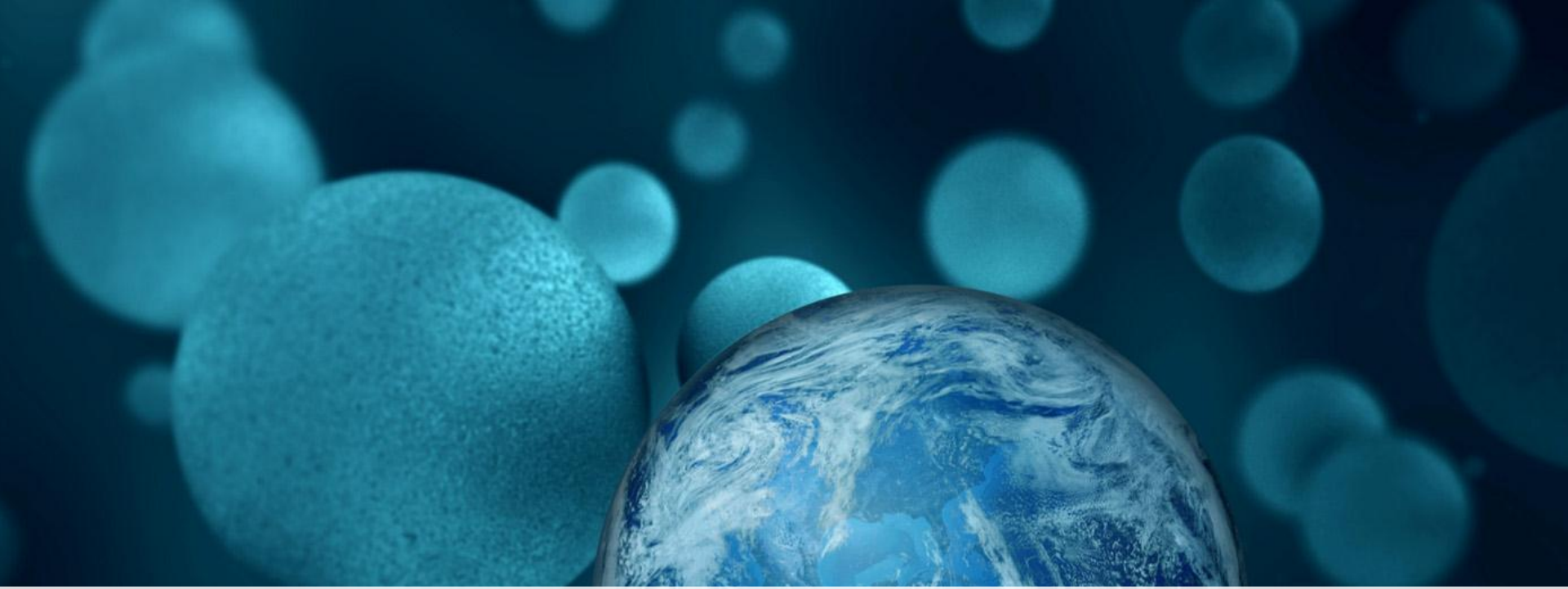

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Applied Biosystems QuantStudio™ 3 Real-Time PCR System之原理與應用介紹

蔡如芸 (Judy Tsai, Ph.D.)
Field Application Scientist

Polymerase Chain Reaction (PCR)

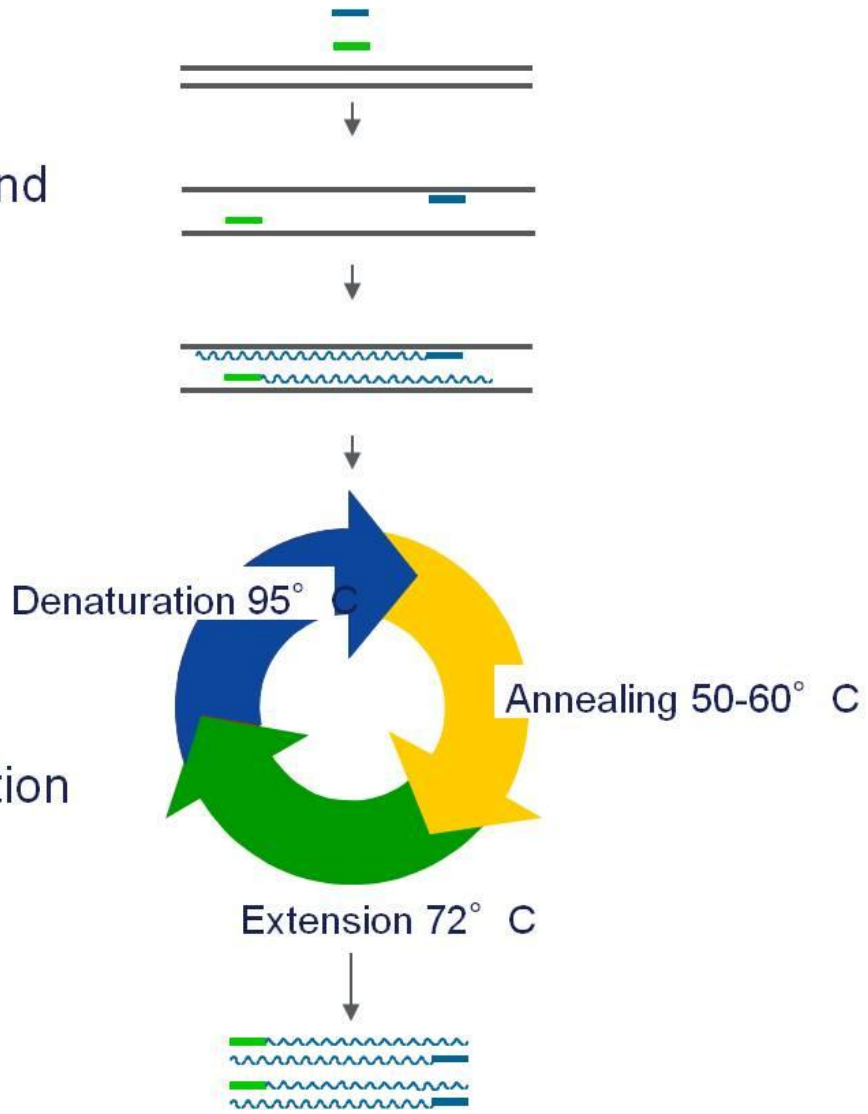
Primers and DNA

Strand denaturation and primer annealing

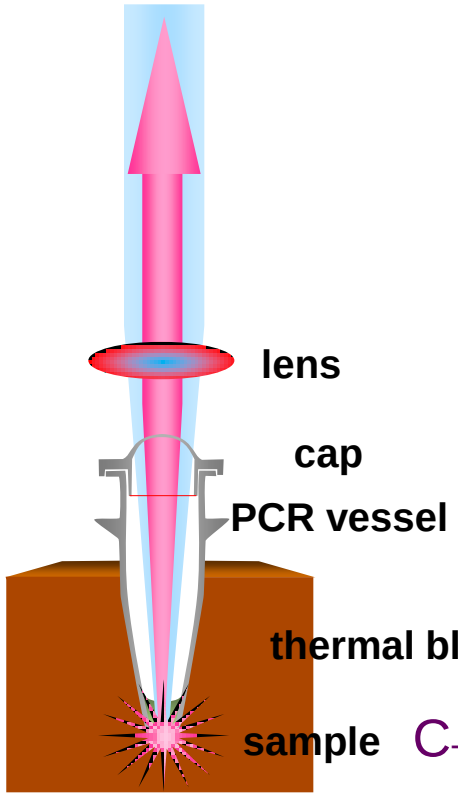
Primer extension

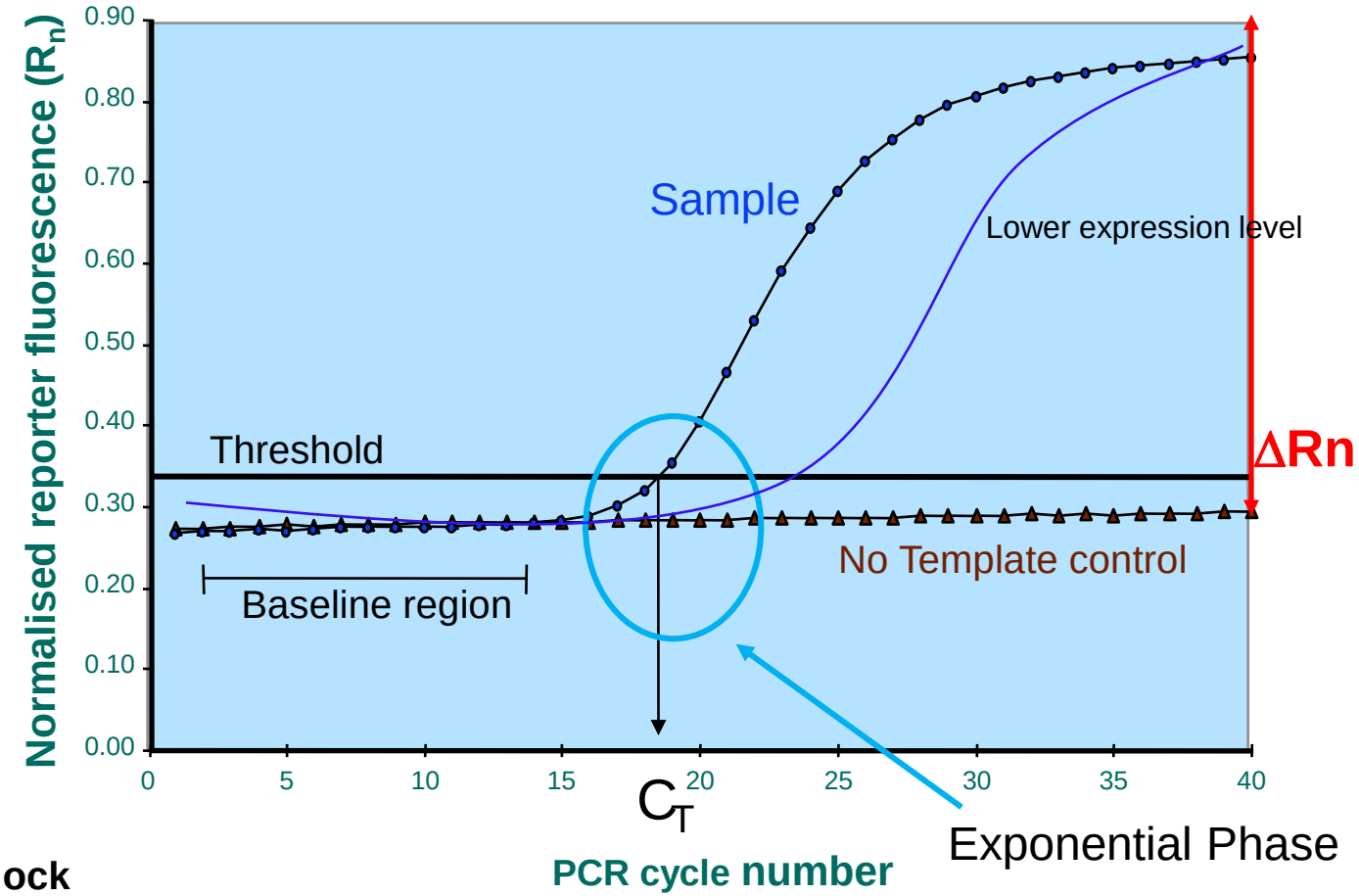
Cycling

Exponential amplification of PCR products



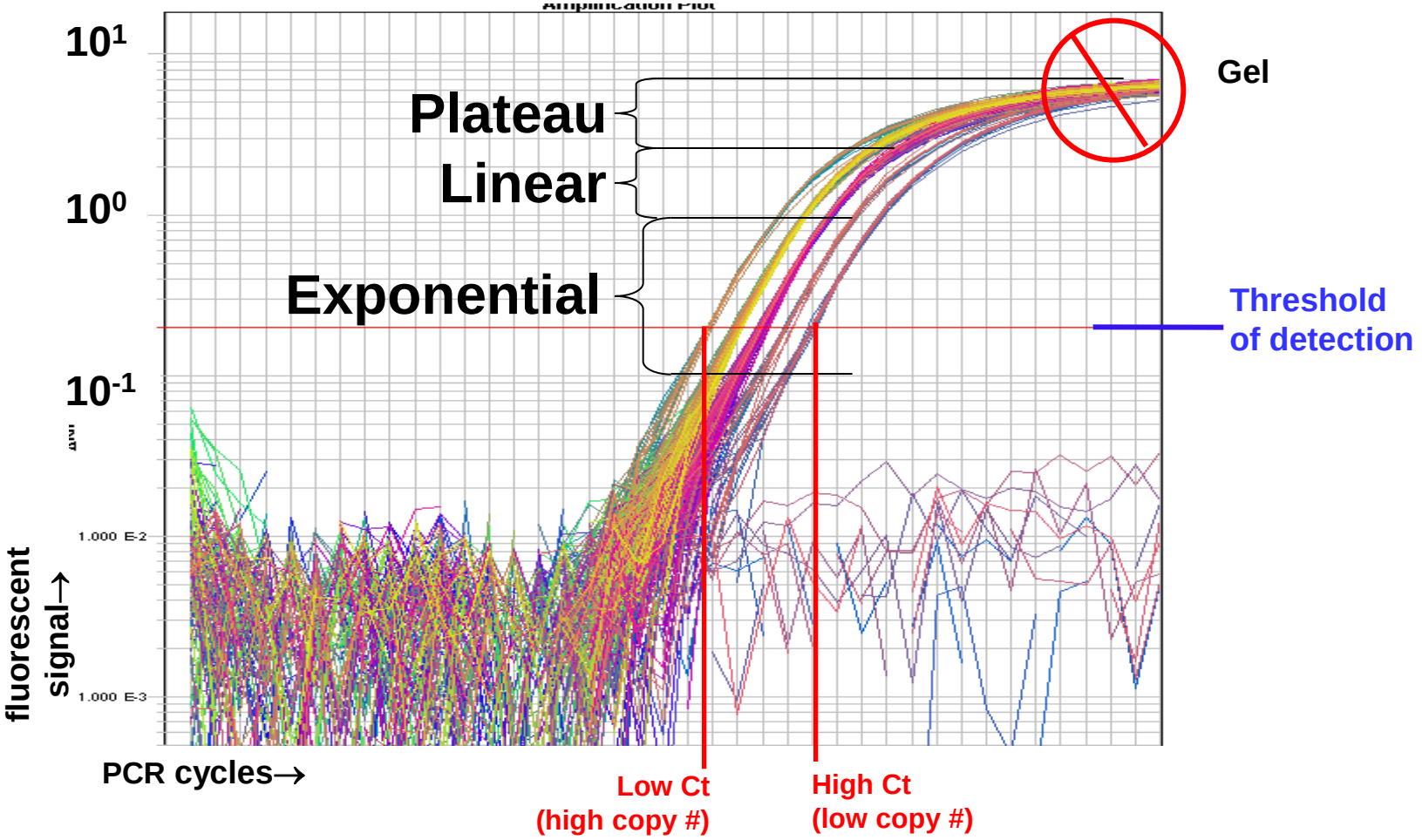
Principle of Real-time PCR





C_T = threshold cycle: **calculated fractional cycle number at which PCR amplification curve crosses the threshold of detection**

Real-time PCR Signal Detection: Exponential Phase



Y= N0 2^n，C_T 與起始濃度之對數值成反比

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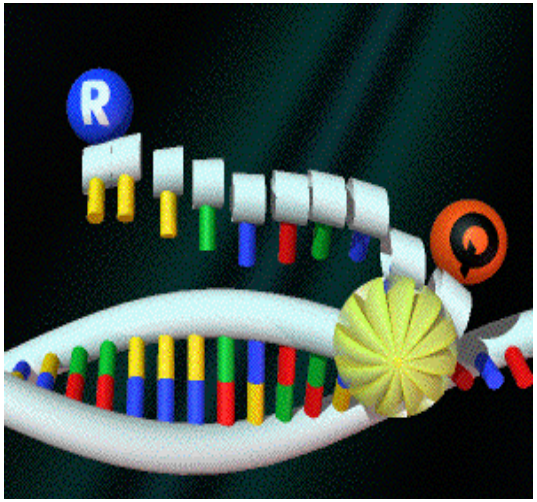
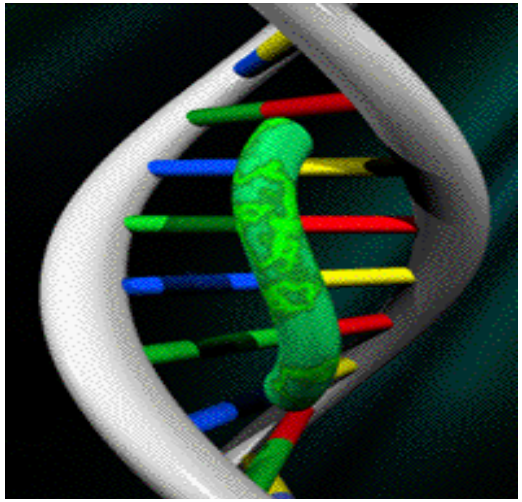
Real-time PCR Chemistries

TaqMan® and TaqMan® MGB



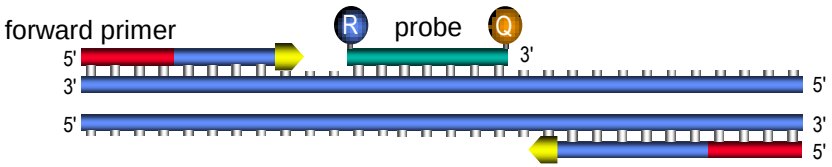
Fluorogenic 5’ Nuclease Assay

SYBR® Green I dye

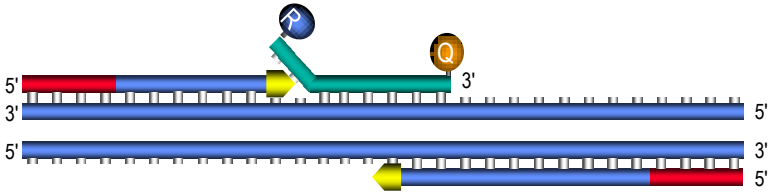


Binds Double-stranded DNA

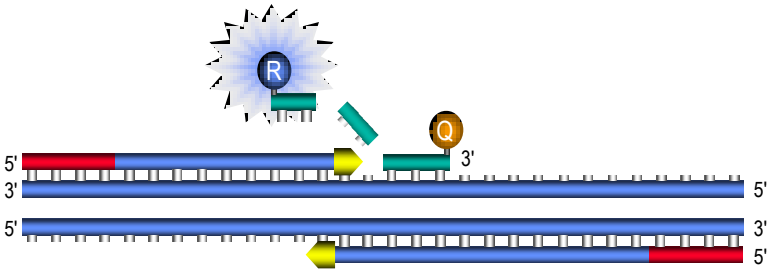
TaqMan® Assay: Fluorogenic 5‘-nuclease Assay



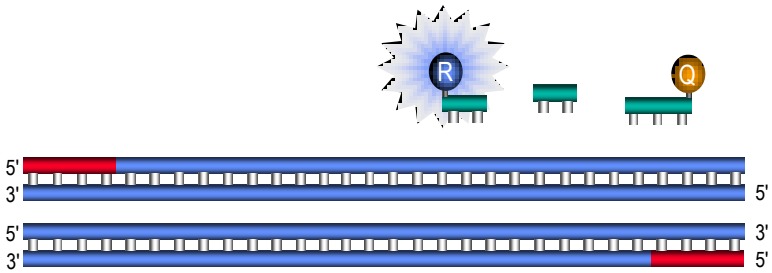
1. Polymerization

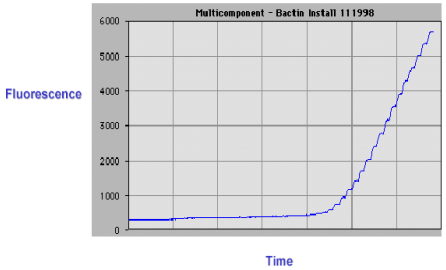


2. Strand displacement



3. Cleavage





4. Polymerization completed

R = Reporter (FAM, VIC, etc.)
Q = Quencher (NFQ/MGB, etc.)

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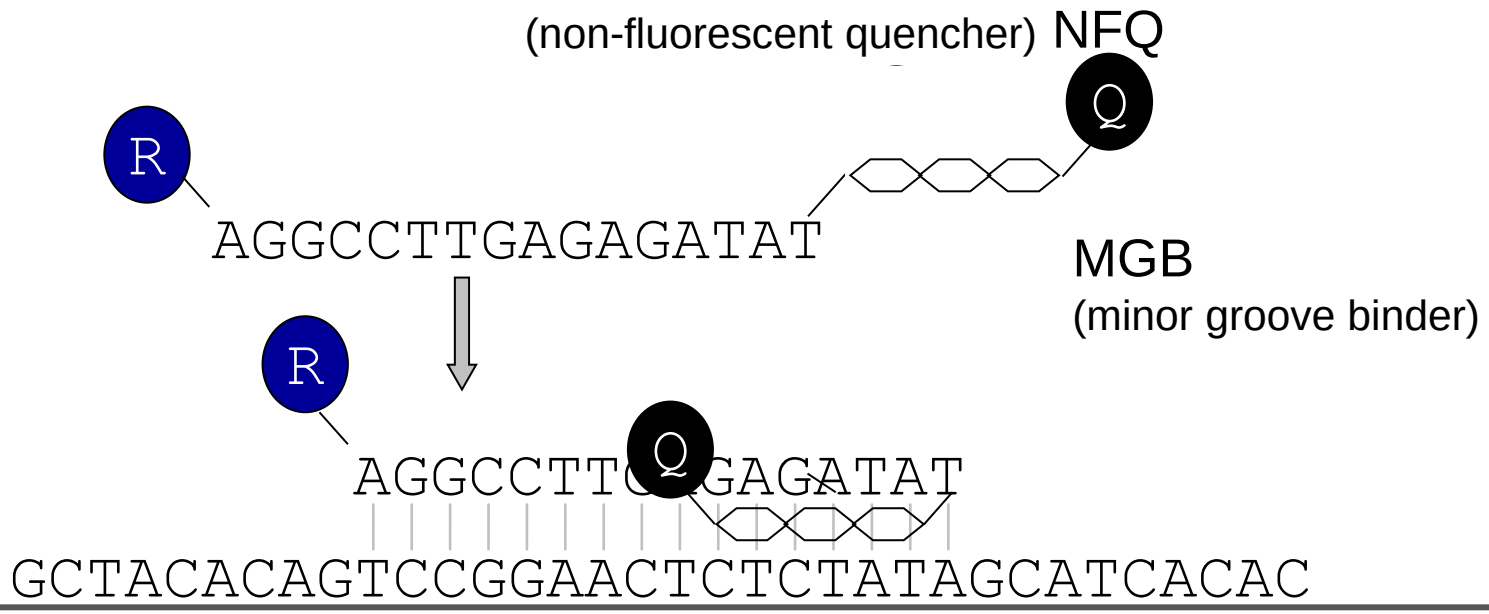
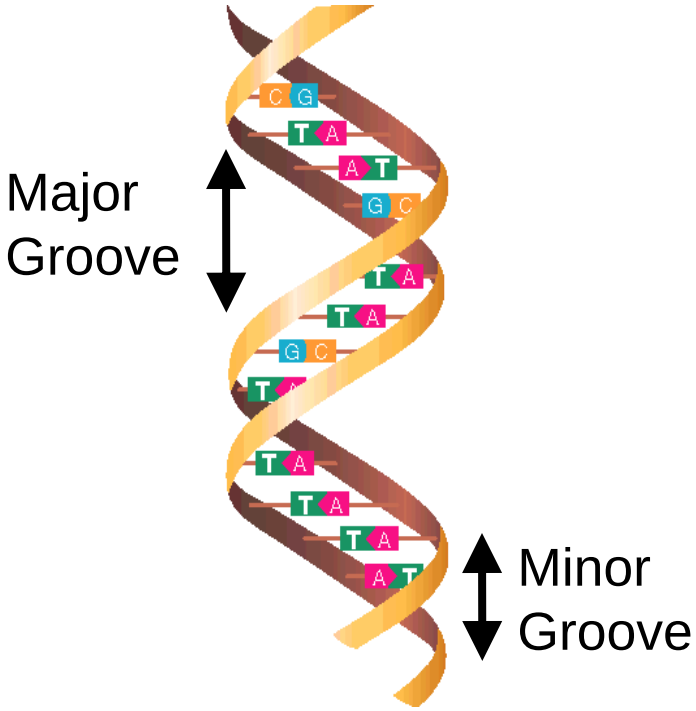
TaqMan® Probe: TaqMan® MGB/NFQ Probes

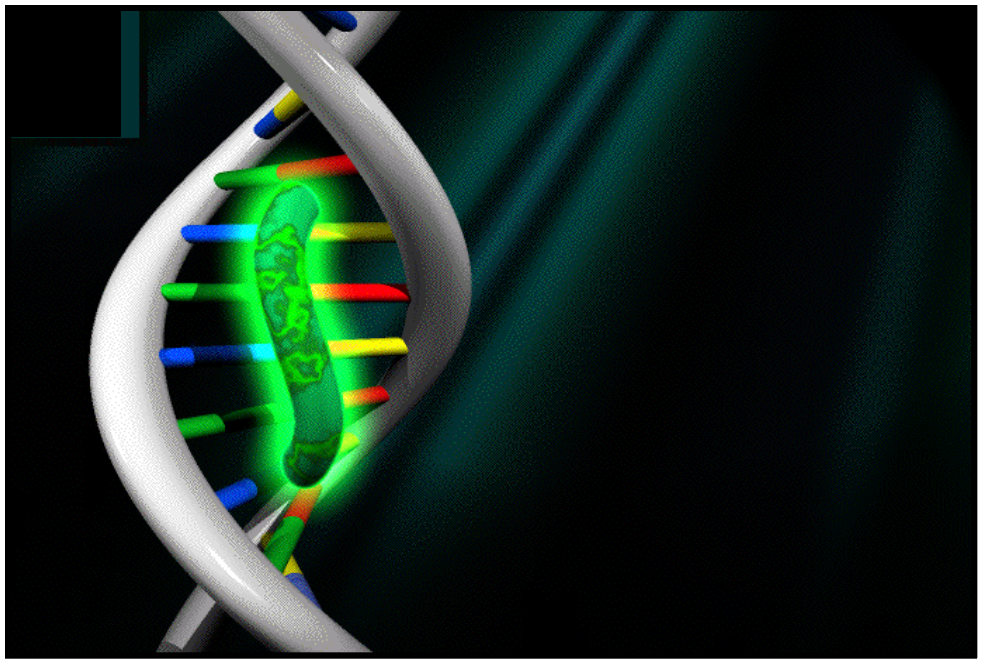
- Minor Groove Binder (MGB)
  - Small molecule that fits snugly into minor groove of duplex DNA
  - Stabilizes probe annealing
- Non-fluorescent Quencher (NFQ)
  - “Dark” quencher acts as energy transfer acceptor that doesn’t emit a detectable fluorescent signal
  - MGB probe design uses a special algorithm in Primer Express® Software
- Shorter probe length (13-25-mers)



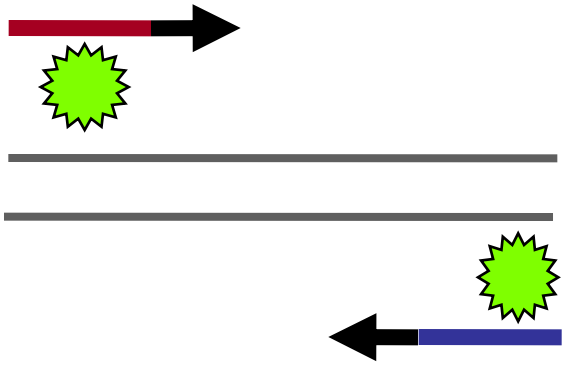
Real-time PCR Chemistries: SYBR® Green I Dye

- A ‘minor groove’-binding molecule specific to the minor groove of double-stranded DNA
- Fluoresces at an increased intensity when bound

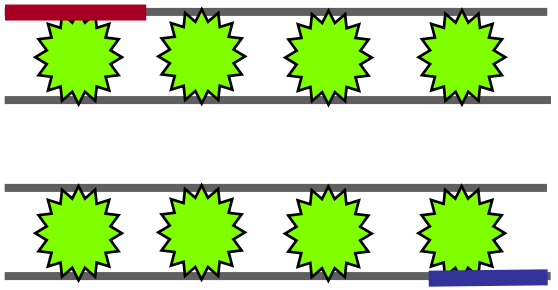




SYBR® Green I Dye: Melting Curve Analysis

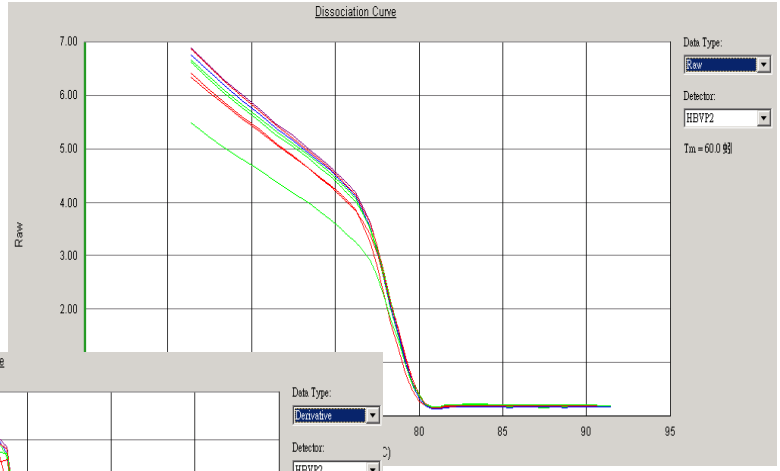


PCR

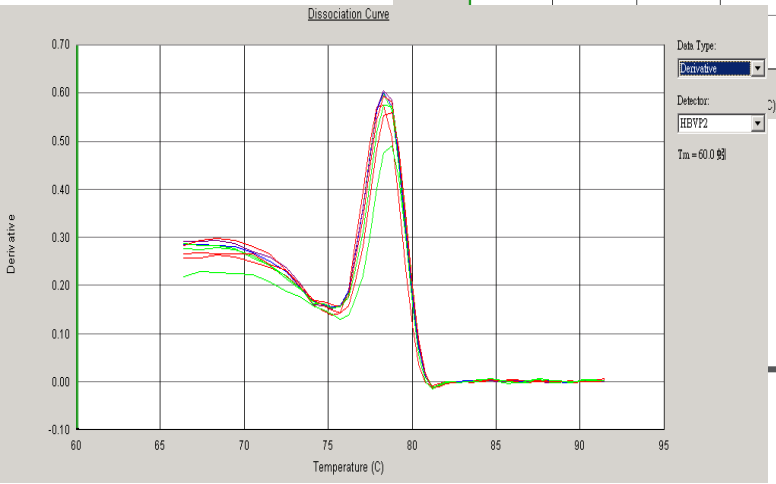


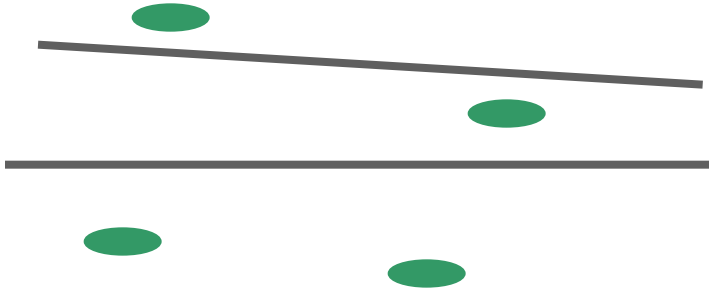
Polymerization Complete

Polymerization



Denaturation





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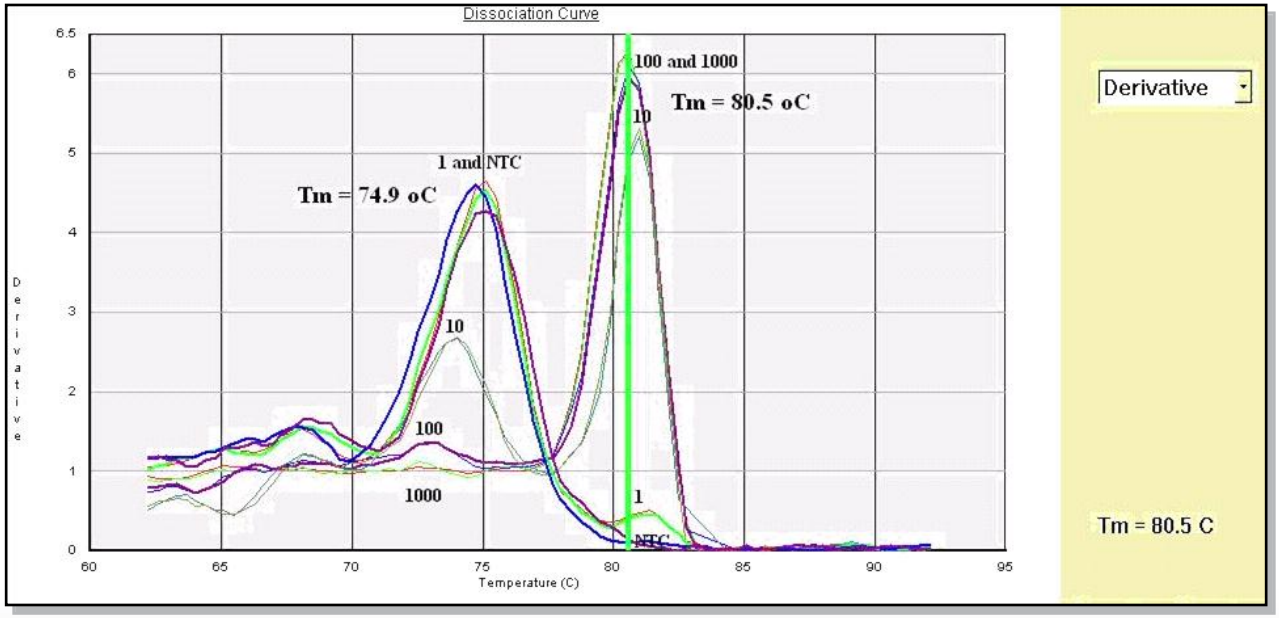
SYBR® Green I Dye: Melting Curve Analysis



- **Use NTC to check whether non-specific product is primer dimer**
- **If the non-specific product is primer dimer:**
  - Optimize primer concentration
  - Re-design primer pair

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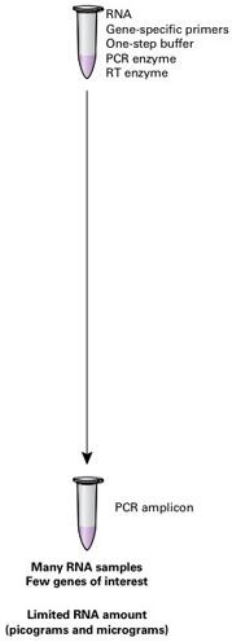
Real-time PCR Chemistries

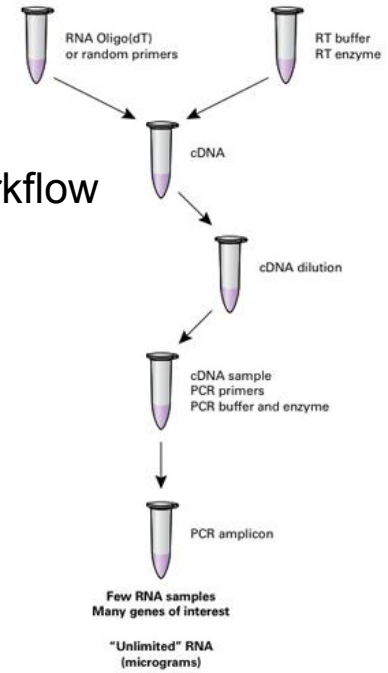
	TaqMan® Assay	SYBR® Green I Dye
Specificity	More specific	Less specific
	Probe hybridization	
Sensitivity	Very high	Very high
Flexibility	Multiplex PCR	No probe required
	SNP detection	Screening tool
	+/- application	
Optimization	Ready to use 20x primer/probe mix - no need to optimize	Need to optimize PCR program
	Gold standard for MAQC	Need to check primer-dimer info
	PCR efficiency 100±10%	Need to check PCR efficiency

Reverse Transcription and Real-time PCR Reaction

One-step vs Two-step Workflows

- One-step Technology
  - RT and PCR are performed in single buffer system
    - √ One tube, one step
    - √ Reduce chance of cross-contamination
    - √ Easy for high throughput workflow
    - √ Cost effective when few targets/sample analyzed
    - √ Uses gene-specific primers
    - X cDNA can not be stored





- Two-step Technology
  - RT and PCR are performed in two separate reactions
    - √ Cost advantaged when interrogating multiple targets
    - √ cDNA can be stored and used for further experiments
    - √ Best choice if RNA is limiting
    - X Multiple steps, longer time to result

1-step qRT-PCR: Real-time PCR Reactions

Component	Volume for one reaction	Notes
4X TaqMan® Fast Virus 1-Step Master Mix	5 µL	—
TaqMan® Gene Expression Assay (20X)	1 µL	If you are not using pre-formulated TaqMan® Gene Expression Assays, Applied Biosystems recommends primer concentrations of 400 to 900 nM and a probe concentration of 100 to 250 nM.
Sample	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-PCR Grade Water	Variable	Fill to the total reaction volume.
Total volume per reaction	**20 µL**	—

For sample volumes ≤30 µL

Run mode	Default†				
Thermal cycling conditions	**Step**	**Stage**	**No. of cycles**	**Temperature**	**Time**
	Reverse transcription	1	1	50 °C‡	5 minutes
	RT inactivation/initial denaturation	2	1	95 °C	20 seconds
	Amplification	3	40	95 °C	3 seconds
				60 °C	30 seconds

† Use the default run mode for your system and sample block module (that is, Fast mode on Fast instruments and standard mode on standard instruments).

‡ Reverse transcription works best between 48 °C and 55 °C.

1-step qRT-PCR: Master Mixes

- TaqMan® Fast Virus 1-Step Master Mix (PN 4444434)
  - 4X master mix to amplify both RNA and DNA
  - Formulated to handle common RT-PCR inhibitors found in blood, stool, and other difficult samples
  - Up to triplex (ROX as passive reference)

- TaqPath™ 1-Step Multiplex Master Mix (PN A28522)
  - 4X master mix to amplify both RNA and DNA
  - Tolerant to common RT-PCR inhibitors
  - Manufactured in an ISO 13485 certified facility
  - Up to quadruplex (does not include passive reference)

2-step qRT-PCR: Real-time PCR Reactions

Reverse Transcription : High Capacity RNA-to-cDNA Kit

2X RT Buffer	10µl
20X RT Enzyme Mix	1µl
Sample (up to 2µg)	Up to 9µl
Nuclease-Free water	To 20µl

	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4
Time	60 min	5 min	∞

Real-time PCR:

TaqMan Chemistry

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

SYBR Chemistry

2x Power SYBR Master Mix	1x	10µl
F Primer	optimized	NA
R Primer	optimized	NA
Water		NA
cDNA	1-100 ng	5-10µl
		20µl

Standard mode

PCR condition:
50°C, 2min
95 °C, 10 min
95 °C, 15 sec] 40 cycles
60 °C, 1min

Fast mode

PCR condition:
95 °C, 20 sec
95 °C, 1 sec] 40 cycles
60 °C, 20 sec

SYBR Green:

- Check Primer Concentration
- Add Melt Curve Program

2-step qRT-PCR: Master Mixes

Standard Mode

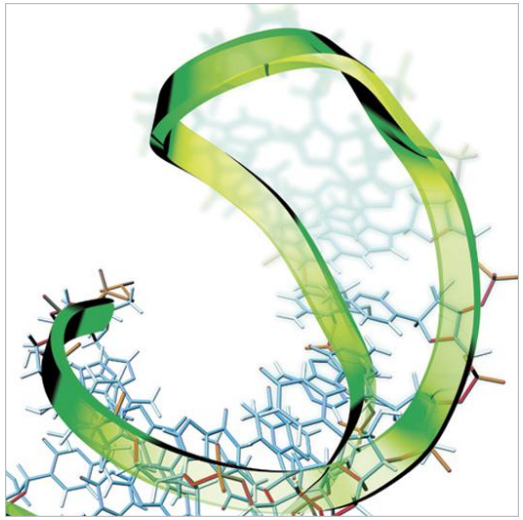
- TaqMan® Chemistry
  - TaqMan® Universal Master Mix II (PN 4440038)
  - TaqMan® Gene Expression Master Mix (PN 4369016)
- SYBR® Green Chemistry
  - Power SYBR® Green PCR Master Mix (PN 4367659)

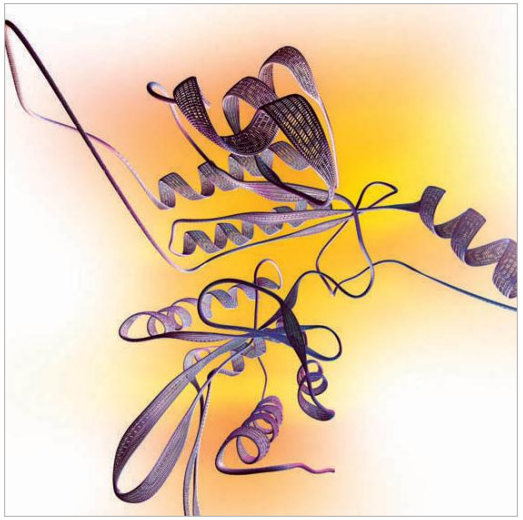
Fast Mode

- TaqMan® Chemistry
  - TaqMan® Fast Universal Master Mix (PN 4366072)
  - TaqMan® Fast Advanced Master Mix (PN 4444557)
- SYBR® Green Chemistry
  - Fast SYBR® Green Master Mix (PN 4385612)
  - PowerUp™ SYBR® Green Master Mix (PN A25742)

Real-time PCR Quantification Methods

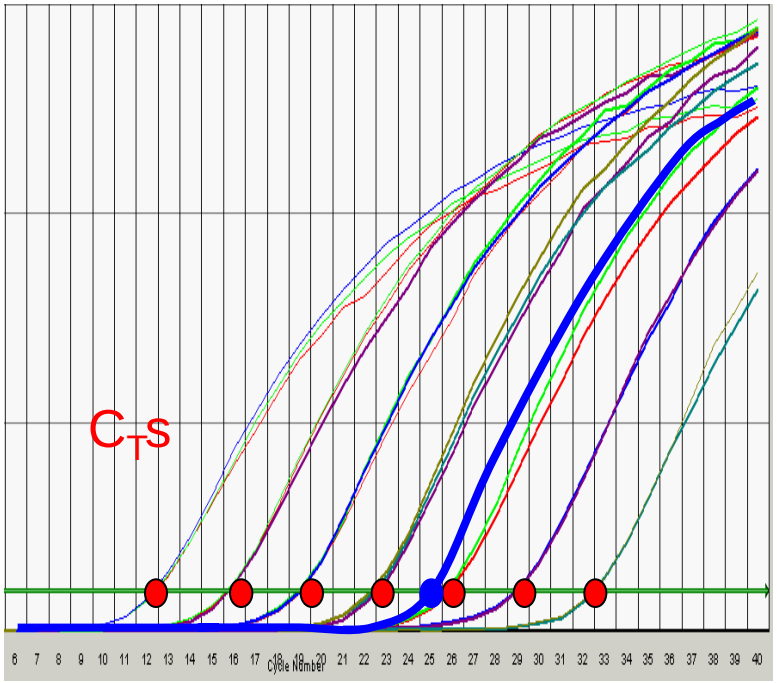
- Absolute Quantification vs. Relative Quantification

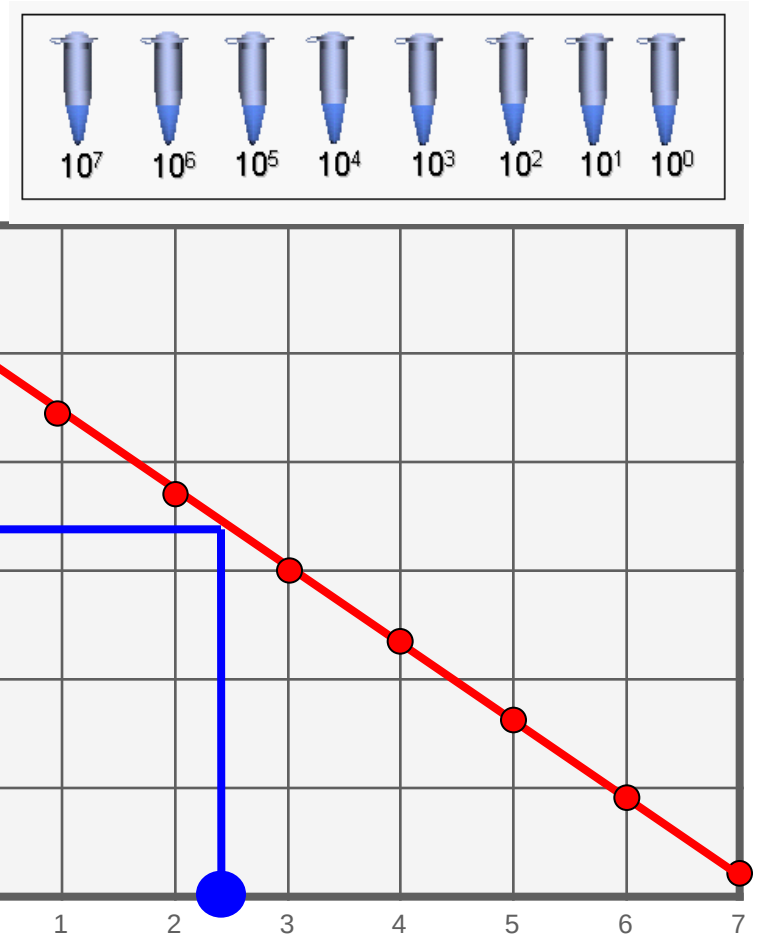




絕對定量 (Absolute Quantification)

- 主要應用於病毒量及病原菌偵測
- To determine the actual number of copies of a target nucleic acid within a sample with statistical confidence.





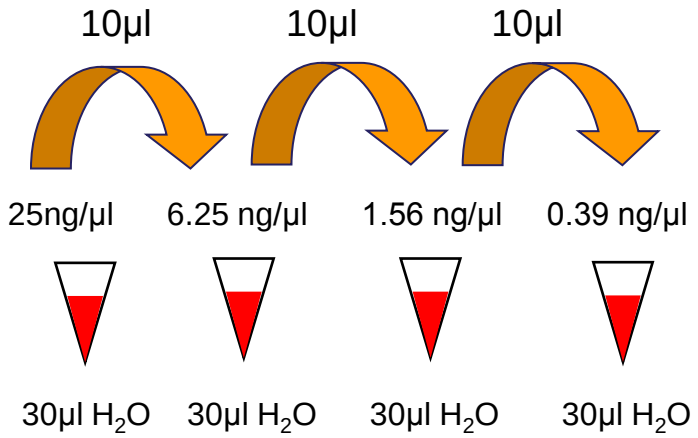
C_T is directly proportional to log of amount of input template

相對定量 (Relative Quantification)

- To determine fold differences of a target nucleic acid in a starting material with statistical confidence.
  - ΔΔCt analysis (most common)
  - Relative standard curve
- Need endogenous gene normalizes the amount of sample added
  - Endogenous control (*e.g.* GAPDH, β-actin, etc.)
- Most powerful and widely used method
- Check primer PCR efficiency if using SYBR Green Dye

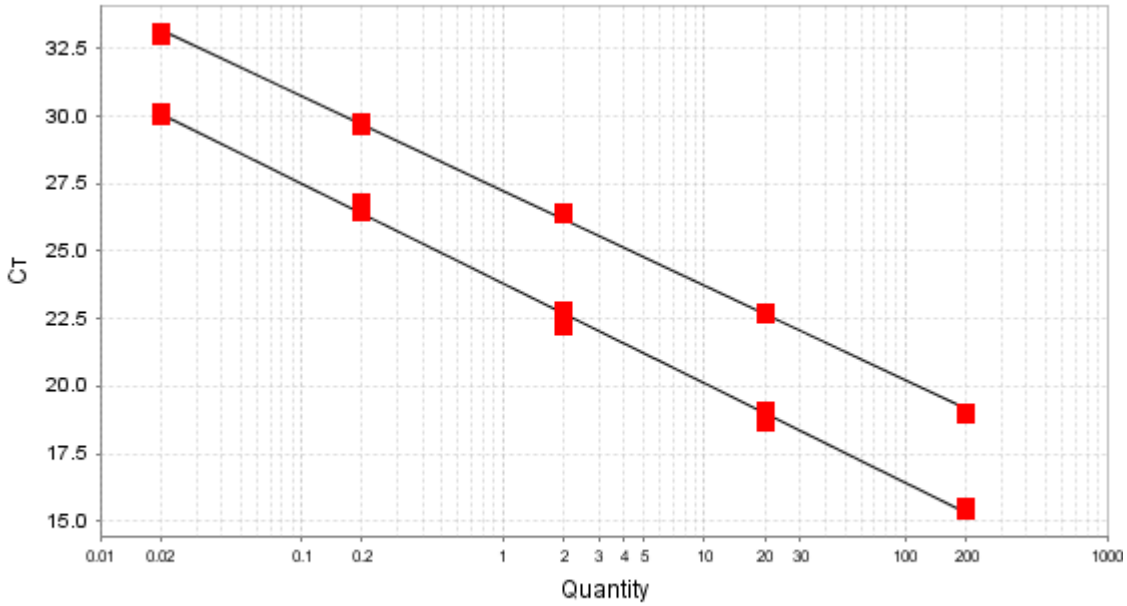
相對定量 (Relative Quantification): PCR Efficiency Validation

- 2μg RNA in 20μl RT = 100ng cDNA/μl
- Gene name: C-Myc and GAPDH
- cDNA 4-fold serial dilution: 10μl cDNA + 30μl H_2O (25ng/μl)
  - 1. 25ng/μl
  - 2. 6.25 ng/μl
  - 3. 1.56 ng/μl
  - 4. 0.39ng/μl
  - 5. NTC (duplicate for each sample)
- 每個濃度點各做二重複



- Prepare a Premix for each gene
- Aliquot 15μl of Premix to each well
- Add 5μl of RT product to the well
- Real-time PCR reaction

相對定量 (Relative Quantification): PCR Efficiency Validation



Target: GAPDH Slope: -3.506 Y-Inter: 27.226 R^2: 0.999 Eff%: 92.853

Target: c-myc Slope: -3.696 Y-Inter: 23.82 R^2: 0.998 Eff%: 86.438

90 ≦ Eff% ≦ 110 → ΔΔ Ct
Eff% < 90 → Relative standard curve

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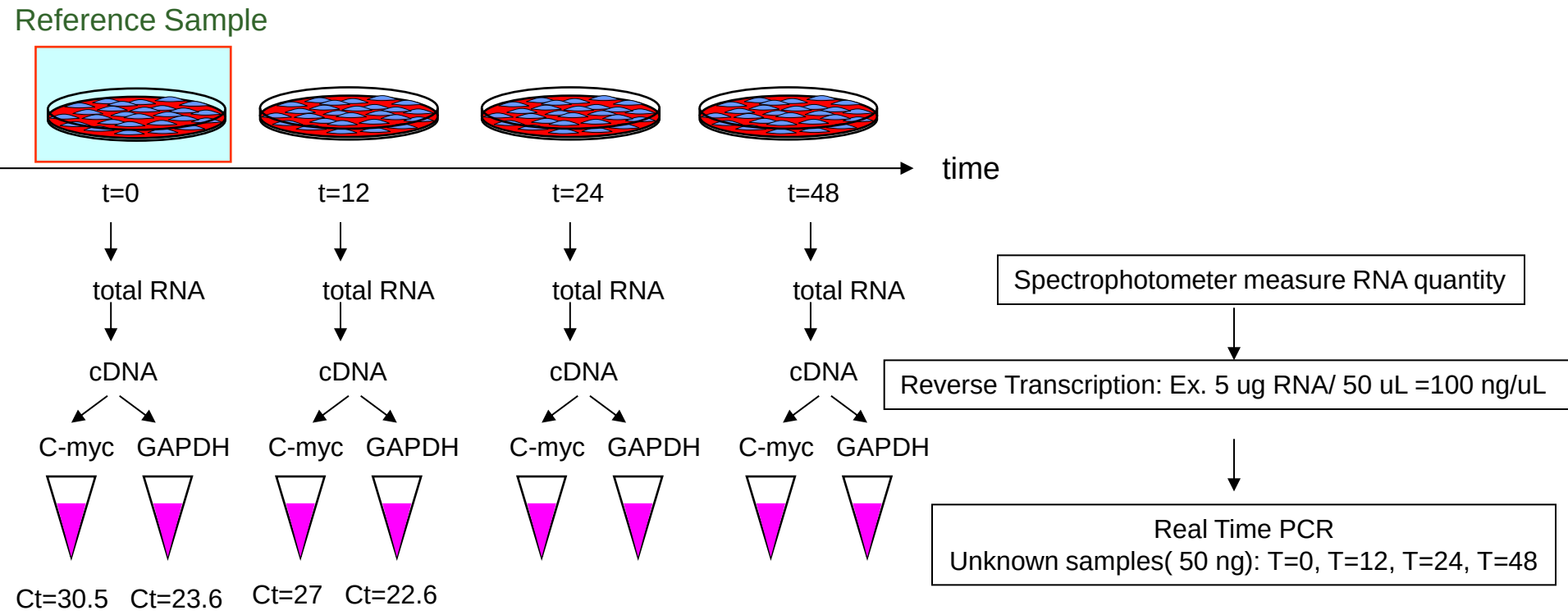
相對定量 (Relative Quantification): Comparative Ct (ΔΔCt)

Comparison of the c-myc expression level
in T=0, T=12, T=24, T=48 time course study



相對定量 (Relative Quantification): Comparative Ct (ΔΔCt)

step 1: Normalization to endogenous control

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

Reference: Ct c-Myc – Ct GAPDH = ΔCt reference sample

step 2: Normalization to reference sample

ΔCt sample – ΔCt reference sample = ΔΔCt

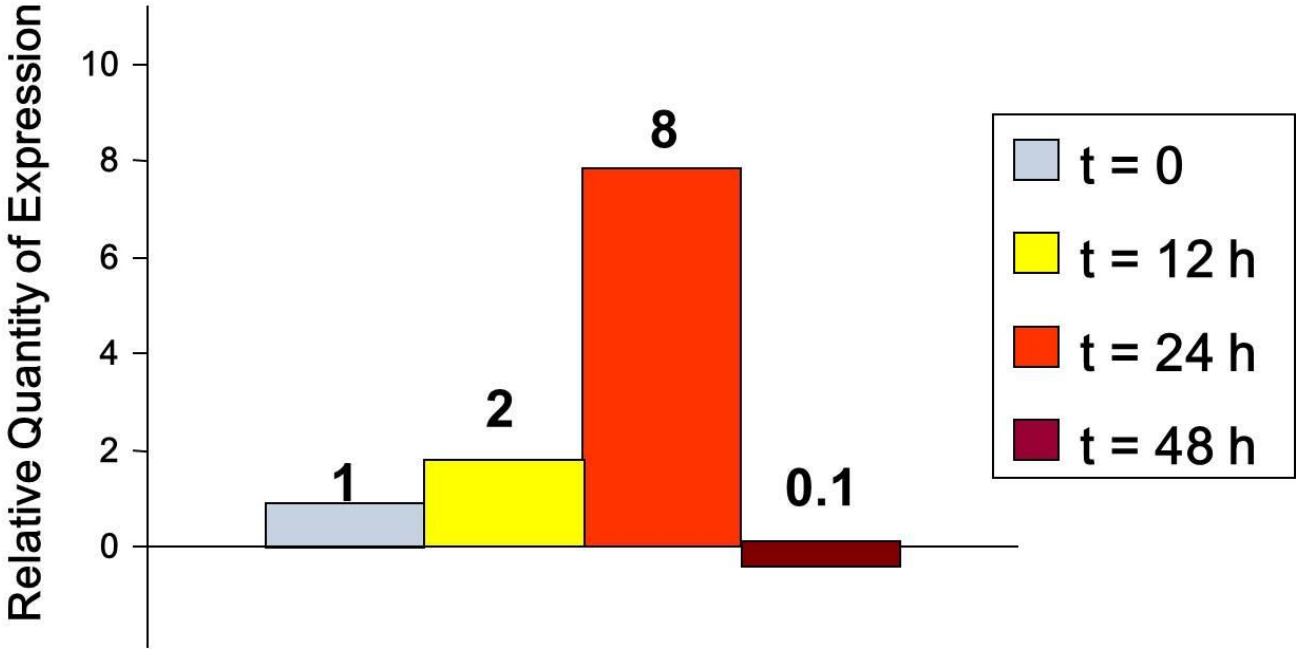
step 3: use the formula

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

A reference sample is a sample to which unknown samples are compared (*e.g.* untreated sample or control).

相對定量 (Relative Quantification): Comparative Ct (ΔΔCt)

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



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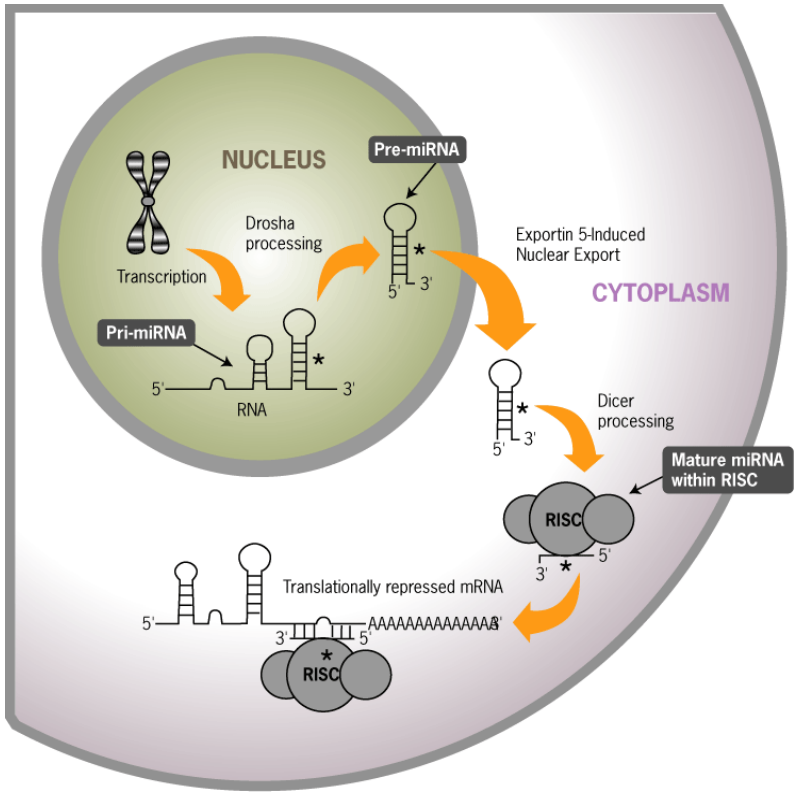
Introducing TaqMan™ Advanced miRNA Assays

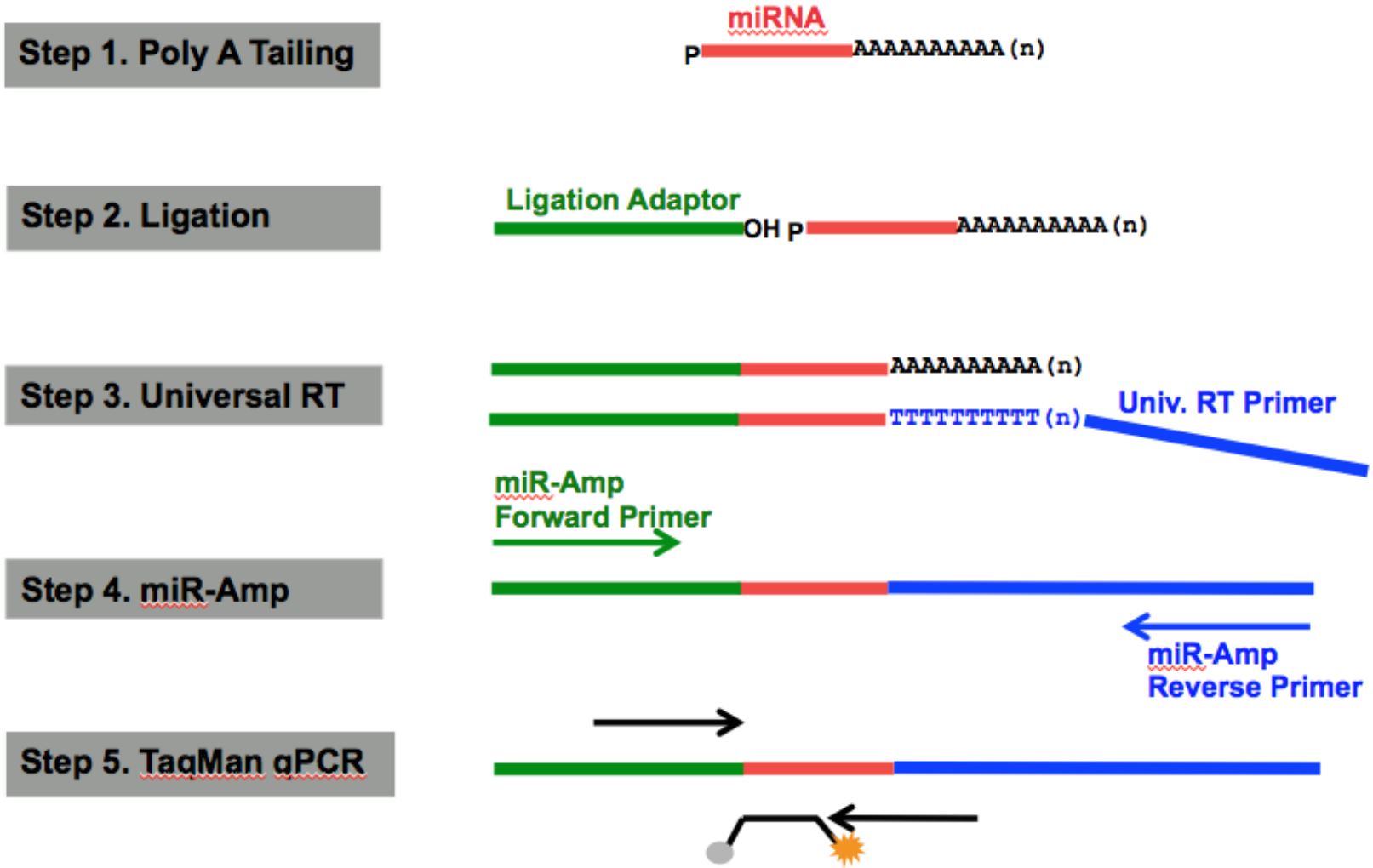


- Excellent sensitivity in biological samples (serum/plasma, tissue)
- Low input amount (2µl) saves precious samples; no need to run multiple RTs and split sample
- Universal cDNA can be used for any miRNA assay
- cDNA can be archived for future miRNA studies

Mature miRNA

- TaqMan™ advanced miRNA assays
  - SKU A25576, Size S, 250 reactions
- TaqMan™ advanced miRNA cDNA synthesis kit

TaqMan™ Advanced miRNA Assays: How it Works



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TaqMan™ Advanced miRNA Assays: High Specificity

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
	* * * *** *

Let-7 miRNA family: differences as small as single base mismatches

	Synthetic Template							
TaqMan Advanced miRNA Assays	Let7a	Let7b	Let7c	Let7d	Let7e	Let7f	Let7g	Let7i
Let7a	100%	0%	0%	0%	4%	2%	0%	0%
Let7b	0%	100%	3%	0%	0%	0%	0%	0%
Let7c	1%	2%	100%	0%	0%	0%	0%	0%
Let7d	0%	0%	0%	100%	0%	0%	0%	0%
Let7e	0%	0%	0%	0%	100%	0%	0%	0%
Let7f	1%	0%	0%	0%	0%	100%	0%	0%
Let7g	0%	0%	0%	0%	0%	0%	100%	4%
Let7i	0%	1%	0%	0%	0%	0%	0%	100%

Extremely low cross-reactivity, usually 1% or lower

Copy Number Variation (CNV)

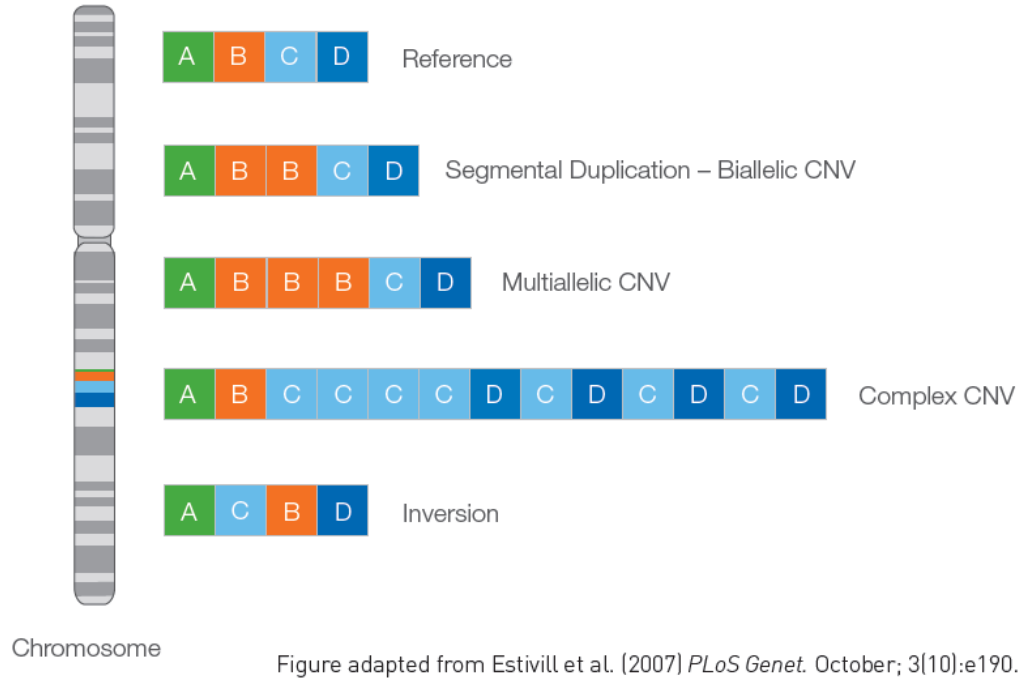


Figure adapted from Estivill et al. (2007) *PLoS Genet.* October; 3(10):e190.

- **Pre-designed human and mouse assays for copy number analysis**

Human

- Over 1.6 million pre-designed assays available for genome-wide coverage
- Genes (exons, introns, and junctions)
- Known copy number variations (CNVs)
- Extragenic/non-gene regions

Mouse

- Over 180,000 pre-designed assays available
- Gene exon coverage

Copy Number Variation (CNV)

- A structural genomic variant involving copy number changes in comparison to a reference genome
- Deletion or duplication events involving >1 kb of DNA. Most are <10 Kb; some rare CNVs >1 Mb

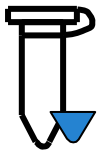
CNVs are found in normal individuals and have also been associated with disease and other phenotypes

Workflow of TaqMan® Copy Number Variation Assays

★ > 1.6M Pre-Designed TaqMan Copy Number Assays available

FAM™-labeled TEST ASSAY — 1

VIC®-labeled CONTROL ASSAY (i.e.: RNase P) — 2

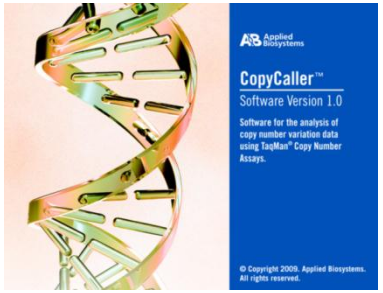


gDNA 1 ng / µL PCR rxn — 3

TaqMan® Genotyping Master Mix — 4

Standard TaqMan® protocol

CopyCaller™

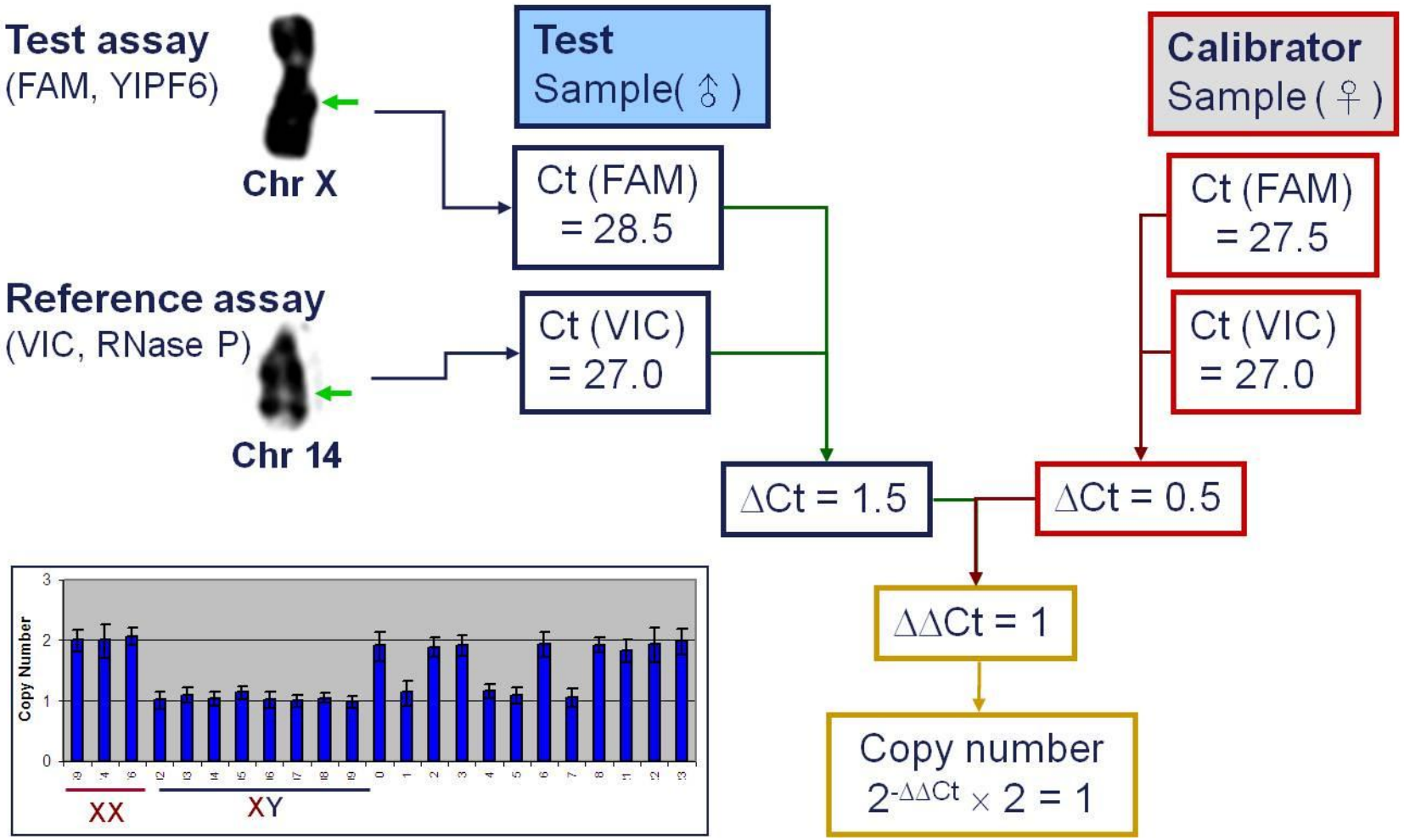


qPCR

4 replicates per gDNA sample

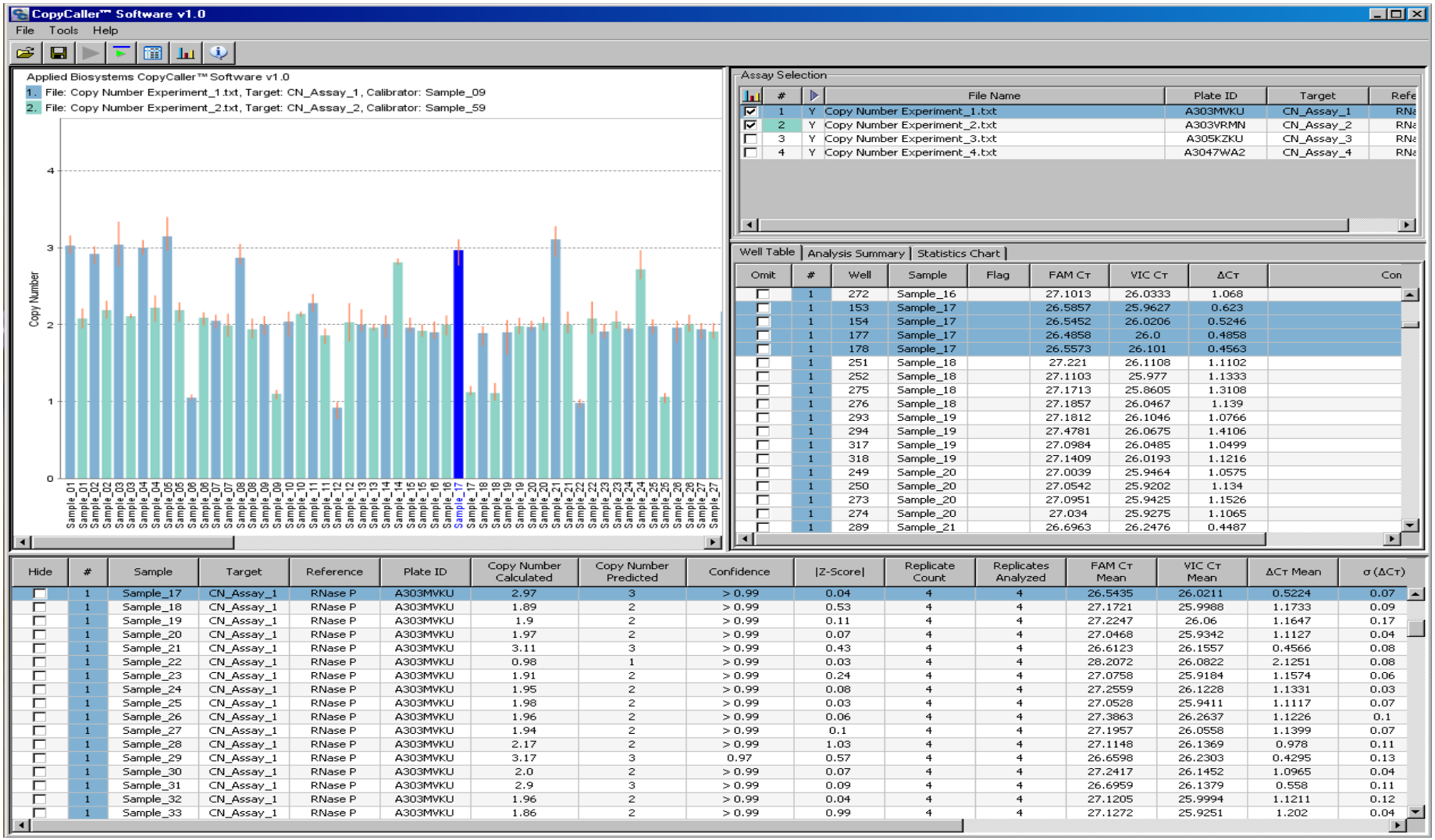
ThermoFisher SCIENTIFIC

Determination of DNA Copy Number



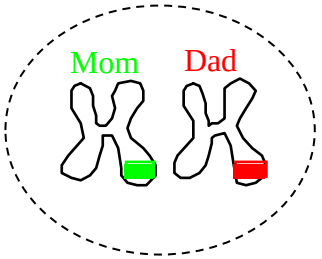
ThermoFisher SCIENTIFIC

CopyCaller™ Software-輕鬆獲得CNV結果



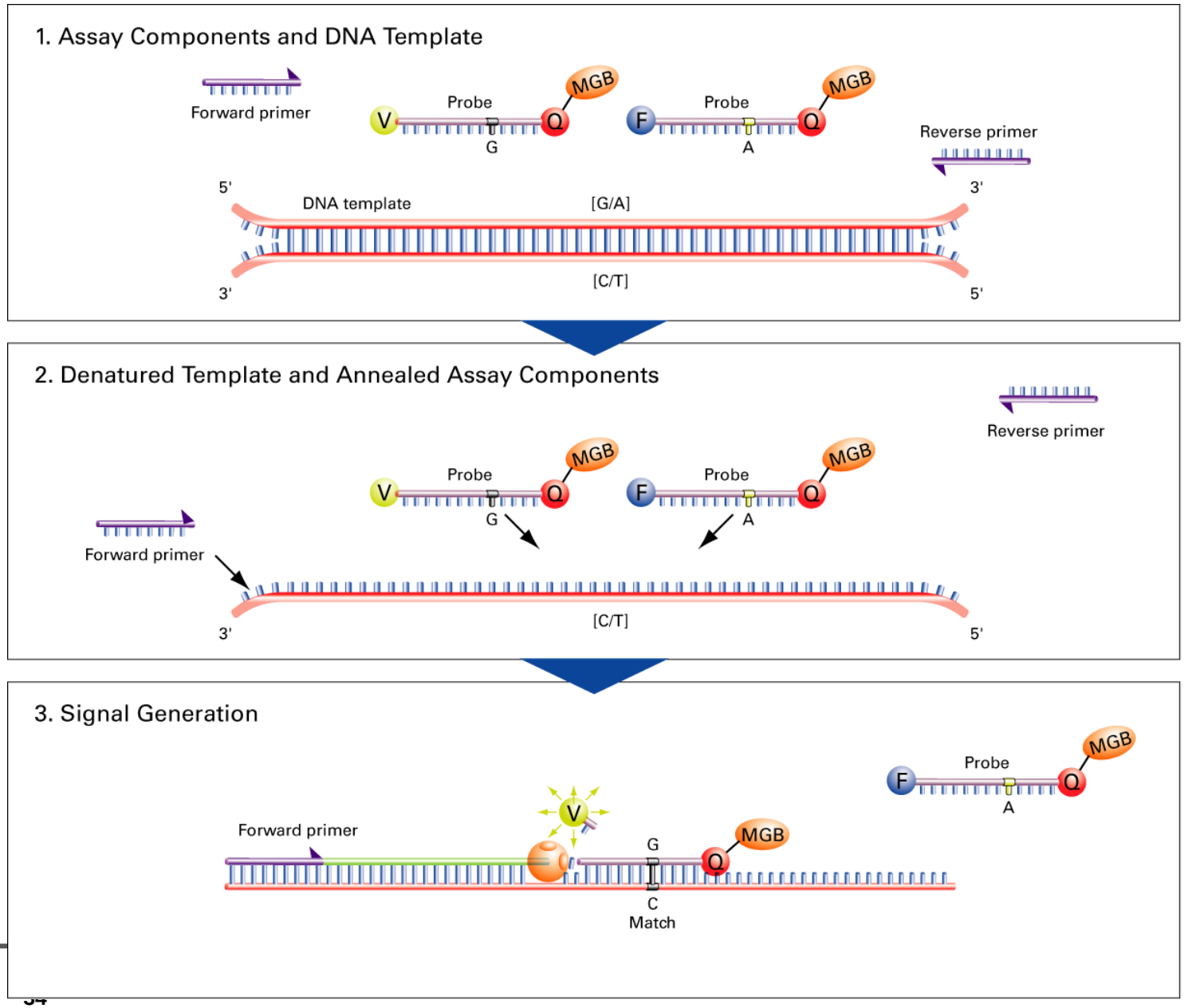
- Flexible 不需要已知拷貝數的 樣品當control
- Free 免費下載分析軟體
- Easy to use 幾分鐘內完成分析，搭配圖形化介面，輕鬆了解判讀結果
- Results with confidence value 軟體內建統計運算邏輯，提供值得信賴的結果

What are SNPs?

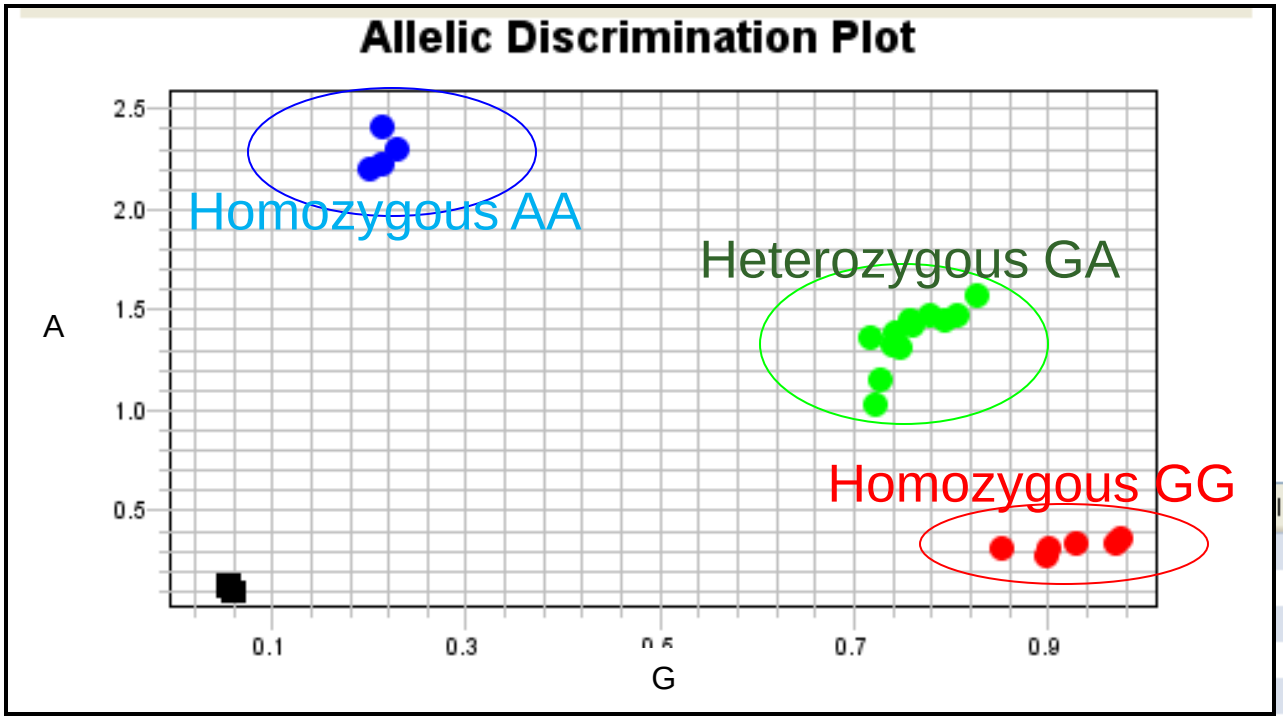


- ***Diploid*** organisms
  - 2 sets of chromosomes
- Each person has 2 copies or ***2 alleles*** of each gene – *1 allele on each chromosome*.
- Each person receives 1 allele from each parent.
- If both alleles are the same, the person is ***homozygous*** for that gene.
- If the alleles differ, then the person is ***heterozygous*** for that gene.

TaqMan® SNP Genotyping Assay Overview



Allelic Discrimination (SNP) Data



Sample 12	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.782	1.5	3,991.875	Heterozygous 1/2
Sample 13	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.716	1.206	3,942.024	Heterozygous 1/2
Sample 14	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.815	1.624	3,956.087	Heterozygous 1/2
Sample 15	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.767	1.526	3,849.214	Heterozygous 1/2
Sample 16	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.748	1.478	3,793.905	Heterozygous 1/2
Sample 17	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.709	1.086	3,820.435	Heterozygous 1/2
Sample 18	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.736	1.371	3,945.303	Heterozygous 1/2
Sample 19	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.795	1.528	4,026.388	Heterozygous 1/2
Sample 2	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.84	0.364	3,737.053	Homozygous 1/1
Sample 20	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.888	0.37	4,099.657	Homozygous 1/1
Sample 3	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.189	2.251	3,643.652	Homozygous 2/2
Sample 4	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.963	0.421	3,826.976	Homozygous 1/1
Sample 5	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.705	1.418	3,982.397	Heterozygous 1/2
Sample 6	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.729	1.386	4,048.287	Heterozygous 1/2
Sample 7	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.916	0.393	4,015.545	Homozygous 1/1
Sample 8	C__11711420_30	...	A	C	VIC-NFQ...	FAM-NF...	0.73	1.437	3,797.601	Heterozygous 1/2

lele 2 Δ...	Pass.Ref	Call
0.152	3,617.946	Negative Control (NC)
0.186	3,784.869	Negative Control (NC)
0.334	4,068.745	Homozygous 1/1
2.282	3,774.144	Homozygous 2/2
0.392	4,004.767	Homozygous 1/1

Types of Cancer Mutations

- Germline mutations
  - Inherited mutations
  - Present in all cells
  - Heterozygote (50%) or homozygote (100%) profile
  - Single gene to multi-genes
  - SNP to large chromosome rearrangement

- Somatic mutations
  - Mutations associated with the cancer itself
  - Present in some somatic cells (*i.e.* CTC)
  - Require sensitive methods to detect minor allelic frequency
  - Single gene to multi-gene
  - SNP to large chromosome rearrangement

Example: BRCA1

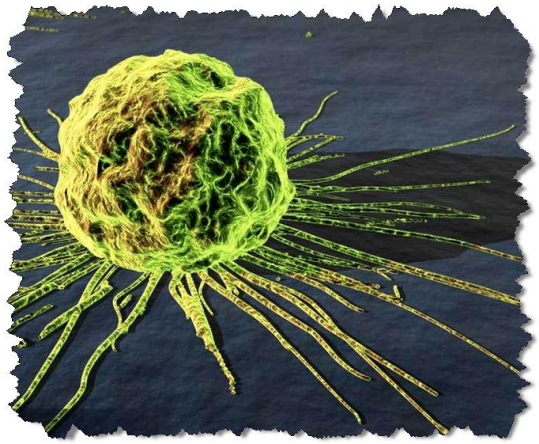
Breast Cancer

Example: PI3K

Breast, Colon Cancer

TaqMan® Mutation Detection Assays (TMDA)

- Somatic Mutation Detection by castPCR™ Technology
- TMDA Product Line Summary
  - Assays for 778 key mutations from 46 cancer genes
  - Corresponding gene reference assays
  - Wild-type assays for a subset of mutation targets
  - Internal Positive Control Reagents (IPC kit)
  - Mutation Detector™ Software
- Somatic mutations reported in the important genes related to biological pathways such as EGFR, Ras-Raf, KIT, FLT3, and PDGFRA
- High sensitivity (0.1-1%) for use with FFPE samples and biopsies
- High specificity for generating accurate results

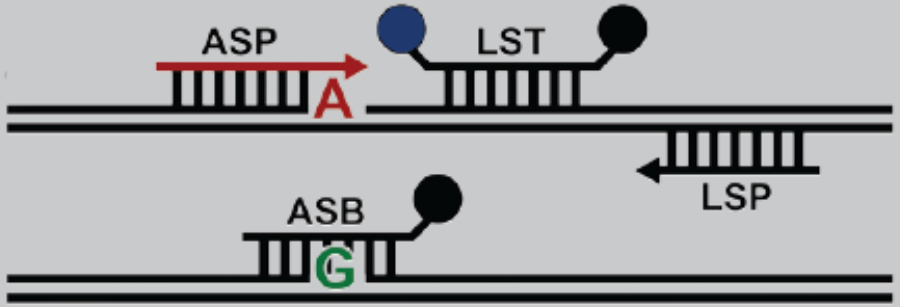


- Gene List
  - AKT1
  - ALK
  - APC
  - BRAF
  - CDKN2A
  - CTNNB1
  - EGFR
  - FGFR3
  - FLT3
  - GNAS
  - HRAS
  - IDH1
  - JAK2
  - KRAS
  - KIT
  - MPL
  - NPM1
  - NRAS
  - PDGFRA
  - PIK3CA
  - PTEN
  - TP53
  - VHL

TaqMan® Mutation Detection Assays (TMDA)

- Superior Sensitivity – 0.1 %
- High Specificity
- Simple and scalable workflow – 3 hrs from sample to results

Competitive Allele-Specific TaqMan® PCR - castPCR

Description	Schematic
• Detects specific or multiple mutant alleles • An allele-specific primer detects the mutant allele • An MGB blocker oligonucleotide suppresses the wild type allele	 ASP = Allele-specific primer ASB = Allele-specific blocker (MGB) LST = Locus-specific TaqMan® probe LSP = Locus-specific primer

Mutation Detector Software

Mutation Detector™ Software

File Help

Current Study: EXAMPLE_CellLine_dCt_cutoff

Show % Mutation | Perform Analysis

Well Data | Replicates Average | Assay Attributes

	Plate	Well	Assay	Sample	Control	Sample Ct	Quantity	Omitted	Well Flag
1	KRAS_gDNA_Titration_Run1_plate1_quant...	74	KRAS_516_mu	G12C_1		22.81	20.0		
2	KRAS_gDNA_Titration_Run1_plate1_quant...	73	KRAS_516_mu	G12C_1		22.82	20.0		
3	KRAS_gDNA_Titration_Run1_plate1_quant...	50	KRAS_516_mu	G12C_1		22.8	20.0		
4	KRAS_gDNA_Titration_Run1_plate1_quant...	49	KRAS_516_mu	G12C_1		22.93	20.0		
5	KRAS_gDNA_Titration_Run1_plate1_quant...	76	KRAS_516_mu	G12C_2		23.96	20.0		
6	KRAS_gDNA_Titration_Run1_plate1_quant...	75	KRAS_516_mu	G12C_2		24	20.0		
7	KRAS_gDNA_Titration_Run1_plate1_quant...	52	KRAS_516_mu	G12C_2		24.03	20.0		
8	KRAS_gDNA_Titration_Run1_plate1_quant...	51	KRAS_516_mu	G12C_2		24.02	20.0		
9	KRAS_gDNA_Titration_Run1_plate1_quant...	78	KRAS_516_mu	G12C_3		24.84	20.0		
10	KRAS_gDNA_Titration_Run1_plate1_quant...	77	KRAS_516_mu	G12C_3		24.98	20.0		
11	KRAS_gDNA_Titration_Run1_plate1_quant...	54	KRAS_516_mu	G12C_3		24.9	20.0		
12	KRAS_gDNA_Titration_Run1_plate1_quant...	53	KRAS_516_mu	G12C_3		24.95	20.0		
13	KRAS_gDNA_Titration_Run1_plate1_quant...	80	KRAS_516_mu	G12C_4		25.98	20.0		
14	KRAS_gDNA_Titration_Run1_plate1_quant...	79	KRAS_516_mu	G12C_4		26.08	20.0		
15	KRAS_gDNA_Titration_Run1_plate1_quant...	56	KRAS_516_mu	G12C_4		26.26	20.0		
16	KRAS_gDNA_Titration_Run1_plate1_quant...	55	KRAS_516_mu	G12C_4		26.04	20.0		
17	KRAS_gDNA_Titration_Run1_plate1_quant...	82	KRAS_516_mu	G12C_5		26.96	20.0		
18	KRAS_gDNA_Titration_Run1_plate1_quant...	81	KRAS_516_mu	G12C_5		27.07	20.0		
19	KRAS_gDNA_Titration_Run1_plate1_quant...	58	KRAS_516_mu	G12C_5		27.15	20.0		
20	KRAS_gDNA_Titration_Run1_plate1_quant...	57	KRAS_516_mu	G12C_5		27.1	20.0		
21	KRAS_gDNA_Titration_Run1_plate1_quant...	84	KRAS_516_mu	G12C_6		28.64	20.0		
22	KRAS_gDNA_Titration_Run1_plate1_quant...	83	KRAS_516_mu	G12C_6		27.97	20.0		
23	KRAS_gDNA_Titration_Run1_plate1_quant...	60	KRAS_516_mu	G12C_6		28.15	20.0		
24	KRAS_gDNA_Titration_Run1_plate1_quant...	59	KRAS_516_mu	G12C_6		28.03	20.0		

General | Current Study Settings

Study name: EXAMPLE_CellLine_dCt_cutoff

Operator:

Date: 6/24/11

Enter comments:

Results

	Plate	Sample	Quantity	Assay	Detect...	Detecte...	#...	Avg C...	Std D...	Ref Assay	#...	Avg C...	Std D...	ΔCt	Calibr...	ΔCt_{norm}	Flag
1	KRAS_gDNA_Titration_R...	G12S_4	20.0	KRAS_517_mu	0.1%	**6.41%**	4	28.53	0.26	KRAS_517_wt	4	23.45	0.04	5.08	1.21	3.87	
2	KRAS_gDNA_Titration_R...	G12V_7	20.0	KRAS_520_mu	0.1%	**1.16%**	4	30.48	0.42	KRAS_520_wt	4	22.76	0.03	7.73	1.315	6.41	
3	KRAS_gDNA_Titration_R...	G12C_4	20.0	KRAS_516_mu	0.1%	**14.6%**	4	26.09	0.12	KRAS_516_wt	4	23.41	0.09	2.68	0.135	2.54	
4	KRAS_gDNA_Titration_R...	KRAS_wt	20.0	KRAS_517_mu	0.1%	**0%**	4	40	0	KRAS_517_wt	4	22.94	0.02	17.06	1.21	15.85	MUNEG
5	KRAS_gDNA_Titration_R...	KRAS_wt	20.0	KRAS_516_mu	0.1%	**0%**	4	40	0	KRAS_516_wt	4	22.77	0.16	17.23	0.135	17.09	MUNEG
6	KRAS_gDNA_Titration_R...	G12C_8	20.0	KRAS_516_mu	0.1%	**0.67%**	4	30.81	0.8	KRAS_516_wt	3	23.47	0.07	7.34	0.135	7.21	
7	KRAS_gDNA_Titration_R...	G12V_8	20.0	KRAS_520_mu	0.1%	**0.59%**	3	31.56	0.08	KRAS_520_wt	4	22.84	0.05	8.72	1.315	7.4	
8	KRAS_gDNA_Titration_R...	G12R_6	20.0	KRAS_518_mu	0.1%	**6.23%**	4	27.54	0.11	KRAS_518_wt	4	23.15	0.03	4.39	0.473	3.91	
9	KRAS_gDNA_Titration_R...	G12S_6	20.0	KRAS_517_mu	0.1%	**1.17%**	4	30.91	0.26	KRAS_517_wt	4	23.3	0.06	7.61	1.21	6.4	
10	KRAS_gDNA_Titration_R...	G12C_5	20.0	KRAS_516_mu	0.1%	**7.39%**	4	27.07	0.08	KRAS_516_wt	4	23.29	0.02	3.78	0.135	3.65	
11	KRAS_gDNA_Titration_R...	G12V_6	20.0	KRAS_520_mu	0.1%	**3.93%**	4	28.73	0.26	KRAS_520_wt	4	22.8	0.05	5.93	1.315	4.61	
12	KRAS_gDNA_Titration_R...	G12R_8	20.0	KRAS_518_mu	0.1%	**1.05%**	4	30.07	0.49	KRAS_518_wt	4	23.03	0.05	7.04	0.473	6.56	
13	KRAS_gDNA_Titration_R...	G12R_4	20.0	KRAS_518_mu	0.1%	**23.8%**	4	25.4	0.13	KRAS_518_wt	4	23.24	0.05	2.16	0.473	1.68	
14	KRAS_gDNA_Titration_R...	G12V_9	20.0	KRAS_520_mu	0.1%	**0.14%**	4	33.52	0.64	KRAS_520_wt	4	22.68	0.05	10.84	1.315	9.52	
15	KRAS_gDNA_Titration_R...	G12S_9	20.0	KRAS_517_mu	0.1%	**0%**	4	37.04	1.42	KRAS_517_wt	4	23.26	0.04	13.78	1.21	12.57	
16	KRAS_gDNA_Titration_R...	G12C_9	20.0	KRAS_516_mu	0.1%	**0%**	4	33.22	0.22	KRAS_516_wt	4	22.85	0.06	10.36	0.135	10.23	
17	KRAS_gDNA_Titration_R...	G12R_9	20.0	KRAS_518_mu	0.1%	**0.23%**	4	32.33	0.57	KRAS_518_wt	4	23.08	0.06	9.25	0.473	8.77	
18	KRAS_gDNA_Titration_R...	G12S_1	20.0	KRAS_517_mu	0.1%	**>99.9%**	4	25.48	0.06	KRAS_517_wt	2	37.42	0.49	-11.94	1.21	-13.15	
19	KRAS_gDNA_Titration_R...	G12C_7	20.0	KRAS_516_mu	0.1%	**53.6%**	4	24	0.03	KRAS_516_wt	4	24.08	0.1	-0.07	0.135	-0.21	

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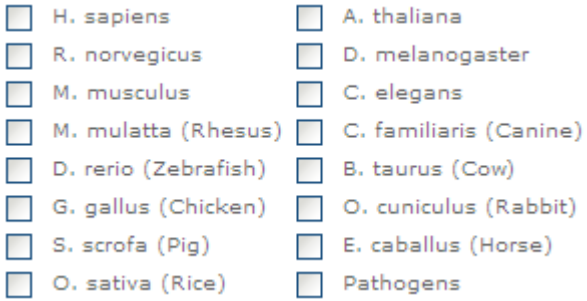
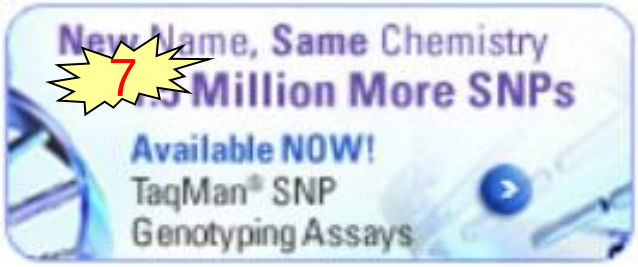
Applied Biosystems 提供Primers/Probe設計的全方位解決方案



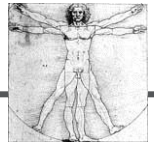


Custom TaqMan Assays Service

- *TaqMan Gene Expression Assays*
  - > 1,300,000 個已設計及測試過的基因定量試劑組
  - 提供所有相關生物資訊 (23 species)
- *TaqMan microRNA and primary microRNA Assays*
- *TaqMan SNP Genotyping Assays*
- *TaqMan Copy Number Assays*
- *TaqMan Mutation Detection Assays*

- Custom TaqMan Assays
  - All-in One tube TaqMan-based Assay

- Primer Express Software
- 上機條件皆相同~~不用再花時間測試primer溫度了



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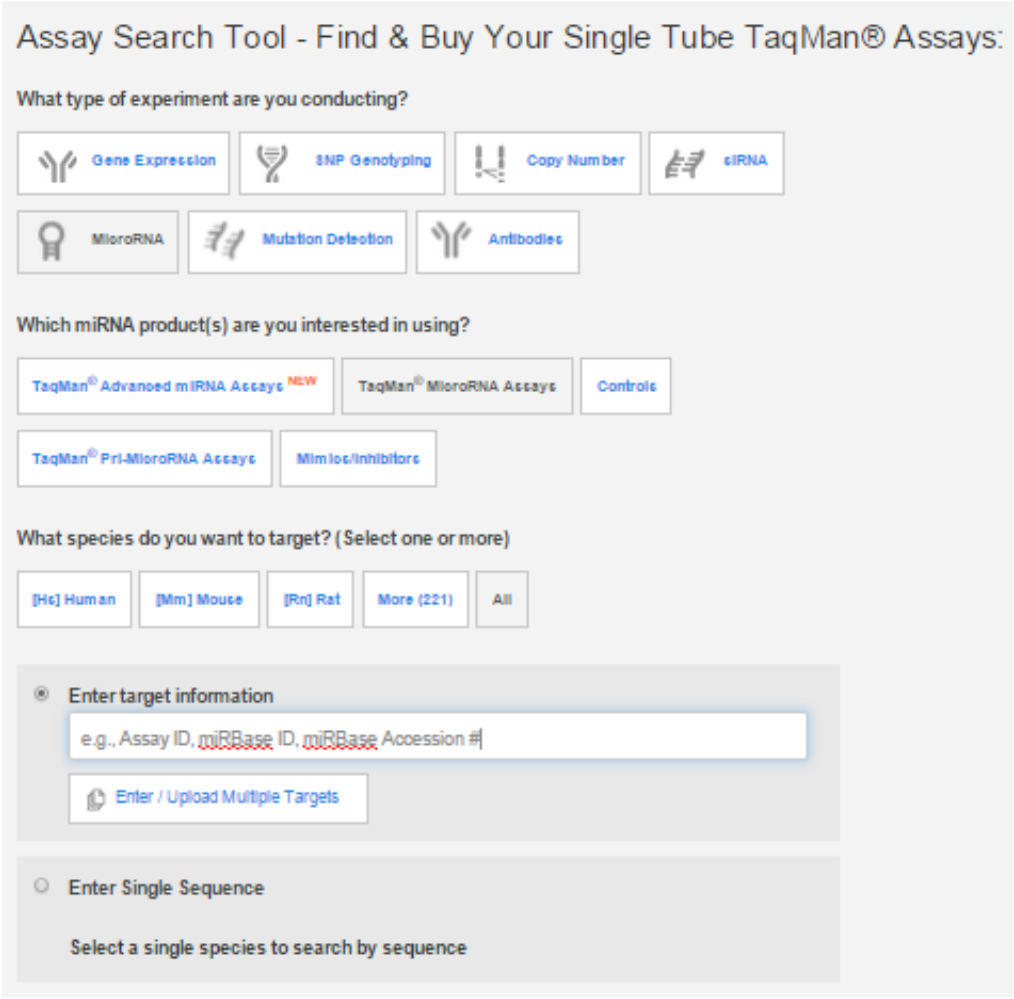
Finding the Right Assay for Your Research

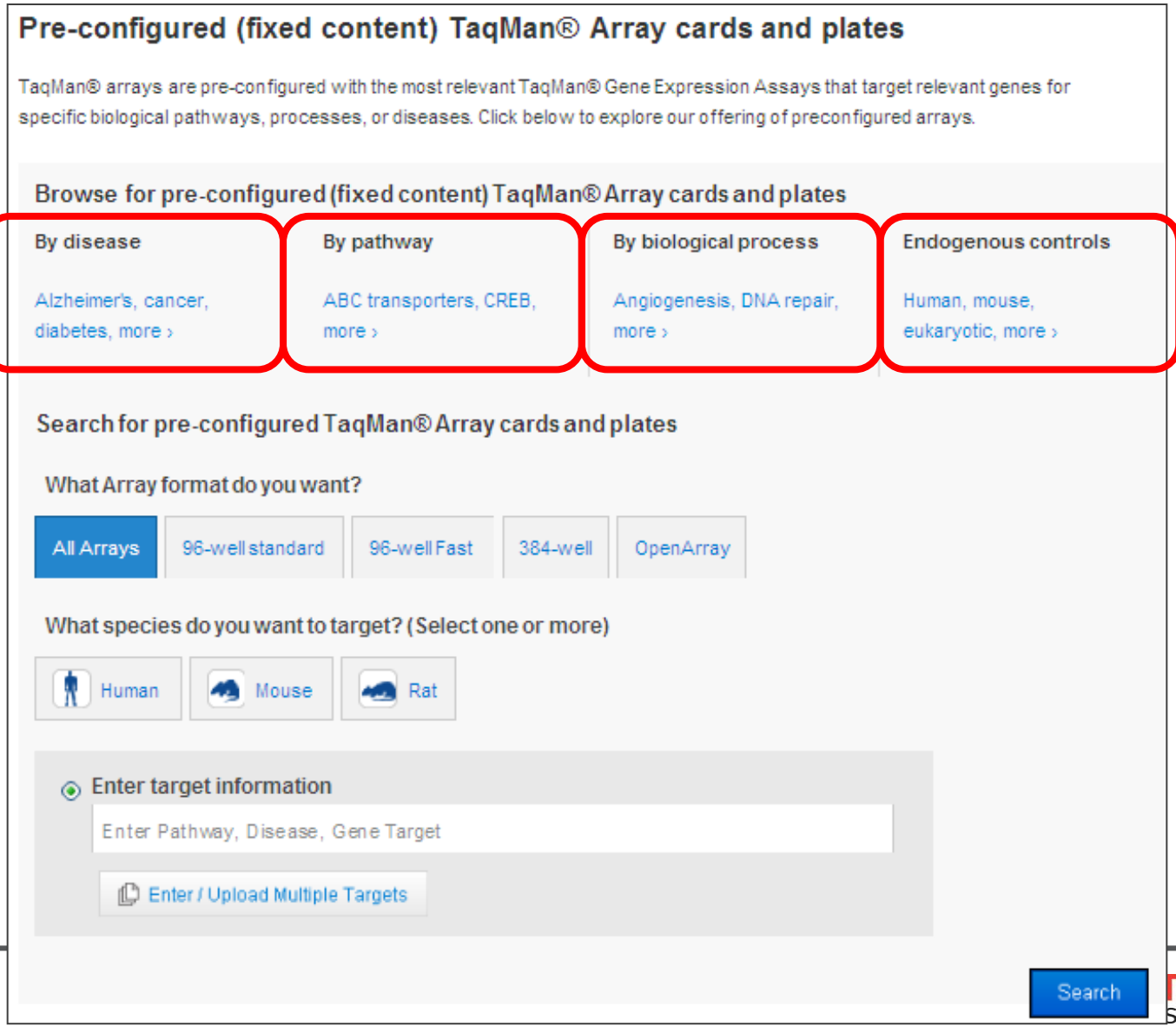


- Search for the assay you need quickly and easily
  - Powerful search engine
  - Streamlined search interface
  - Flexibility to search by gene name, gene alias or assay ID

http://www.thermofisher.com/tw/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays.html

TaqMan® Gene Expression Array Plates

https://www.thermofisher.com/tw/en/home/life-science/pcr/real-time-pcr/real-time-pcr-assays/taqman-gene-expression/real-time-pcr-taqman-arrays.html



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Targets and Pathway Information

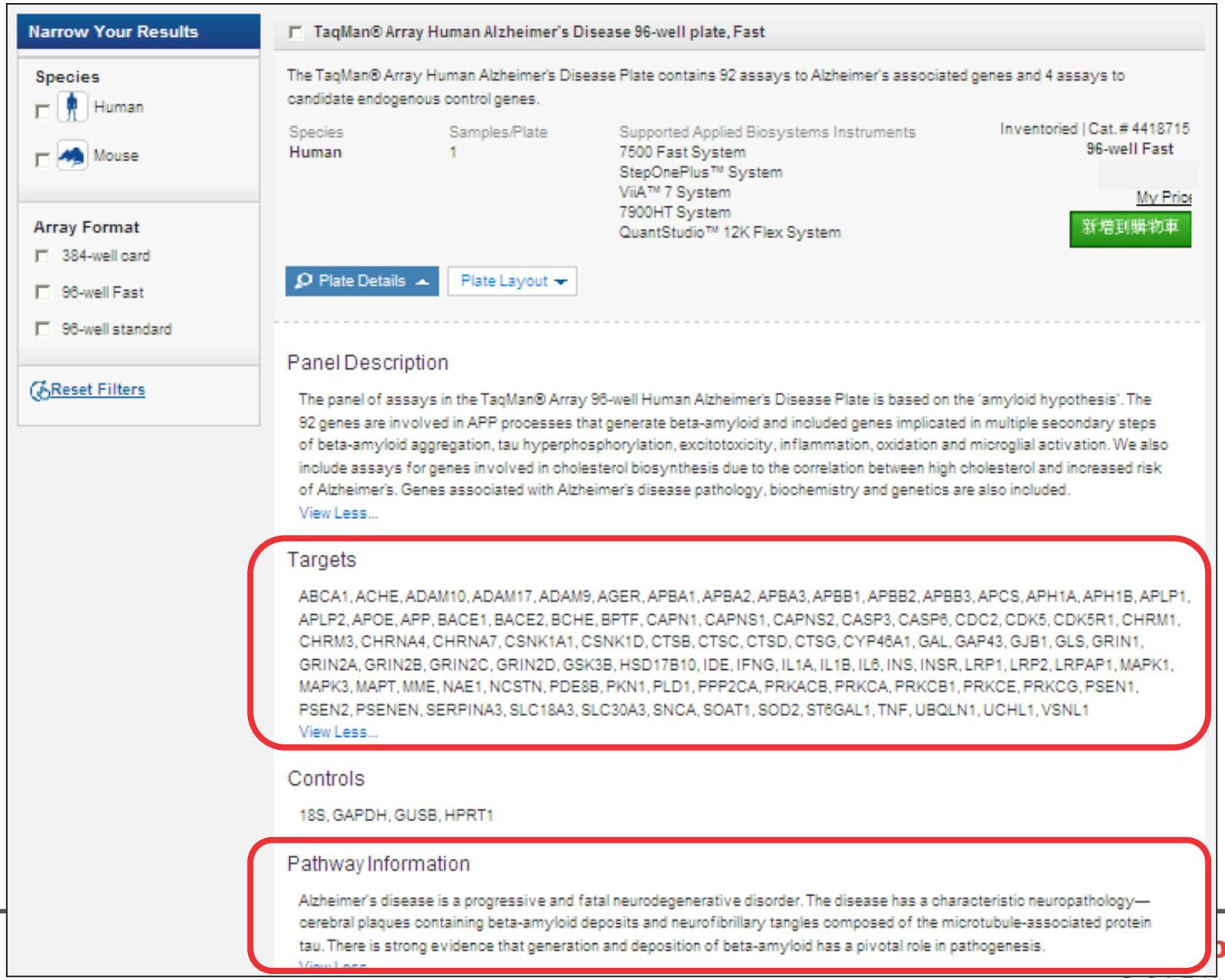
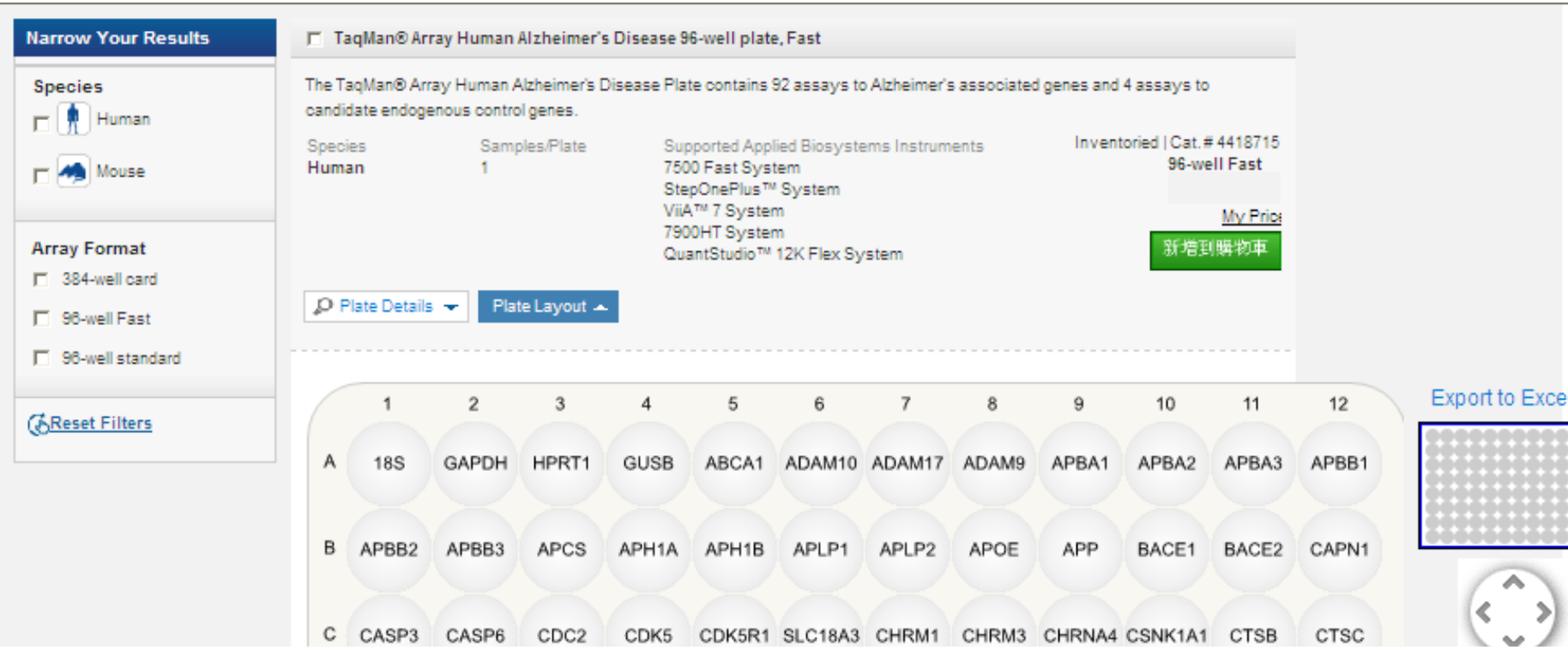


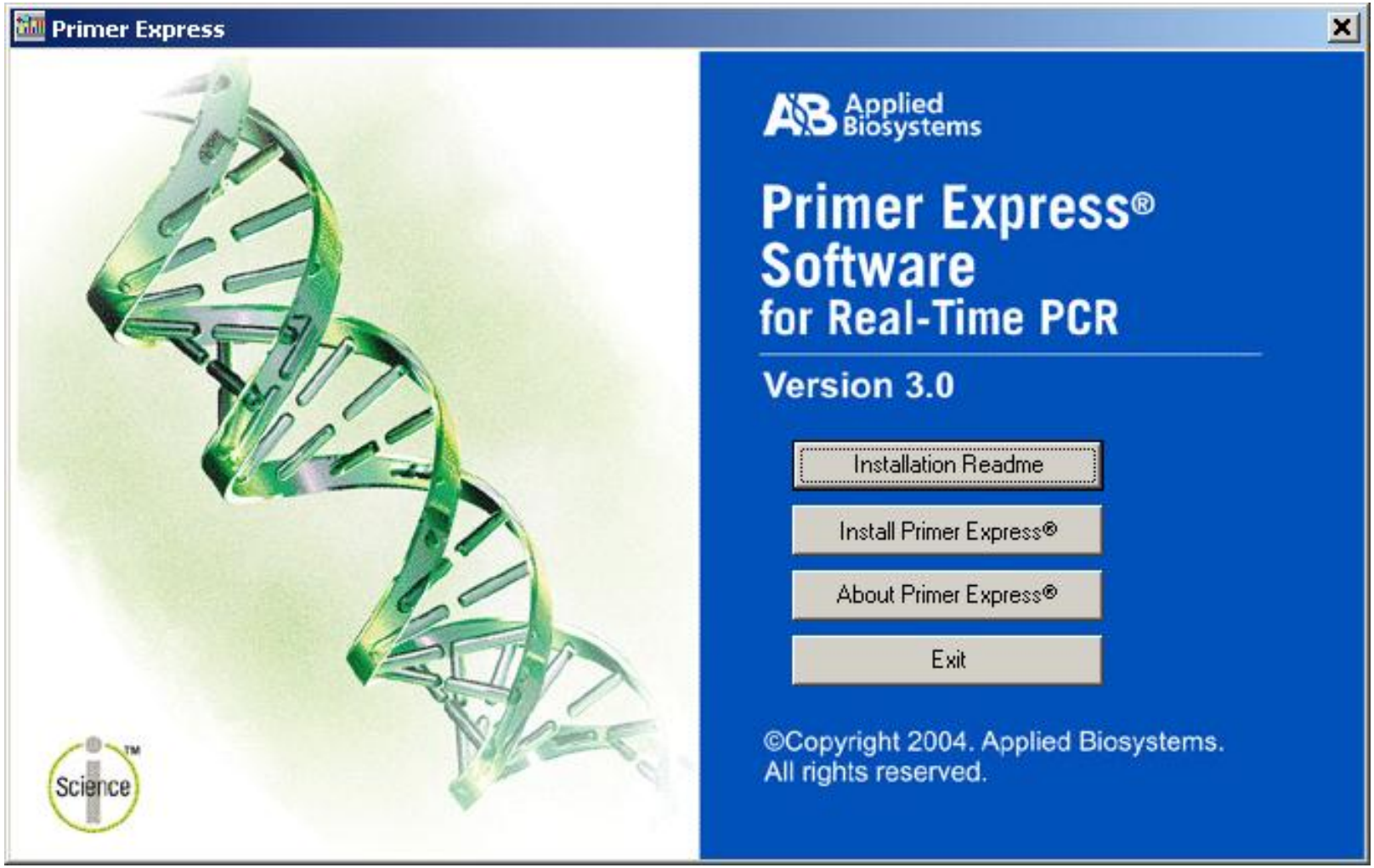
Plate Layout and Assay ID



Assay ID	1	2	3	4	5	6	7	8	9	10	11	12
A	Hs99999901_s1	Hs99999905_m1	Hs99999909_m1	Hs99999908_m1	Hs00194045_m1	Hs00153853_m1	Hs00234224_m1	Hs00177638_m1	Hs00154104_m1	Hs00194072_m1	Hs00191660_m1	Hs00377427_m1
B	Hs00300268_m1	Hs00195923_m1	Hs00356632_g1	Hs00211268_m1	Hs00229911_m1	Hs00193069_m1	Hs00155778_m1	Hs00171168_m1	Hs00169098_m1	Hs00201573_m1	Hs00273238_m1	Hs00559804_m1
C	Hs00263337_m1	Hs00154250_m1	Hs00364293_m1	Hs00358991_g1	Hs00243655_s1	Hs00268179_s1	Hs00265195_s1	Hs00265216_s1	Hs00181247_m1	Hs00793391_m1	Hs00157194_m1	Hs00175188_m1
D	Hs00157205_m1	Hs00175195_m1	Hs00189461_m1	Hs00702141_s1	Hs00248163_m1	Hs00609557_m1	Hs00168219_m1	Hs00168230_m1	Hs00181352_m1	Hs00275656_m1	Hs00189576_m1	Hs00610438_m1
E	Hs00174143_m1	Hs00174092_m1	Hs00174097_m1	Hs00174131_m1	Hs00355773_m1	Hs00169631_m1	Hs00233856_m1	Hs00189742_m1	Hs00158875_m1	Hs00177066_m1	Hs00385075_m1	Hs00213491_m1
F	Hs00153519_m1	Hs00299716_m1	Hs00405493_m1	Hs00708570_s1	Hs00160118_m1	Hs00427259_m1	Hs00176944_m1	Hs00176973_m1	Hs00176998_m1	Hs00178455_m1	Hs00177010_m1	Hs00177028_m1
G	Hs00997789_m1	Hs01577197_m1	Hs00153674_m1	Hs00240906_m1	Hs00162077_m1	Hs00167309_m1	Hs00260517_s1	Hs00174128_m1	Hs00188233_m1	Hs00374305_m1	Hs00544355_m1	Hs01085739_g1
H	Hs00542592_g1	Hs01000370_m1	Hs00992319_m1	Hs00998426_m1	Hs01063373_m1	Hs01017895_m1	Hs01042347_m1	Hs00967138_m1	Hs01016626_m1	Hs00900696_m1	Hs00949382_m1	Hs00923840_m1

Gene Symbol	1	2	3	4	5	6	7	8	9	10	11	12
A	18S	GAPDH	HPRT1	GUSB	ABCA1	ADAM10	ADAM17	ADAM9	APBA1	APBA2	APBA3	APBB1
B	APBB2	APBB3	APCS	APH1A	APH1B	APLP1	APLP2	APOE	APP	BACE1	BACE2	CAPN1
C	CASP3	CASP6	CDC2	CDK5	CDK5R1	SLC18A3	CHRM1	CHRM3	CHRNA4	CSNK1A1	CTSB	CTSC
D	CTSD	CTSG	BPTF	GJB1	GLS	GRIN1	GRIN2A	GRIN2B	GRIN2D	GSK3B	HSD17B10	IDE
E	IFNG	IL1A	IL1B	IL6	INS	INSR	LRP1	LRP2	LRPAP1	MAPK1	MAPK3	MAPT
F	ORC3L	NCSTN	PDE8B	PSENEN	PLD1	PPP2CA	PRKACB	PRKCA	PRKCB1	PRKCE	PRKCG	PKN1
G	PSEN1	PSEN2	SERPINA3	SNCA	SOAT1	SOD2	CAPNS2	TNF	UCHL1	VSNL1	GAL	ACHE
H	AGER	NAE1	BCHE	CAPNS1	CHRNA7	CSNK1D	CYP46A1	GAP43	GRIN2C	SLC30A3	ST6GAL1	UBQLN1

定量PCR Primers/ Probe設計軟體



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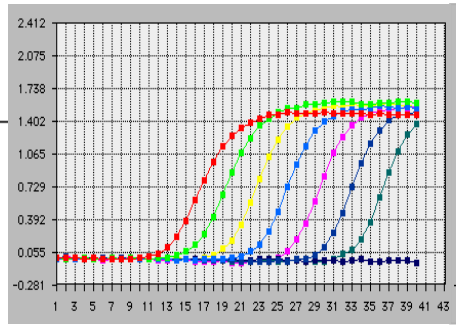
清楚明確的 TaqMan Probe and Primer 設計規範

TaqMan Probe	Primer
Probe 與 Primer 的距離愈近愈好, PCR 產物大小建議在 50-150 bp 為最佳	
G/C % 為 30-80 %	
避免有重複序列的出現，尤其避免 4 個以上 G 的出現	
Tm 值: 68-70℃ (Quantification assay) 65-67℃ (Allelic Discrimination assay)	Tm 值: 58-60℃
Probe 長度: 13~25 bases (TaqMan MGB probe) 13~30 bases (TaqMan probe)	**Primer 長度:** 20 bases (Optimal)
避免連續 6 個 A 的序列出現	3'端的前五個序列裡不能超過 2 個 C+G
5'端第一個序列不能為 G (如果選擇 FAM-dye 在 5'端第二個序列也不能為 G)	
選擇 C 比 G 多的 strand 當作 probe [b]	
避免 3'端的前 4 個序列裡含有 3 個或以上 G (GGG-MGB-3' or GGAG-MGB-3') [a]	
避免 probe 的中間區域含有 2 個或以上的 CC di-nucleotides [a]	

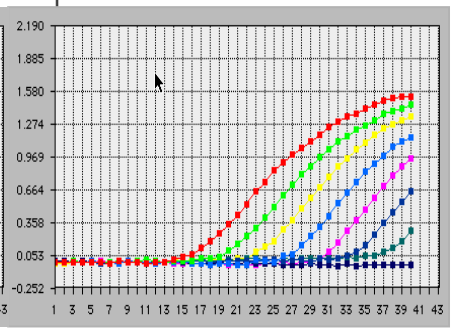
a: 針對 TaqMan MGB probe

b: 參數可選擇設定

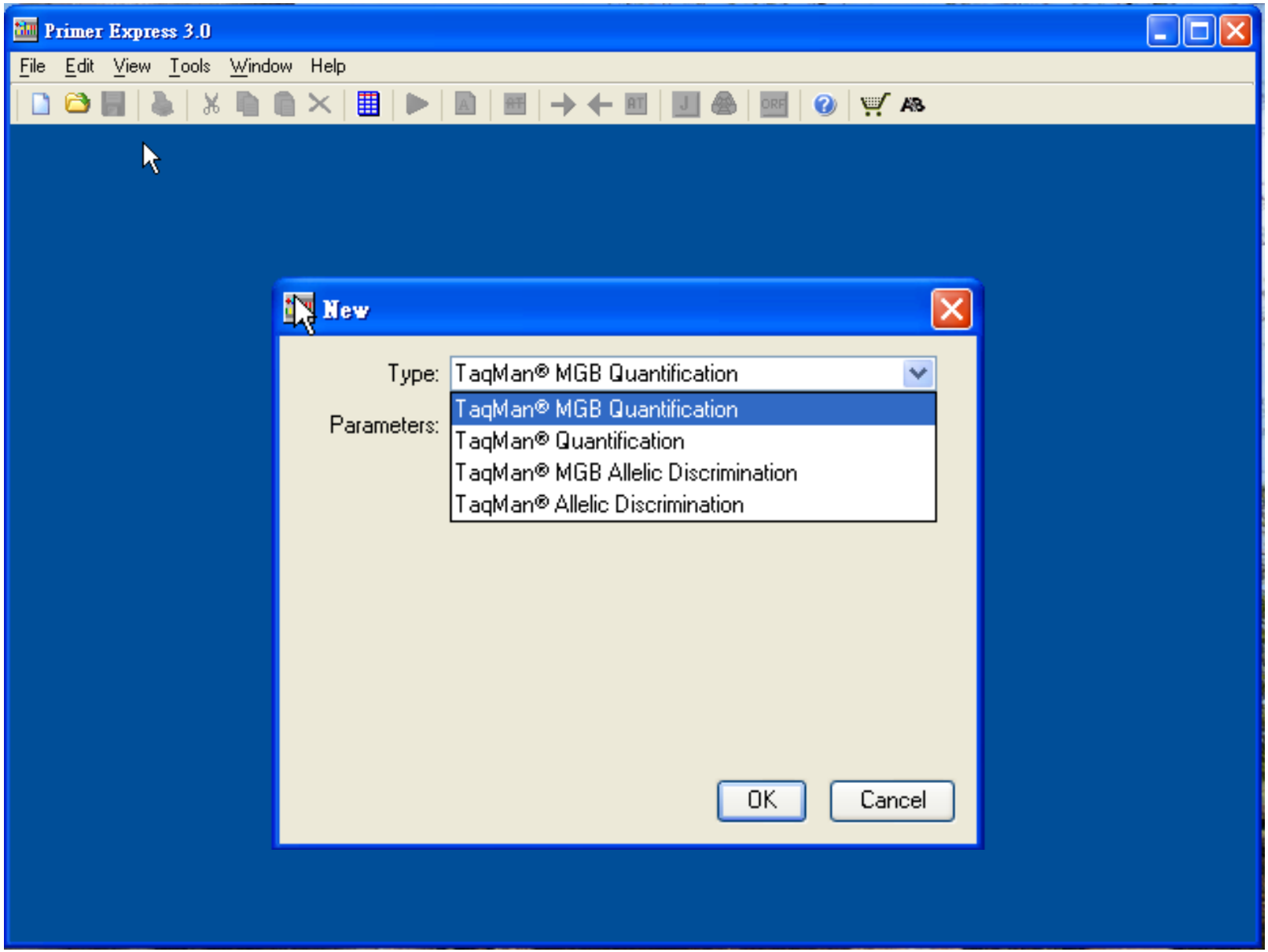
200 bp amplicon



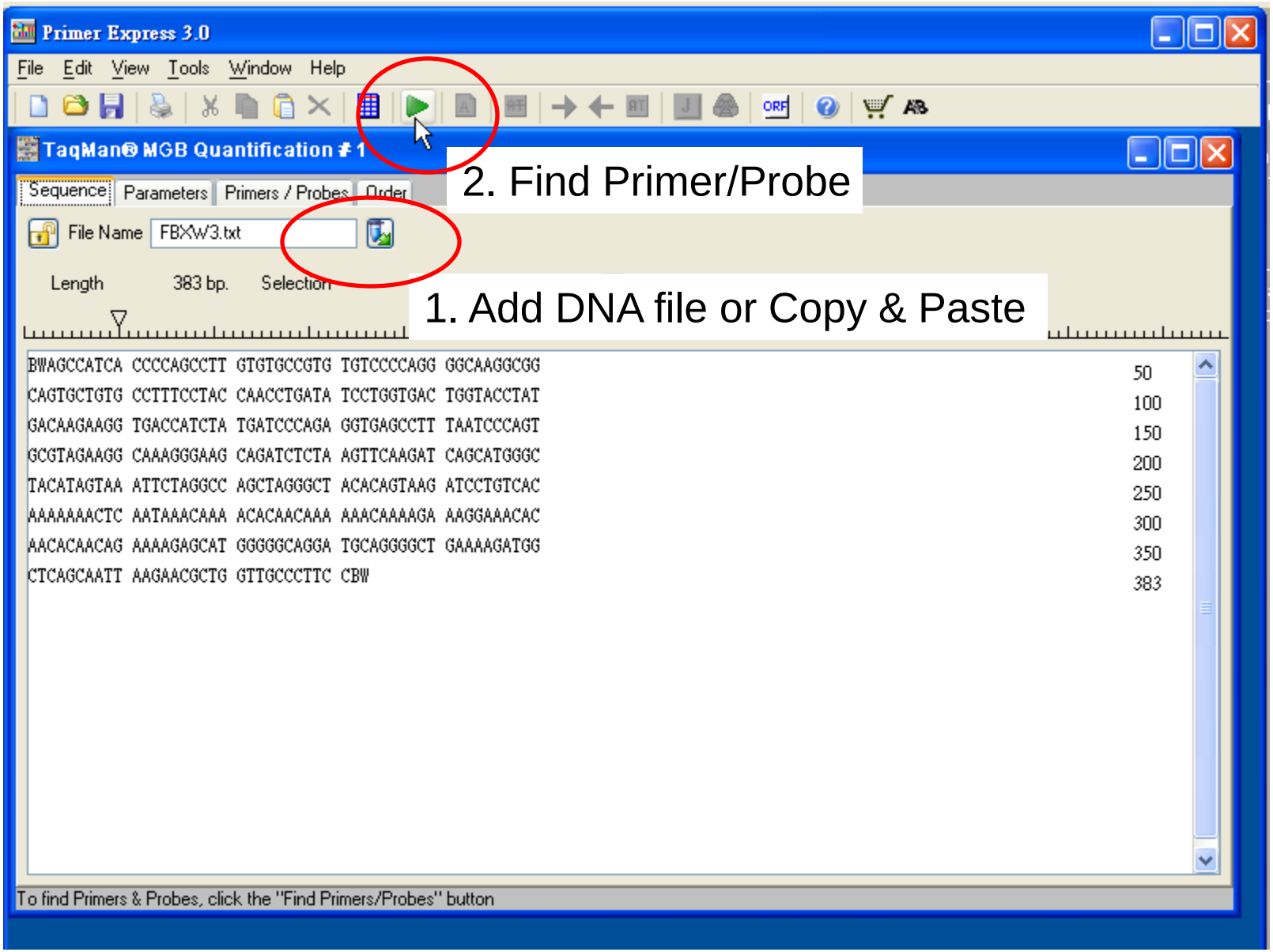
500 bp amplicon



ThermoFisher SCIENTIFIC



Sequence



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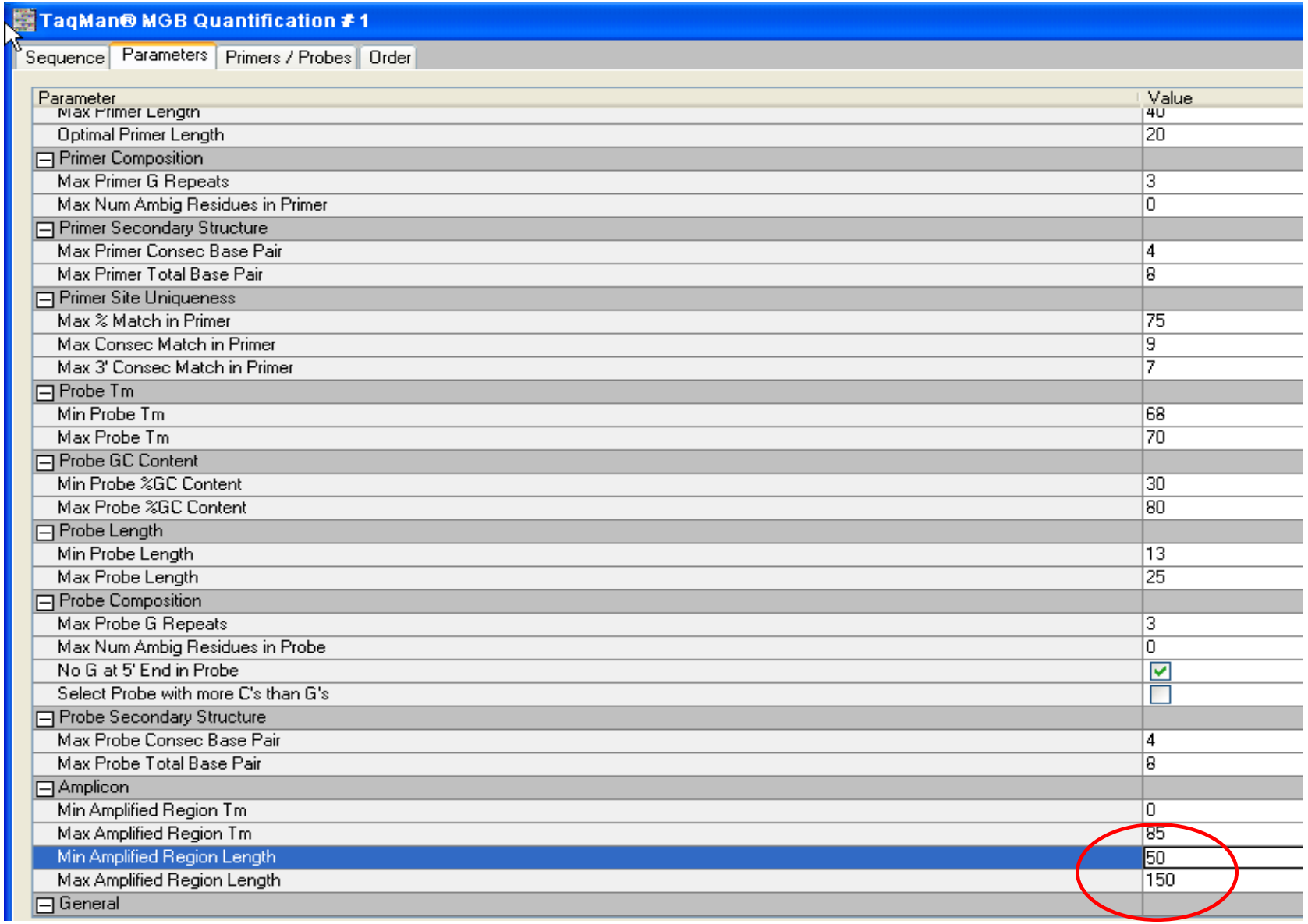
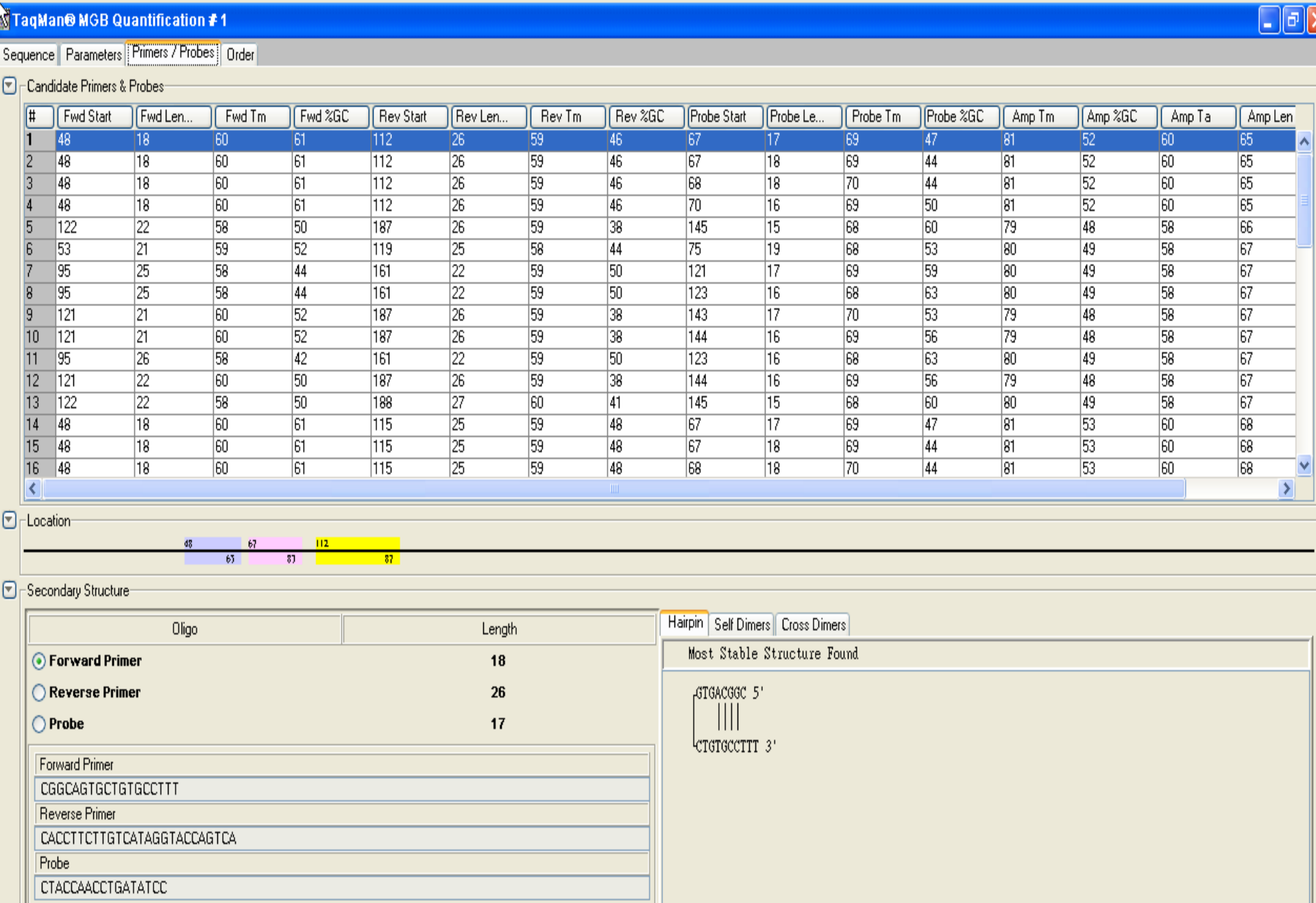
Design Parameter



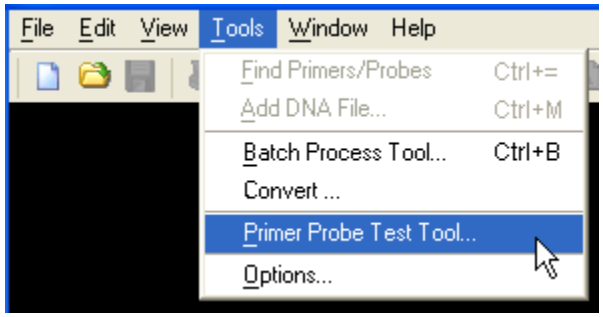
Parameter	Value
Max Primer Length	40
Optimal Primer Length	20
Primer Composition	
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniqueness	
Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
Probe Tm	
Min Probe Tm	68
Max Probe Tm	70
Probe GC Content	
Min Probe %GC Content	30
Max Probe %GC Content	80
Probe Length	
Min Probe Length	13
Max Probe Length	25
Probe Composition	
Max Probe G Repeats	3
Max Num Ambig Residues in Probe	0
No G at 5' End in Probe	☑
Select Probe with more C's than G's	☐
Probe Secondary Structure	
Max Probe Consec Base Pair	4
Max Probe Total Base Pair	8
Amplicon	
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	50
Max Amplified Region Length	150
General	

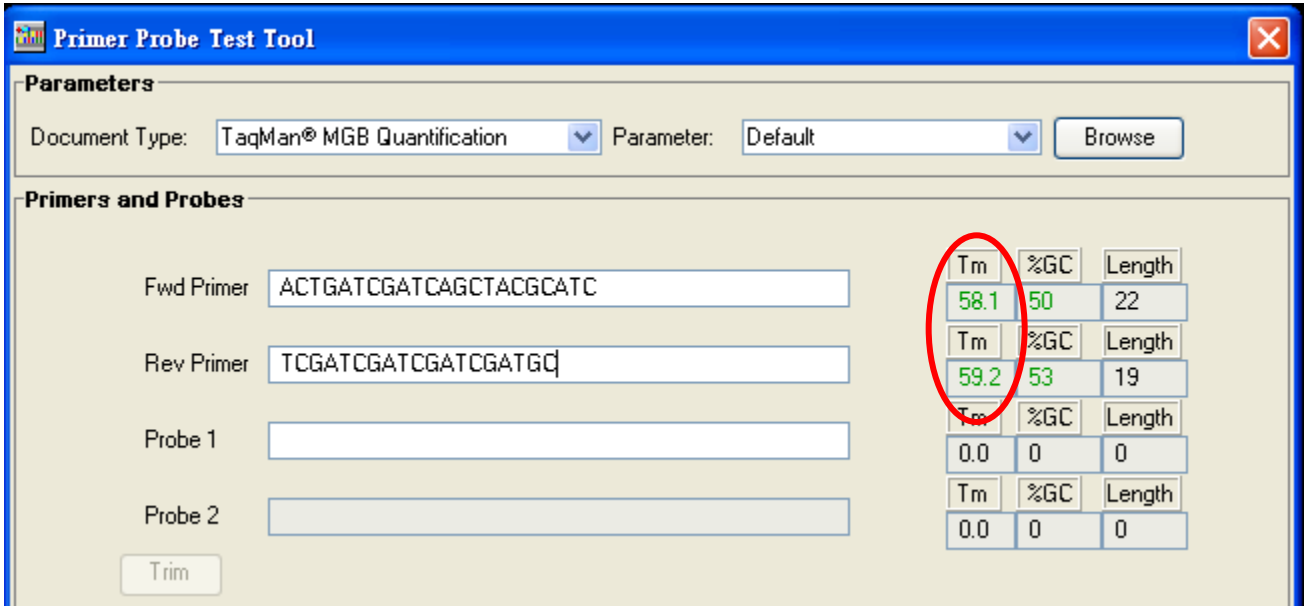
Results



#	Fwd Start	Fwd Len...	Fwd Tm	Fwd %GC	Rev Start	Rev Len...	Rev Tm	Rev %GC	Probe Start	Probe Le...	Probe Tm	Probe %GC	Amp Tm	Amp %GC	Amp Ta	Amp Len
1	48	18	60	61	112	26	59	46	67	17	69	47	81	52	60	65
2	48	18	60	61	112	26	59	46	67	18	69	44	81	52	60	65
3	48	18	60	61	112	26	59	46	68	18	70	44	81	52	60	65
4	48	18	60	61	112	26	59	46	70	16	69	50	81	52	60	65
5	122	22	58	50	187	26	59	38	145	15	68	60	79	48	58	66
6	53	21	59	52	119	25	58	44	75	19	68	53	80	49	58	67
7	95	25	58	44	161	22	59	50	121	17	69	59	80	49	58	67
8	95	25	58	44	161	22	59	50	123	16	68	63	80	49	58	67
9	121	21	60	52	187	26	59	38	143	17	70	53	79	48	58	67
10	121	21	60	52	187	26	59	38	144	16	69	56	79	48	58	67
11	95	26	58	42	161	22	59	50	123	16	68	63	80	49	58	67
12	121	22	60	50	187	26	59	38	144	16	69	56	79	48	58	67
13	122	22	58	50	188	27	60	41	145	15	68	60	80	49	58	67
14	48	18	60	61	115	25	59	48	67	17	69	47	81	53	60	68
15	48	18	60	61	115	25	59	48	67	18	69	44	81	53	60	68
16	48	18	60	61	115	25	59	48	68	18	70	44	81	53	60	68

Check Tm of Primers





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SYBR Green Experiment Notes

1. **Primer Concentration Optimization**
   - Primer final concentration
   - No primer dimer or non-specific product involved
2. **PCR Primer Efficiency Validation**
   - Serially-diluted sample to generate standard curve for target gene and endogenous control gene
3. **Test with samples that are comparable to real experiment for each gene**

ThermoFisher SCIENTIFIC

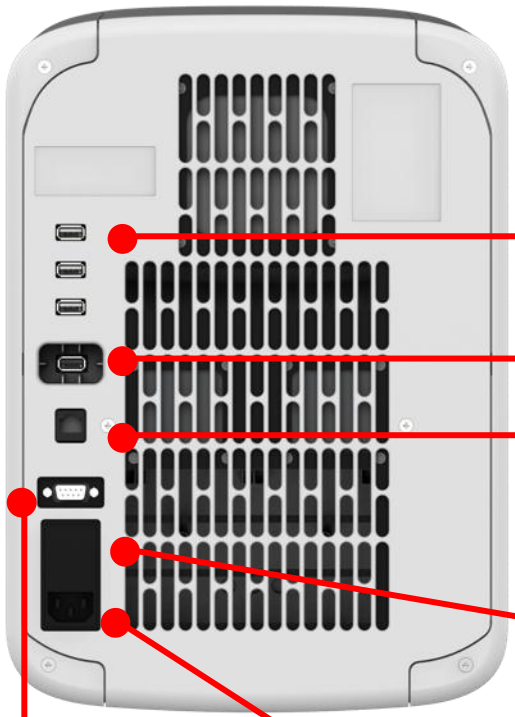
Applied Biosystems QuantStudio™ 3 Real-Time PCR System

QuantStudio™ 3 Real-Time PCR Systems: The Basics

Touchscreen (stand-alone capabilities, PIN-protected user accounts, and dye calibration/RNaseP functionality)

USB port for template upload and data download

Motorized block drawer (controlled by touchscreen)



USB ports

WiFi adapter port (optional use)

Ethernet port : RJ45 (10/100Mbps)

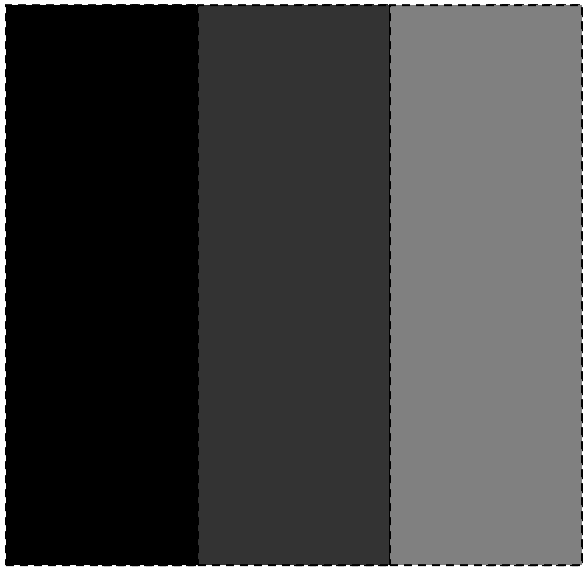
Fuse cover

Power port: 100/240 VAC

RS232 port (Service only)

QuantStudio™ 3 Real-Time PCR System: The Basics

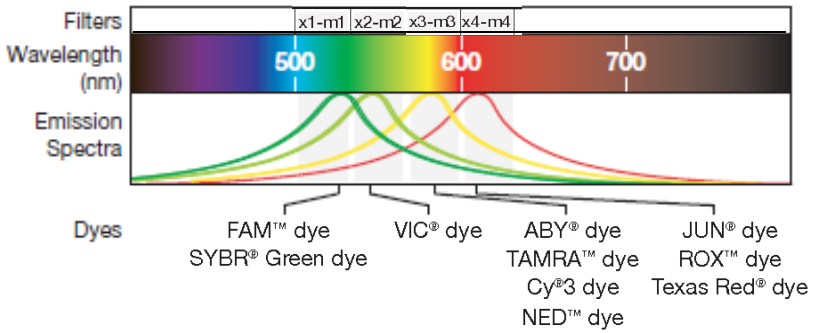
- VeriFlex™ Block with 3 programmable zones
  - Independent temperature control in each zone (more precise than gradient)
  - Can program at will, including multiple zones with same temp
  - Great for optimization and also running multiple assays at the same time



Multiplexing Capabilities

- OptiFlex™ System with Bright White LED
- Four color locked filter system
- Factory calibrated

Peak channel	Color	Filter wavelength (nm)[1]		Pre-calibrated dyes	Example custom dyes
		Excitation	Emission		
x1-m1	Blue	470 ± 15	520 ± 15	FAM™ and SYBR® Green	SYT09
x2-m2	Green	520 ± 10	558 ± 12	VIC®	HEX™, TET™, and JOE™[2]
x3-m3	Yellow	550 ± 10	587 ± 10	ABY®, NED™, and TAMRA™	Cy®3
x4-m4	Orange	580 ± 10	623 ± 14	JUN® and ROX™	Texas Red®



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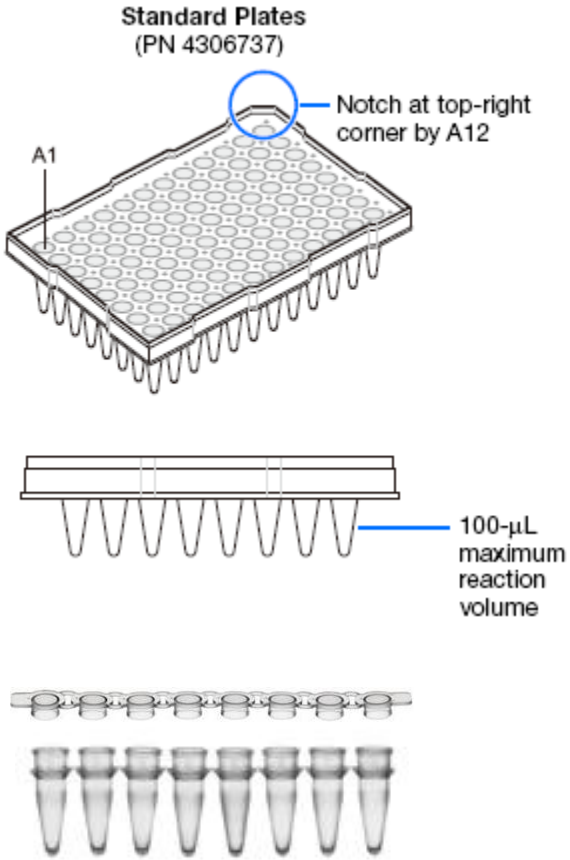
QuantStudio™ 3 Real-Time PCR System: Consumables

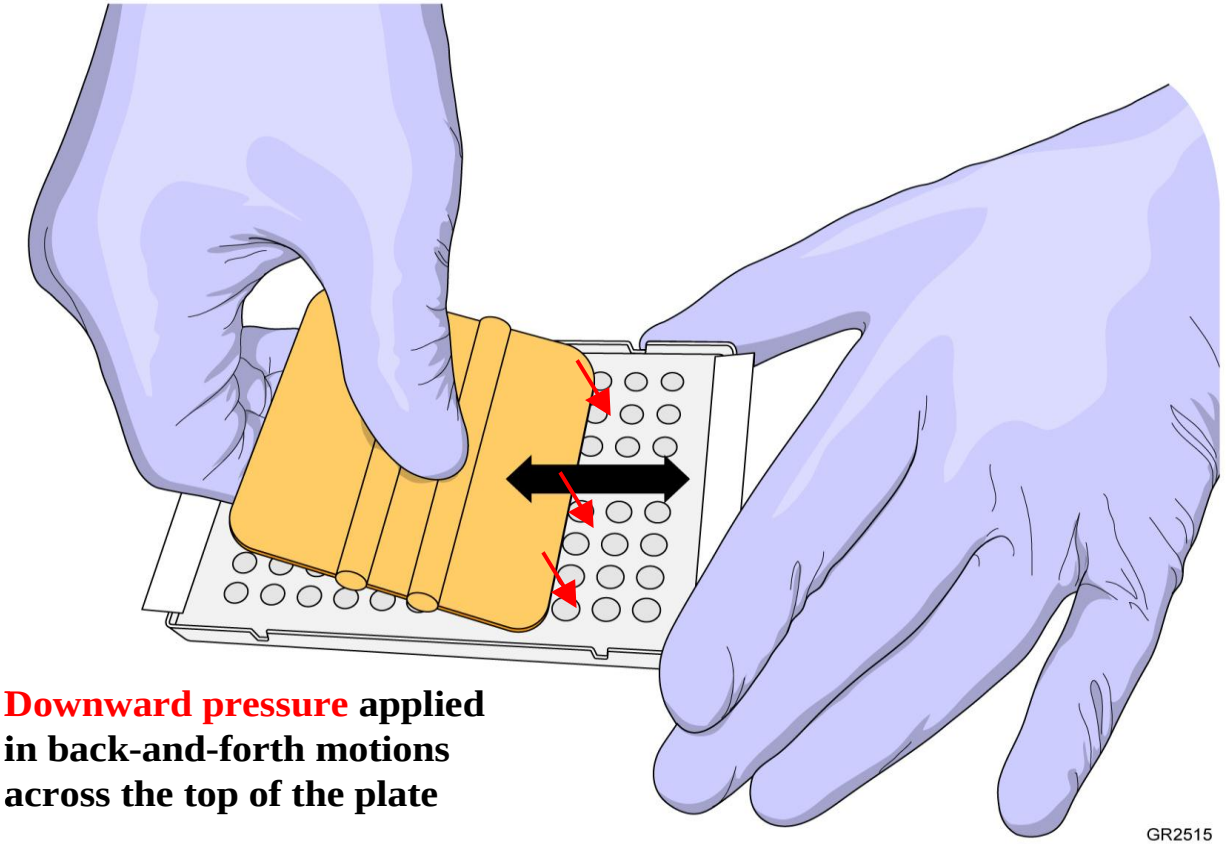
- 樣品量多時
  - MicroAmp Optical 96-Well Reaction Plate (0.2ml) -10 plates (P/N N8010560)
  - ABI PRISM™ Optical Adhesive Covers - 100 films (P/N 4311971)
- 樣品量少時
  - ABI PRISM™ Optical 8 Tubes/Strip (0.2ml) - 125 strips (P/N 4316567)
  - MicroAmp Optical 8 Caps/Strip - 300 strips (P/N 4323032)



★ Load at least 16 tubes with tray

Sealing the Plate

The flat edge of an applicator is rubbed back-and-forth along the length of the plate with a significant downward pressure to form a complete seal on top the wells

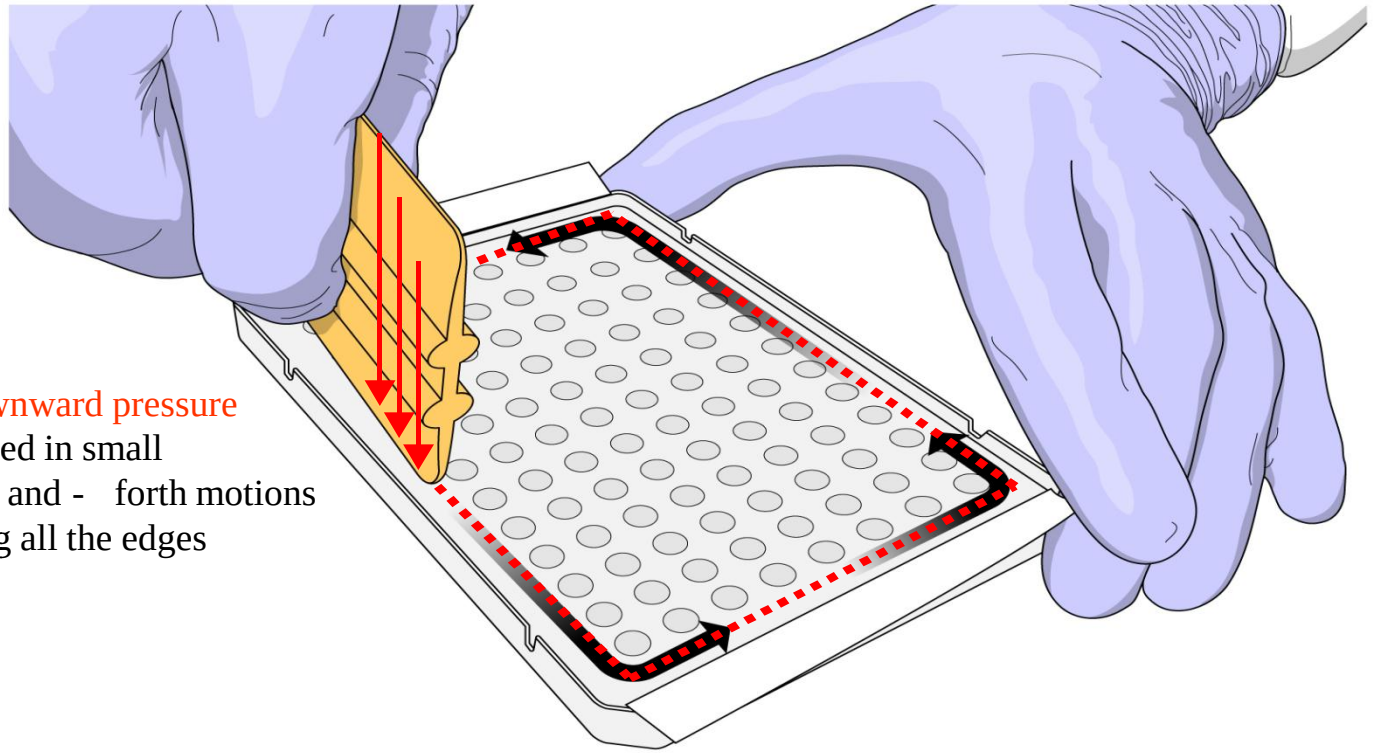


Downward pressure applied in back-and-forth motions across the top of the plate

Note: Pressure is required to activate the adhesive on the optical cover

Sealing the Plate

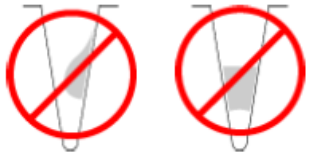
The end of an applicator is rubbed around all the outside edges of the plate with a significant downward pressure to form a complete seal around the outside wells



Note: Pressure is required to activate the adhesive on the optical cover

QuantStudio™ 3 Real-Time PCR System: Operation Notes

- Use a tray for 8-tube strips
- Do not label on the consumables
  - This may increase the background signal
- Avoid bubbles when pipetting into each well
  - Centrifuge samples

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

Stand-alone, Desktop, or Online

Connected Laptop with QuantStudio Design and Analysis desktop software

WiFi

LAN

USB

Connect to the Design and Analysis Cloud software using any device with a compatible web browser

Note: You can start an experiment run only from the instrument touchscreen or from the Desktop Software

QuantStudio™ 3 Touch Screen

Open/Close door

Help

Sign In or Create a Local User Account

Connection type

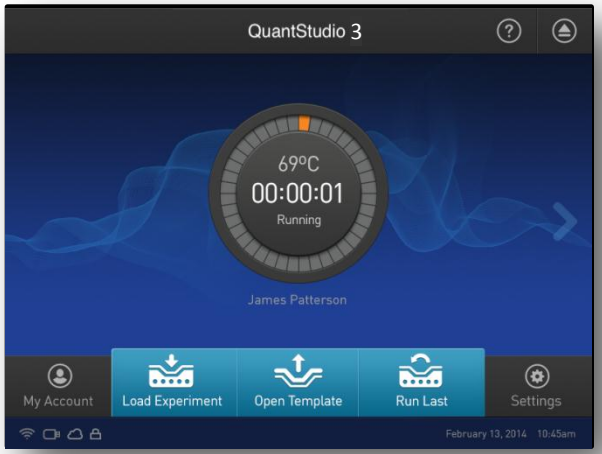
Settings:
- Calibration
- Runs
- Logs
- Ship Prep

Edit Run Protocol

Full method editing capabilities on the touch screen, including VeriFlex, Pause, and Melt

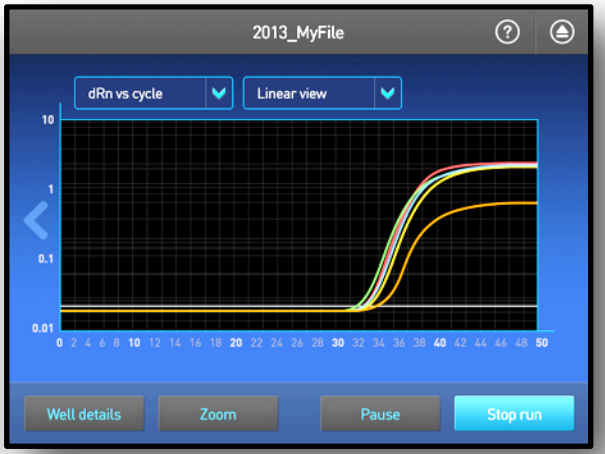
Monitor Progress During the Run

Time Remaining

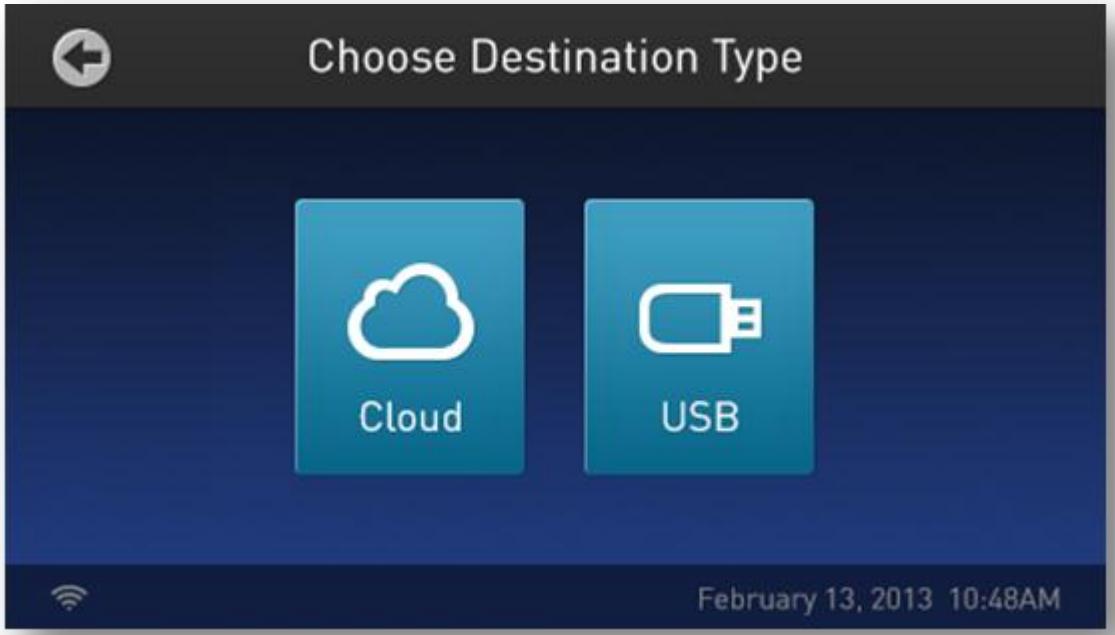


Thermal Protocol Status

Live Amplification Curves



Options to Upload Data



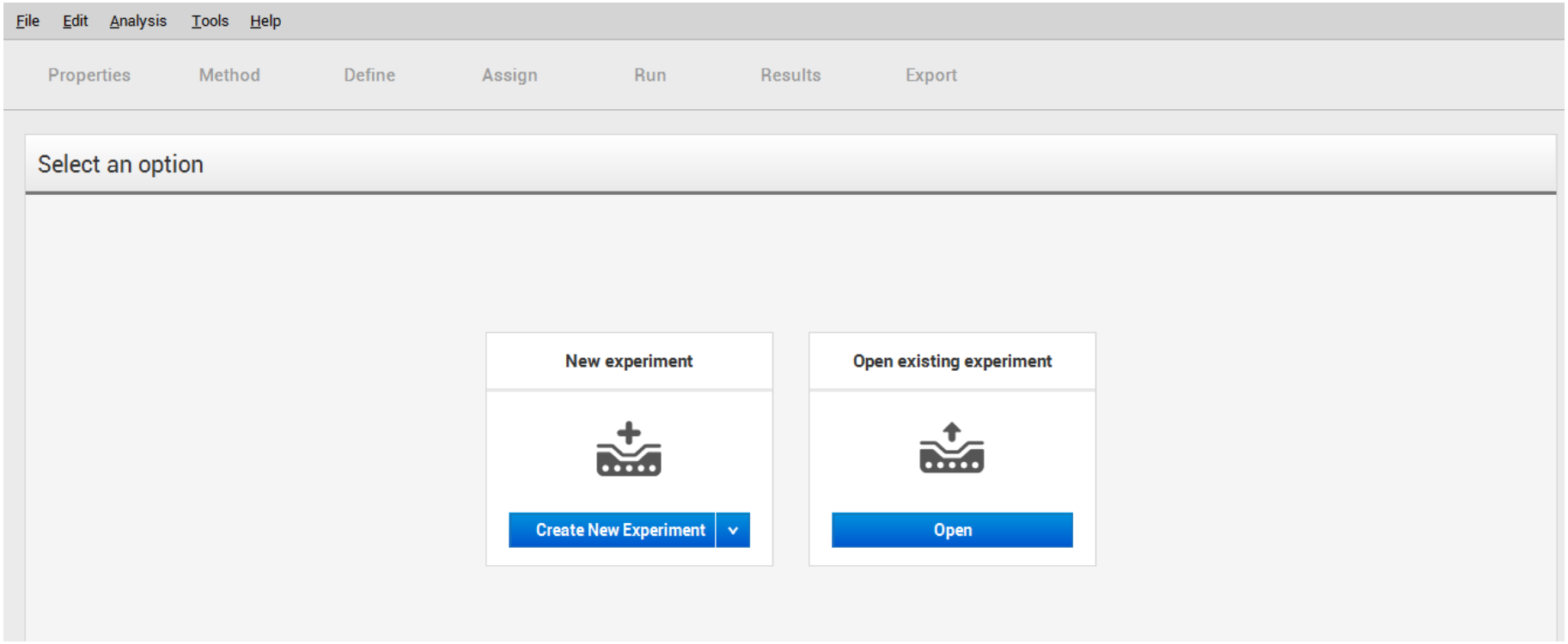
1. **Cloud = Data saved to user's online account**
2. **USB = Data saved to attached USB drive**
3. **Desktop = Data automatically saves back to desktop if run started from desktop**

QuantStudio™ Design and Analysis Software

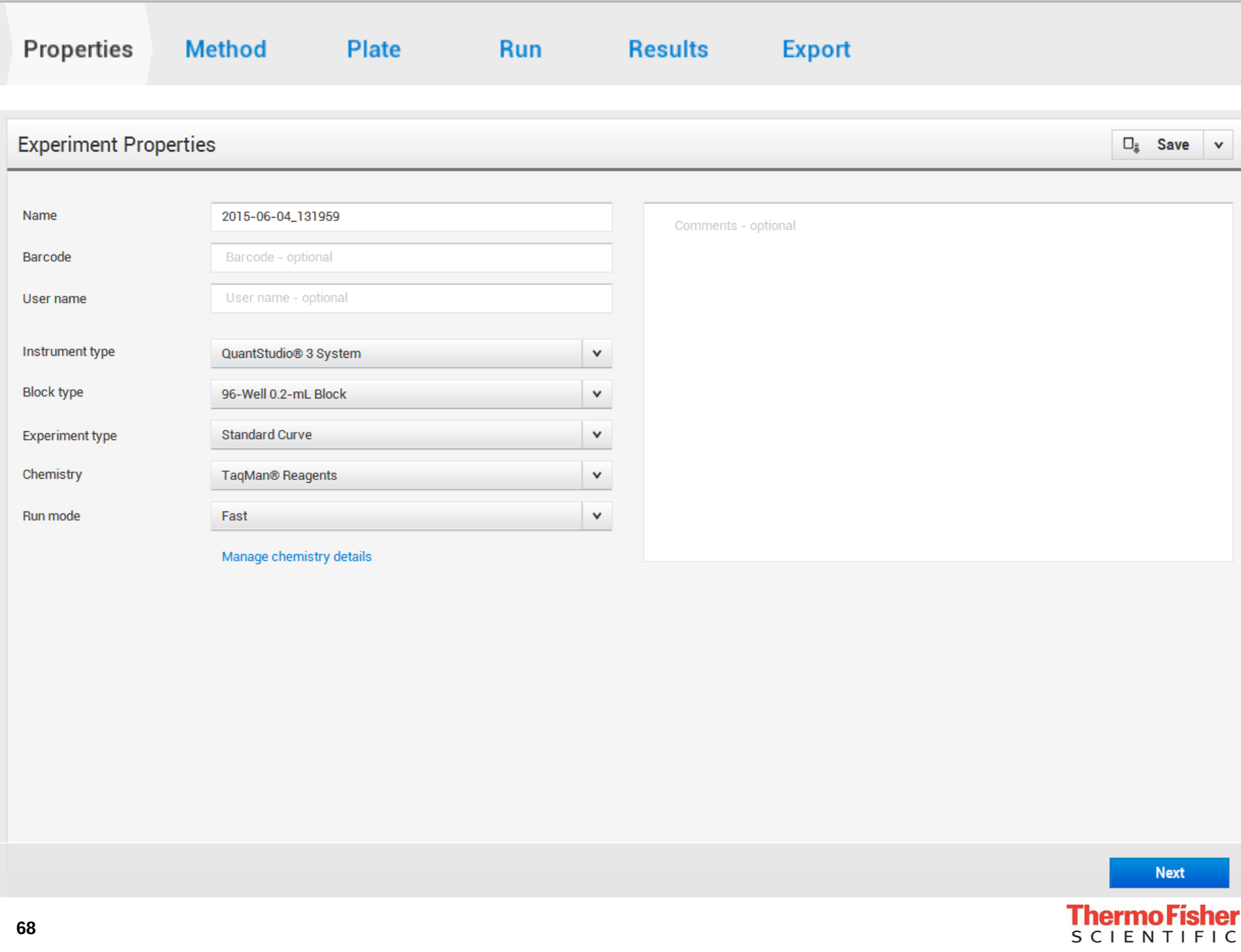
- QuantStudio™ Design and Analysis Software supports a variety of analysis methods, including:
  - Absolute Quantitation
    - Standard Curve
  - Relative Quantitation
    - Relative Standard Curve
    - Comparative CT (ΔΔ CT)
  - Presence/absence (Plus/Minus) assays with an internal positive control
  - Melt curve analysis
  - Genotyping (including real-time amplification)

- Multiplate GEx analysis available online on the QuantStudio Design and Analysis **Cloud** Software (https://www.thermofisher.com/tw/en/home/cloud.html)

QuantStudio™ Design and Analysis Software



- Similar look and feel as online software
- http://www.thermofisher.com/tw/en/home/technical-resources/software-downloads/ab-quantstudio-3-and-5-real-time-pcr-system.html



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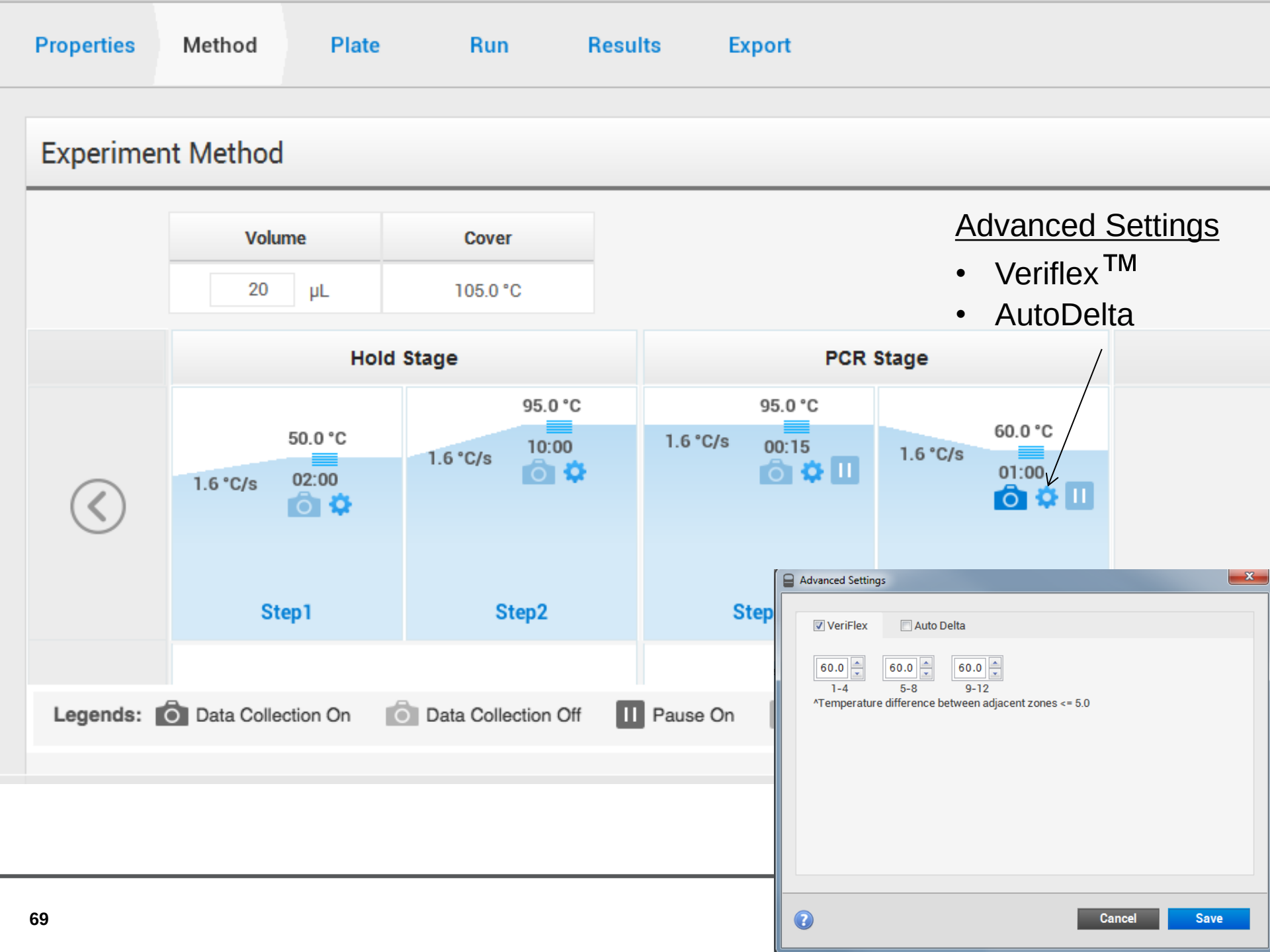
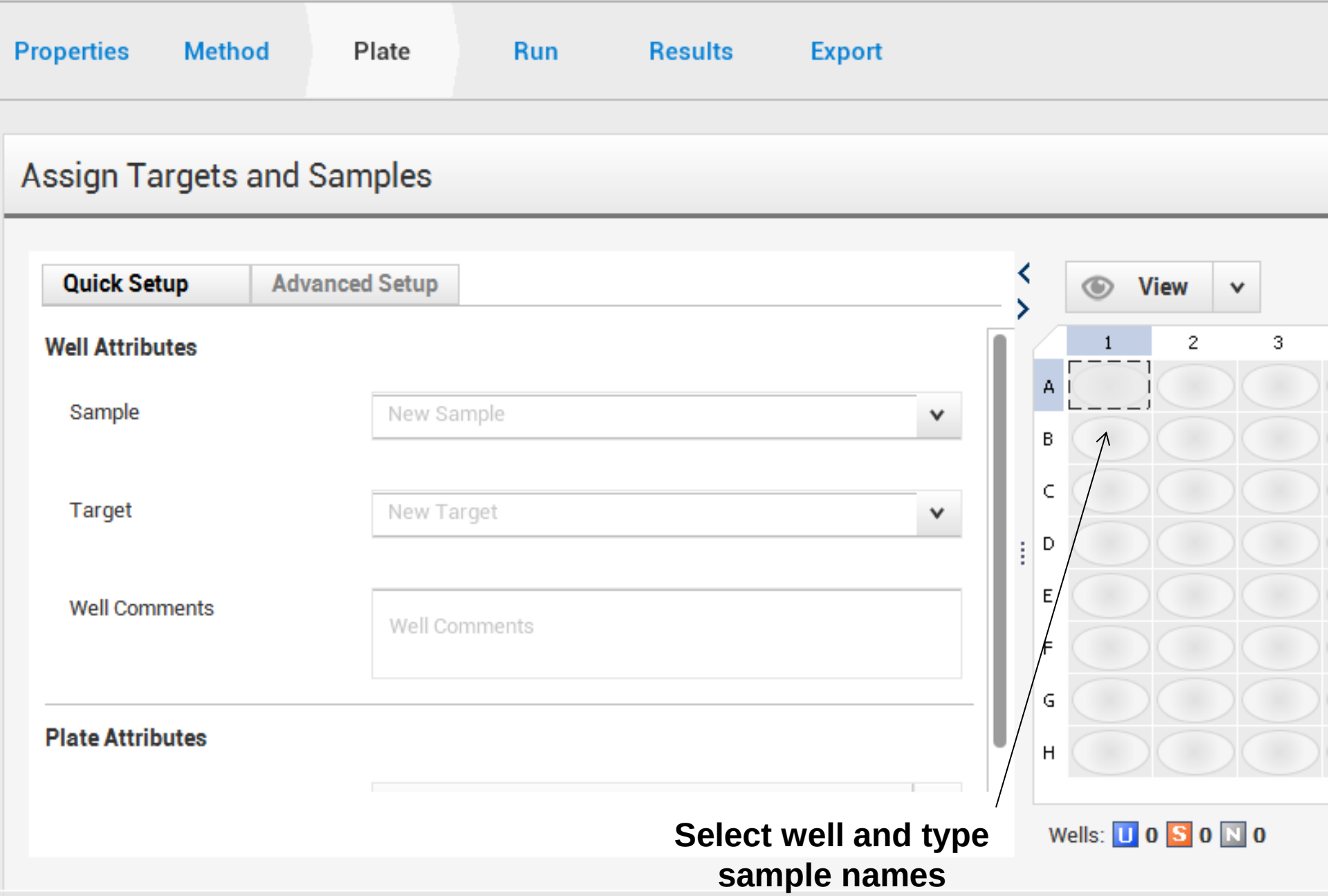
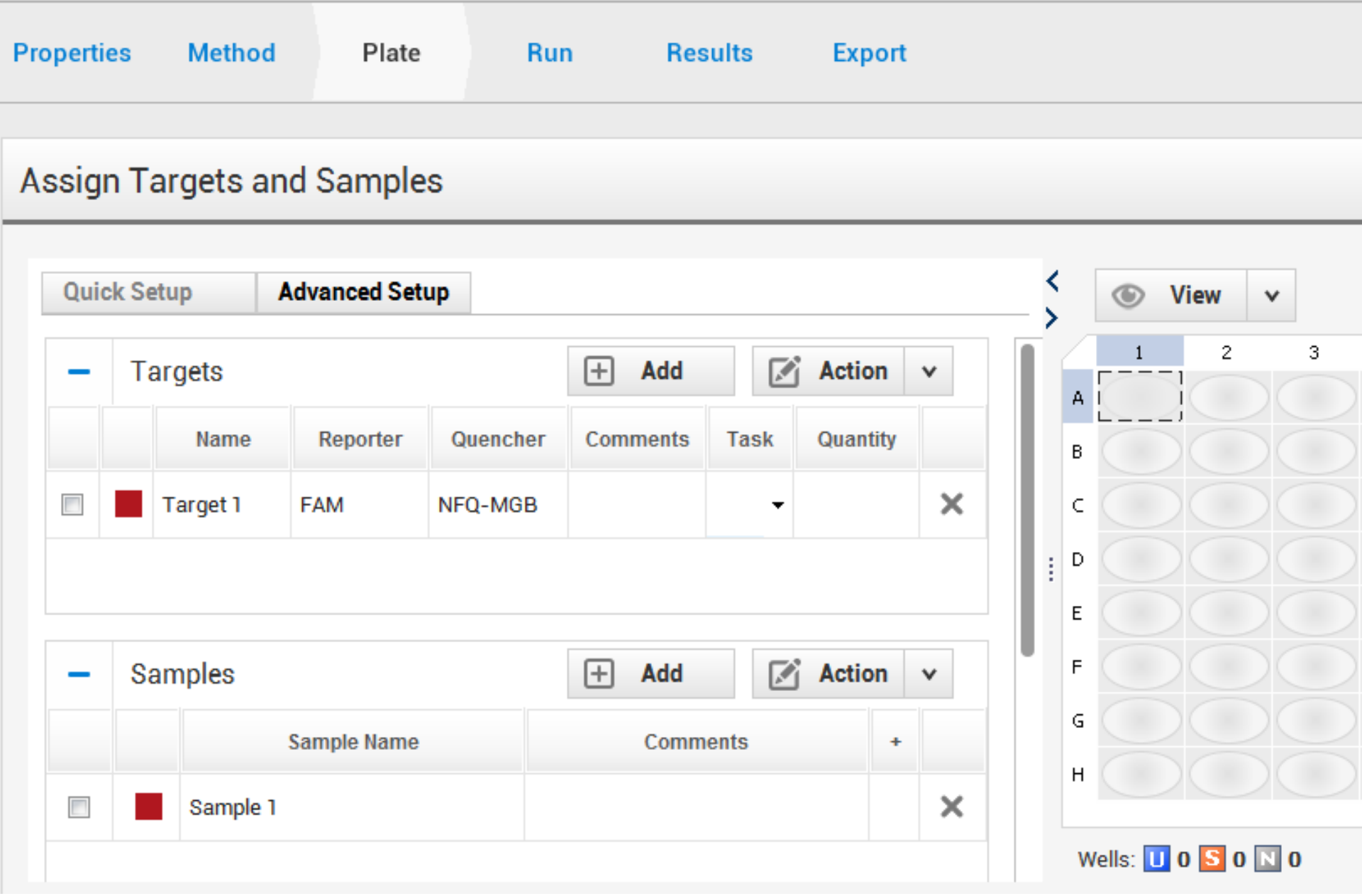

Properties
Method
Plate
Run
Results
Export
Experiment Method
Volume
Cover
20
µL
105.0 °C
Advanced Settings
• Veriflex TM
• AutoDelta
Hold Stage
PCR Stage
50.0 °C
1.6 °C/s
02:00
Step1
95.0 °C
1.6 °C/s
10:00
Step2
95.0 °C
1.6 °C/s
00:15
Step
60.0 °C
1.6 °C/s
01:00
Legends:
Data Collection On
Data Collection Off
Pause On
Advanced Settings
VeriFlex
Auto Delta
60.0
1-4
60.0
5-8
60.0
9-12
^Temperature difference between adjacent zones <= 5.0
Cancel
Save





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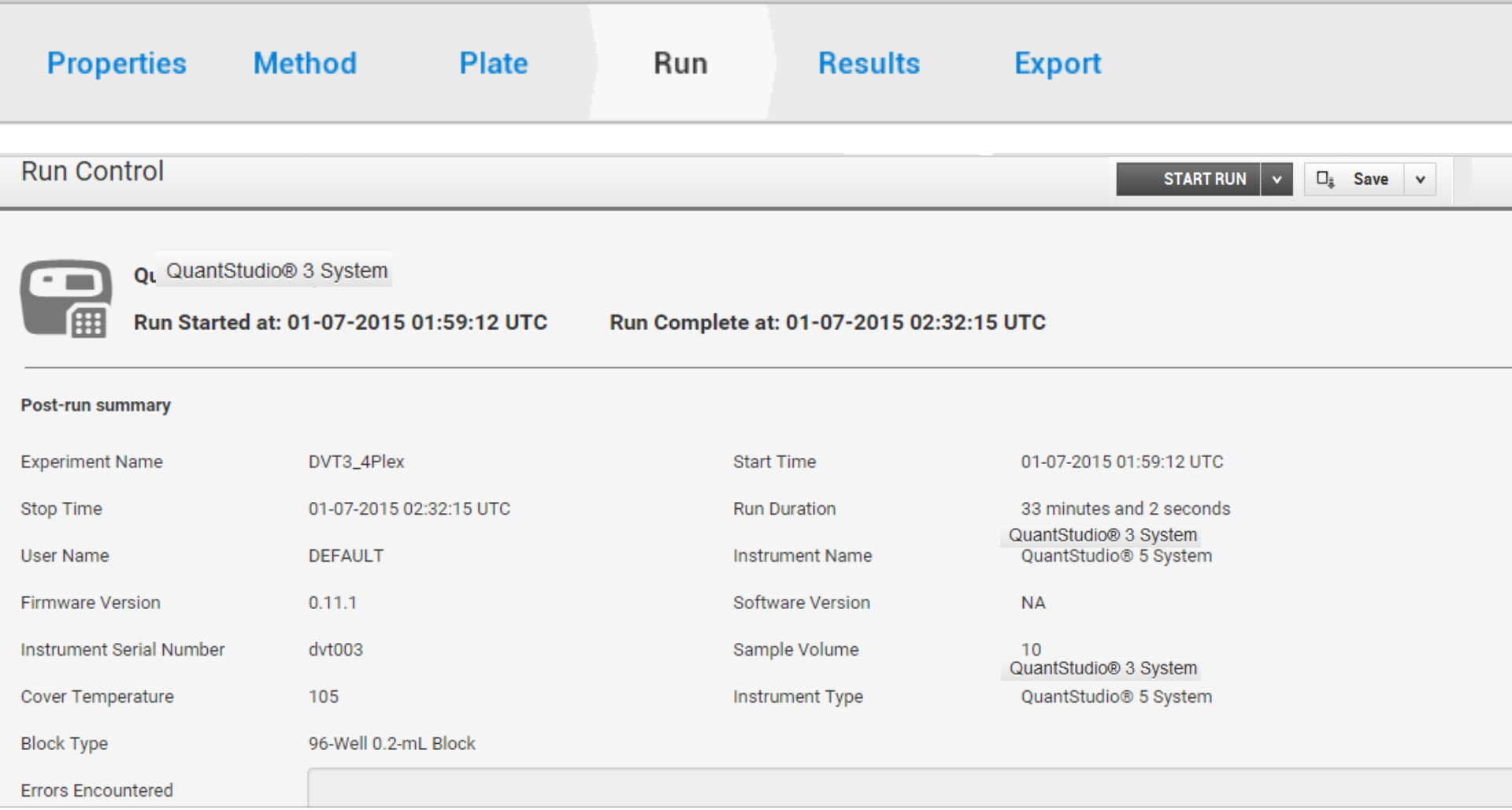
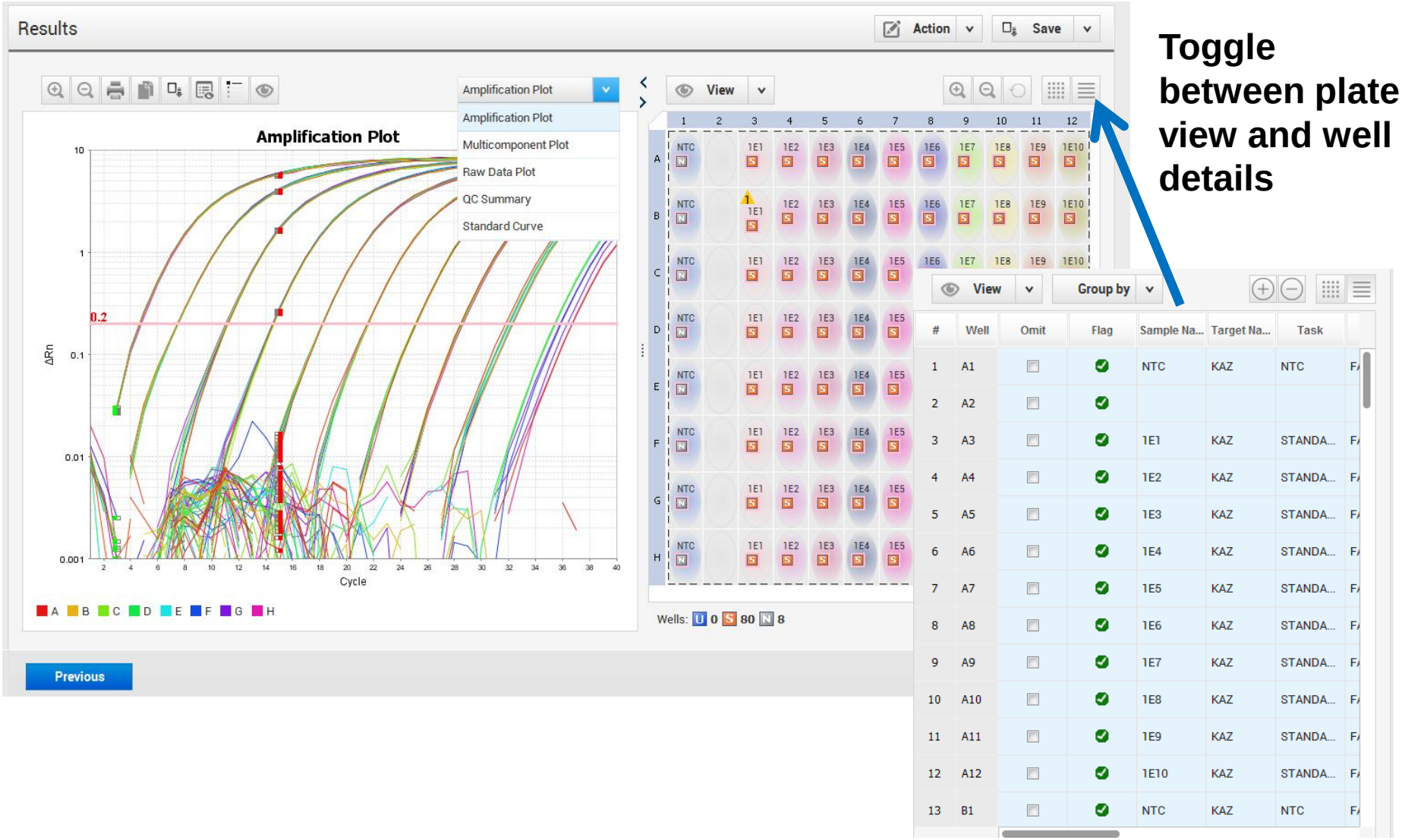


Start Run from touchscreen or desktop



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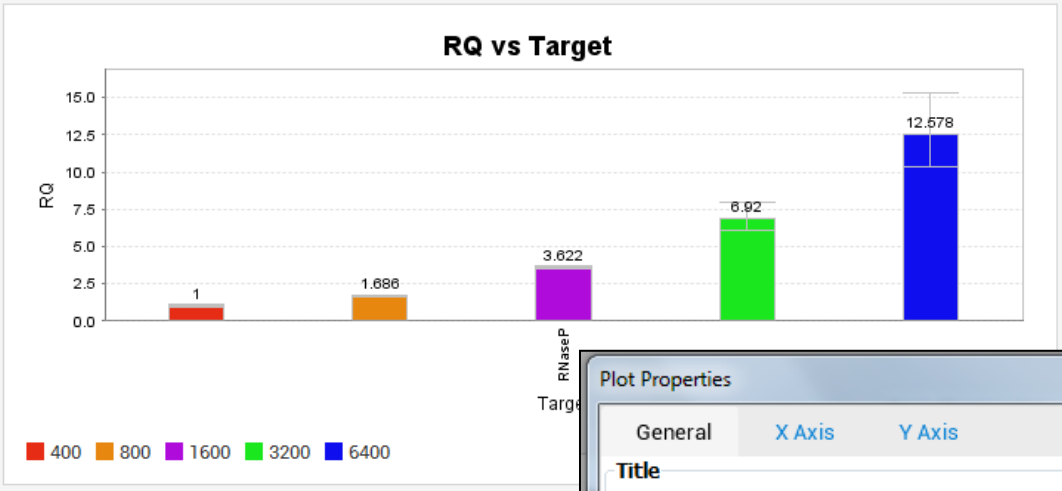
Properties Method Plate Run Results Export

Results

Action Save

Gene Expression View



#	Cт Mean	ΔCт Mean	ΔCт SD	ΔΔCт	RQ	RQ Min	RQ Max
1	25.455	-4.47	0.103	-3.653	12.578	10.308	15.3
2	29.925						
3	29.354	-0.817	0.068	0	1	0.903	1.1
4	27.379	-2.674	0.018	-1.857	3.622	3.528	3.7
5	28.391	-1.57	0.018	-0.753	1.686	1.641	1.7
6	26.38	-3.608	0.091	-2.791	6.92	6.041	7.9

Plot Properties

General X Axis Y Axis

Title

Text RQ vs Target

Font SansSerif.bold, 18 Select

Color 0, 0, 0

Show Title

Bar

Show Error Bar

Show Value Label

Show Target Label

Show Sample Label

20 Group Spacing in %

60 Bar Width in %

Cancel Save

Font Chooser

Roboto Regular

Tw Cen MT Condensed Extra Bold
Utsaah
Vani
Verdana
Vijaya
Viner Hand ITC
Vivaldi
Vladimir Script
Vrinda
Webdings
Wide Latin
Wingdings
Wingdings 2
Wingdings 3
Roboto Regular

PLAIN

Plain
Bold
Italic
Bold/Italic

13

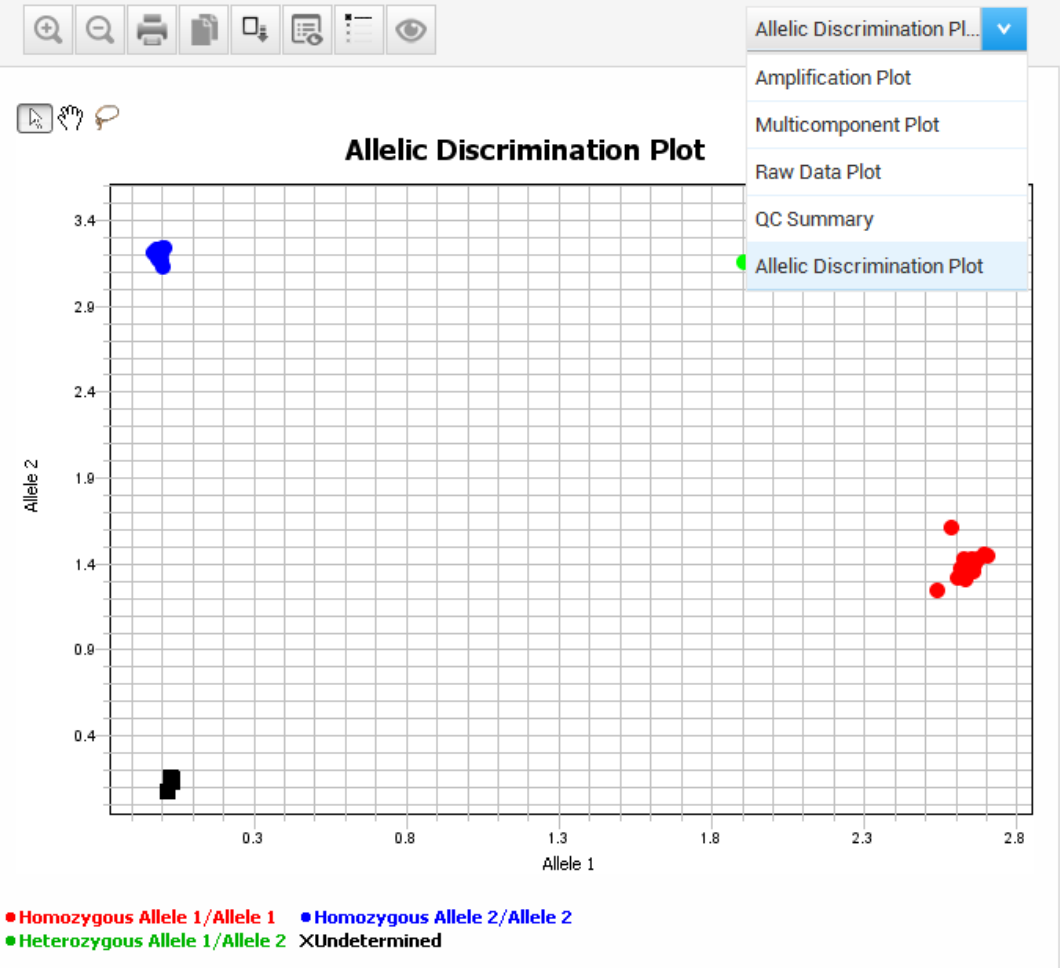
13 14 15 16 17 18 19 20 22 24 27 30 34 39 45

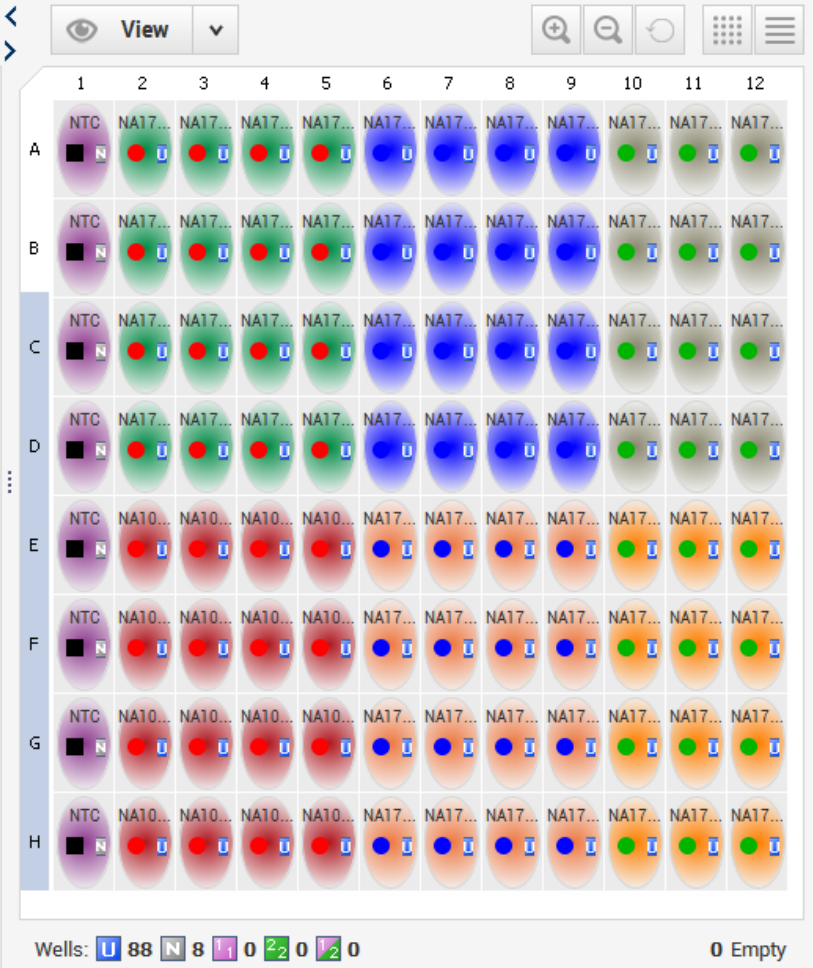
The quick brown fox jumps over the lazy dog.

OK Cancel

Results

Action Save





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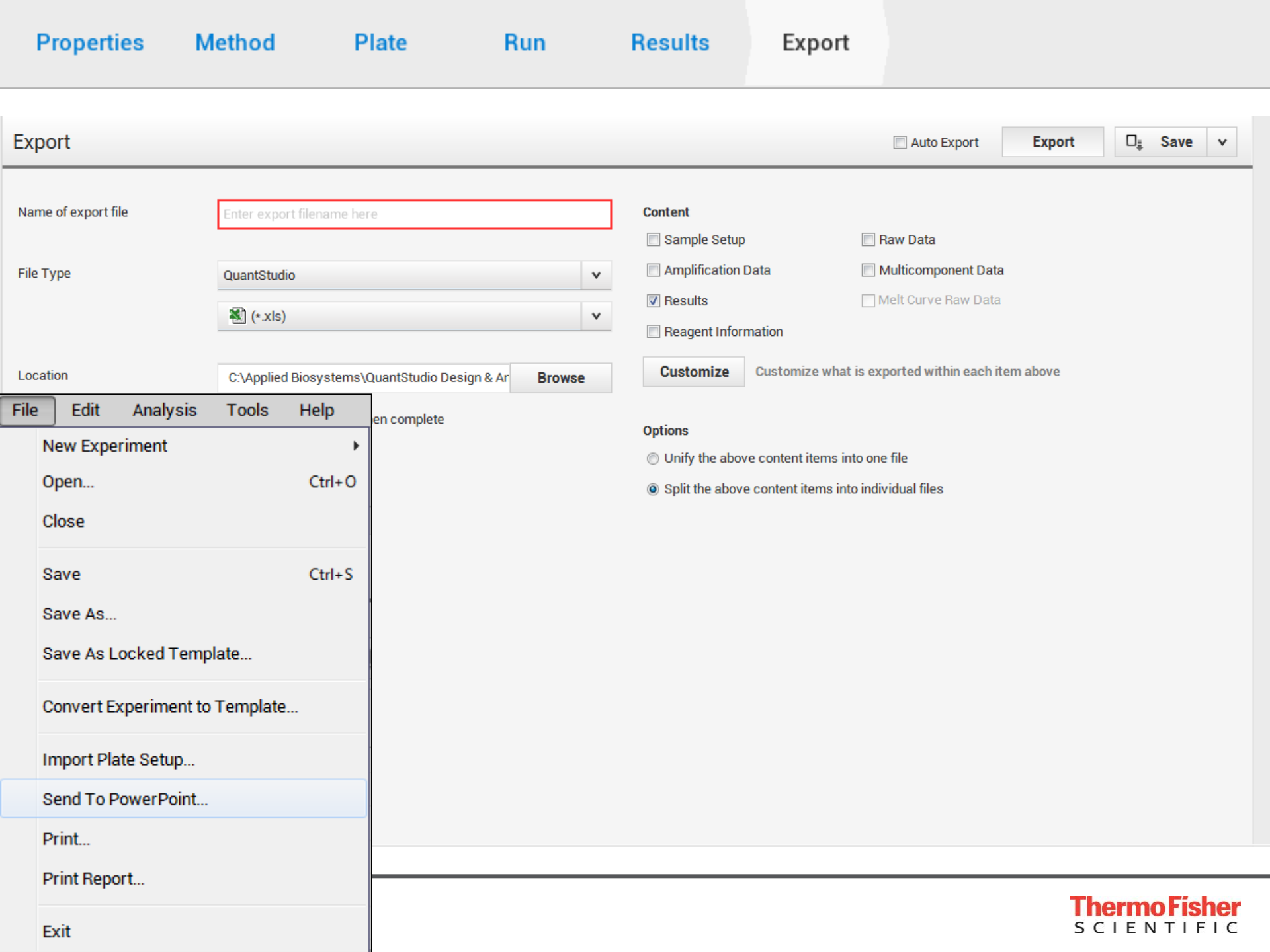
Properties
Method
Plate
Run
Results
Export
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Auto Export
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Name of export file
Enter export filename here
File Type
QuantStudio
(*.xls)
Location
C:\Applied Biosystems\QuantStudio Design & Ar
Browse
Content
Sample Setup
Raw Data
Amplification Data
Multicomponent Data
Results
Melt Curve Raw Data
Reagent Information
Customize
Customize what is exported within each item above
Options
Unify the above content items into one file
Split the above content items into individual files
File
Edit
Analysis
Tools
Help
New Experiment
Open... Ctrl+O
Close
Save Ctrl+S
Save As...
Save As Locked Template...
Convert Experiment to Template...
Import Plate Setup...
Send To PowerPoint...
Print...
Print Report...
Exit
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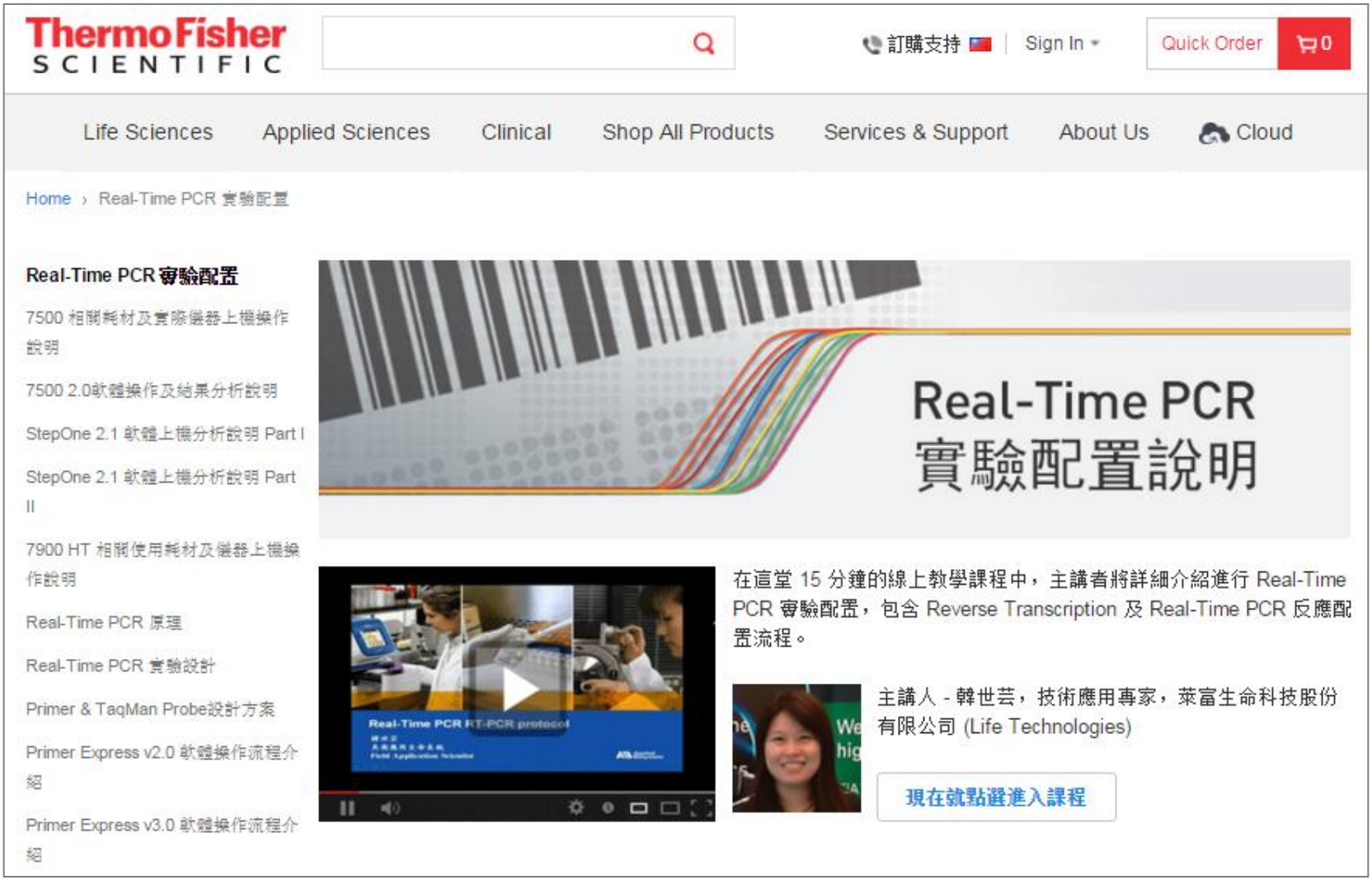
Real-time PCR 中文線上講座

http://www.thermofisher.com/tw/en/home/taiwan/real-time-pcr-webinars/real-time-pcr-experimental-configuration.html



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Thank You!

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