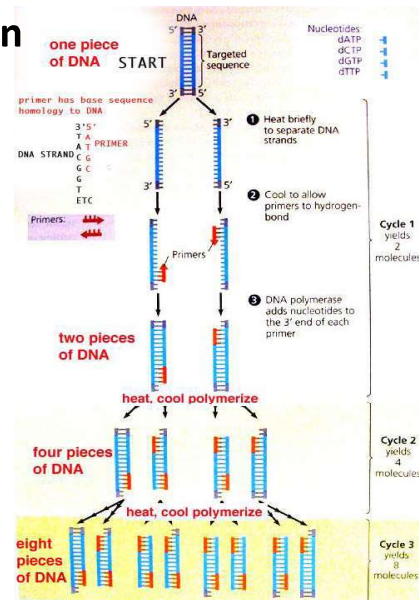
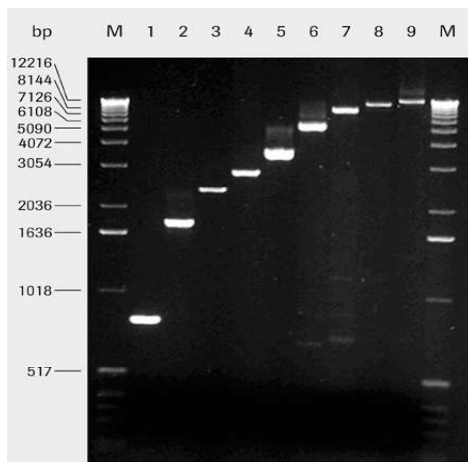
applied biosystems
by Thermo Fisher Scientific

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

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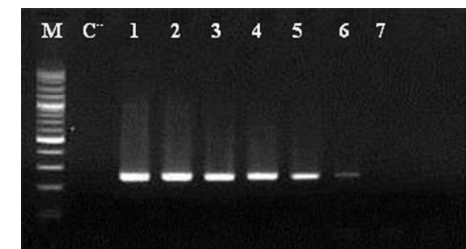
Polymerase Chain Reaction (PCR)



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by Thermo Fisher Scientific

Conventional PCR Quantitative System

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



M: Marker

C: Control

Lane 1: 42 pg

Lane 4: 42 fg

Lane 2: 4.2 pg

Lane 5: 4.2 fg

Lane 3: 420 fg

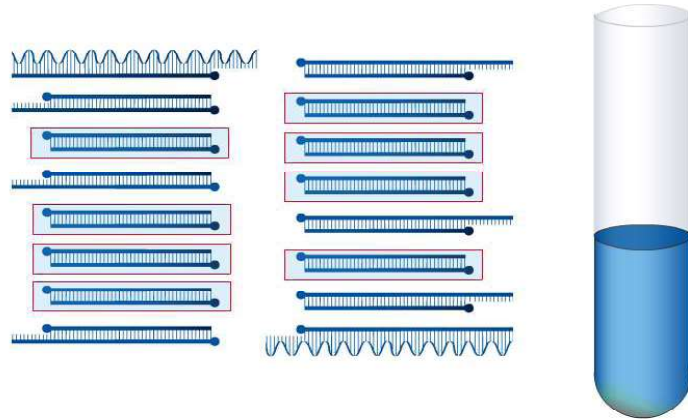
Lane 6: 0.42 fg

Lane 7: 0.042 fg

applied biosystems 4
by Thermo Fisher Scientific

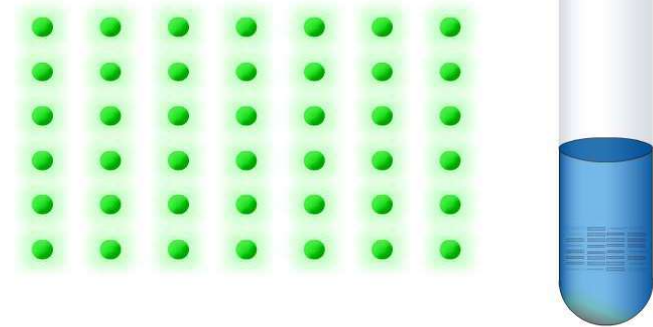
Real-Time PCR System

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



Real-Time PCR System

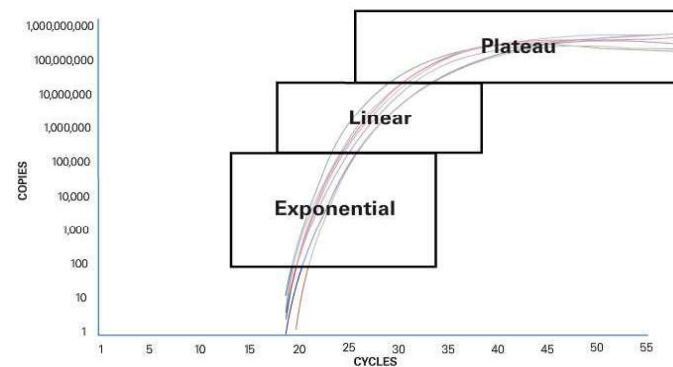
Add fluorescent labels to the PCR mix. A light source in the Real-Time PCR instrument excites the fluorescence. A camera captures the fluorescent signals.



Polymerase Chain Reaction

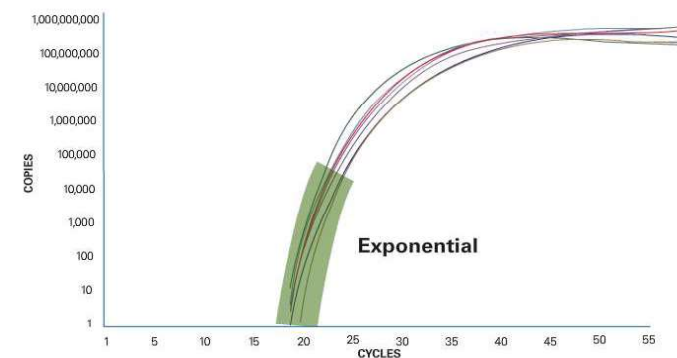
With Real-Time PCR, there are three amplification stages:

1) Exponential, 2) Linear, and 3) Plateau.



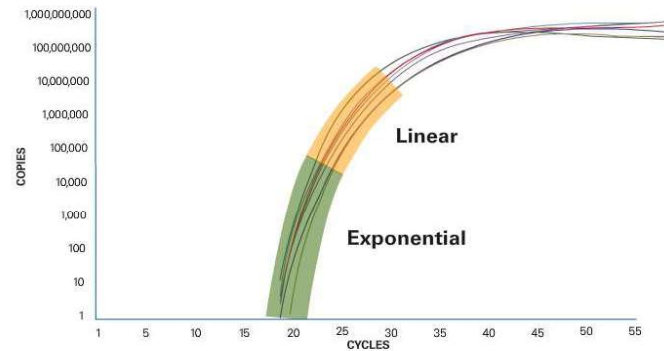
Polymerase Chain Reaction

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



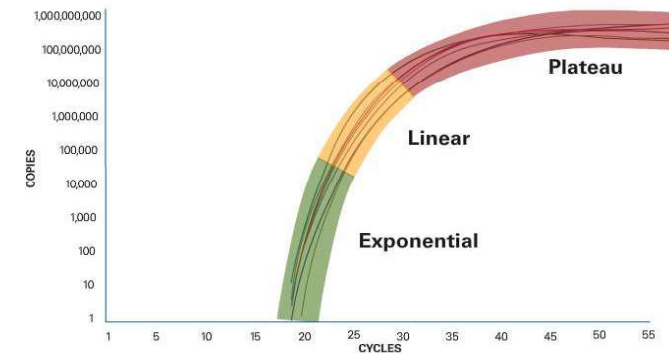
Polymerase Chain Reaction

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.

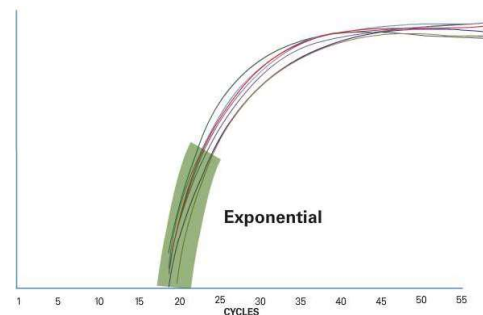


Real-Time PCR System

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

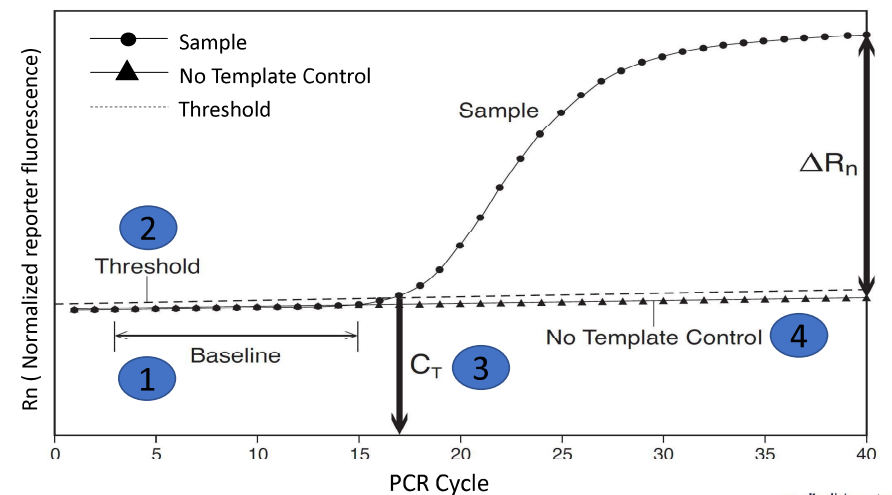
$$Y = X(1 + e)^n$$

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

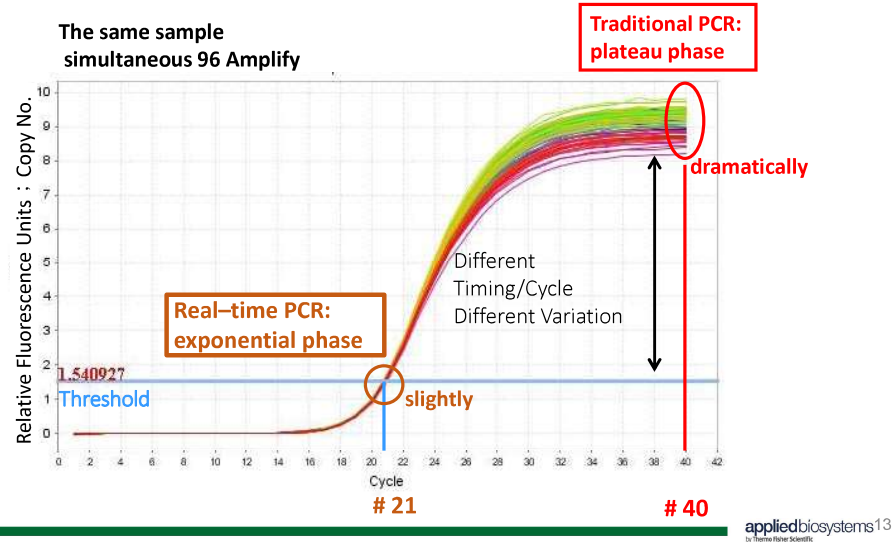


Real-Time PCR System

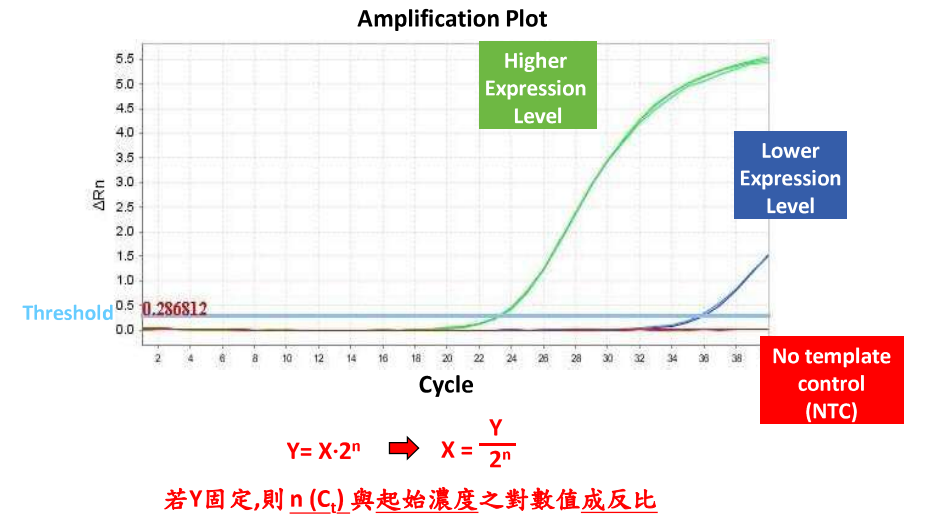
Amplification plot (linear scale)



Real-time PCR signal detection: Exponential phase



Real-Time PCR System

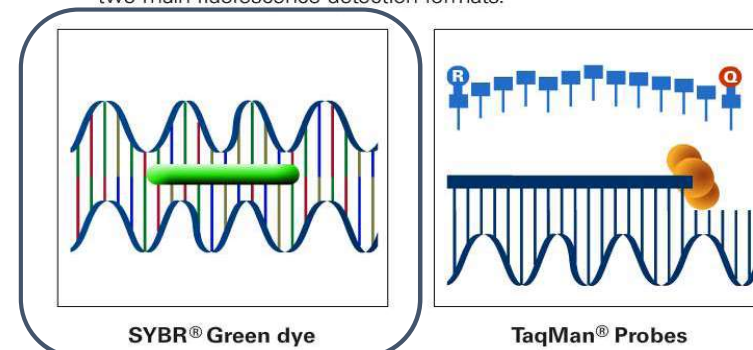


Real-time PCR chemistries

applied biosystems¹⁵
by Thermo Fisher Scientific

Real-Time PCR Chemistries

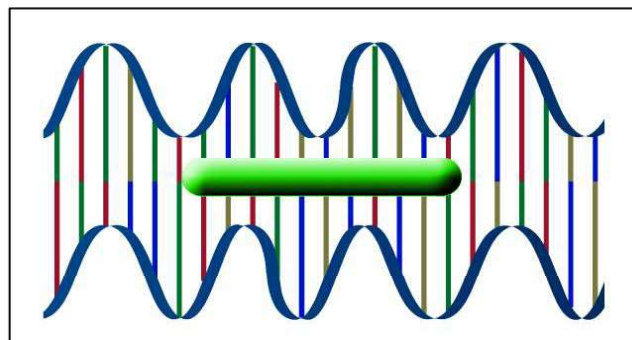
Real-Time PCR systems from Applied Biosystems use two main fluorescence detection formats.



applied biosystems¹⁶
by Thermo Fisher Scientific

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.

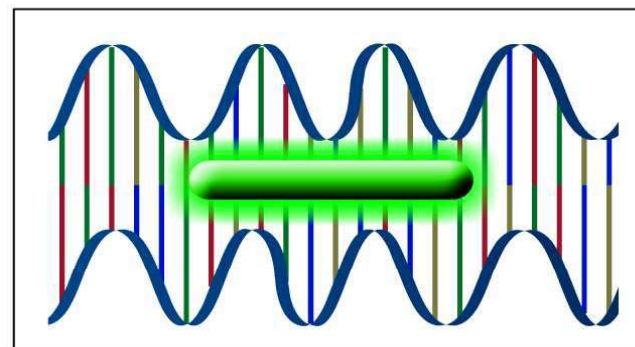


SYBR® Green dye

appliedbiosystems¹⁷
by Thermo Fisher Scientific

Real-Time PCR System – SYBR® Green System

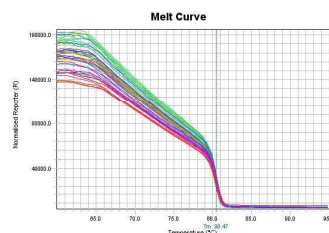
The DNA-dye complex emits green light, which is recorded by the Real-Time PCR instrument.



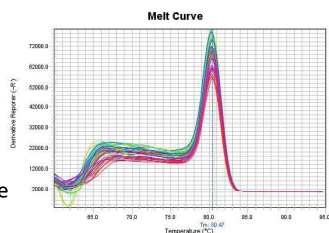
SYBR® Green dye

appliedbiosystems¹⁸
by Thermo Fisher Scientific

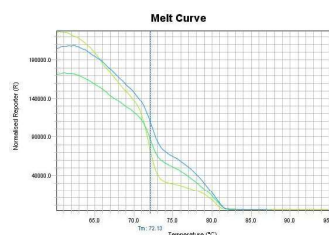
Real-Time PCR System – SYBR® Green System (Melting Curve)



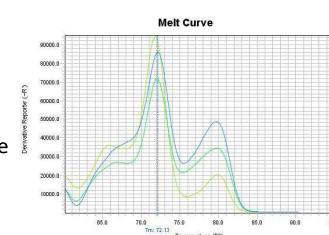
Derivative



Single Tm peak



Derivative

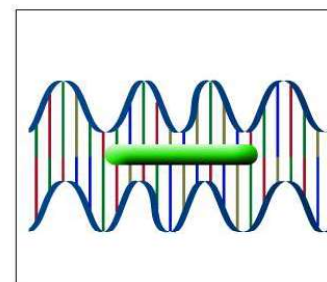


Multiple Tm peak
- Contamination
- Primer dimer

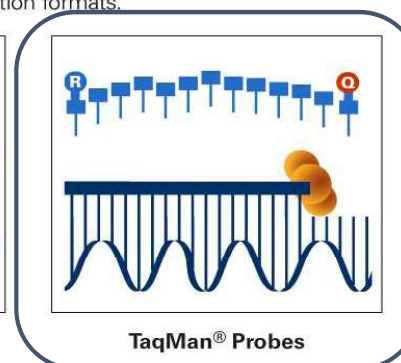
appliedbiosystems¹⁹
by Thermo Fisher Scientific

Real-Time PCR System

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



SYBR® Green dye

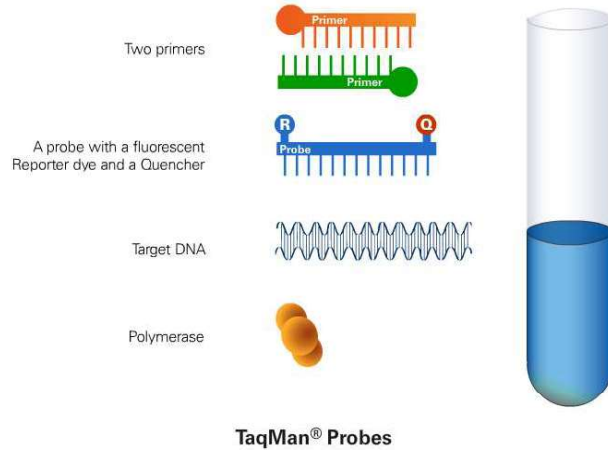


TaqMan® Probes

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by Thermo Fisher Scientific

Real-Time PCR System – TaqMan® Probe System

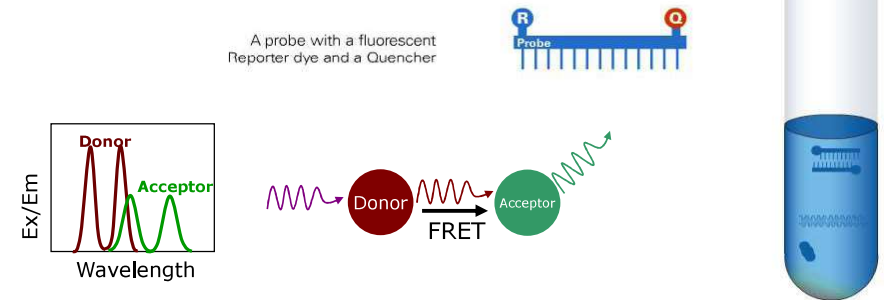
The TaqMan® Probe fluorescence format uses:



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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.

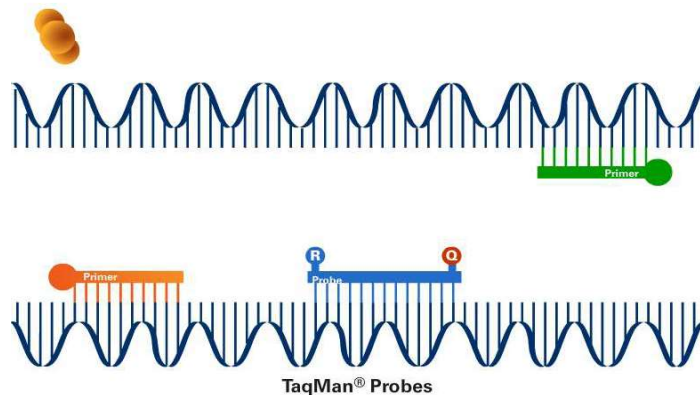


Fluorescence Resonance Energy Transfer (FRET)

appliedbiosystems²²
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Real-Time PCR System – TaqMan® Probe System

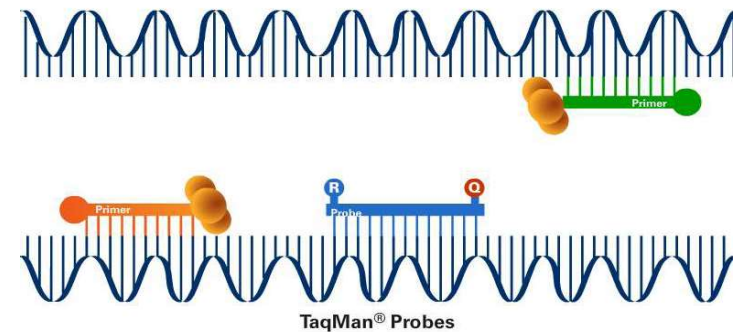
While the dye and quencher are intact, there is no fluorescence.



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by Thermo Fisher Scientific

Real-Time PCR System – TaqMan® Probe System

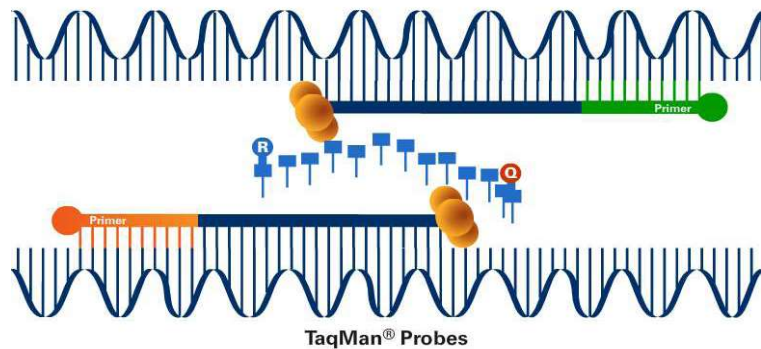
While the dye and quencher are intact, there is no fluorescence.



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by Thermo Fisher Scientific

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.



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by Thermo Fisher Scientific

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



TaqMan®
Probe
System



SYBR® Green I
Dye
System

Specificity

- **Highly specific**
- Probe Hybridization

Sensitivity

- Very High

Flexibility

- **Multiplex PCR**
- SNP detection
- +/- application

Optimization

- Universal Guideline
- Optimized 20x probe/primer mix
- PCR efficiency 100±10%

- Less specific

- Very High

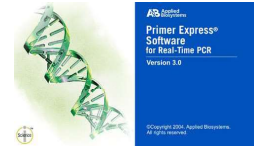
- **No Probe is required**

- **Screening tool**

- Universal Guideline

- Need to **optimize PCR program**、**PCR efficiency**

- Need to check primer-dimer information
(**Melting curve**)



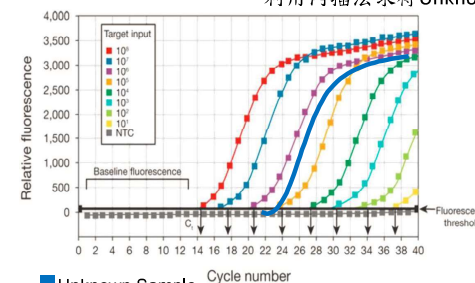
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by Thermo Fisher Scientific

Real-time PCR Quantitation methods and applications

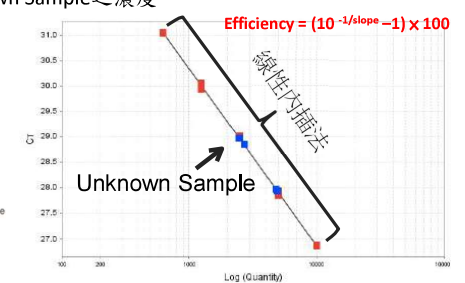
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by Thermo Fisher Scientific

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



Unknown Sample



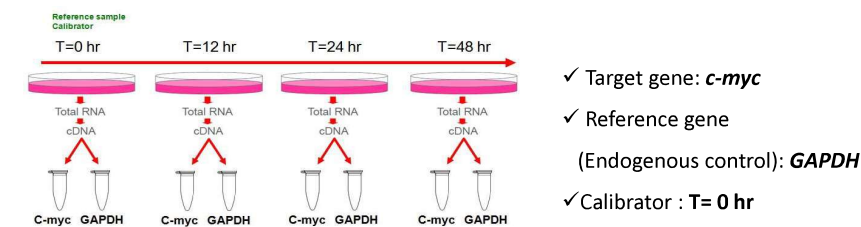
Target: RNase P Slope: -3.477 Y-inter: 40.768 R²: 0.999 Eff: 93.912
Standard Unknown Unknown (Flagged)

Standard Sample

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

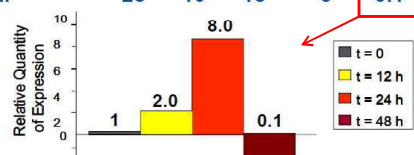
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Relative Quantitation (comparative Ct)



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

	c-Myc	GAPDH	ΔC_t	$\Delta\Delta C_t$	$2^{-\Delta\Delta C_t}$
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: $Ct\ c-myc - Ct\ GAPDH = \Delta C_t\ sample$

Calibrator: $Ct\ c-myc - Ct\ GAPDH = \Delta C_t\ calibrator$

step 2: Normalization to calibrator sample

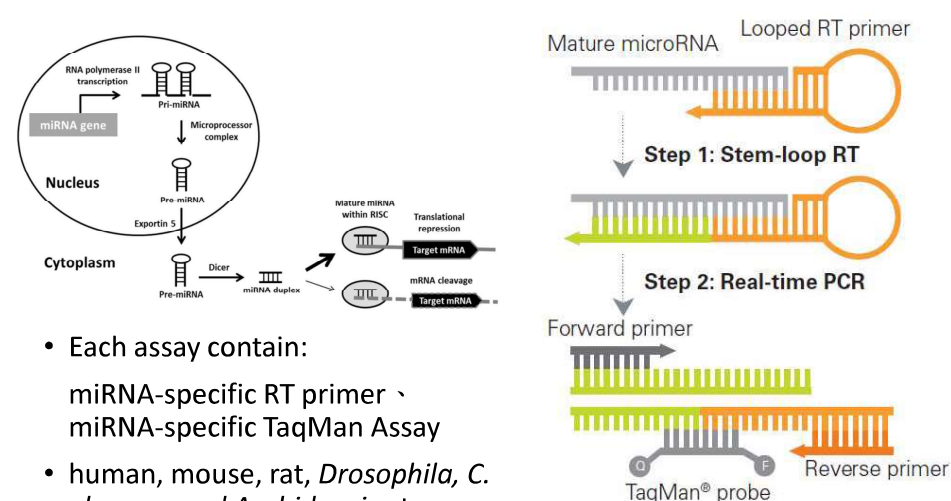
$\Delta C_t\ Sample - \Delta C_t\ Calibrator = \Delta\Delta C_t$

step 3: use the formula

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

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by Thermo Fisher Scientific

TaqMan microRNA Assays



Front Cell Neurosci, Oct 2013 vol.7, article178
appliedbiosystems³⁰
by Thermo Fisher Scientific

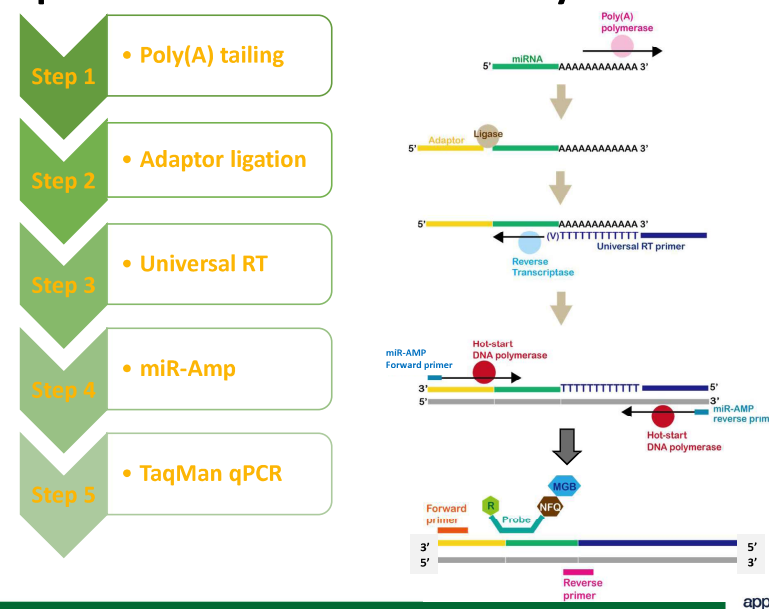
TaqMan Advanced miRNA Assays

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



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by Thermo Fisher Scientific

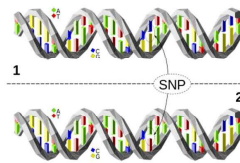
TaqMan Advanced miRNA Assays: workflow



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by Thermo Fisher Scientific

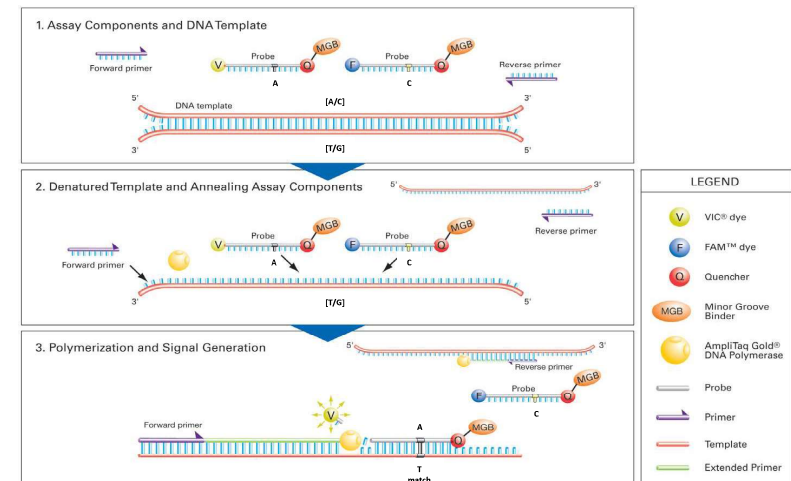
What are Single Nucleotide Polymorphism (SNP)?

- 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%.



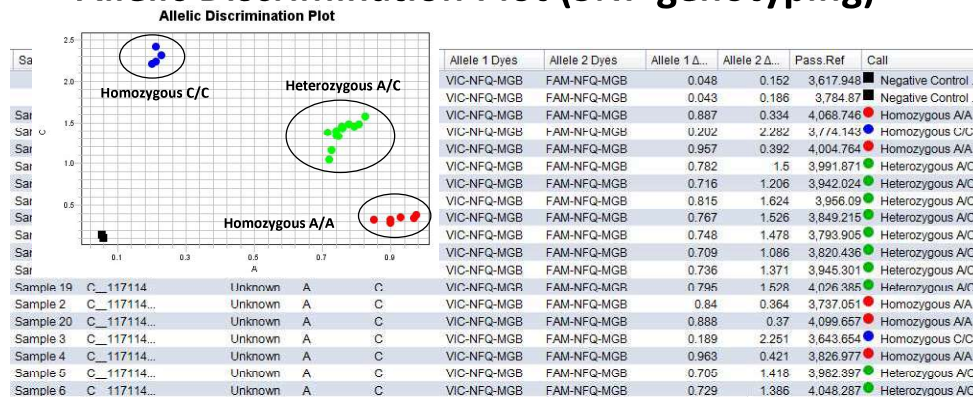
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by Thermo Fisher Scientific

TaqMan Probe System (Single Nucleotide Polymorphism)



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by Thermo Fisher Scientific

Allelic Discrimination Plot (SNP genotyping)



appliedbiosystems³⁵
by Thermo Fisher Scientific

Application of TaqMan Assays and SYBR systems

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

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by Thermo Fisher Scientific

The StepOnePlus Real-Time PCR System

簡易三步驟!!



appliedbiosystems³⁷
by Thermo Fisher Scientific

Design and Feature



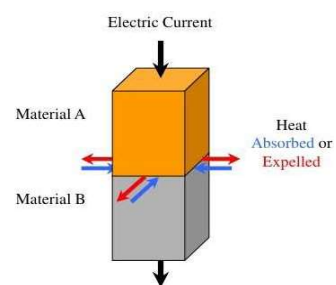
StepOnePlus 96 well



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)

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by Thermo Fisher Scientific

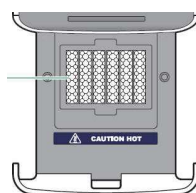
Block



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



CAUTION



StepOnePlus
instrument VeriFlex™
Sample Blocks

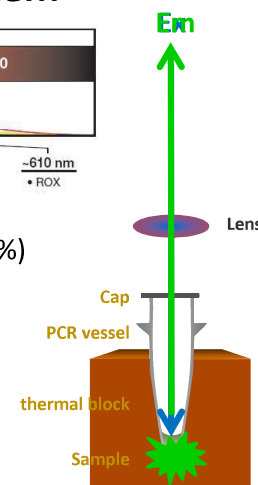
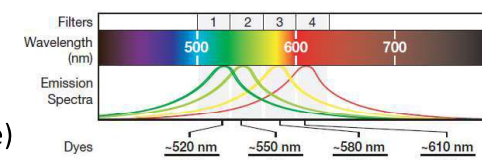
appliedbiosystems³⁹
by Thermo Fisher Scientific

StepOne™ Plus Real-Time PCR System

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

Common Features

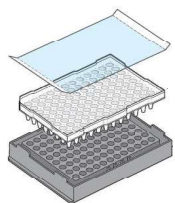
- Fast and Standard ramping in the same block (1-100%)
- Single LED excitation
- Supported volumes are 10-30 µL
- Standalone or co-located
- Remote monitoring - LAN



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by Thermo Fisher Scientific

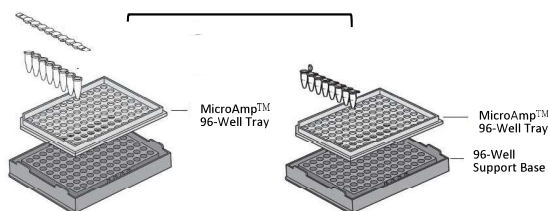
Supported Consumables

- 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



more than 48 reactions/run

- ✓96-well plate +
Optical adhesive film



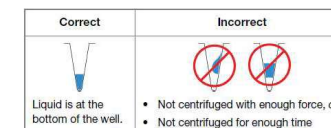
less than 48 reactions/run

- ✓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。

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by Thermo Fisher Scientific

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)
- ✓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。



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by Thermo Fisher Scientific

SetUp

1. PC free



2. PC controlled



or



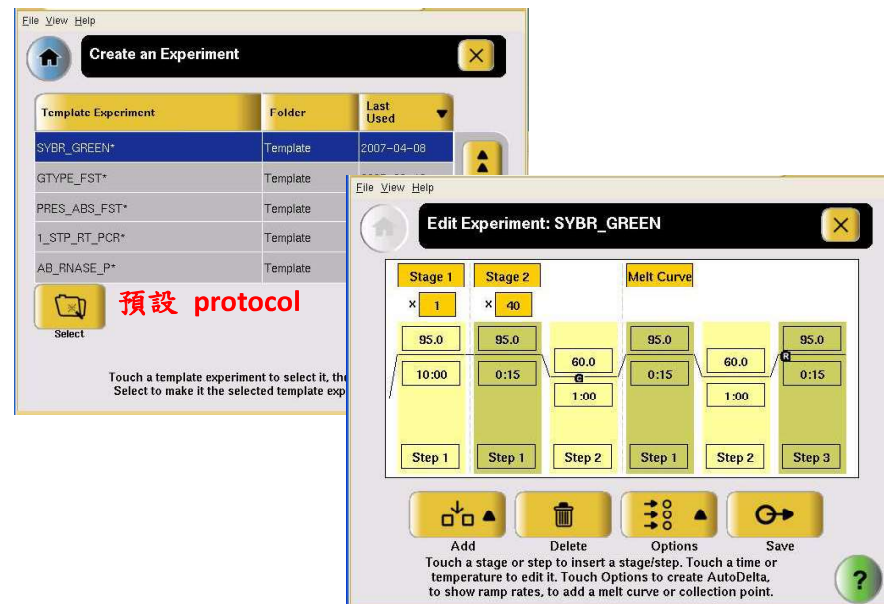
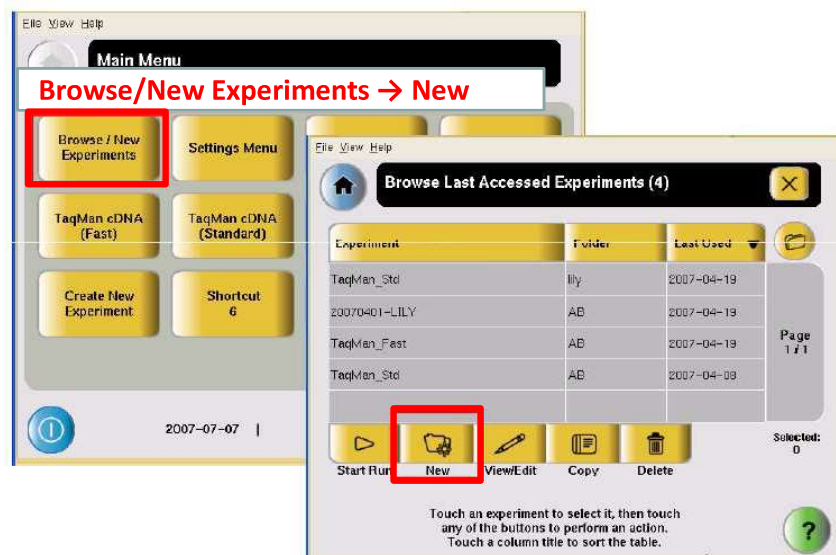
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by Thermo Fisher Scientific

Standalone (PC-free)

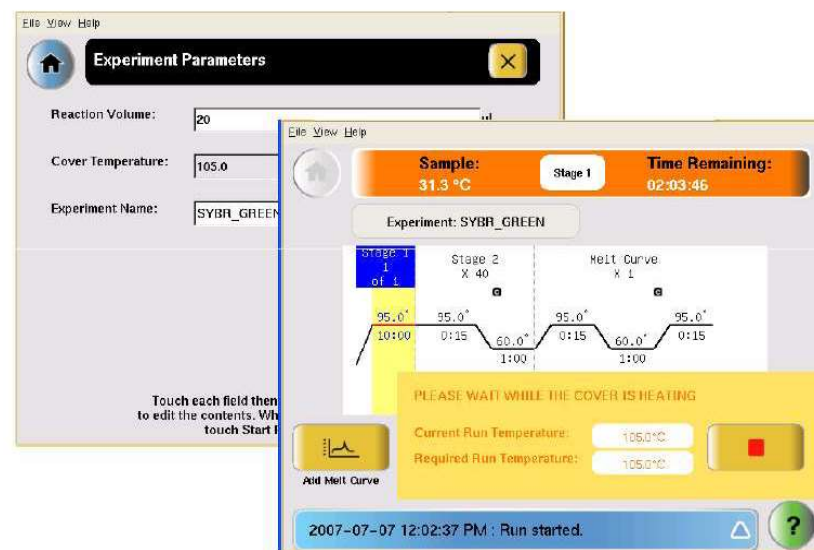
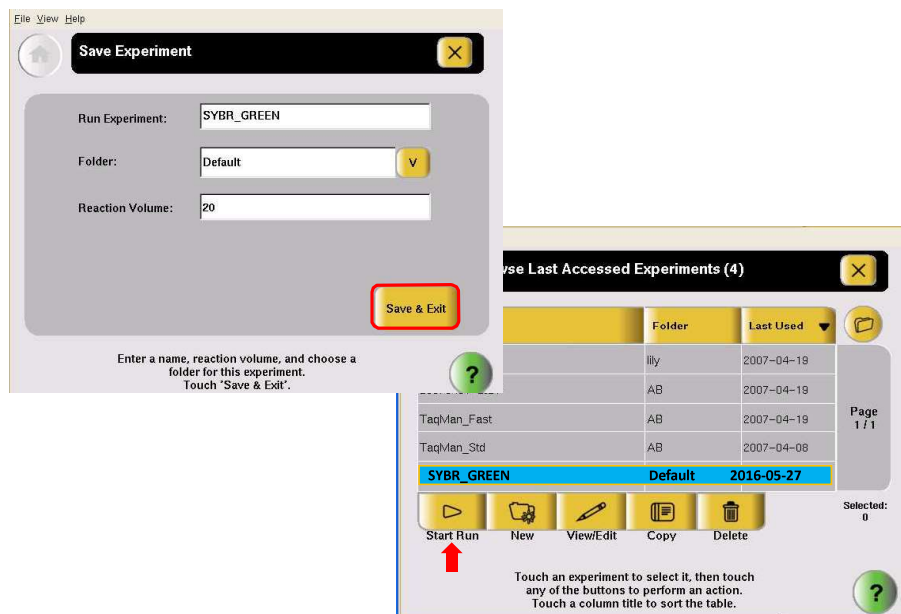


1. **Start the run** from the touch screen.
2. **Download the file (.eds)** to your PC. (主機暫存最後一筆檔
案)
3. **Analyze** your data.

appliedbiosystems⁴⁴
by Thermo Fisher Scientific



Select Experiment → Save





appliedbiosystems⁴⁹
by Thermo Fisher Scientific

SetUp

1. PC free



2. PC controlled



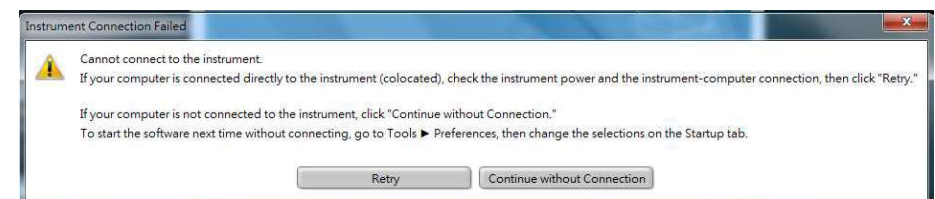
appliedbiosystems⁵⁰
by Thermo Fisher Scientific

StepOne Software v2.3 軟體操作



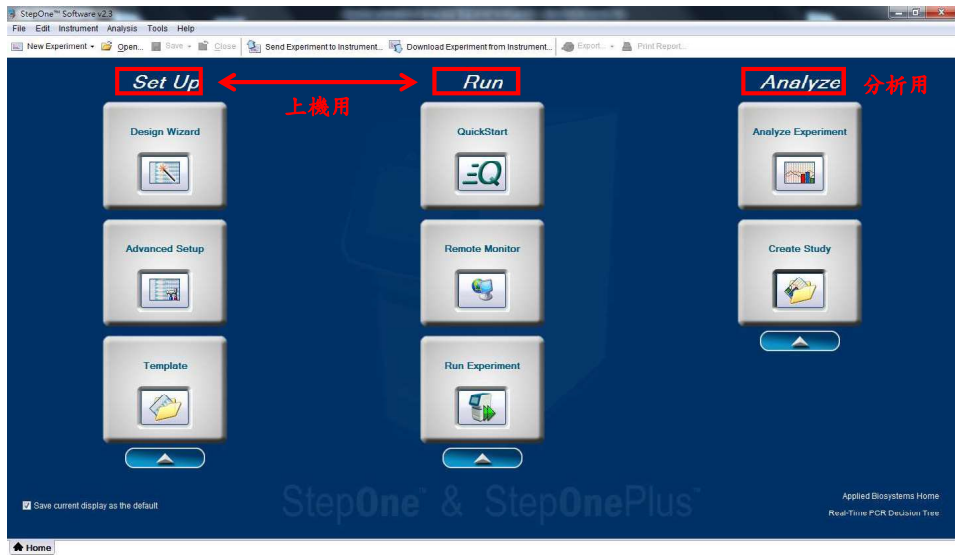
appliedbiosystems⁵¹
by Thermo Fisher Scientific

Login the software

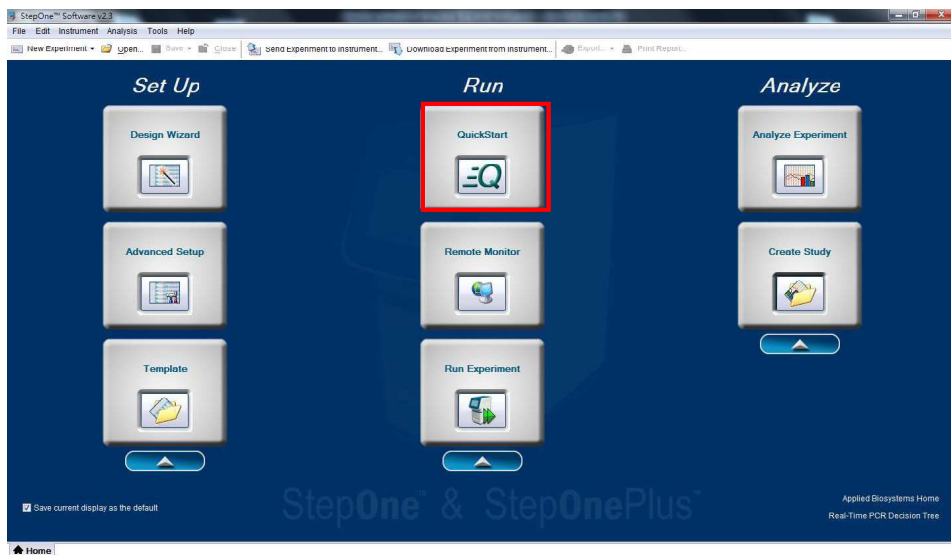


* Analyze data -> Continue without Connection

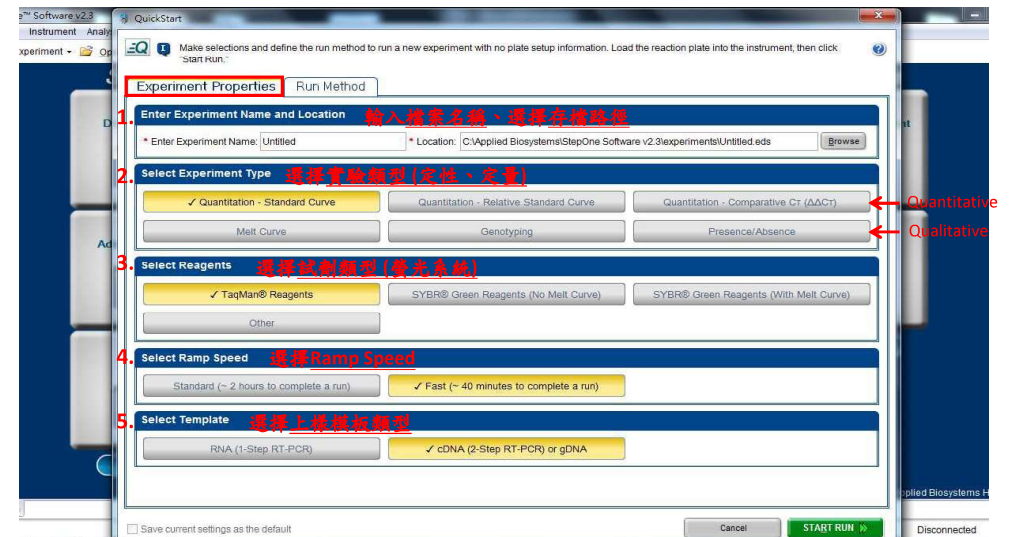
appliedbiosystems⁵²
by Thermo Fisher Scientific



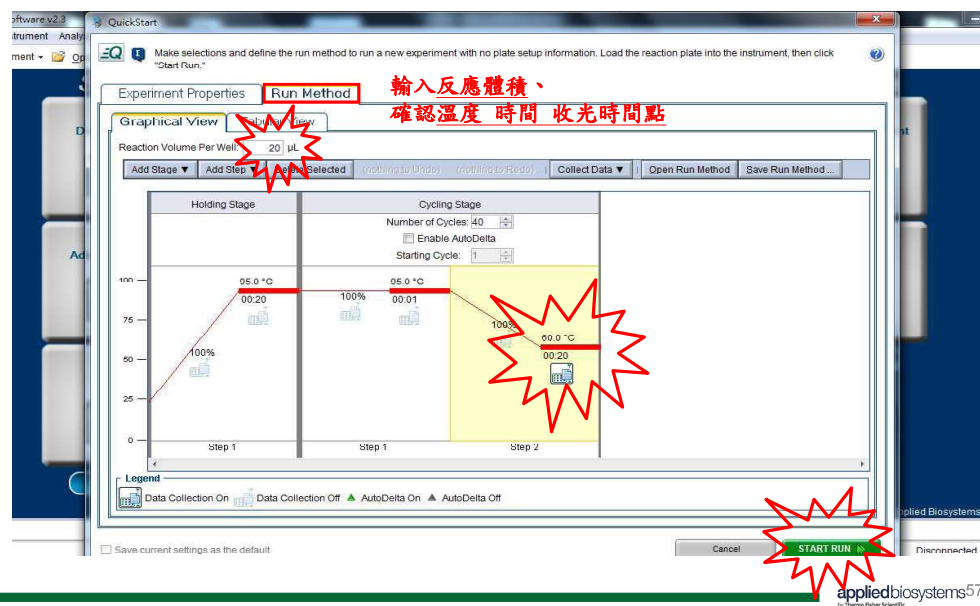
Run the experiment



Run (QuickStart) : Experiment Properties

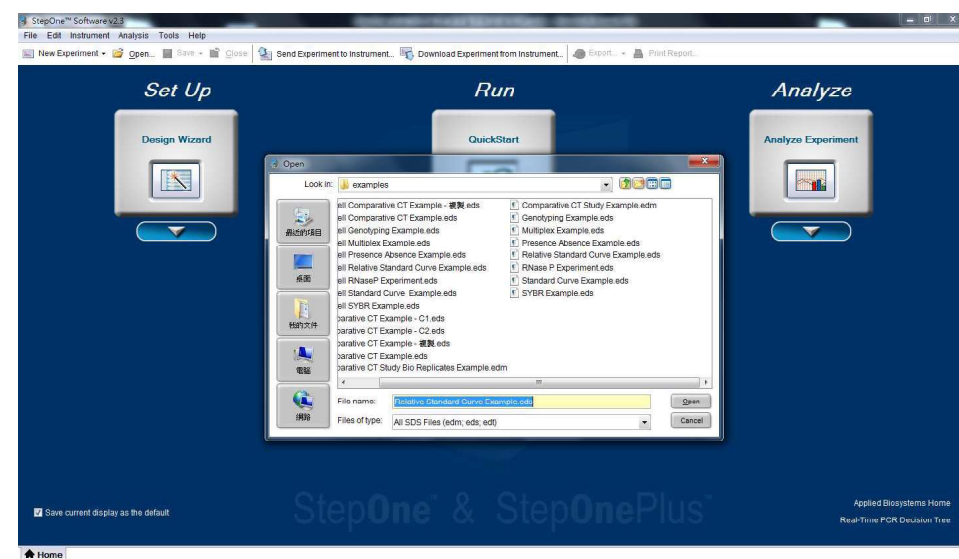
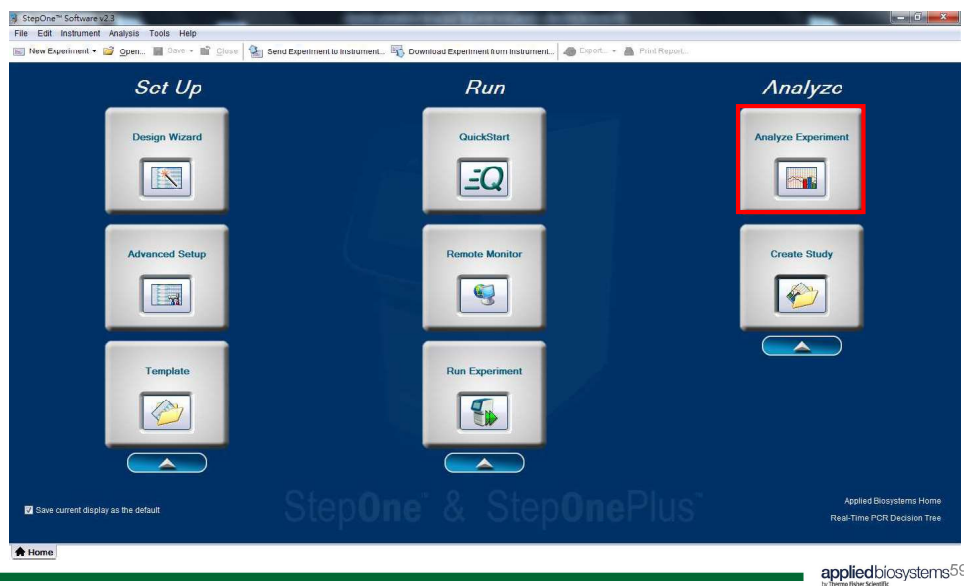


Run (QuickStart) : Run Method



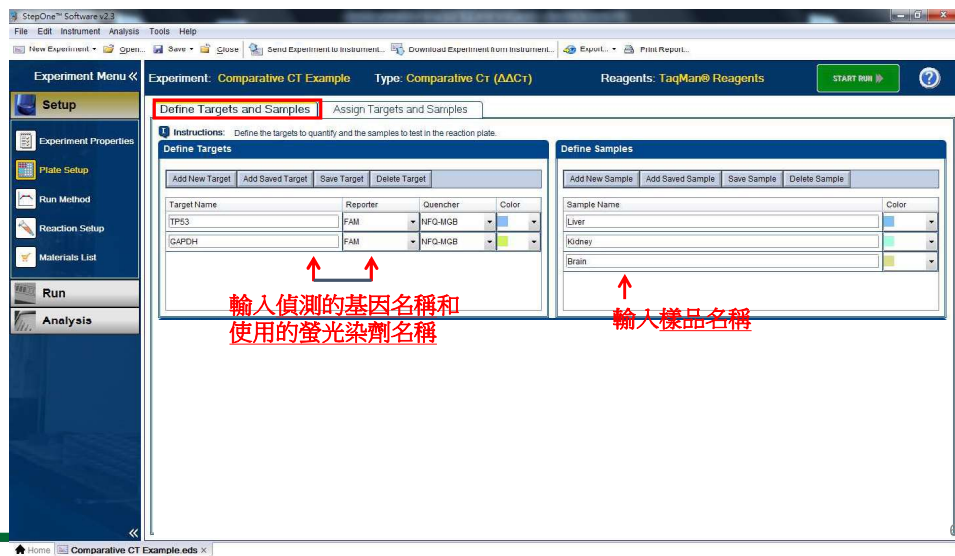
Analyze the data

Analyze: Analyze Experiment



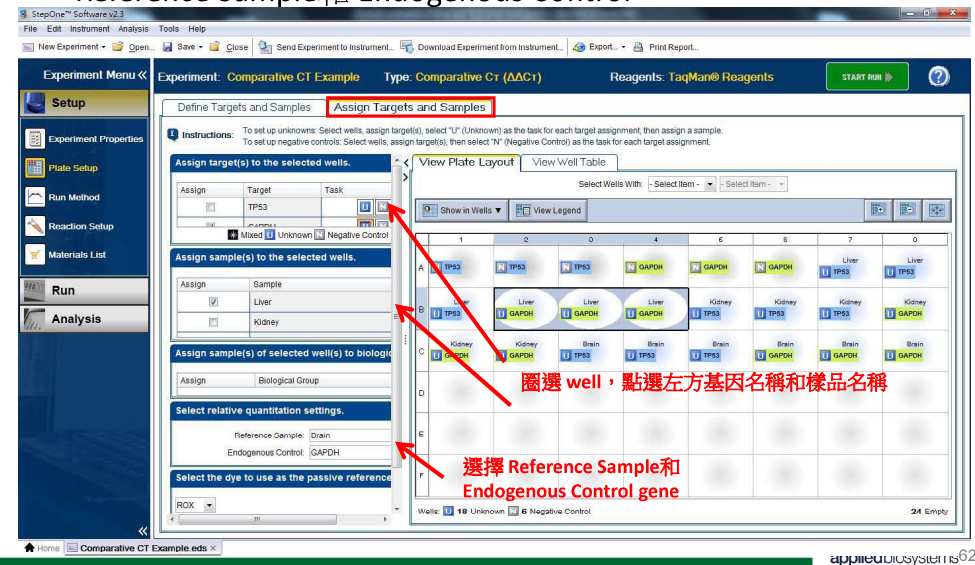
Setup: plate Setup

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



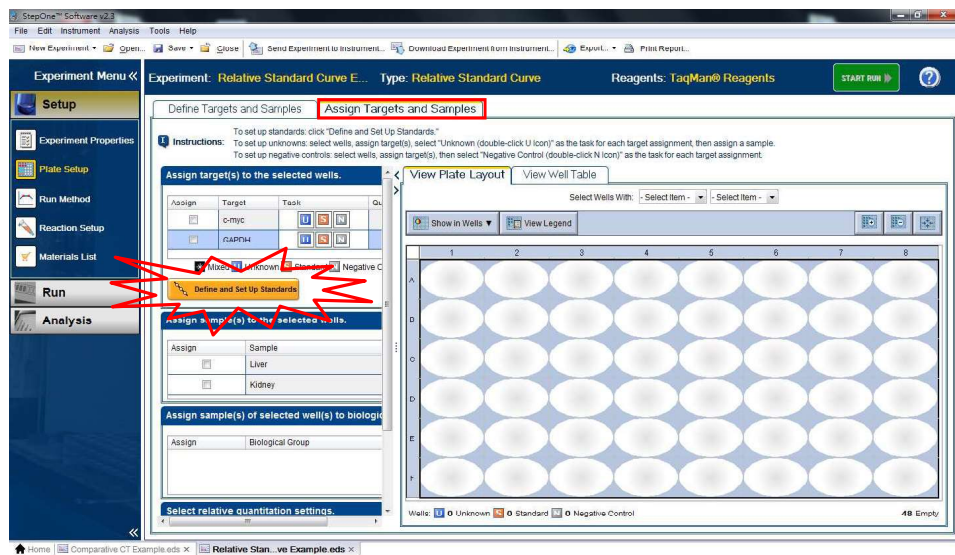
Setup: plate Setup

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



Setup: plate Setup

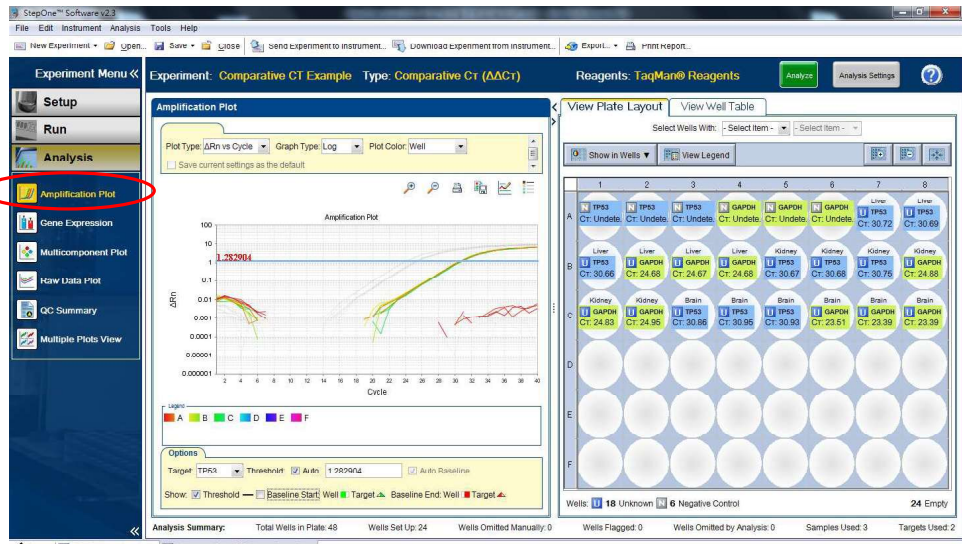
→ Assign Targets and Samples 定義標準品樣品位置、濃度



Analysis: Melt curve (SYBR Green only)

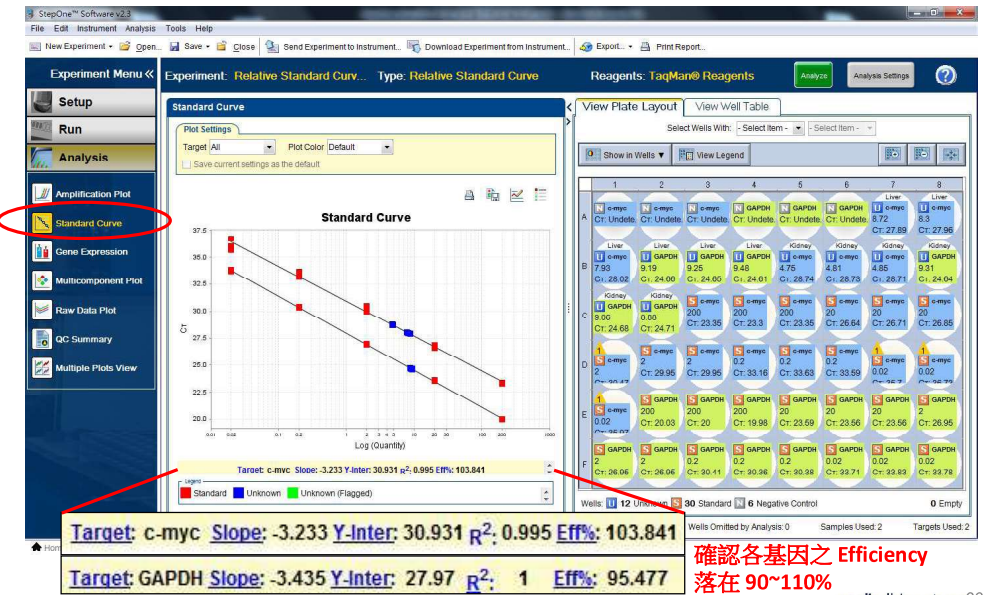


Analysis: Amplification plot

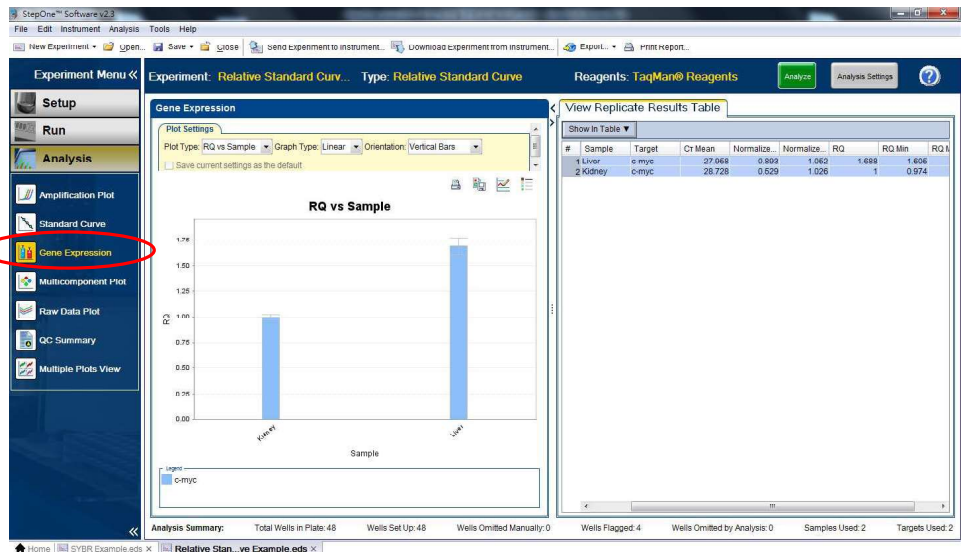


appliedbiosystems⁶⁵
by Thermo Fisher Scientific

Analysis: Standard curve

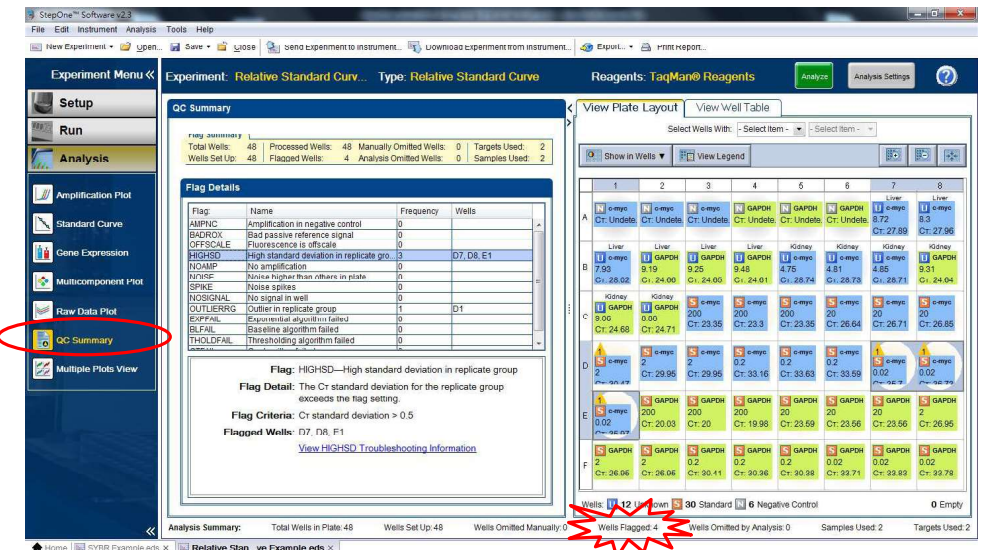


Analysis: Gene Expression



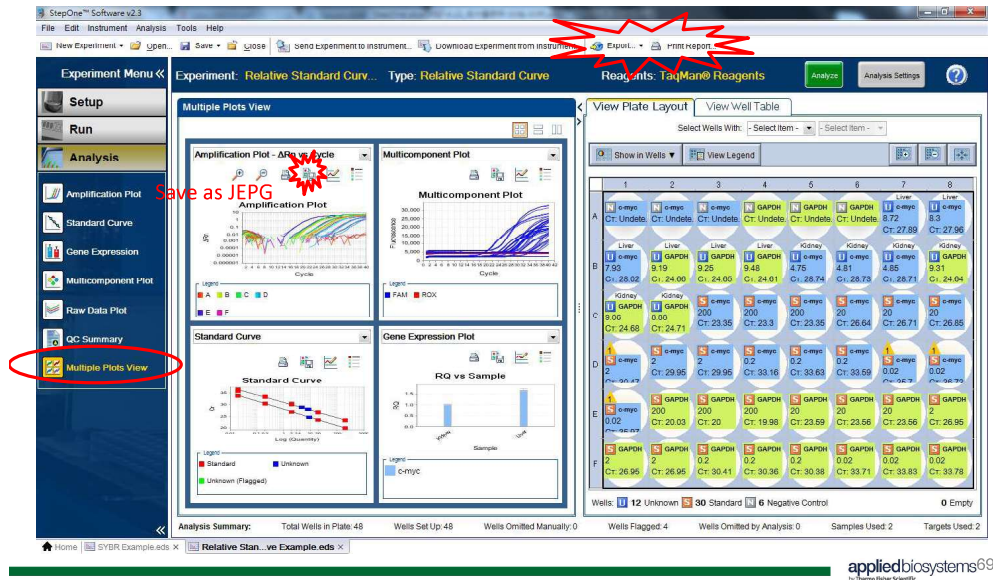
appliedbiosystems⁶⁷
by Thermo Fisher Scientific

Analysis: QC Summary → 協助 troubleshooting



appliedbiosystems⁶⁸
by Thermo Fisher Scientific

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



Thank you for your attention!

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