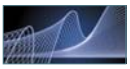

Bio-Rad Real-Time PCR Detection System

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Version 1.0

AMPLIFICATION

Outline

- Part I: What is the real-time PCR?
  - Real-time PCR introduction
- Part II: Bio-Rad Real-Time PCR Detection System

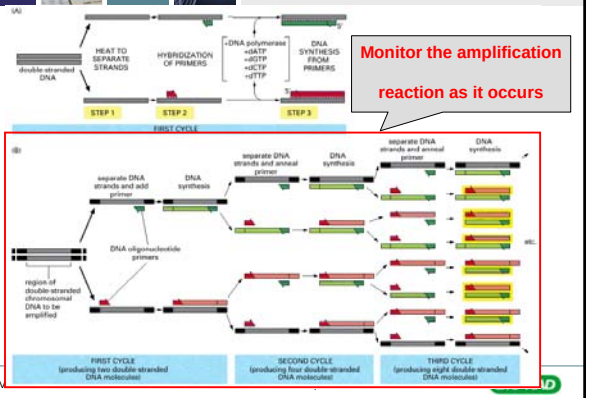
AMPLIFICATION

主要應用領域

- 基因表現
  - 藥物作用
  - 腫瘤指標
  - 基因調控
  - 基因治療
  - 微陣列基因晶片確認
  - 基因改造食品（GMO）
- 病原菌偵測
  - 多重病原菌同時偵測（Up to 5-Target）
  - 適合大量檢體篩選（High throughput）
  - 定性，定量 同時完成
  - 藥物治療成效的監控
- SNP 分型
  - 唯一可用 Taqman, Molecular Beacon 以及 FRET 三種方法的品牌

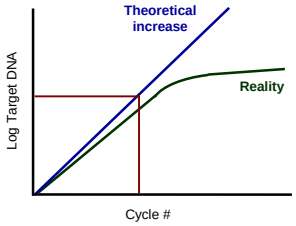
AMPLIFICATION

What is qPCR(Real Time PCR)?

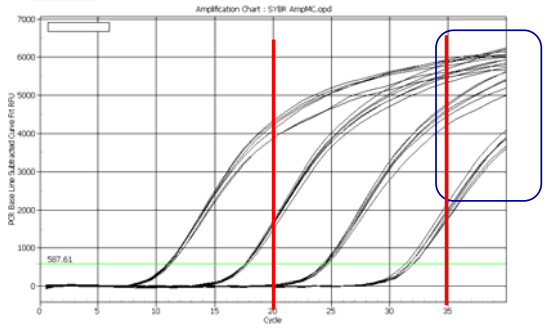


PCR: Theory vs. Reality

- In theory, PCR reactions amplify exponentially, doubling every cycle and grow forever.
- In reality,

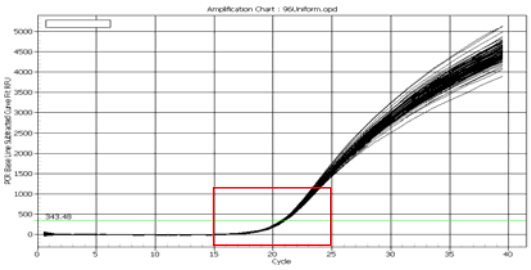


End Point Measurements



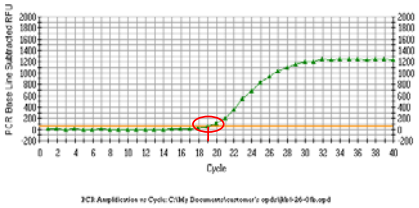
100 fold dilution series

96 Identical replicates

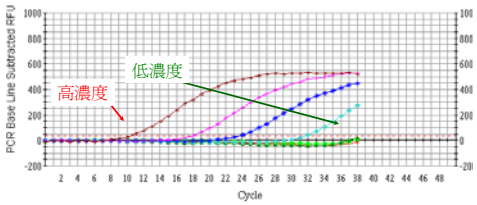


Threshold Cycle, C_T

The point at which the fluorescence rises appreciably above background

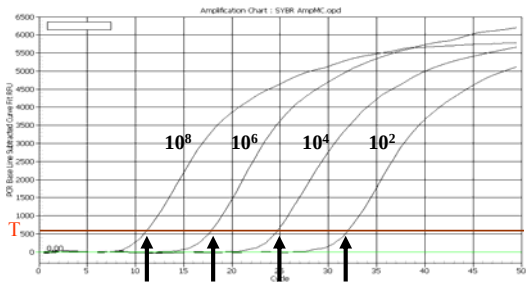


Threshold Cycle (Ct) 與起始濃度的對數成反比

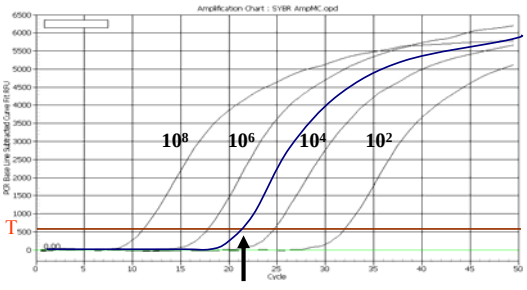


- Assumes 100% efficiency
- 1 cycle = 2 fold difference
- 3.3 cycles ≅ 10 fold difference

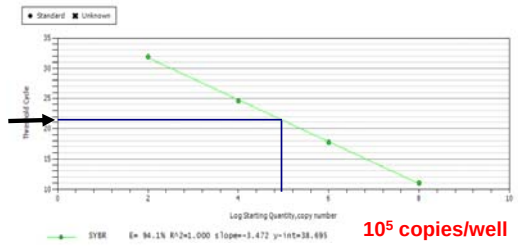
Absolute quantification



Absolute quantification

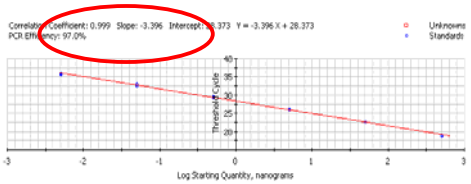


Absolute quantification



10^5 copies/well

C_T is linear with the log of starting copy number



The **slope** of the standard curve can be directly correlated to the **efficiency** of the reactions:

Efficiency (η) = $[10^{(-1/slope)}]$ - 1
when slope = -3.32, Efficiency = 100%

Slope = - [1/log (1+E)]

log (1+E) = - (1/slope)

$E = 10^{[-1/slope]} - 1$

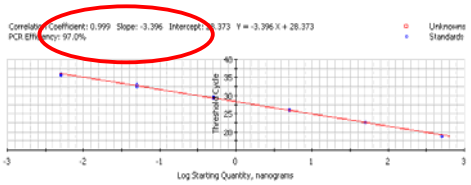
E	Slope
0.5	-5.679
0.6	-4.899
0.7	-4.339
0.8	-3.917
0.9	-3.587
1	-3.322
1.1	-3.103
1.2	-2.920
1.3	-2.765

Aim for Efficiency Values:

Good = 90 – 110%

Fantastic = 95 – 105%

C_T is linear with the log of starting copy number

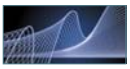


r = is a measure of how well the actual data fit to the standard curve. = (explained variation/total variation)

Aim for R value (Correlation Coefficient) of ≥0.98

Points to Remember

- **Threshold Cycle values (C_T) have a direct relationship to the amount of starting template**
- **100% efficiency - 2^n = fold dilution**
- **Efficiency of reactions between 90-110%**
- **R value should be *≥0.98***



qPCR Chemistry

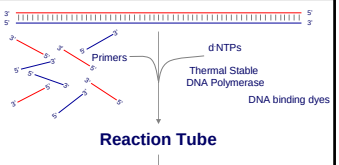
Version 1.0

Real-Time PCR

- These fluorescent molecules can be used
  - Non-specific DNA binding dyes
    - SYBR® Green
    - EvaGreen™
  - Specific Hybridization Probes/Primers
    - TaqMan™
    - molecular beacons
    - dual-oligo FRET pairs
    - Scorpions™/Amplifluor™ /LUX™

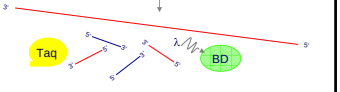
DNA binding dyes

Add Master Mix & Sample



Reaction Tube

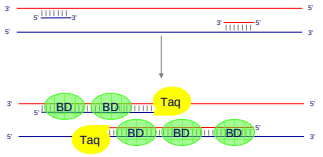
Denaturation



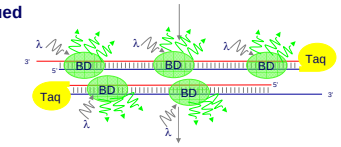
Annealing

DNA binding dyes

Extension



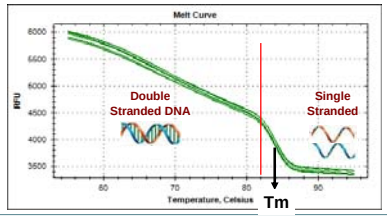
Extension Continued
Apply Excitation Wavelength



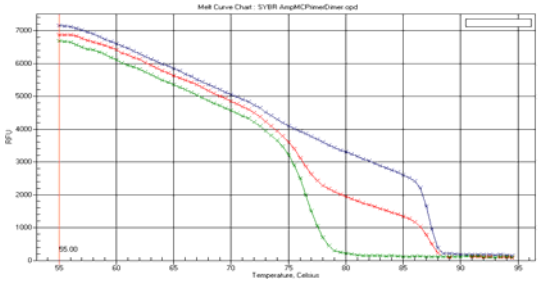
Repeat

Melt Curve Analysis

- After real-time PCR amplification, a melt curve is performed where we monitor fluorescence as temperature increases.
- Melting temperature (Tm)
  - DNA is half double and half single-stranded
  - Depends on nucleotide content (GC-AT ratio) and length

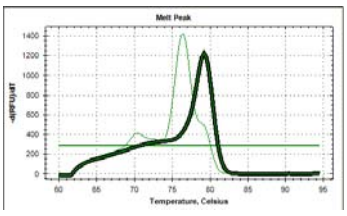


Melt Curve Analysis

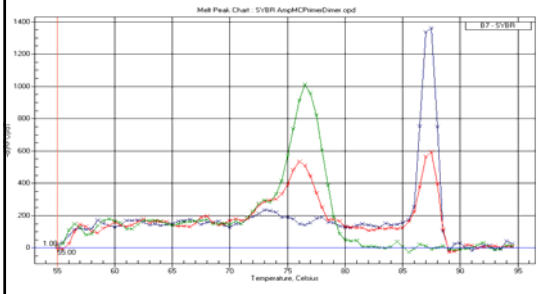


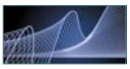
Melt Curve Analysis

- Distinguish products based on their Tms
  - Plot negative rate of change of fluorescence vs. temp (-dI/dT) for easy discrimination of products based on their Tms



Melt Curve Analysis



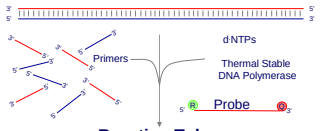


TaqMan –
the “Hydrolysis” probes

Version 1.0

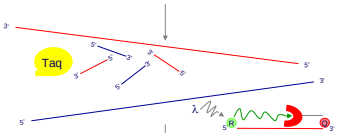
Cleavage-based assay: TaqMan

Add Master Mix & Sample



Reaction Tube

Denaturation



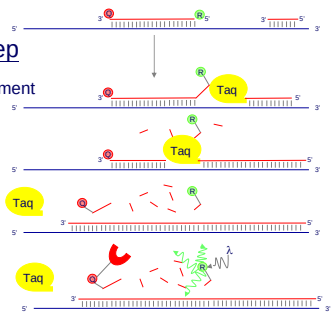
Annealing

Version 1.0 www.bio-rad.com/pcr

Cleavage-based assay: TaqMan

Extension Step

1. Strand Displacement
2. Cleavage
3. Polymerization Complete
4. Detection



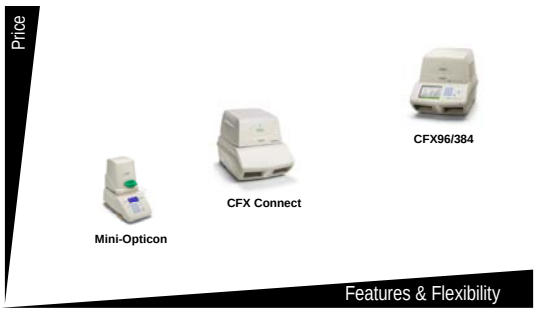
Version 1.0 www.bio-rad.com/pcr

Which Method To Use?

- Each method has advantages and disadvantages.
- One method may be more appropriate for an application over another
  - microarray, SNP, limited sample….
- Bio-Rad Real-Time Instrumentation is equipped to handle all chemistries.

Version 1.0 www.bio-rad.com/pcr

Biorad Real-time PCR Instruments

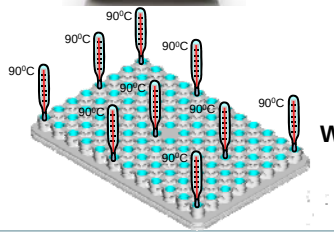


Tips for buying a real-time PCR

- **Instrument performance**
- **Multiplexing capability**
- **Ease of use**
- **Flexibility of the consumables**
- **Customer support**
- **Warranty & ongoing service contract**

Instrument performance

Fast run times



Well-to-well variability

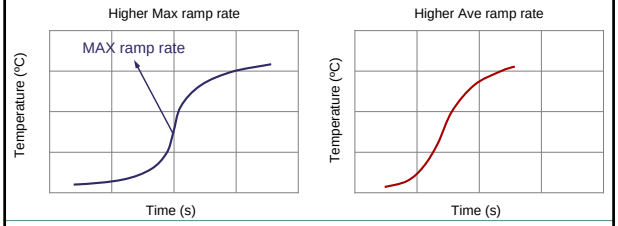
Next Generation Thermal Cycling

- CFX96 builds on the precise thermal control of the C1000
  - Maintain uniformity even while ramping
  - 10 second settling - the time it takes all wells to reach temperature - is unsurpassed in the market

Max ramp rate	5°C/sec
Average ramp rate	3.3°C/sec
Temp Accuracy	± 0.2°C
Temp Uniformity	± 0.4°C in 10 sec
Temp Range	0-100°C

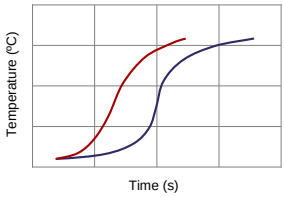
Max vs Average Ramp Rate

- Max ramp rate is achieved only at the steepest section of the curve, it is not maintained during the ramping period
- It takes time to ramp up to the max rate and then time to slow down to the next temperature



Max vs Average Ramp Rate

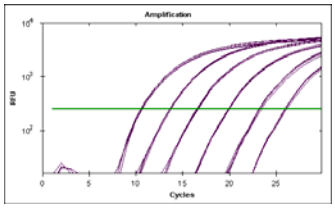
- A cycler ramping with a higher average ramp rate reaches target temperature faster than a cycler ramping with a higher Max ramp rate



FAST PCR with SYBR Green

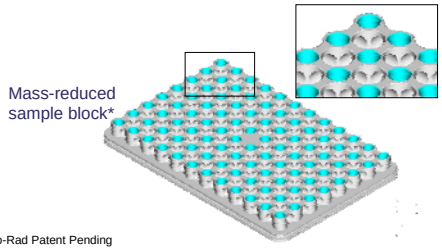
- Serial dilution of target from 10^8 to 10^3 copies
- Target detected using SYBR Green
- Amplification run time: 24 minutes

Protocol
95ºC for 20 sec,
93ºC for 3 sec,
60ºC for 8 sec + Plate Read
GOTO 2, 29 X more times



Next Generation Fast Block Design

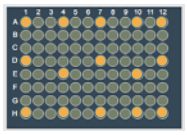
- The honeycomb architecture promotes uniform heating and cooling of all wells of the block, even during ramping



* Bio-Rad Patent Pending

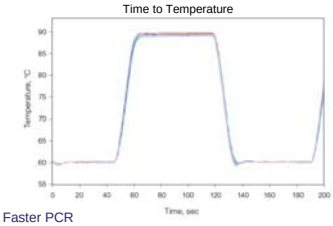
Unparalleled Temperature Uniformity

- Wells heat and cool at the same average rate to ensure block uniformity and to achieve a 10 second settling time
- The minimal overshoot ramping up and undershoot ramping down prevents non-specific amplification due to improper primer binding



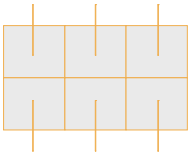
Probe location

Time to Temperature



Uniform ramping + shorter settling times = Faster PCR

Thermal Reaction Module



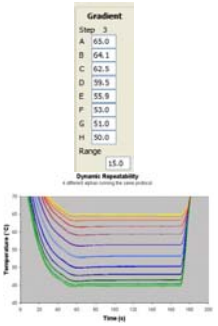
6 Thermo Electrics (TEs) - Independent zones of thermal control, each with its own temperature sensor, provides uniform and reliable control of temperature across the block

Bio-Rad patented O-ring hermetic seal forms an airtight barrier around the TE and extends the life of the heating and cooling elements

*Patented by Bio-Rad

Thermal Cycler Gradient

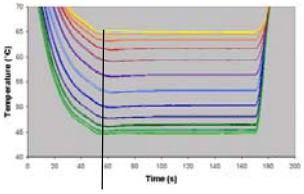
- Annealing temperature is critical for reaction specificity and efficiency
- Save valuable time optimizing annealing in a single experiment
  - Program up to a 24˚C gradient
  - Back-to-front (back row is hotter)
  - 30-100˚C range
  - "Dynamic Ramping" - Wells hit temperature set point together, and then maintain the same dwell time to eliminate time as a reaction variable



Color traces of temperature measurements from different columns on C1000 with 96Fast

Dynamic vs. Steady Slope

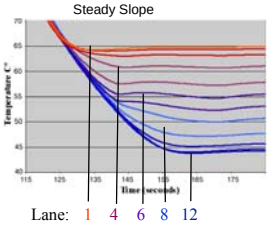
1000 Series Dynamic Gradient



ALL LANES reach target temp. at the same time

End user specifies the temperature **and** time, the ramp rate varies

Steady Slope



Lane: 1 4 6 8 12

Each lane reaches target temp. at a different time, ~30 second difference

End user specifies temperatures but has to use software to figure out ideal temp and hold time

Amplicon Secondary Structures

http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold

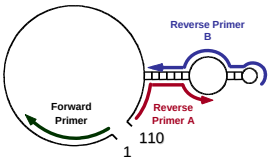
- Bad location for primers

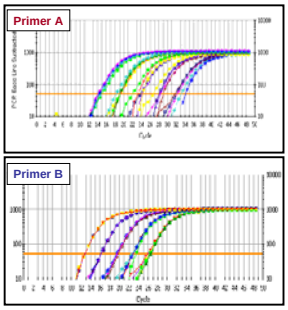
- Good location for primers

Effect of primer location

Primer A: η = 66.3 %

Primer B: η = 95.8 %



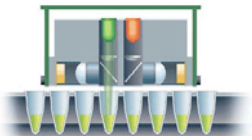


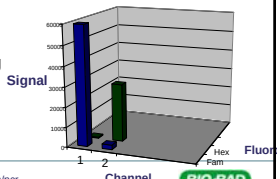
Tips for buying a real-time PCR

- Instrument performance
- **Multiplexing capability**
- Ease of use
- Flexibility of the consumables
- Customer support
- Warranty
- Ongoing service contract

Next Generation Optical Technology

- CFX connect uses a scanning shuttle
  - 3 filtered LEDs for excitation
  - 3 filtered photodiodes for detection
- Save samples and reagent costs by multiplexing up to 2 targets
- Maximal excitation of dyes with a fixed optical path for all wells and no cross talk for better multiplexing
  - One LED fires at a time to independently illuminate and then detect fluorescence in a channel

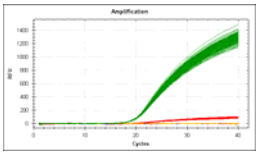




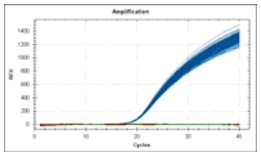
Discrete Excitation and Detection

- Color separation yields pure dye signal in each channel

Non-color separated



Color separated

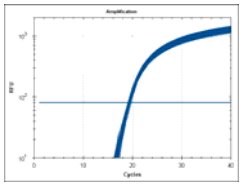


Filter Sets

Channel	Excitation (nm)	Detection (nm)	Calibrated Fluorophores
1	450-490	515-530	FAM™, SYBR Greem I™
2	515-535	560-580	VIC®, HEX™, TET™, Cal Gold 540™
3	450-490	560-580	Accommodates FRET Chemistry

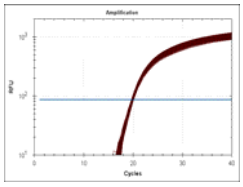
Excellent Uniformity at 10µl

FAM



Ave Ct = 19.29 ± 0.10
Max-Min =0.58

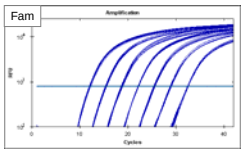
All Channels



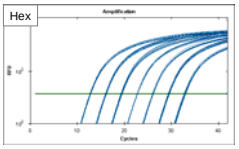
Ave Ct = 19.81 ± 0.10
Max-Min =0.53

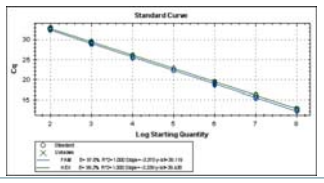
2-Target Multiplexing

Fam



Hex





Reaction efficiencies range from 97-99%

Tips for buying a real-time PCR

- Instrument performance
- Multiplexing capability
- **Ease of use**
- Flexibility of the consumables
- Customer support
- Warranty& ongoing service contract

Start a run in just 3 steps

Protocol setup

Channel setup

Run

Add Repeats

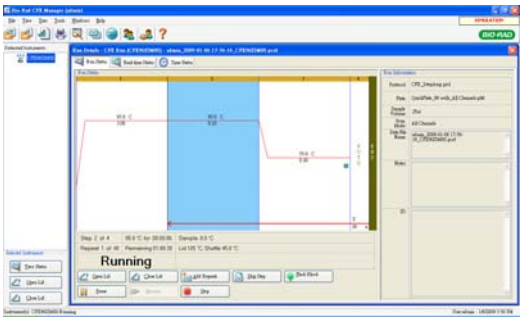
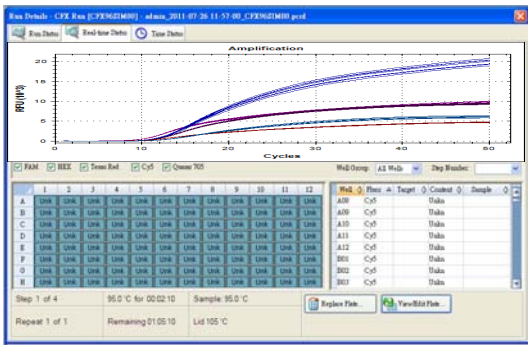
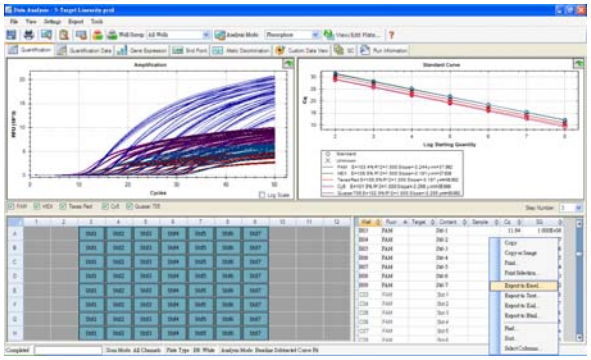


Plate view and setup in anytime



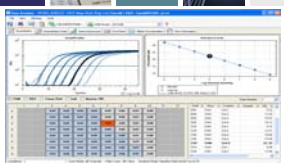
AMPLIFICATION

Click right to export what you want



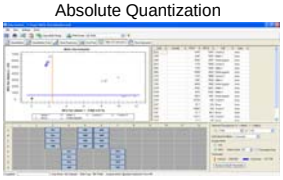
AMPLIFICATION

Data Analysis

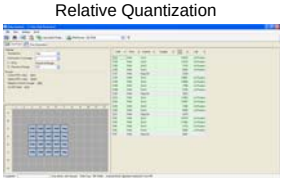


Absolute Quantization

Relative Quantization



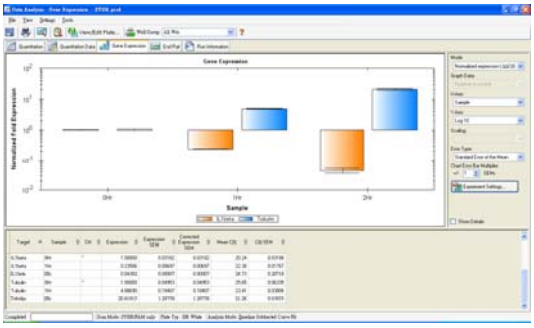
Allelic Discrimination



End Point Analysis

AMPLIFICATION

Relative Quantization



AMPLIFICATION

Gene Expression Normalization - Comparative C_T

- Relative Quantity (Δ CT)
  - Not normalized
  - Normalization accomplished via equal loading of samples
  - Post analysis normalization
- Normalized Expression ($\Delta\Delta$ CT)
  - Accounts for loading differences
  - Usually normalize to reference gene
  - Relative quantity of GOI is normalized by the relative quantity of the reference genes

Normalized Expression

- ΔΔCT
  - Assume 100% efficiency
  - Only one Ref Gene
- Pfaffl Modification
  - Accounts for efficiency differences
  - Only one Ref Gene
- Vandesompele Method
  - Accounts for efficiency differences
  - Allows multiple reference genes for normalization

Simple

Complex

Relative Quantification - ΔCt

	GOI
Tissue #1:	22
Tissue #2:	24
Delta Ct:	24-22 = 2

Fold induction = $2^2 = 4$

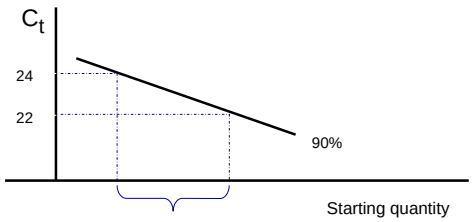
Comparative Ct Method (2-Δ Δ Ct)

		Reference	GOI
	Tissue #1:	21	22
	Tissue #2:	20	24
1st Delta	Delta Ct #1:		22-21 = 1
	Delta Ct #2:		24-20 = 4
2nd Delta	Delta Ct:		4-1 = **3**

Fold induction = $2^3 = 8$

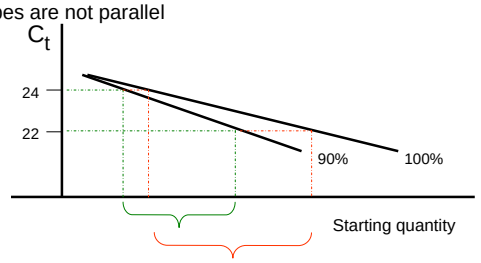
Relative Quantification

- Problem with the ΔΔCT



Relative Quantification

- Problem with the ΔΔCT
- Slopes are not parallel



Relative Quantification

$$\text{Fold induction} = \frac{\text{Efficiency}_{\text{target}}^{\text{deltaCt }_{\text{target}}\text{ (control-sample)}}}{\text{Efficiency}_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\text{ (control-sample)}}}$$

(Pfaffl, 2001; Nucleic Acid Research)

$$\text{Efficiency} = 10^{-1/\text{slope}}$$

Relative Quantification –Pfaffl Modification

		Primer set #1Reference	Primer set #2 GOI
	Tissue #1:	21	22
	Tissue #2:	20	24
(From Standard curve)	Efficiency:	90% = 1.9	100% = 2
	Delta Ct:	20-21 = -1	24-22 = 2

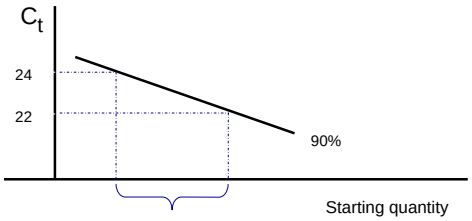
$$\text{Fold induction} = \frac{2_{\text{target}}^{\text{deltaCt }_{\text{target}}\text{ (24-22 = 2)}}}{1.9_{\text{reference}}^{\text{deltaCt }_{\text{reference}}\text{ (20-21 = -1)}}} = \frac{4}{0.53} = 7.5$$

Methods Comparison

- Δ Ct method: (no reference gene)
  - Fold induction : 4
- ΔΔ Ct method: (reference gene)
  - Fold induction : 8
- Pfaffl modification: (reference gene and efficiency)
  - Fold induction : 7.5

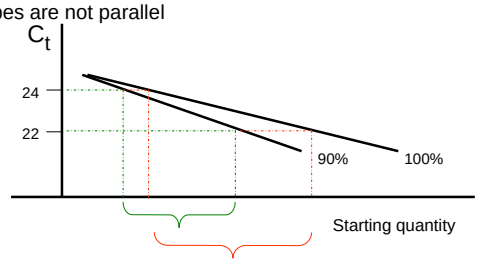
Relative Quantification

- Problem with the ΔΔCT

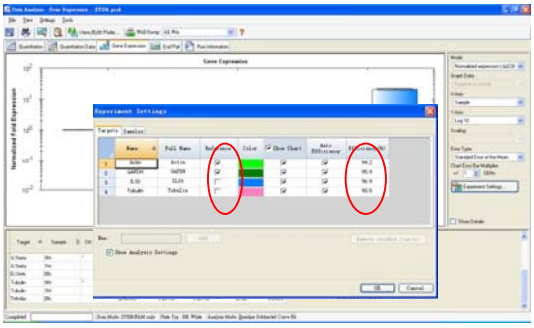


Relative Quantification

- Problem with the ΔΔCT
- Slopes are not parallel

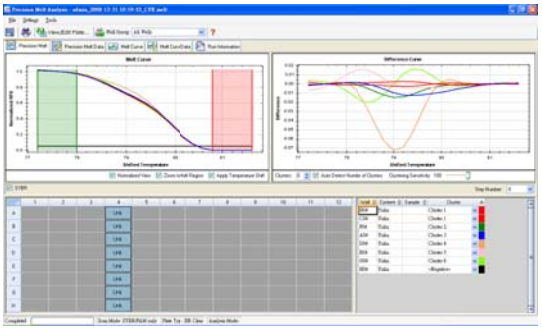


Multiple reference genes and efficiency Normalization in RQ



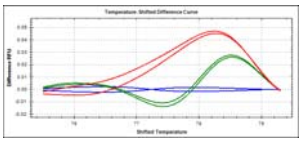
Power of gene study

High Resolution Melting

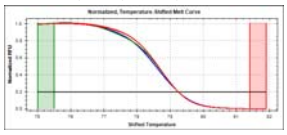


HRM mutation analysis

Lung cancer screen in *KRAS* mutation on CFX96
(Dr Chen in SSH)



SsoFast EvaGreen Supermix Protocol
98°C – 2 mins
(40 cycles)
98°C – 5 sec
61°C – 10 sec
(Melt)
95°C – 30 sec
75°C – 5 sec (+0.2°C/ cycle to 95°C)

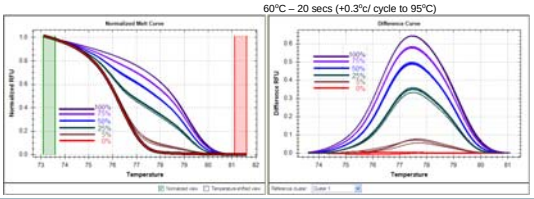


Well	Content	Sample	Cluster	Percent Confidence
A05	Uaku-1	A549-92bp	Cluster 1	99.7
B06	Uaku-1	A549-92bp	Cluster 1	99.7
C06	Uaku-2	HCT116_92bp	Cluster 2	96.8
D06	Uaku-2	HCT116_92bp	Cluster 2	97.2
E06	Uaku-3	48T-92bp	Cluster 3	96.5
F06	Uaku-3	48T-92bp	Cluster 3	97.3
G05	NTC	NTC	<Excluded>	N/A
B06	NTC	NTC	<Excluded>	N/A

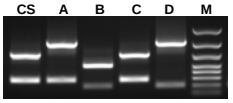
HRM Methylation analysis

Methylation studies on CFX96
(Dr Hua-Chien Chen in CGU)

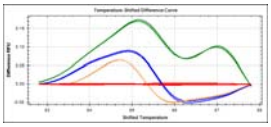
Protocol
95°C – 10 mins
(30 cycles)
95°C – 15 sec
60°C – 30 sec
72°C – 15 sec
(Melt)
95°C – 10 secs
60°C – 20 secs (+0.3°c/ cycle to 95°C)

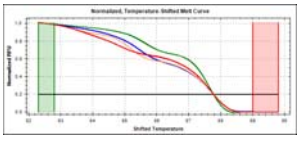


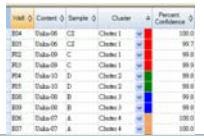
Bio-Rad Crime Scene Investigator PCR kit



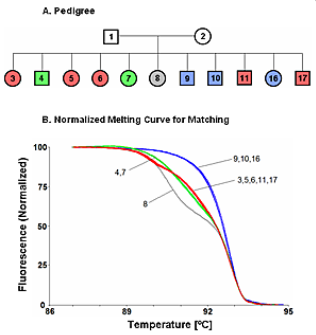
SsoFast EvaGreen Supermix Protocol
94°C – 2 mins
(40 cycles)
94°C – 30 sec
52°C – 30 sec
72°C – 60 sec
(Melt)
65°C – 10 sec (+0.2°C/ cycle to 95°C)





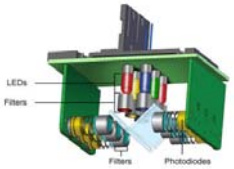


High-Resolution Melting in HLA-A



The figure shows an example of matching 11 siblings (CEPH family UT1331) using the HLA-A locus. The siblings separate into four matched groups.
(*Zhou et al 2004*)

Low maintenances



Hassle free performance, LEDs do not degrade, so no need to change the light source or recalibrate

Auto- self test while turn on and thermal validation service as requested

Tips for buying a real-time PCR

- Instrument performance
- Multiplexing capability
- Ease of use
- **Flexibility of the consumables**
- Customer support
- Warranty & ongoing service contract

Open system

CFX96 fit all kinds of reagents and plastics (low-profile)

Tips for buying a real-time PCR

- Instrument performance
- Multiplexing capability
- Ease of use
- Flexibility of the consumables
- **Customer support**
- **Warranty & ongoing service contract**

Services of instruments

- **Customer supports**
  Bio-Rad Taiwan: Specialist X 2, Engineer X 4
  Genmall Biotech: Specialist X 6, Engineer X4
- **Warranty & ongoing service contract**
  By contract

A new beginning

Clinical Chemistry 55:4
611–622 (2009)

Special Report

The MIQE Guidelines:
Minimum Information for Publication of Quantitative Real-Time PCR Experiments

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]

57 Essential information
(E) must be submitted with the manuscript

28 Desirable information
(D) should be submitted if available

What is MIQE? It's a Checklist

- qPCR community driven guidelines for essential and desired information in litterature;
  - Experimental Design
  - Sample Information
  - Nucleic Acid Extraction
  - Reverse Transcription
  - qPCR Target Information
  - qPCR Oligonucleotides
  - qPCR Protocol
  - qPCR Validation
  - Data Analysis

Data Analysis

Item to check	Importance
Data analysis	
qPCR analysis program (source, version)	E
Method of C_q determination	E
Outlier identification and disposition	E
Results for NTCs	E
Justification of number and choice of reference genes	E
Description of normalization method	E
Number and concordance of biological replicates	D
Number and stage (reverse transcription or qPCR) of technical replicates	E
Repeatability (intraassay variation)	E
Reproducibility (interassay variation, CV)	D
Power analysis	D
Statistical methods for results significance	E
Software (source, version)	E
C_q or raw data submission with RDML	D

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qPCR Validation

qPCR validation	Item to check	Importance
	Evidence of optimization (from gradients)	D
	Specificity (gel, sequence, melt, or digest)	E
	For SYBR Green I, C_q of the NTC	E
	Calibration curves with slope and y intercept	E
	PCR efficiency calculated from slope	E
	CIs for PCR efficiency or SE	D
	r^2 of calibration curve	E
	Linear dynamic range	E
	C_q variation at LOD	E
	CIs throughout range	D
	Evidence for LOD	E
	If multiplex, efficiency and LOD of each assay	E

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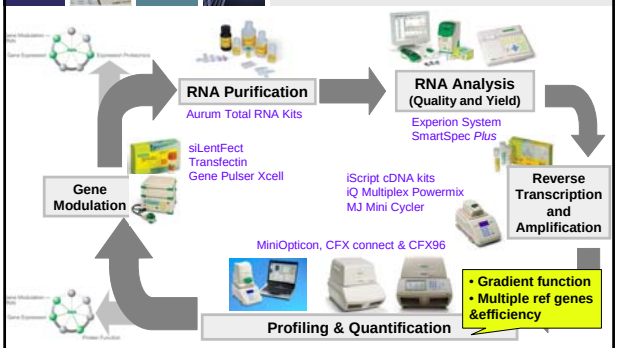
Complete solutions



Mechanical Technology

- CFX384 and CFX96 use the same mechanical technology
- We have performed over 1 million scans on a CFX96
  - 4 runs a day with 40 cycles, 250 working days = 25 years!
  - Shuttle positional shift less than 5 microsteps (~60 microns)
  - Difference is less than the thickness of a piece of paper!