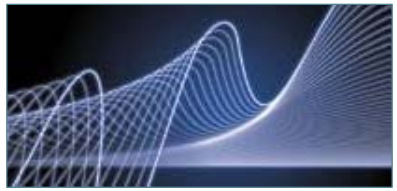

An Introduction to Real-Time PCR

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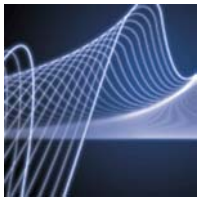
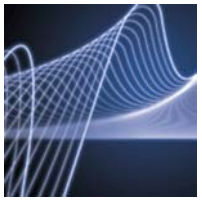
Version 1.0



Outline

- Part I: What is the real-time PCR?
  - Real-time PCR introduction

- Part II: How to use the real-time PCR?
  - Operation and Data analysis

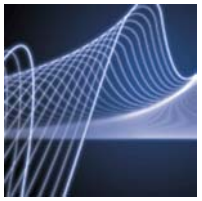


主要應用領域

- 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）

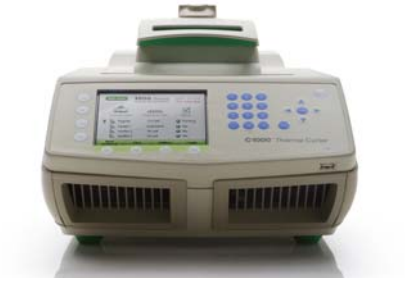
- 病原菌偵測
  - 多重病原菌同時偵測（Up to 5-Target）
  - 適合大量檢體篩選（High throughput）
  - 定性，定量 同時完成
  - 藥物治療成效的監控

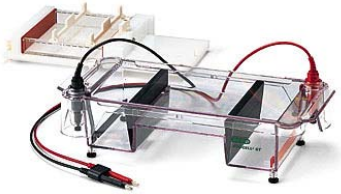
- SNP 分型
  - 唯一可用 Taqman, Molecular Beacon 以及 FRET 三種方法的品牌

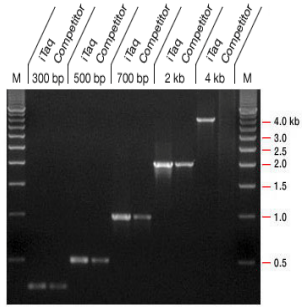


Conventional PCR vs QPCR

+Taq
+dNTP
+$MgCl_2$
+H_2O
+Buffer
+Template
+Primer
(On ice)



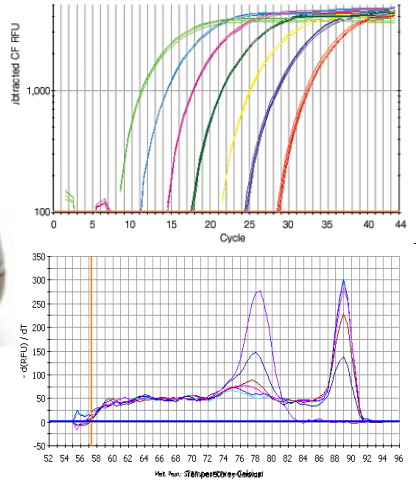




- End point
- Fuzzy data
- Labor intensive

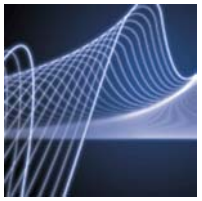
> 3 hrs(1.5 hrs labor)

+2x Master Mix
+H_2O
+Template
+Primer
(Room Temp)



- Online monitoring
- More sensitive
- Automated

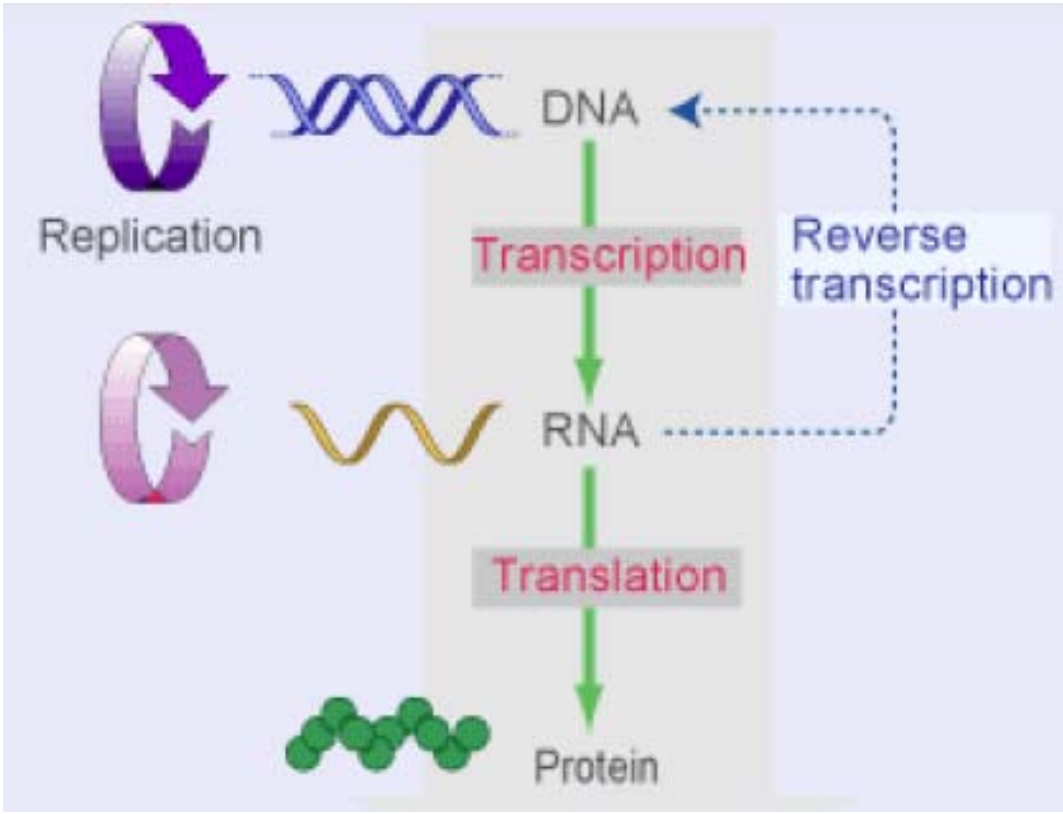
<1 hrs (20 min labor)

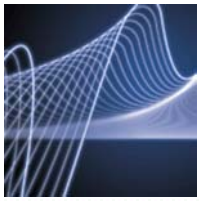


“中心法則Central Dogma”

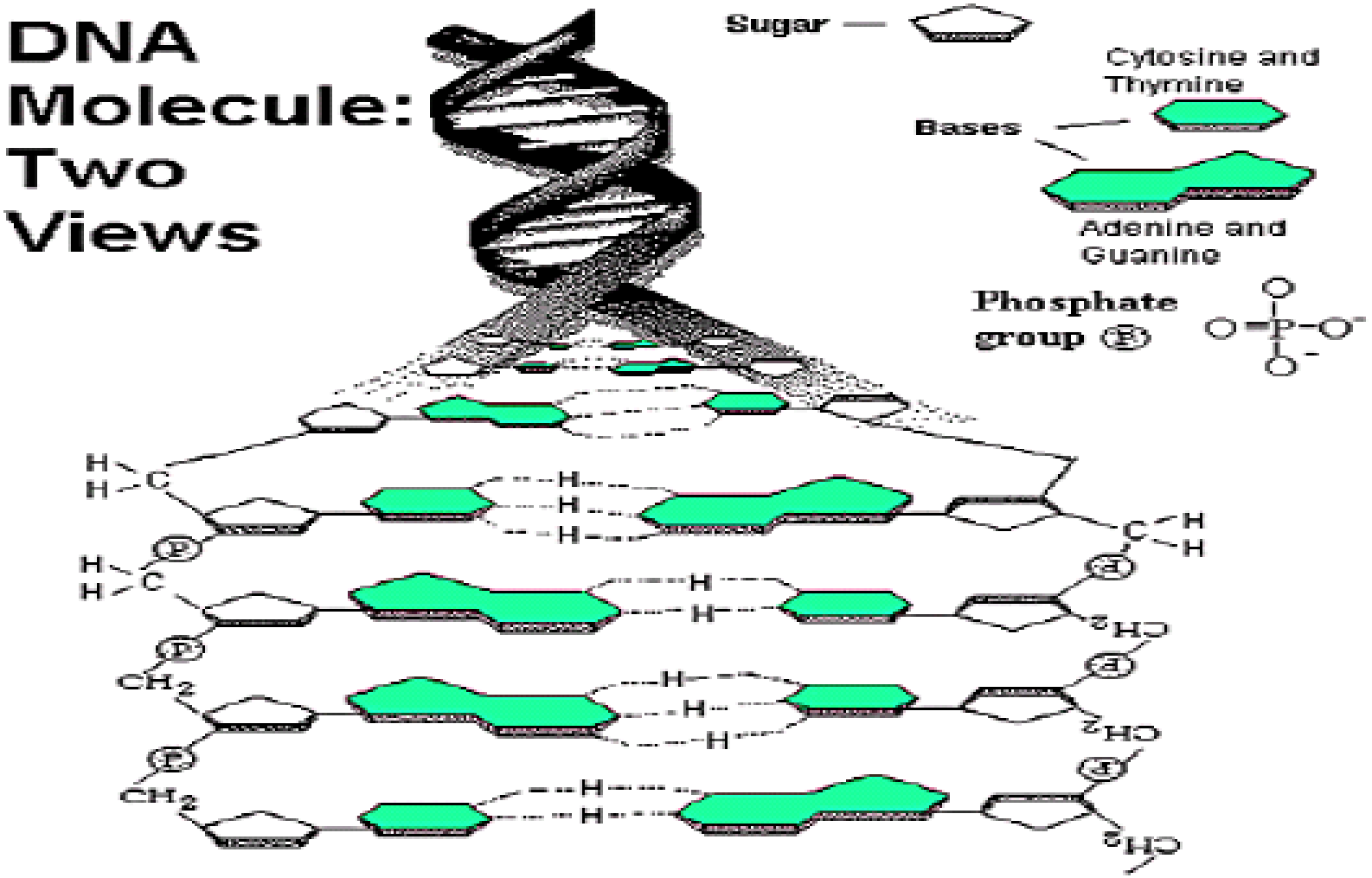
- Central dogma describes information flow from DNA→RNA→protein

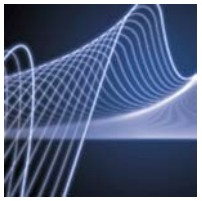
- Protein considered the functional unit within the cell



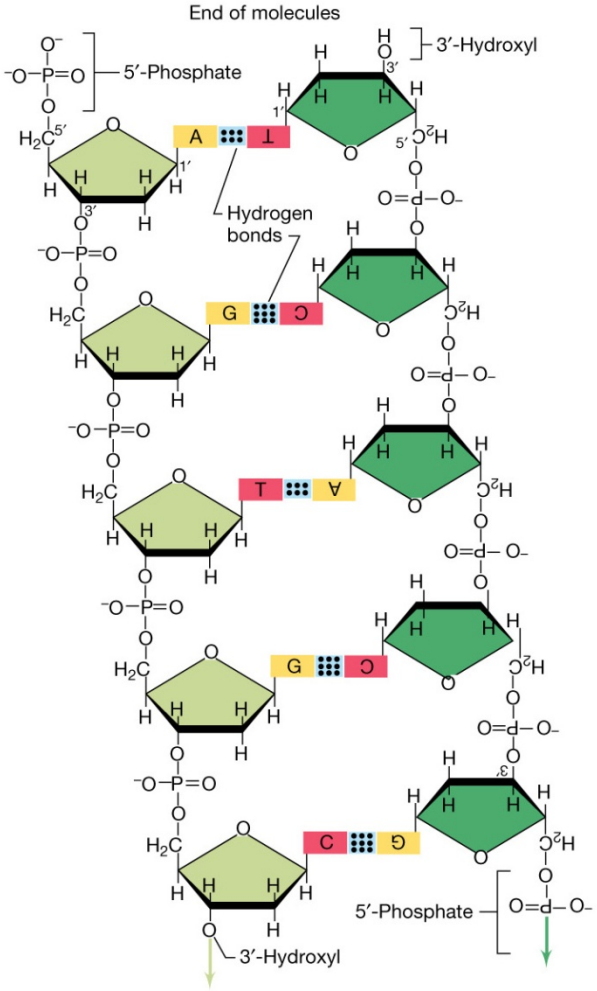


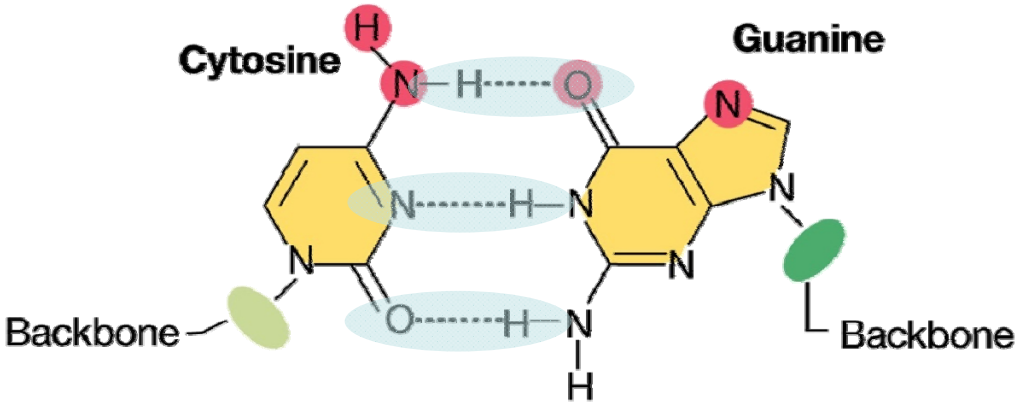
Structure of DNA



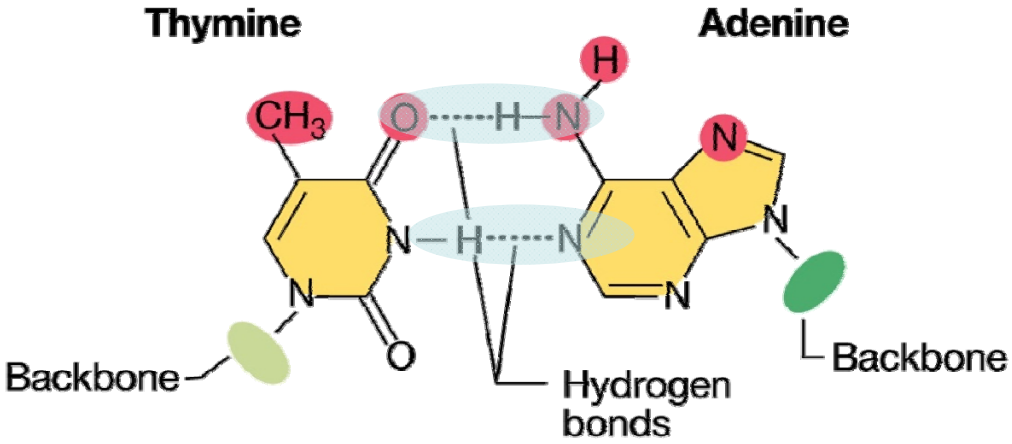


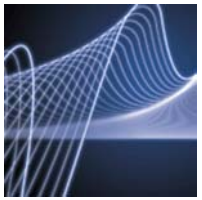
DNA Structure



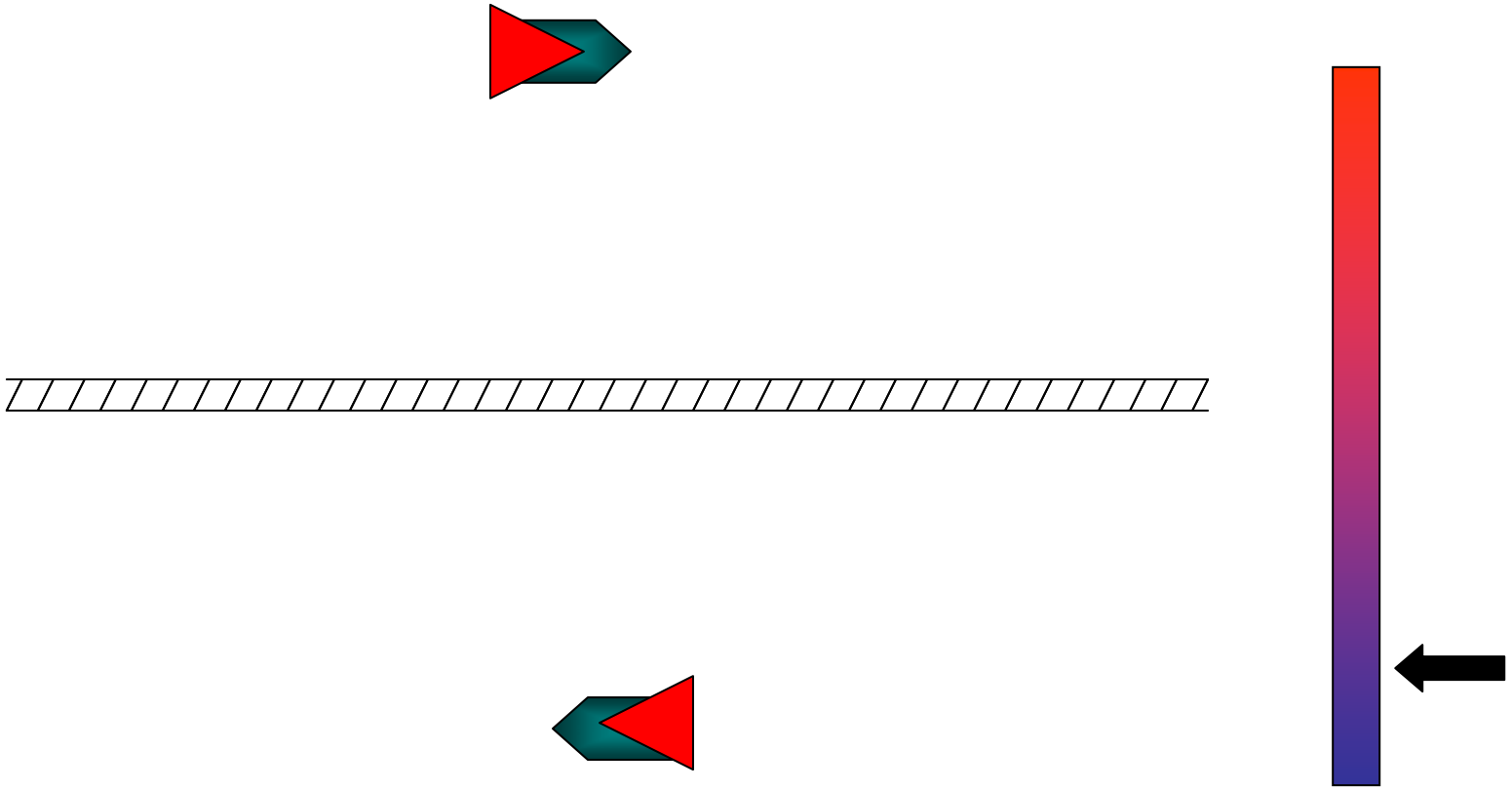


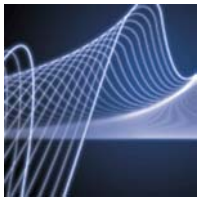
GC pair > AT pair



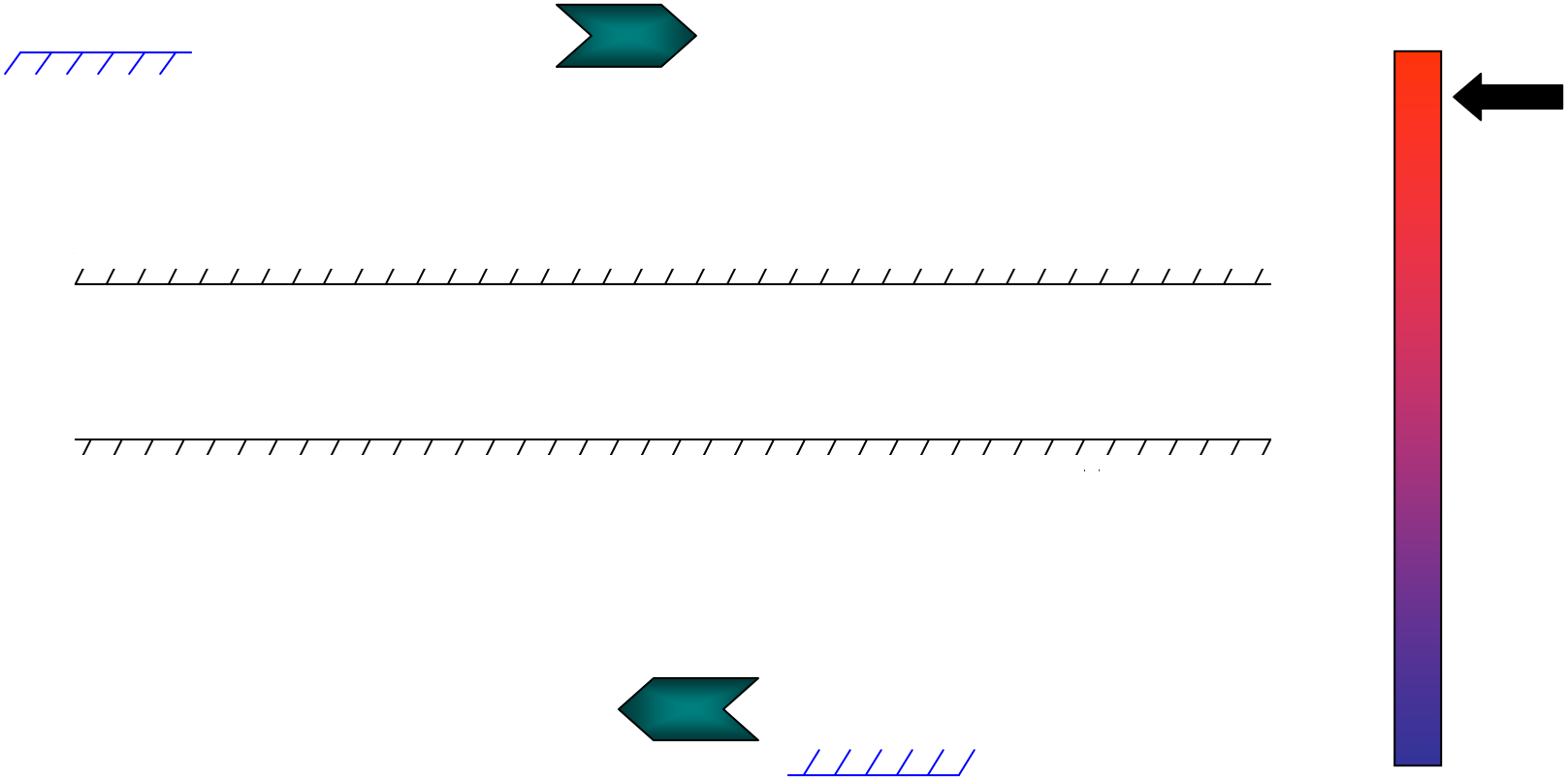


Classic PCR

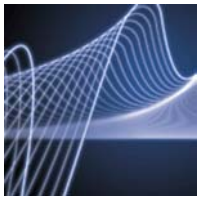




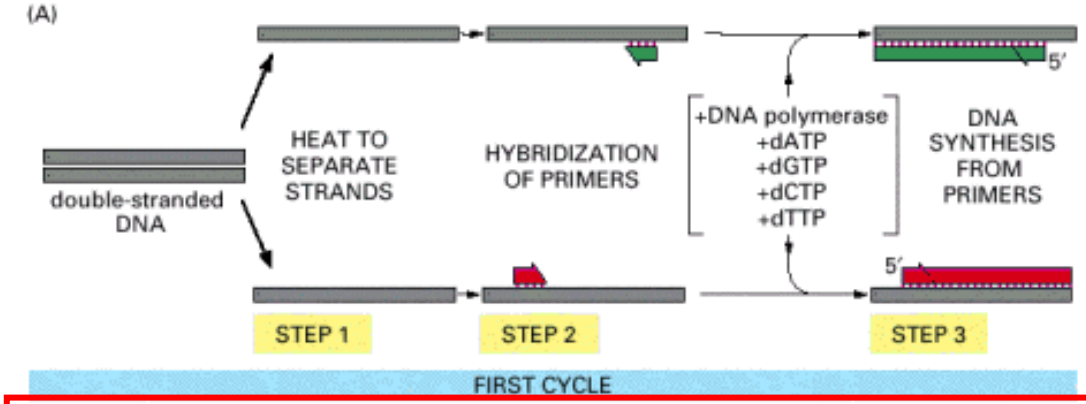
Classic PCR



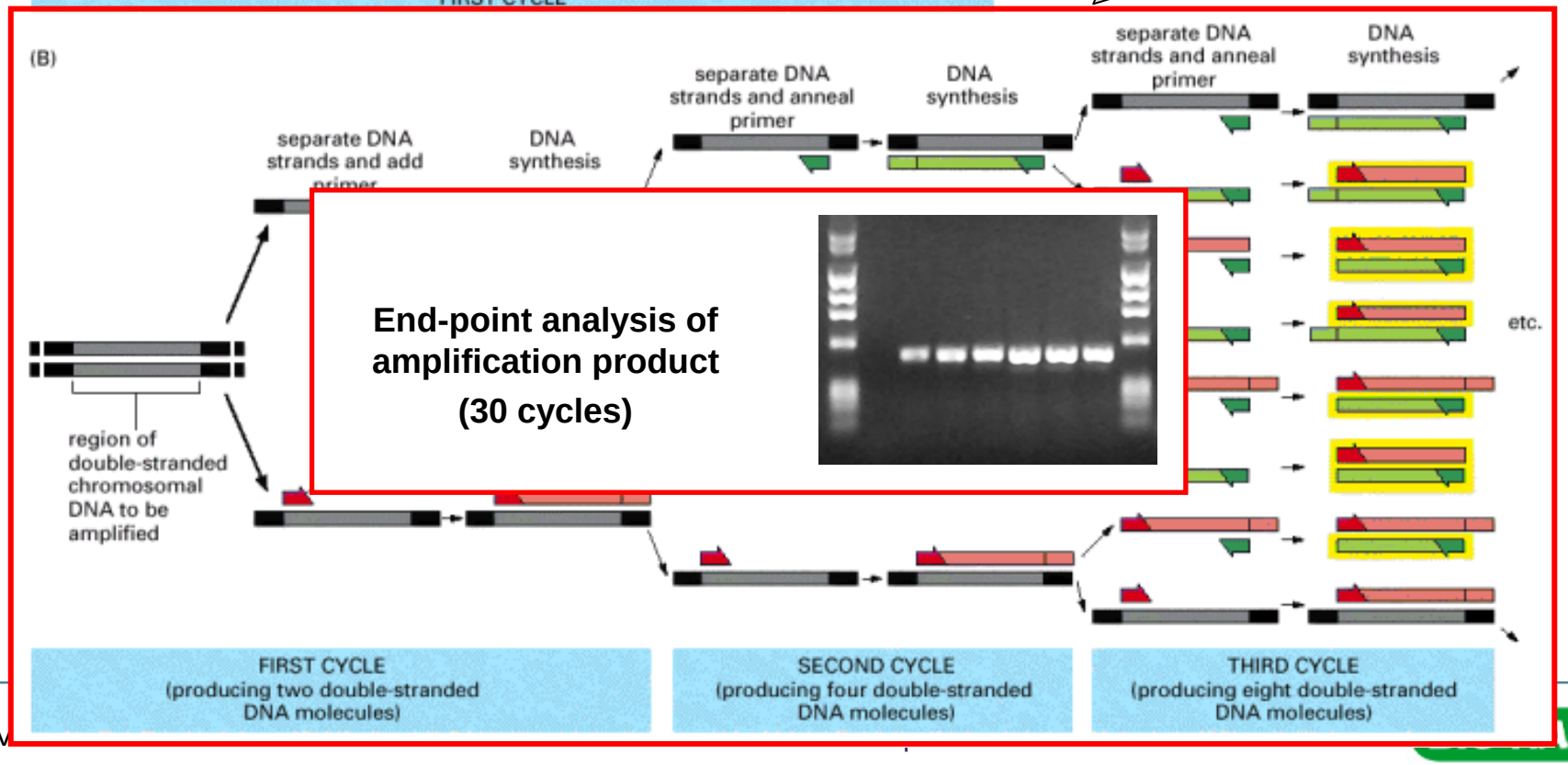
AMPLIFICATION



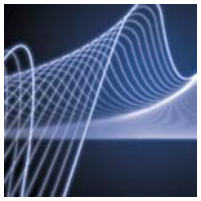
What is qPCR(Real Time PCR)?



Monitor the amplification reaction as it occurs

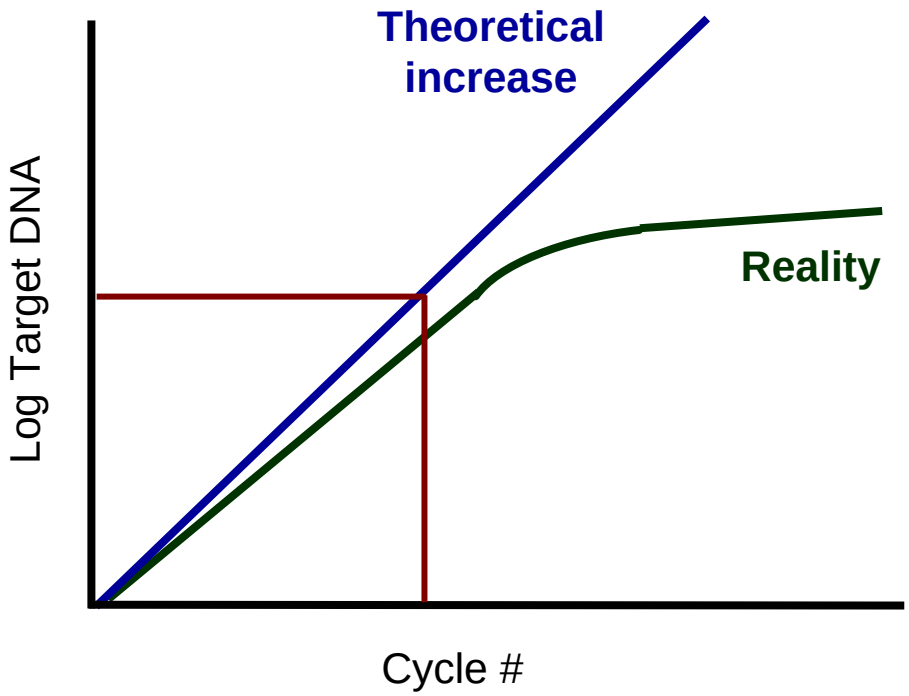


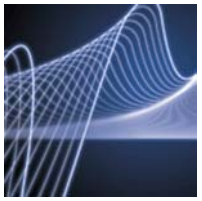
End-point analysis of amplification product (30 cycles)



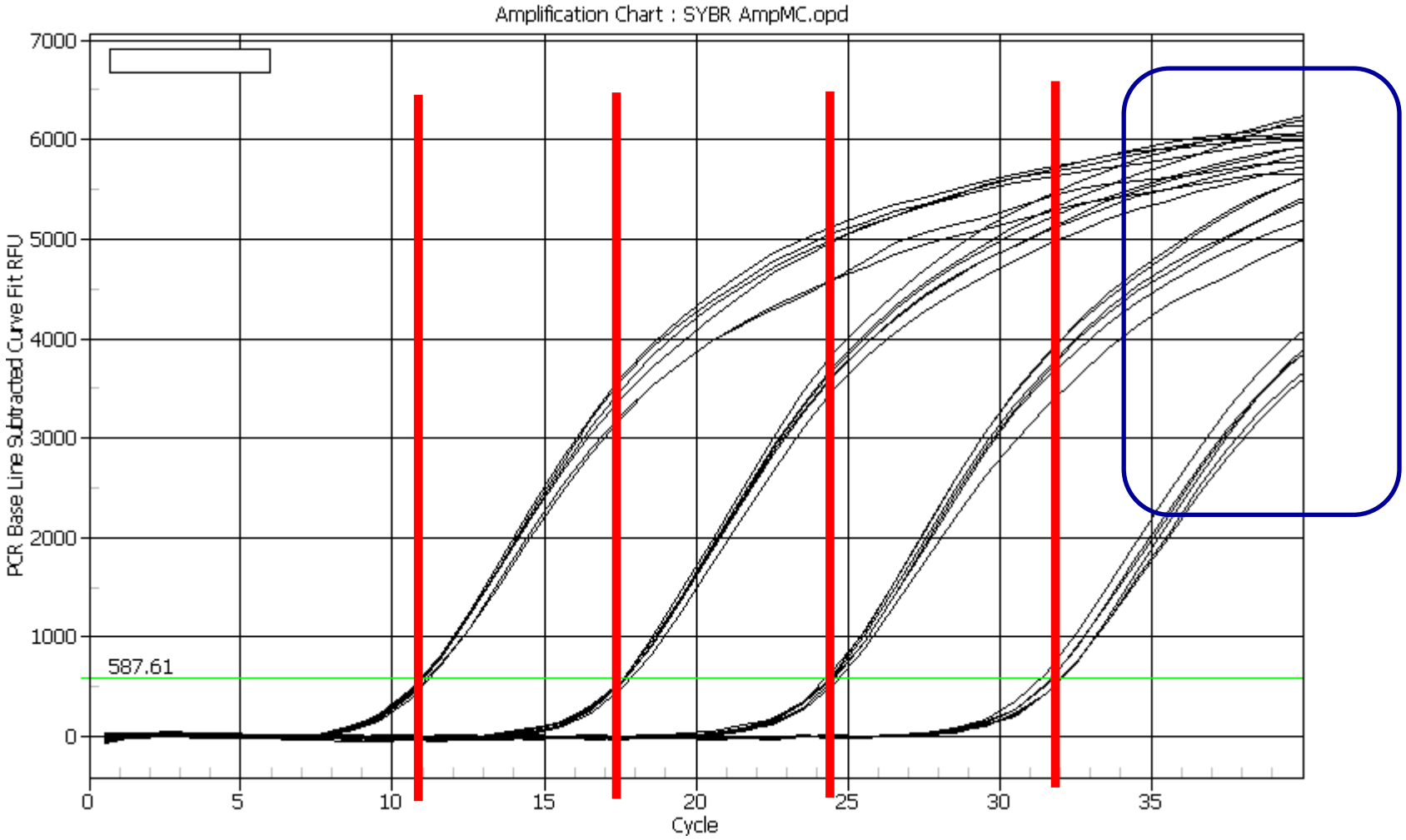
PCR: Theory vs. Reality

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

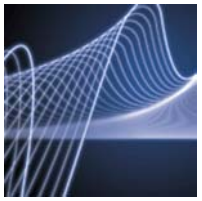




End Point Measurements

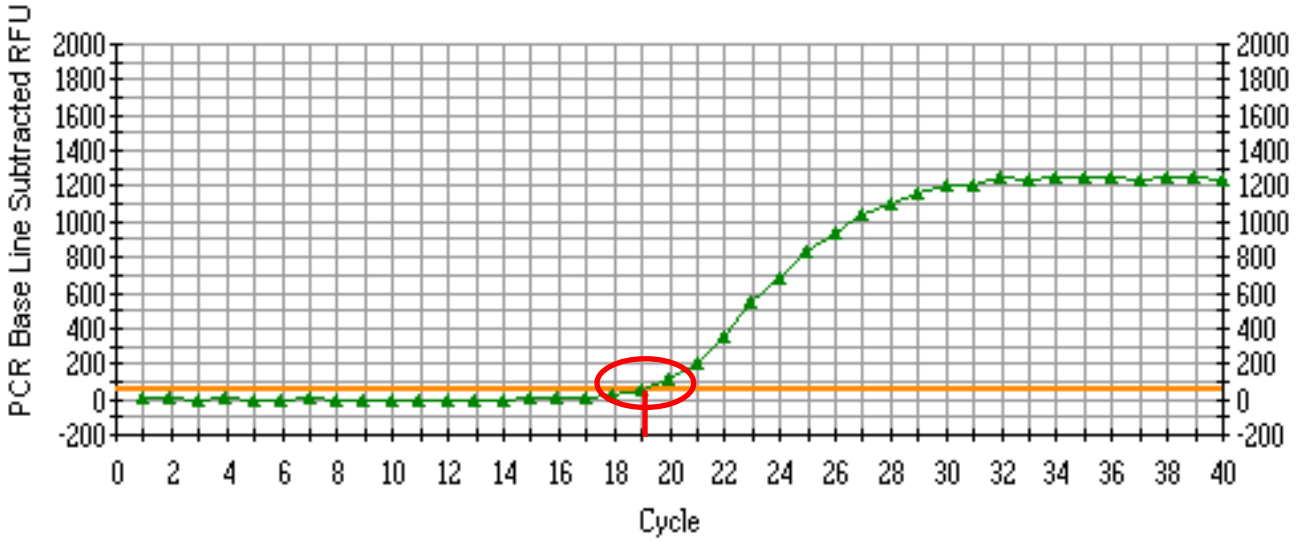


100 fold dilution series

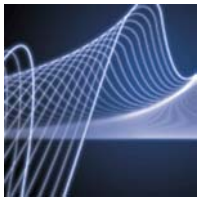


Threshold Cycle, C_T

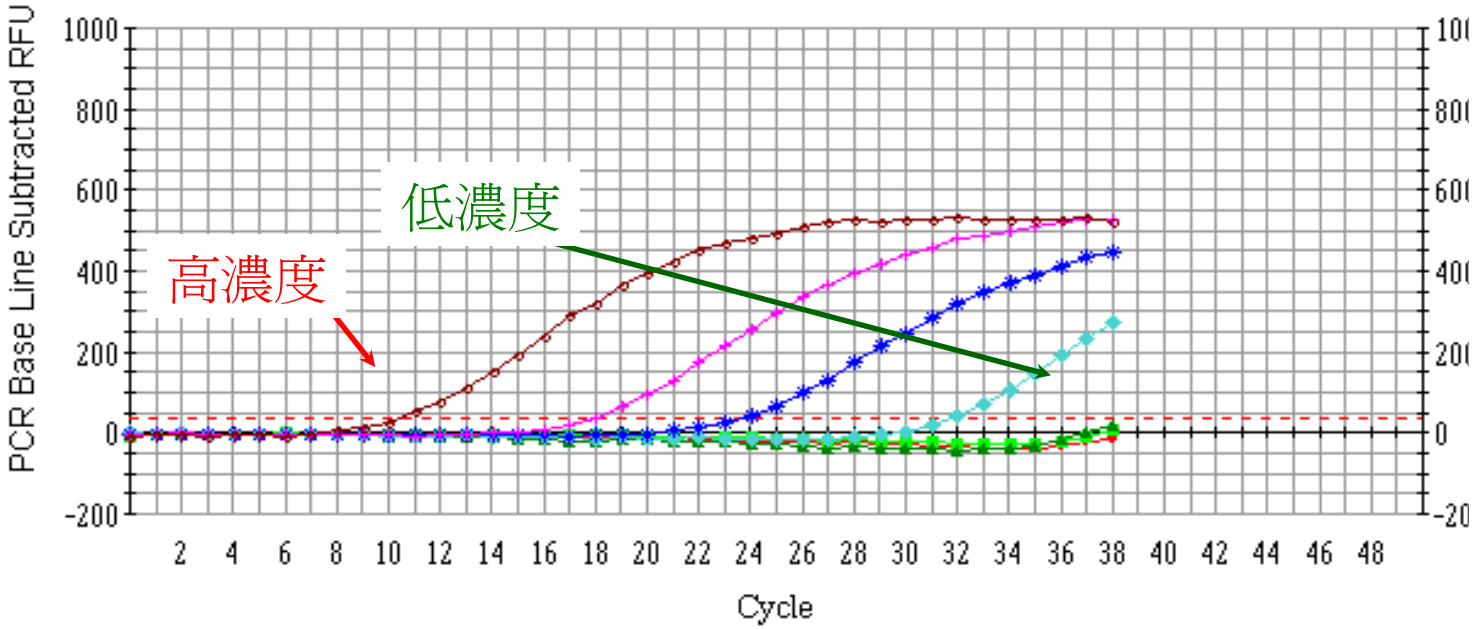
The point at which the fluorescence rises appreciably above background



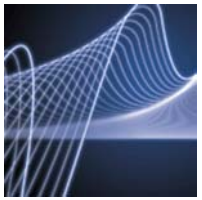
PCR Amplification vs Cycle: C:\My Documents\customer's opds\jkb1-26-01b.opd



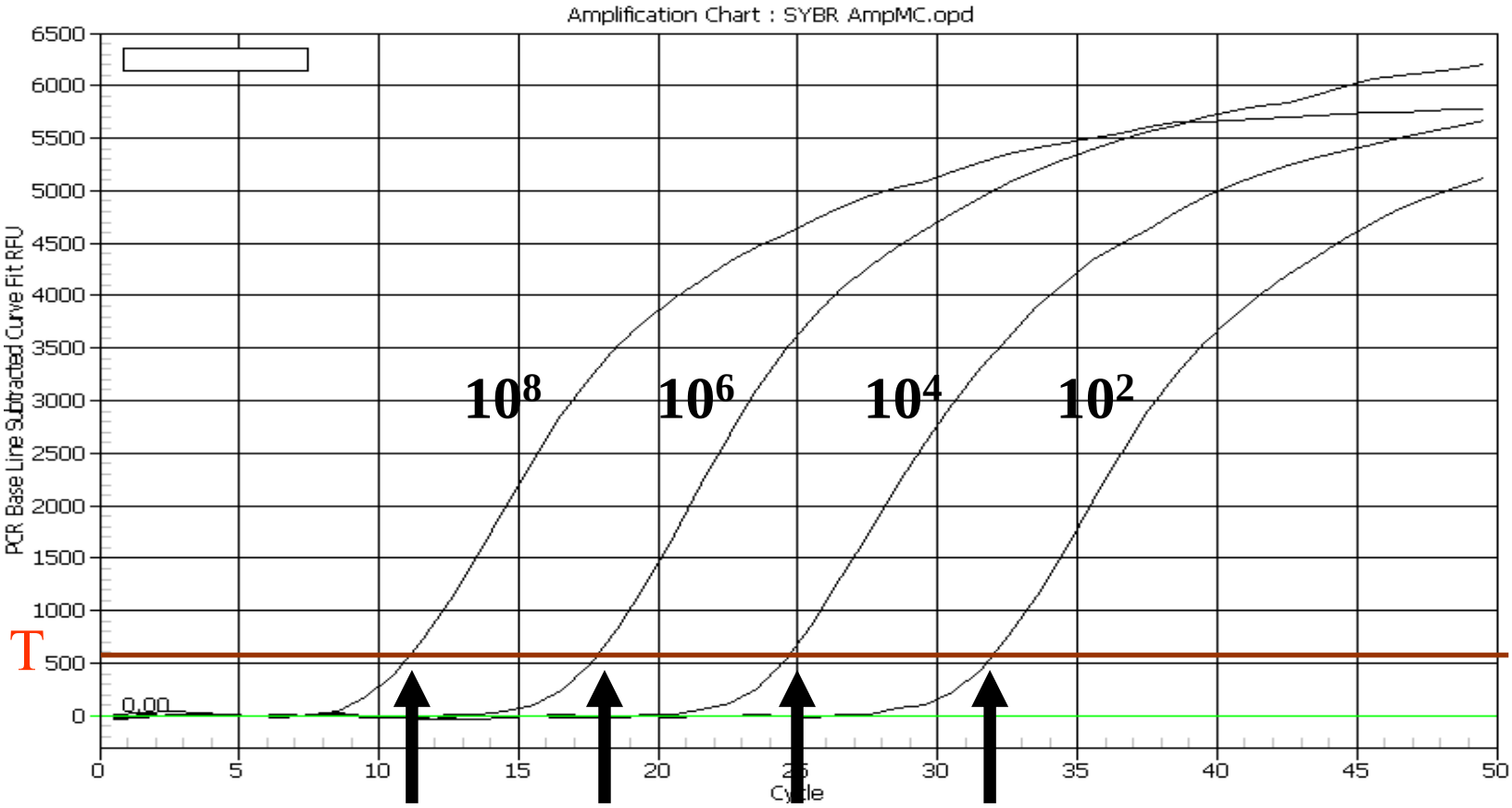
Threshold Cycle (Ct) 與起始濃度的對數成反比

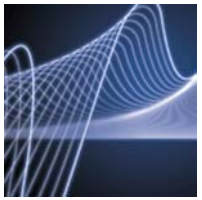


- Assumes 100% efficiency
- 1 cycle = 2 fold difference
- 3.3 cycles ≅ 10 fold difference

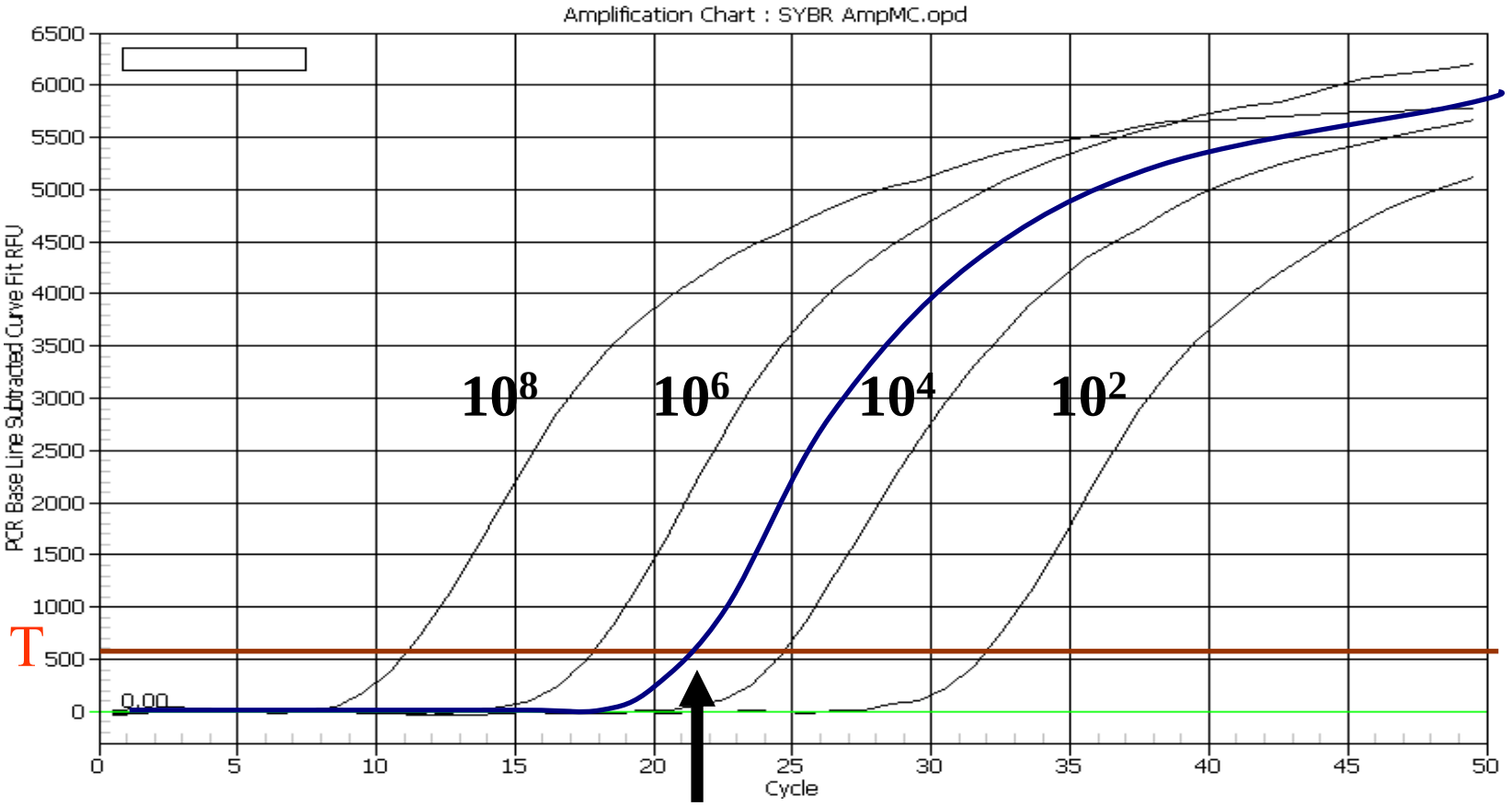


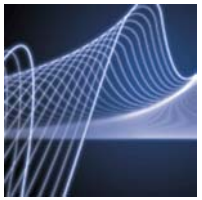
Absolute quantification



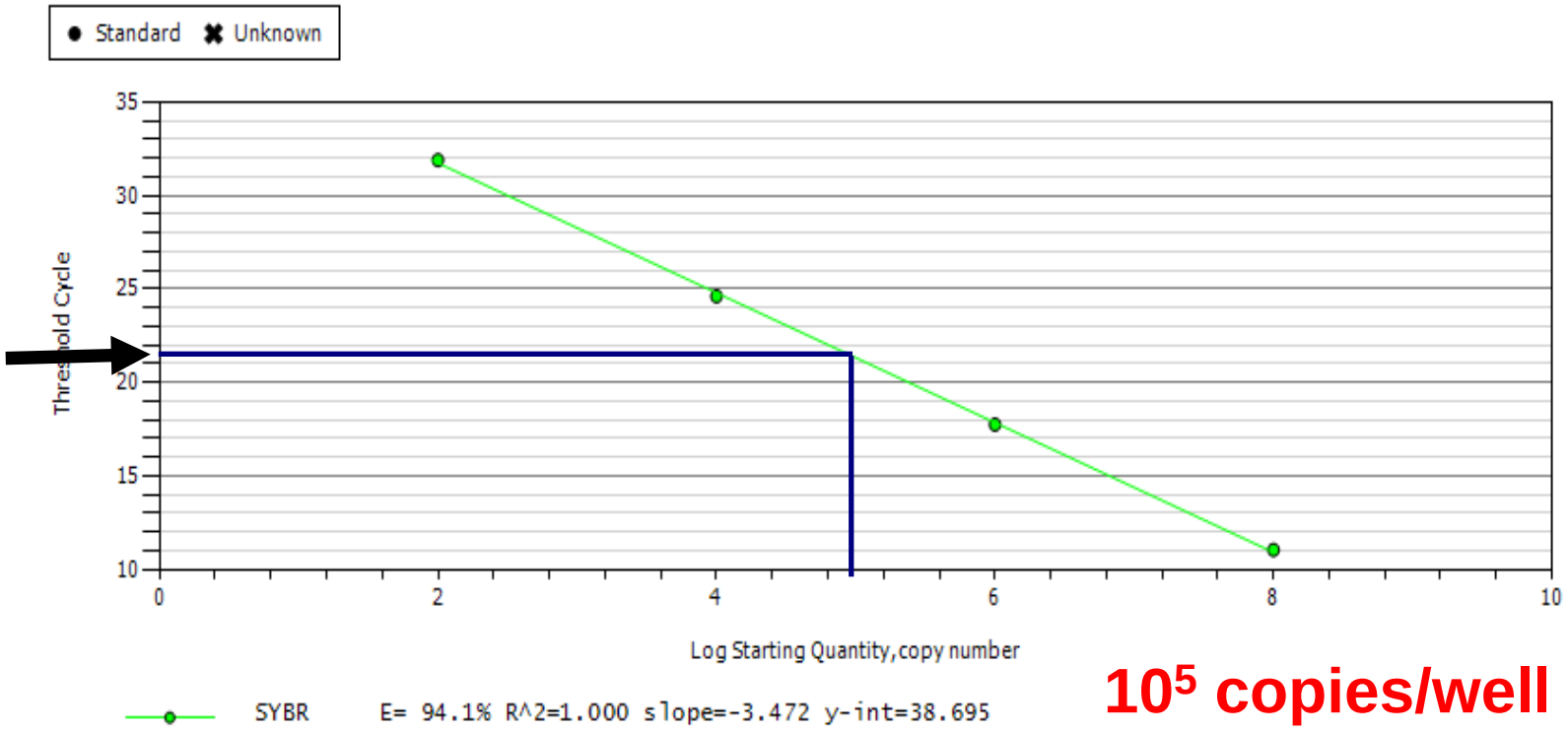


Absolute quantification

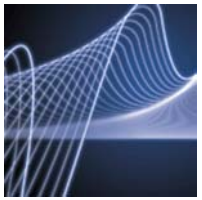




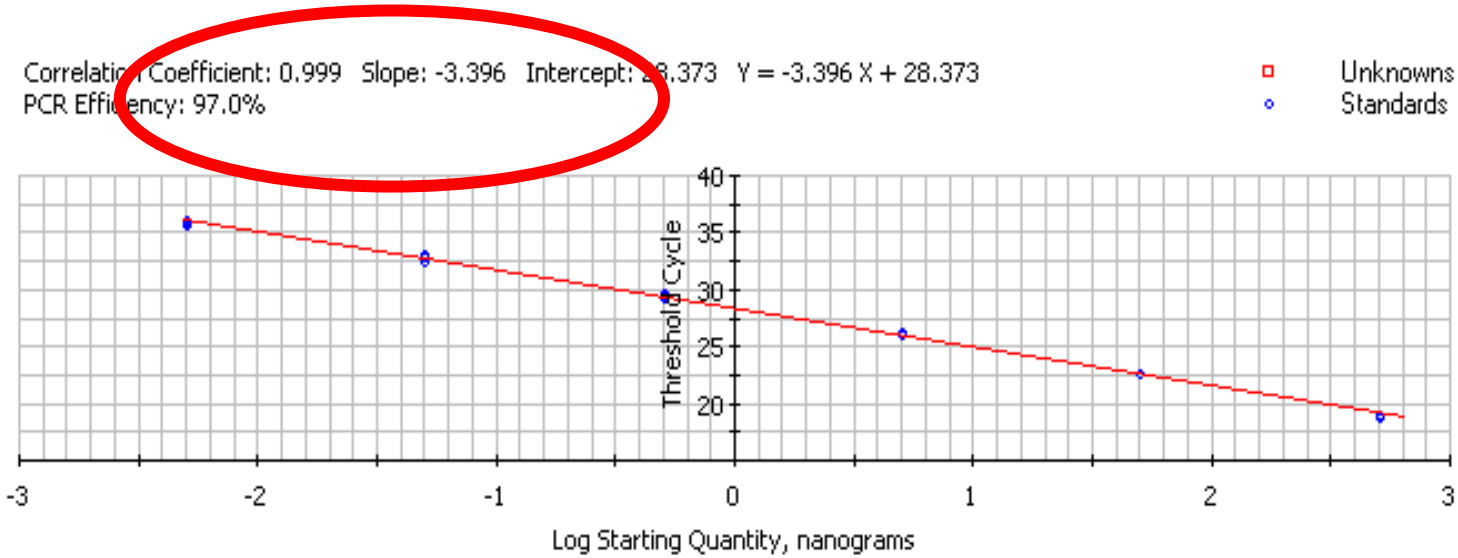
Absolute quantification



10^5 copies/well



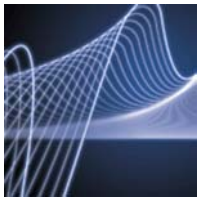
C_T is linear with the log of starting copy number



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

$$\text{Efficiency } (\eta) = [10^{(-1/\text{slope})}] - 1$$

when slope = -3.32, Efficiency = 100%



$$\text{Slope} = -\,[1/\log\,(1+E)]$$

$$\log\,(1+E) = -\,(1/\text{slope})$$

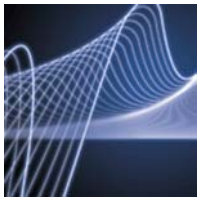
$$E = 10^{[-1/\text{slope}]} - 1$$

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

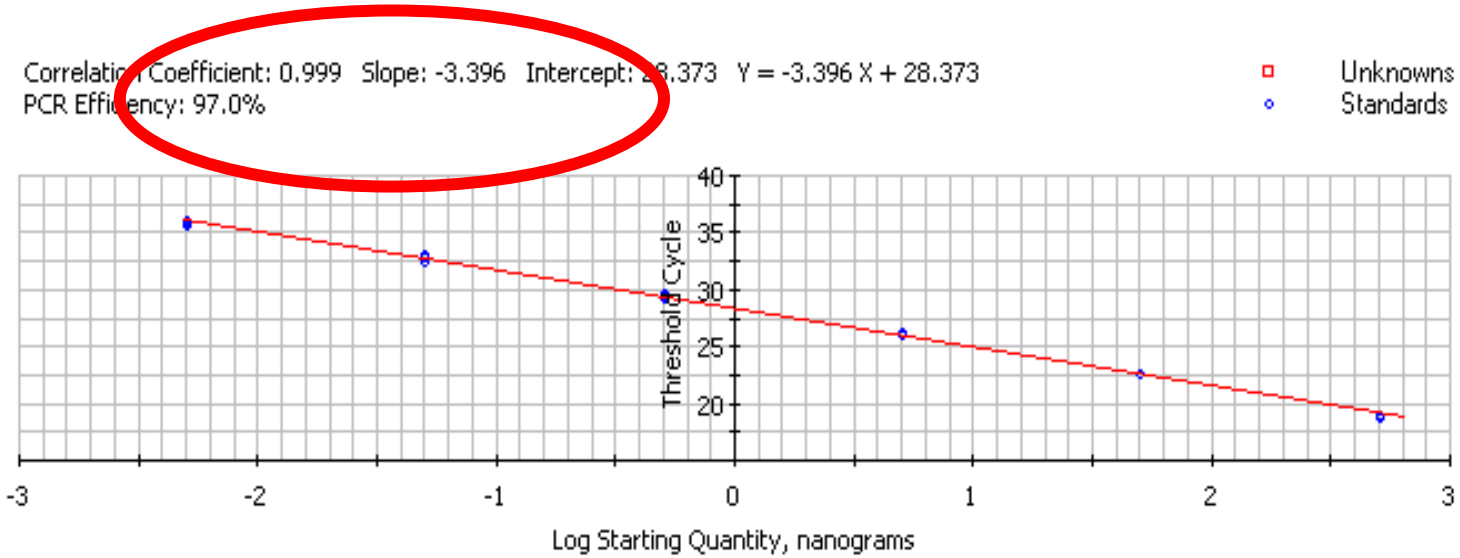
Aim for Efficiency Values:

Good = 90 – 110%

Fantastic = 95 – 105%

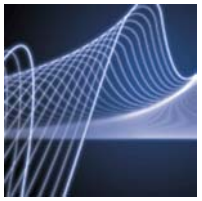


C_T is linear with the log of starting copy number



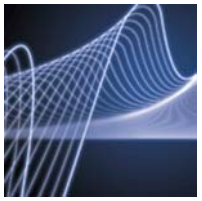
r = is a measure of how well the actual data fit to the standard curve.
= (explained variation/total variation)

Aim for R value (Correlation Coefficient) of ≥0.98



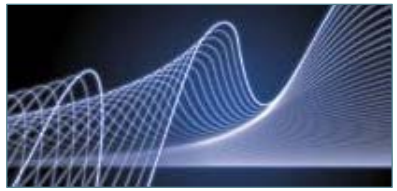
Points to Remember

- **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**



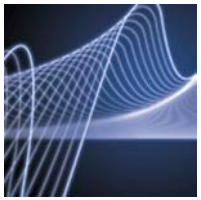
Real-Time PCR

- 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™



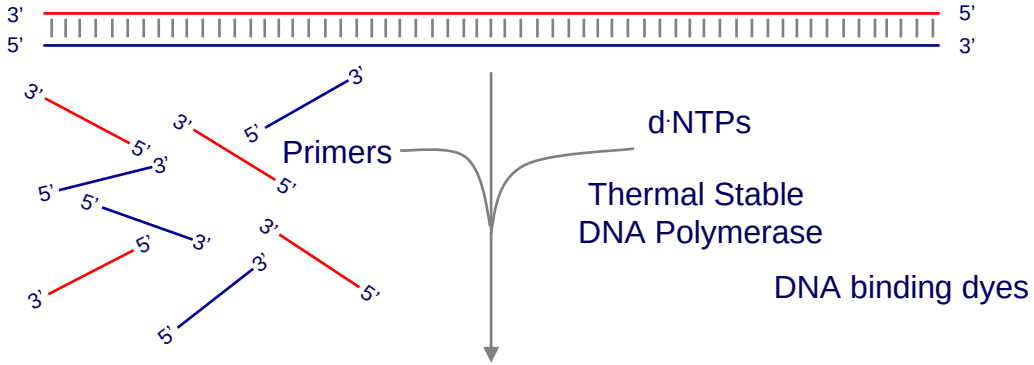
SYBR Green I

Version 1.0



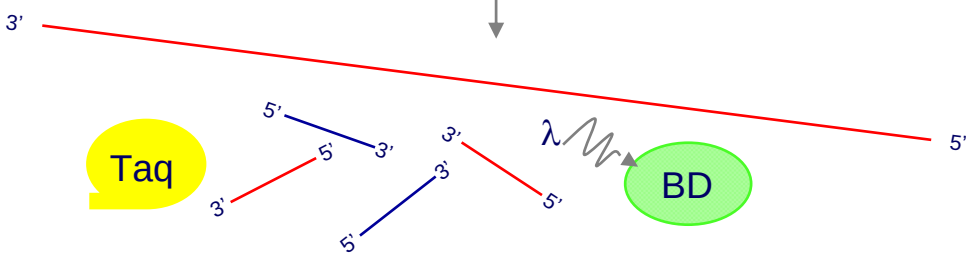
DNA binding dyes

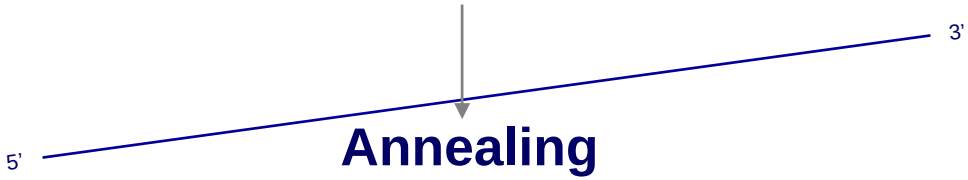
Add Master Mix & Sample



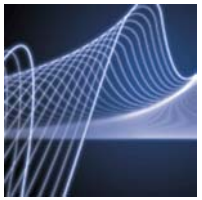
Reaction Tube

Denaturation





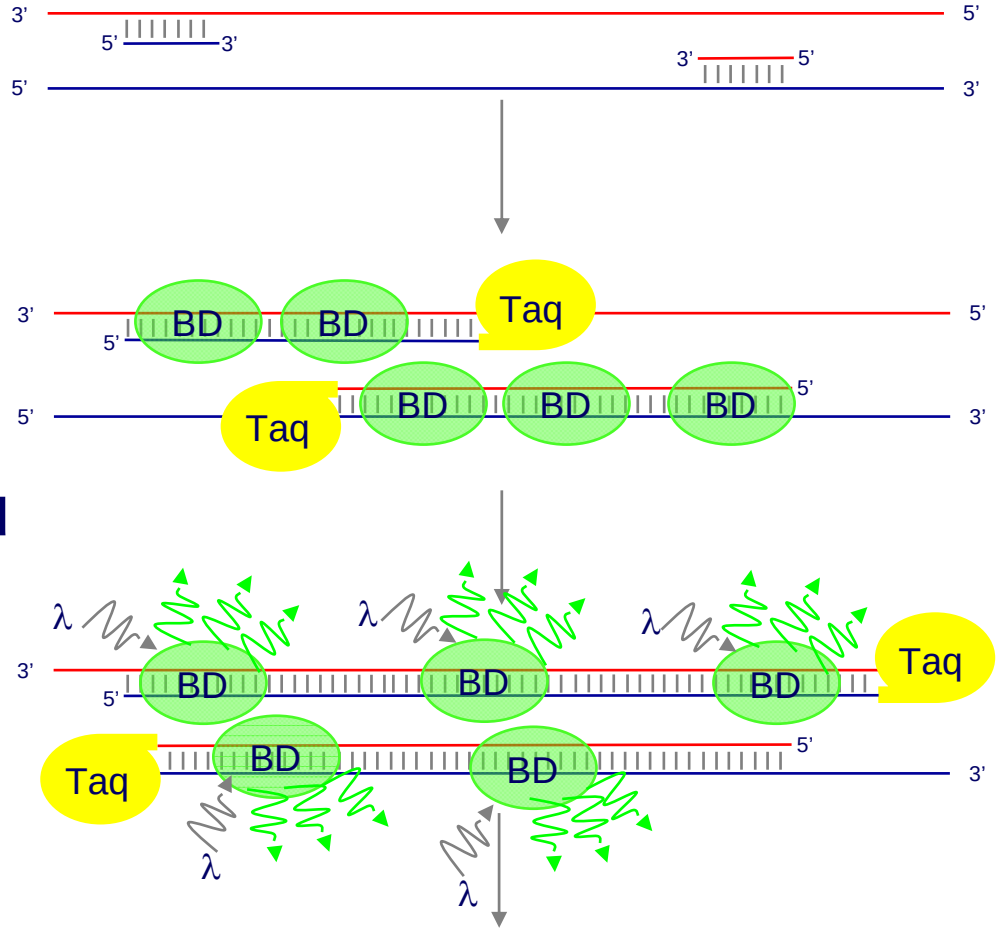
Annealing



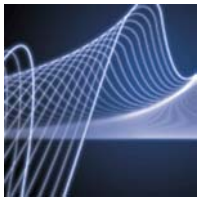
DNA binding dyes

Extension

Extension Continued
Apply Excitation Wavelength



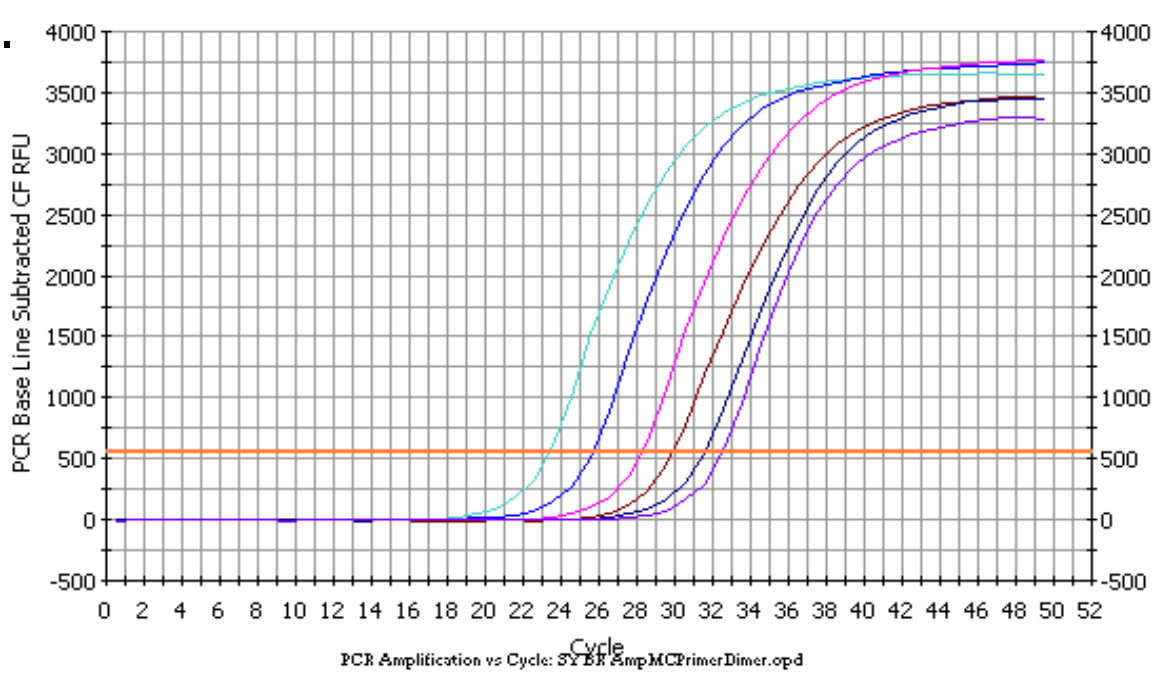
Repeat

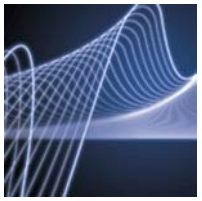


DNA binding dyes

- Easy to start qPCR Exp.
- Inexpensive
- Accurate
- Large dynamic range

- Primer specificity
- Single target

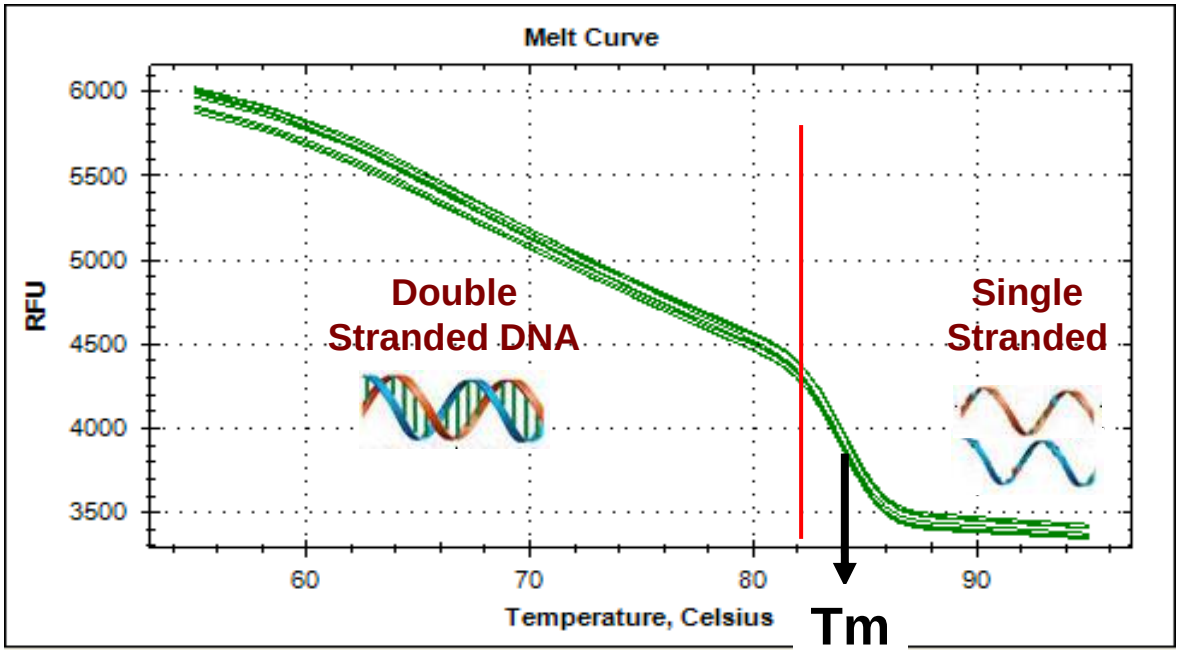


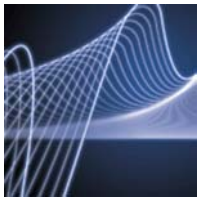


Melt Curve Analysis

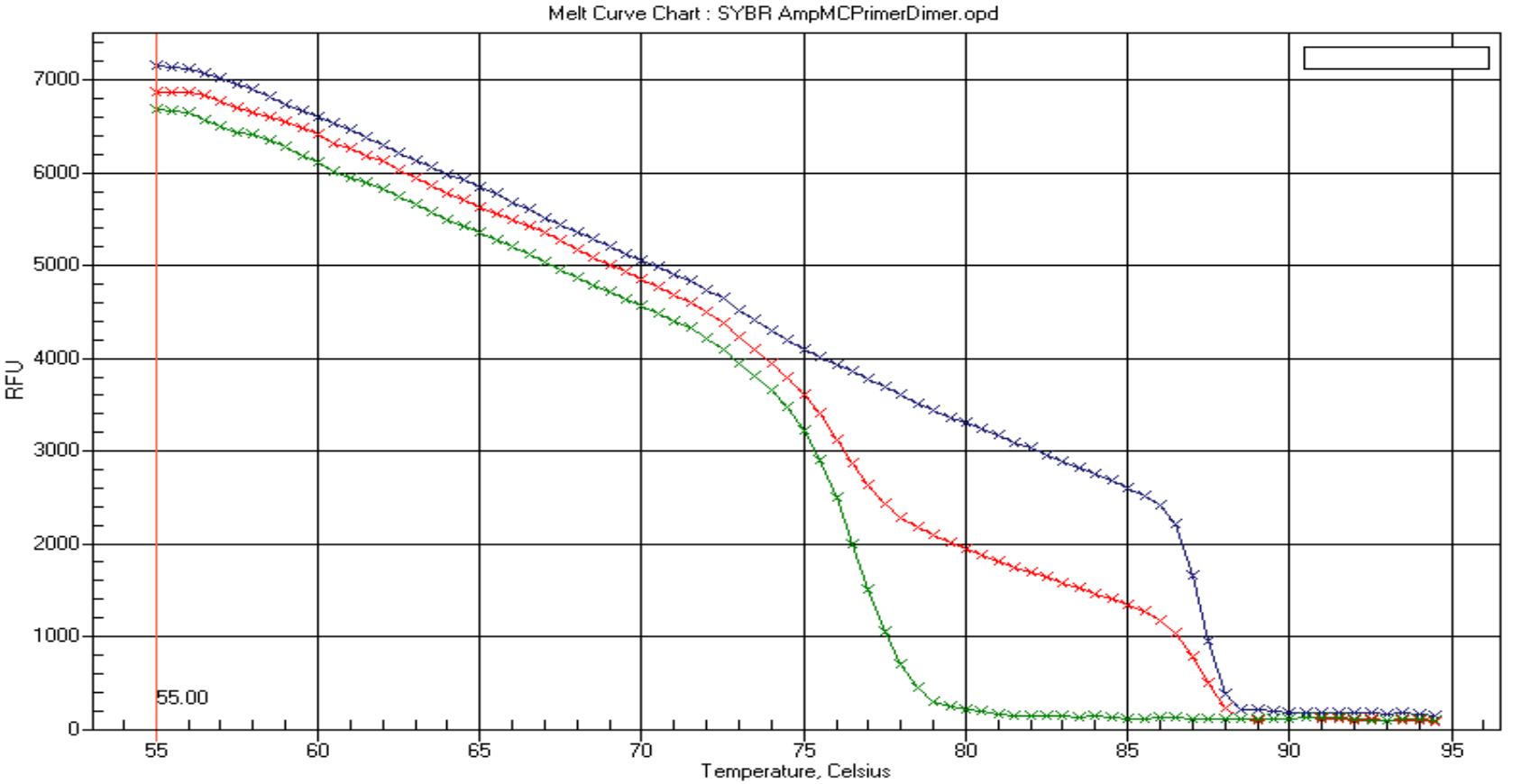
- After real-time PCR amplification, a melt curve is performed where we monitor fluorescence as temperature increases.

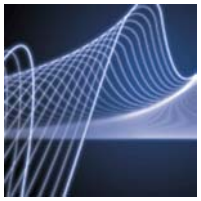
- Melting temperature (Tm)
  - DNA is half double and half single-stranded
  - Depends on nucleotide content (GC-AT ratio) and length





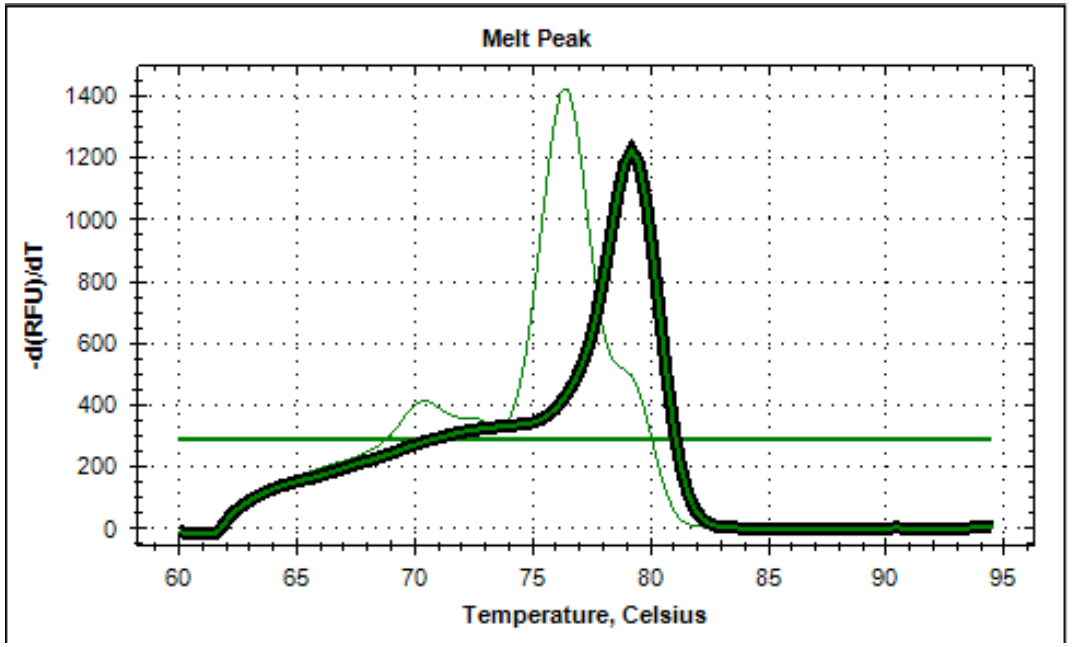
Melt Curve Analysis

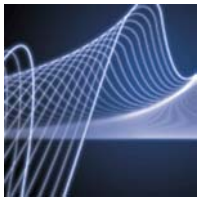




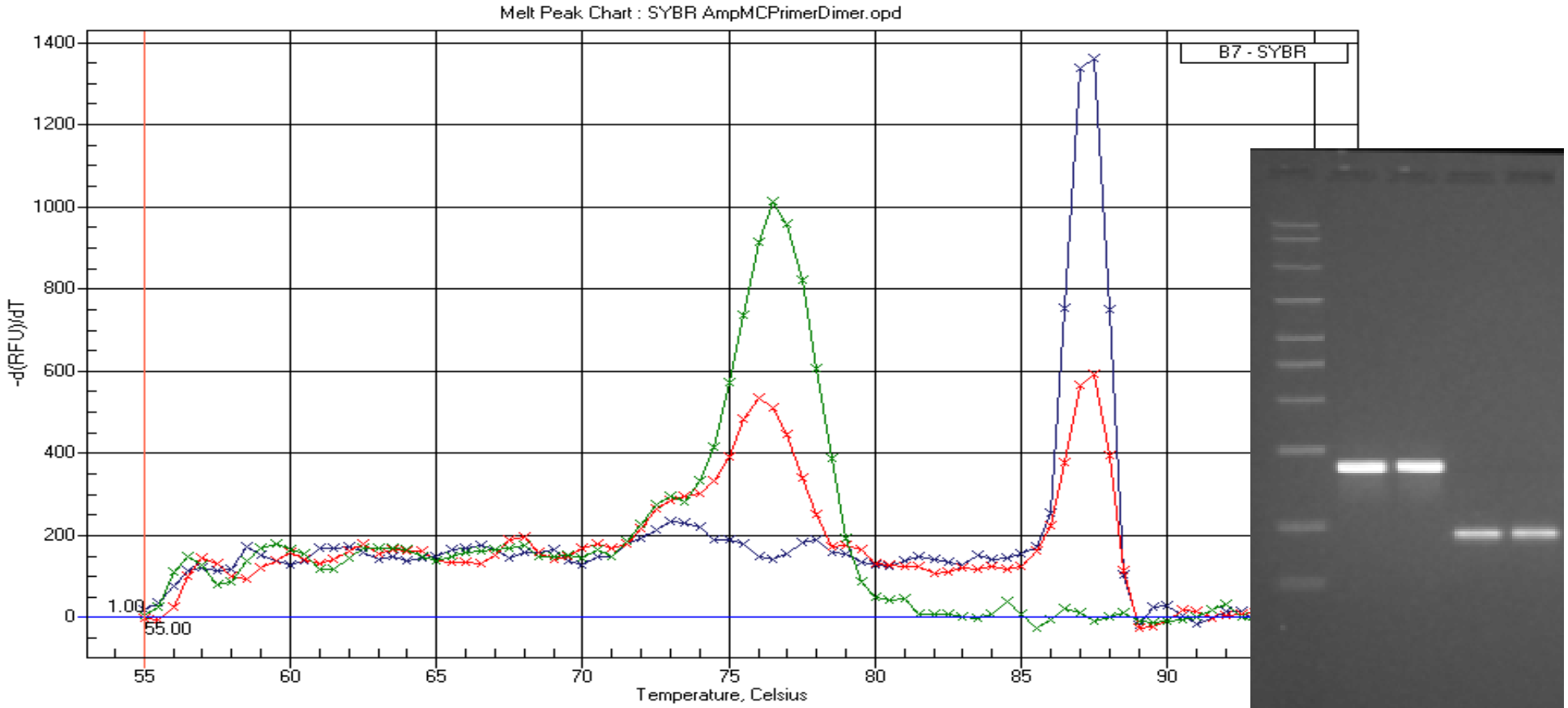
Melt Curve Analysis

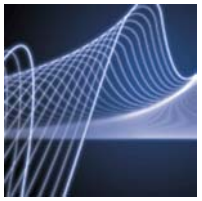
- Distinguish products based on their Tms
  - Plot negative rate of change of fluorescence vs. temp (-dI/dT) for easy discrimination of products based on their Tms





Melt Curve Analysis





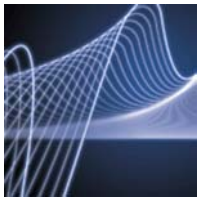
The DNA binding dye reagents of Bio-Rad

Reagents

- **iQ SYBR Green Supermix**
- **Ssofast EvaGreen Supermix**
- **iScript One-Step RT-PCR Kit with SYBR Green**

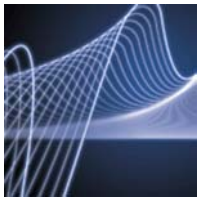
Key Features

- Sensitive SYBR Green or EvaGreen
- Simplified reaction set up with ready-to-use 2x reaction mix
- iTaq hot-start DNA polymerase, hot-start in 30 sec or 3 min at 95°C
- Linear results over 7 orders of magnitude



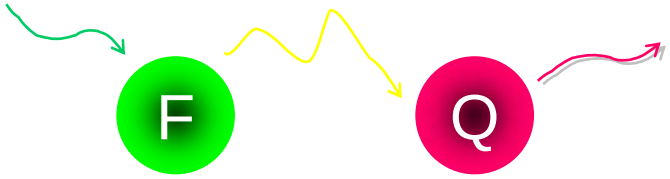
Real-Time PCR

- 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™

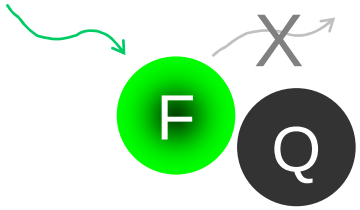


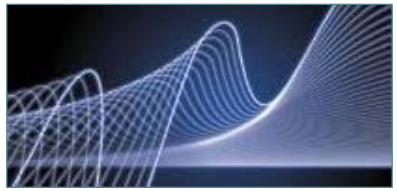
Dyes and Quenchers

FRET: Fluorescence (Förster) Resonance Energy Transfer



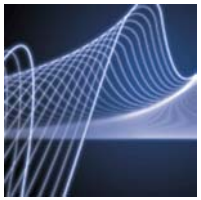
Dark Quenchers : Ground state or static quenching
Energy is dissipated as heat
Molecular interactions inhibit fluorescence





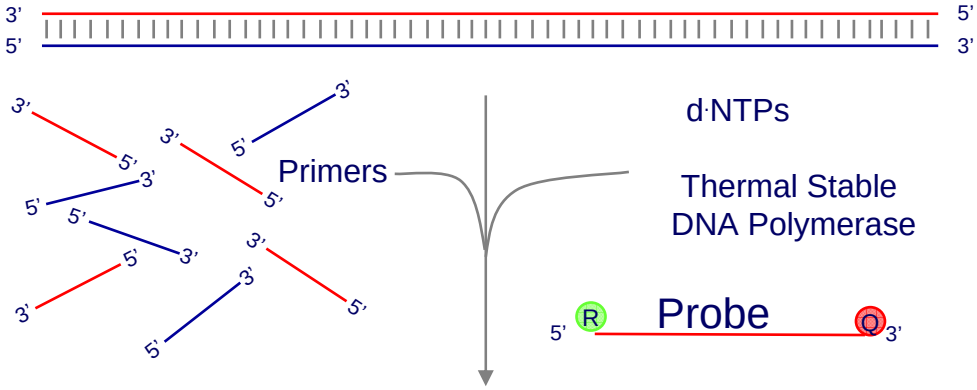
TaqMan –
the “Hydrolysis” probes

Version 1.0



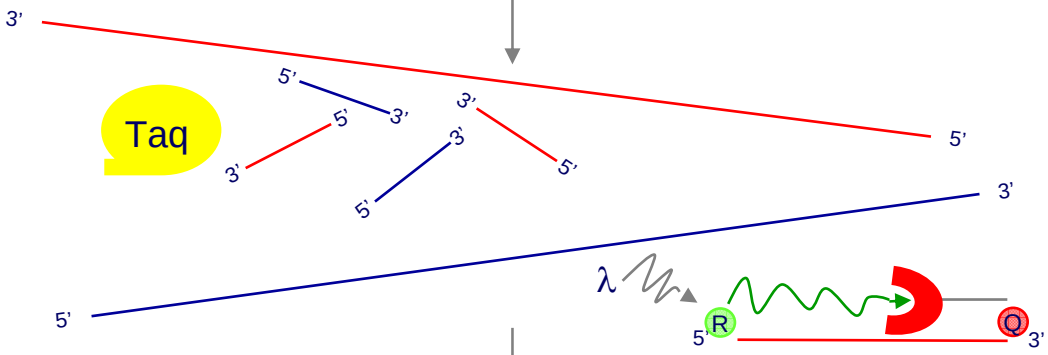
Cleavage-based assay: TaqMan

Add Master Mix & Sample

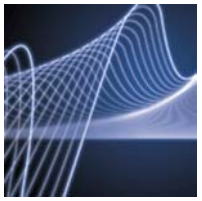


Reaction Tube

Denaturation



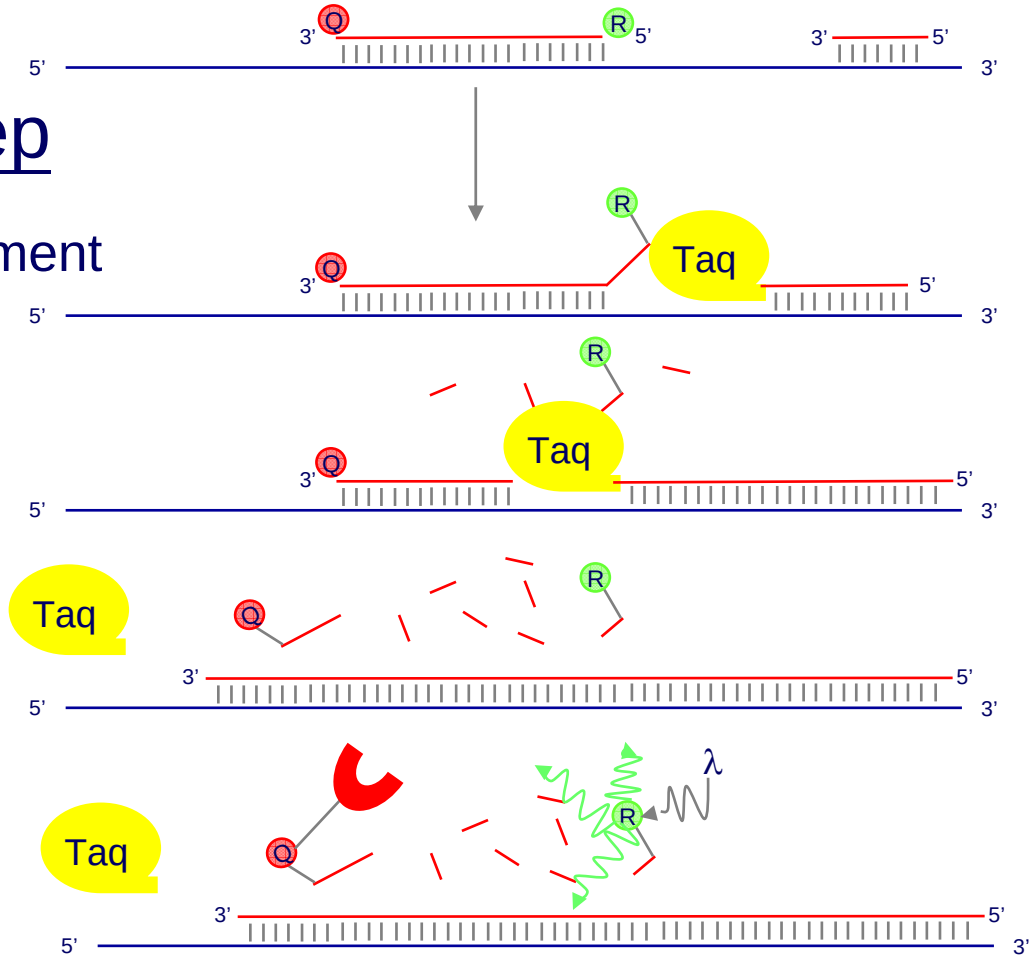
Annealing

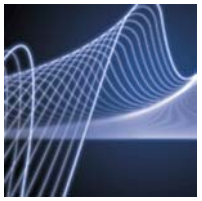


Cleavage-based assay: TaqMan

Extension Step

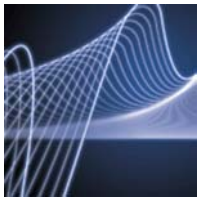
1. Strand Displacement
2. Cleavage
3. Polymerization Complete
4. Detection





Cleavage-based assay: TaqMan

- More specific than dye base method
- Multiplex able using different reporter dye
- SNP genotyping application
- More high cost than dye base method



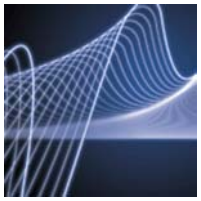
The probes based reagents of Bio-Rad

Reagents

- **iQ Supermix**
- **Ssofast Probes Supermix**
- **iScript One-Step RT-PCR Kit for Probes**
- **iQ Multiplex Powermix**

Key Features

- Simplified reaction set up with ready-to-use 2x reaction mix
- hot-start DNA polymerase, hot-start in 30 sec or 3 min at 95°C
- Linear results over 7 orders of magnitude



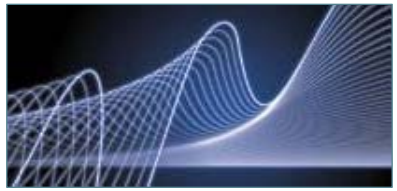
Which Method To Use?

- Each method has advantages and disadvantages.

- Bio-Rad Real-Time Instrumentation is equipped to handle all chemistries.

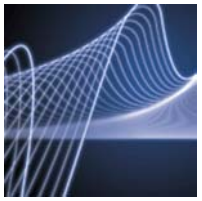
- One method may be more appropriate for an application over another

  -SNP, microarray, limited sample….



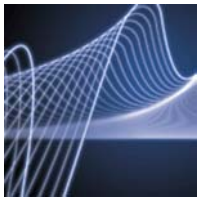
Real Time PCR application

Version 1.0

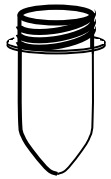


Real Time PCR application

- ***Quantitation***
  - ***Absolute –viral load detection***
  - ***Relative – Gene expression***
- ***Identification***
  - ***Microorganisms***
  - ***Allelic discrimination, SNP***



Types of quantification



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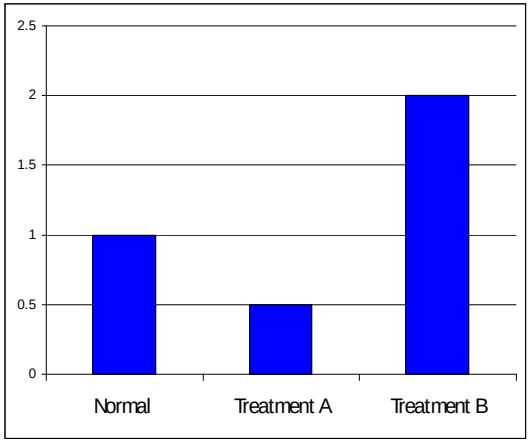
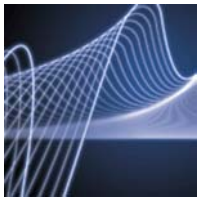
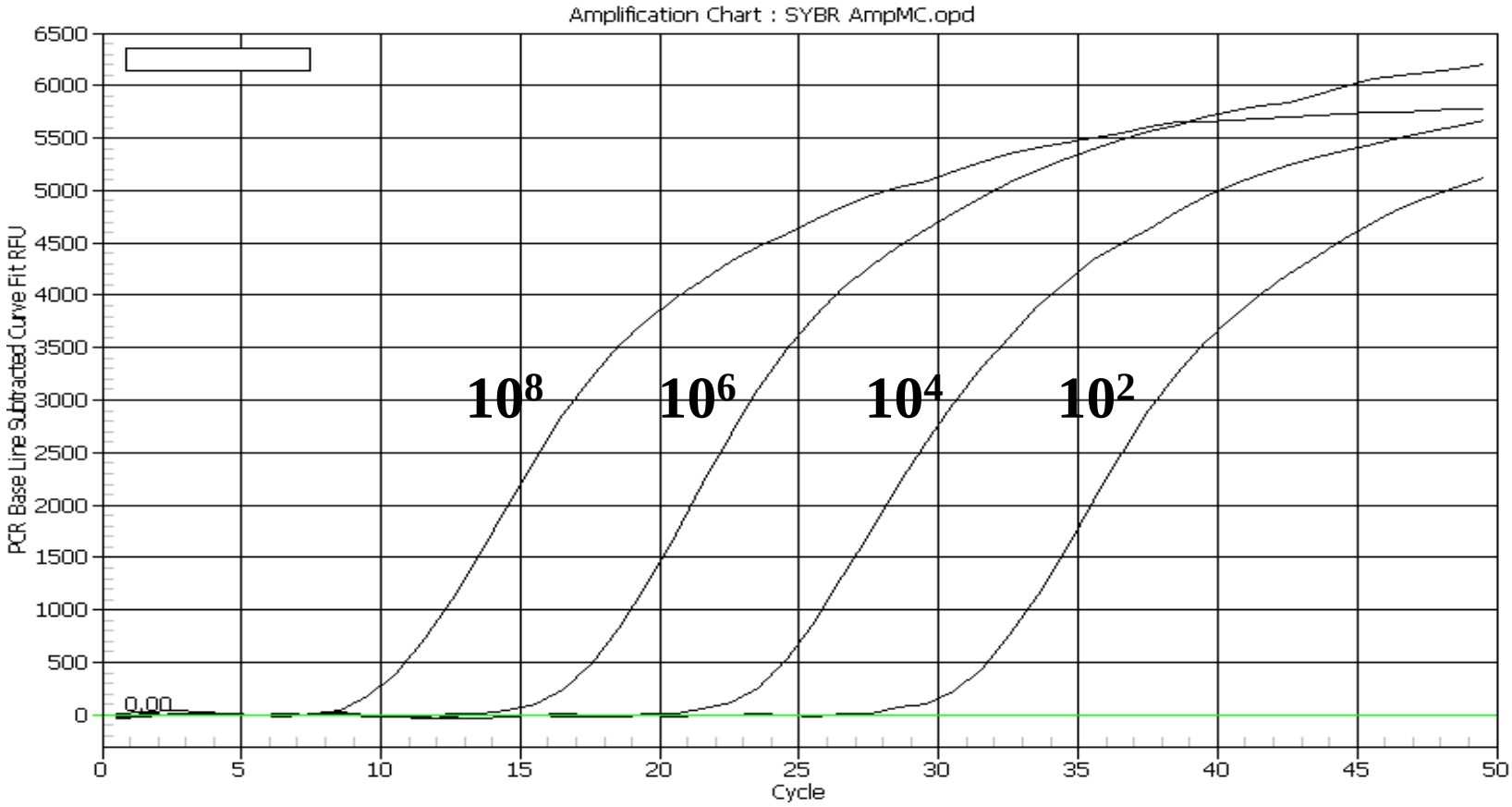
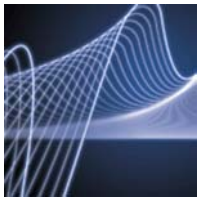


- ***Absolute***
  - Determines input quantity of a nucleic acid target
  - Requires a standard curve
- ***Relative***
  - Determines fold differences in input target nucleic acid quantities between samples
  - Performed with or without a standard curve
  - Typically used for gene expression analysis with a target gene normalized to a reference gene



Absolute quantification





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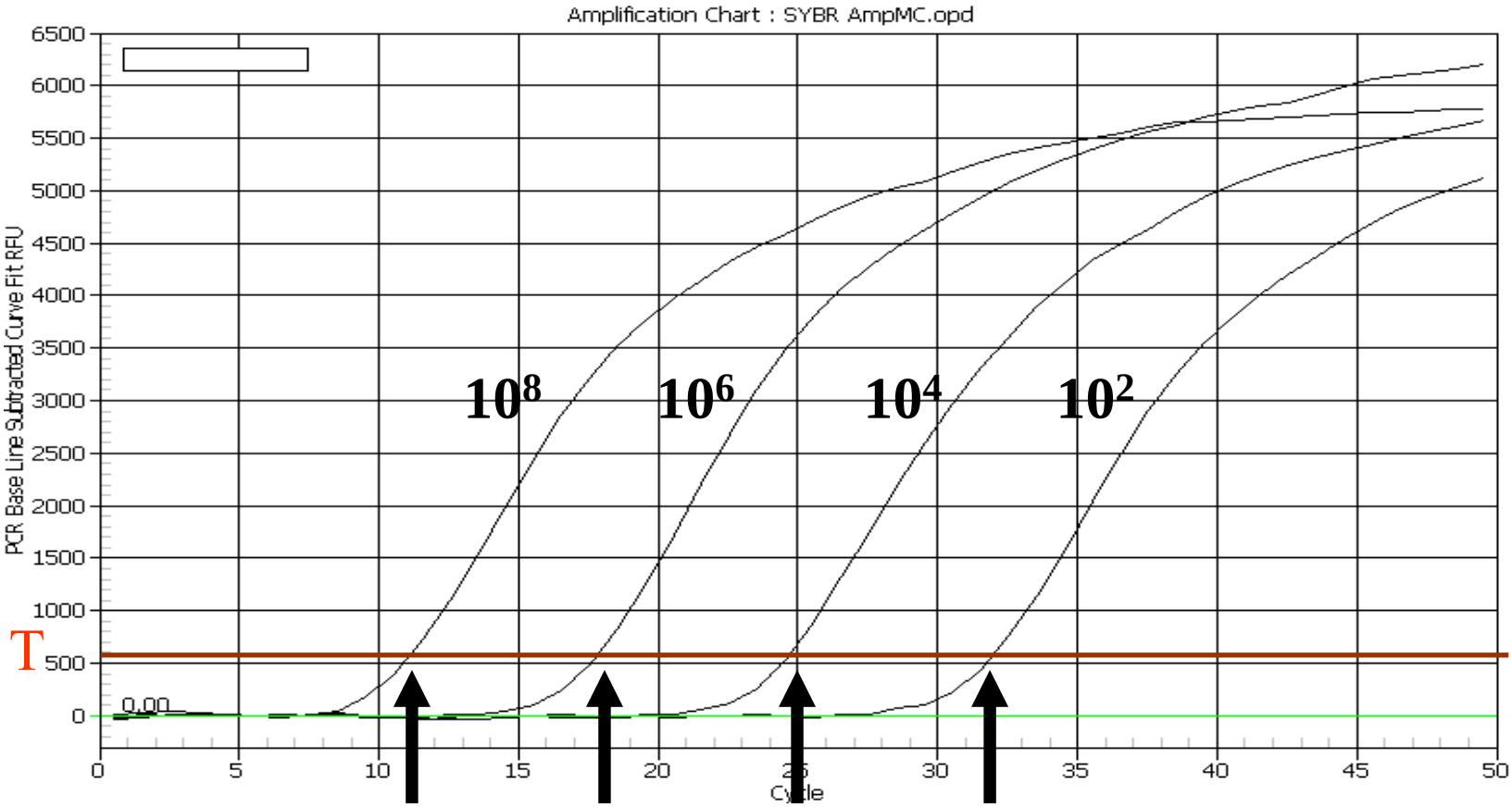

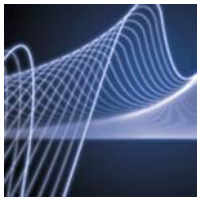

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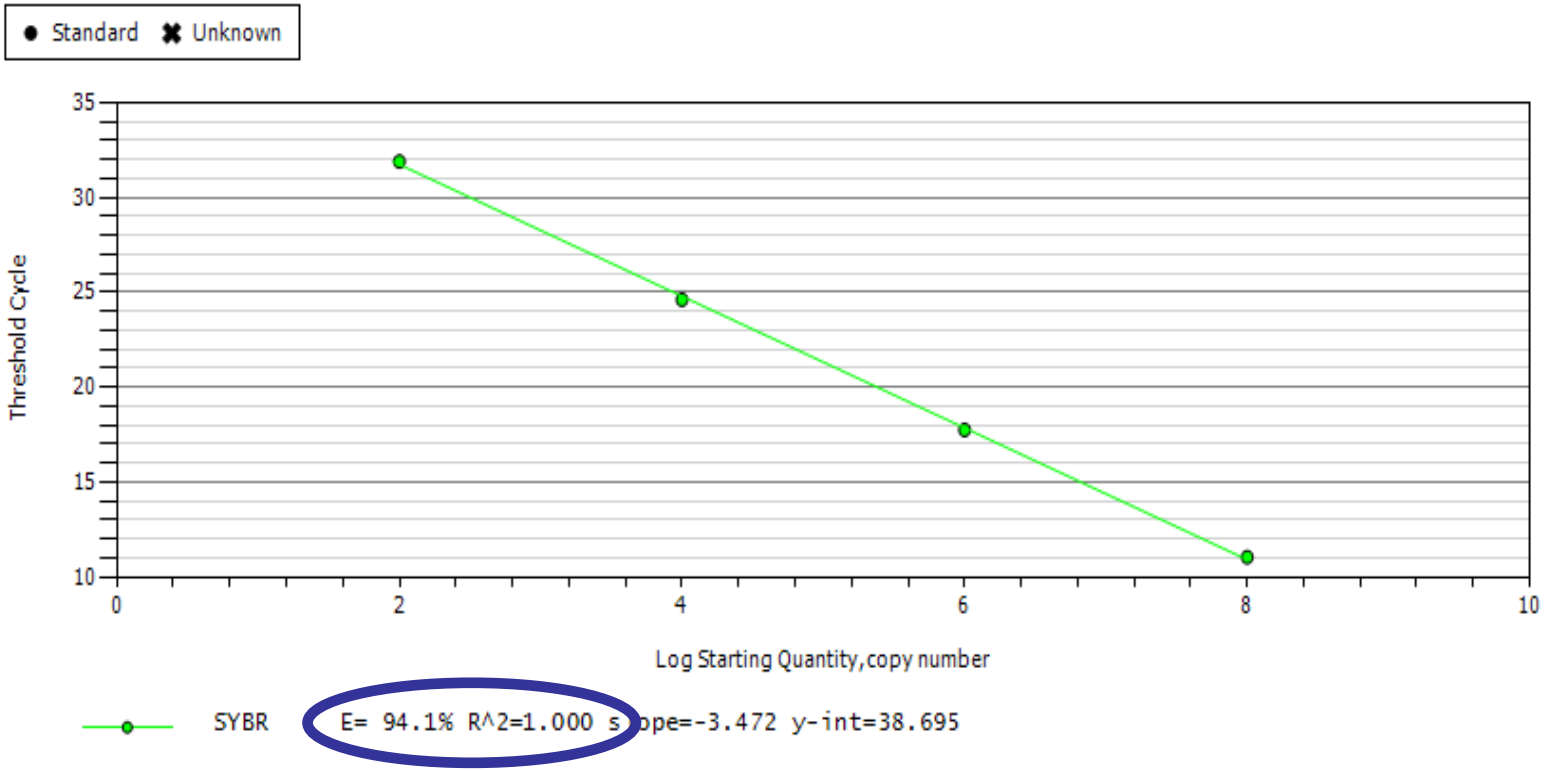

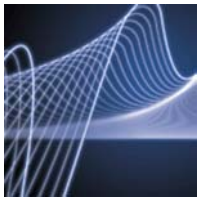

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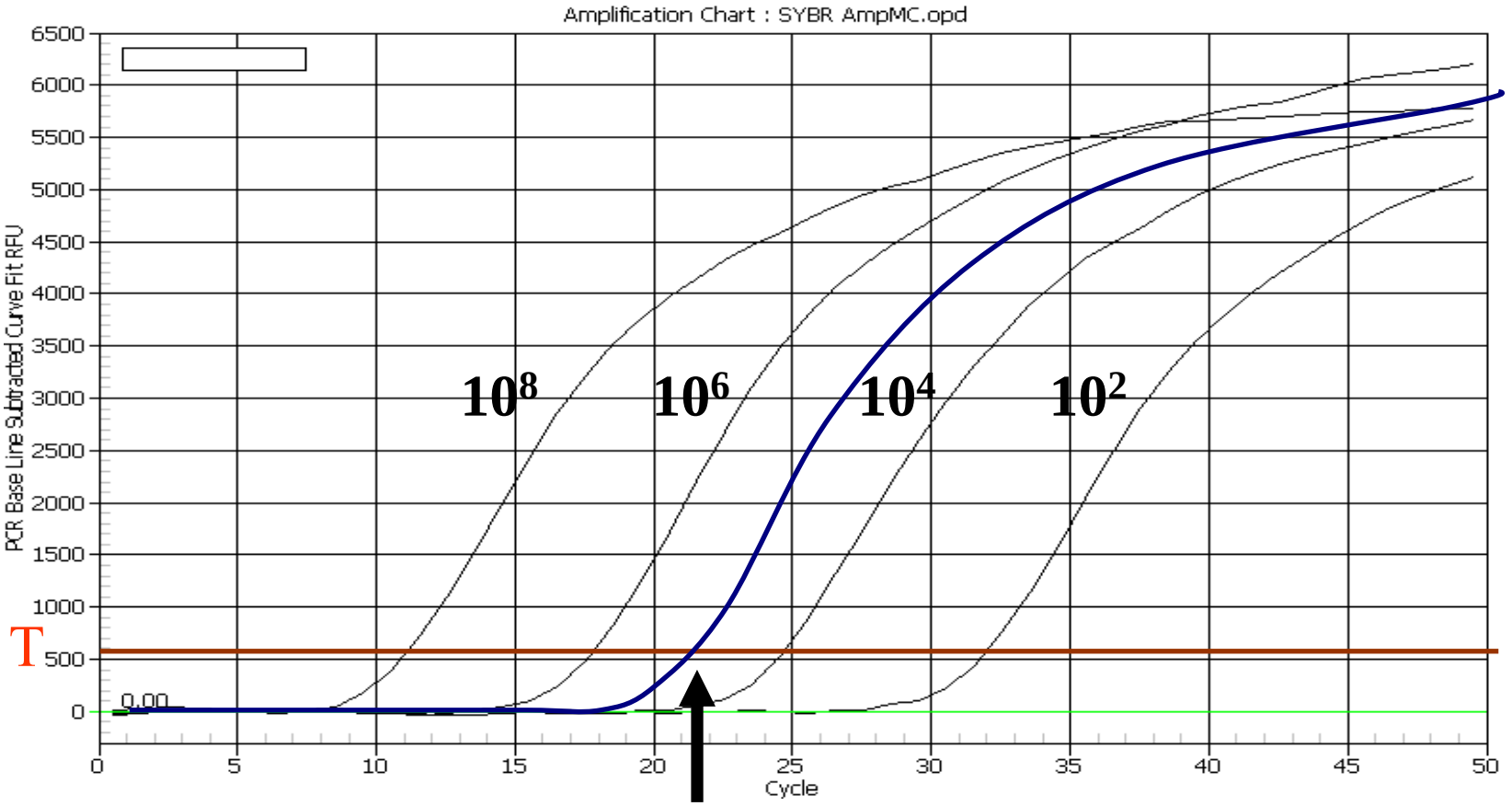

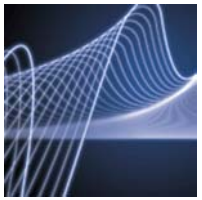

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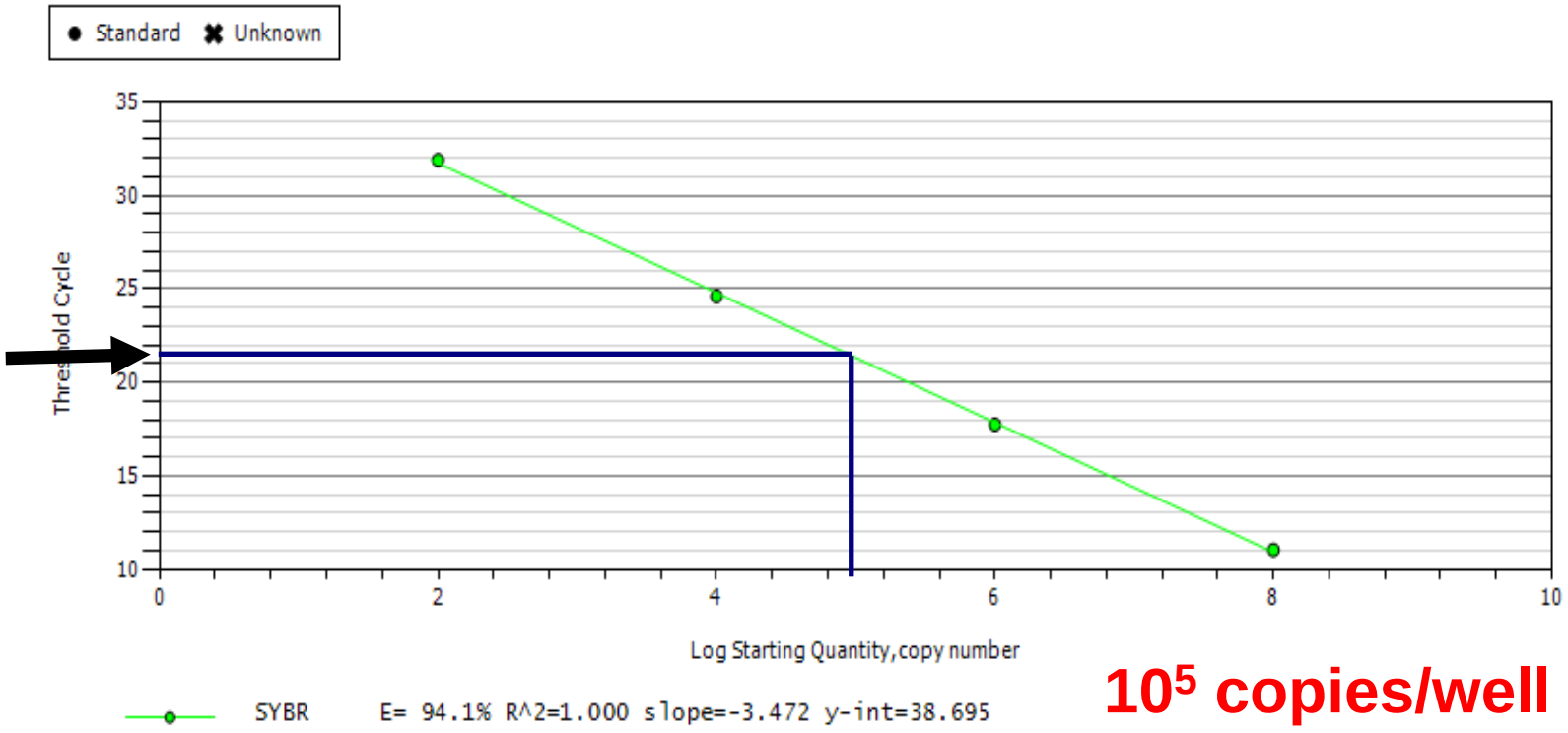
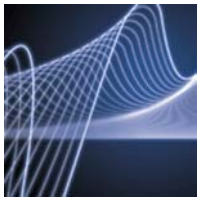


10^5 copies/well



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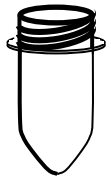

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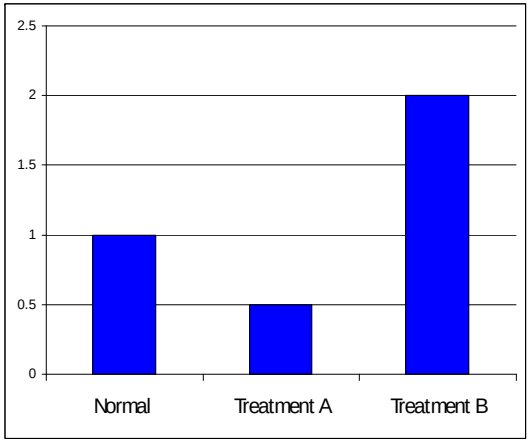

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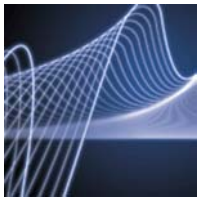
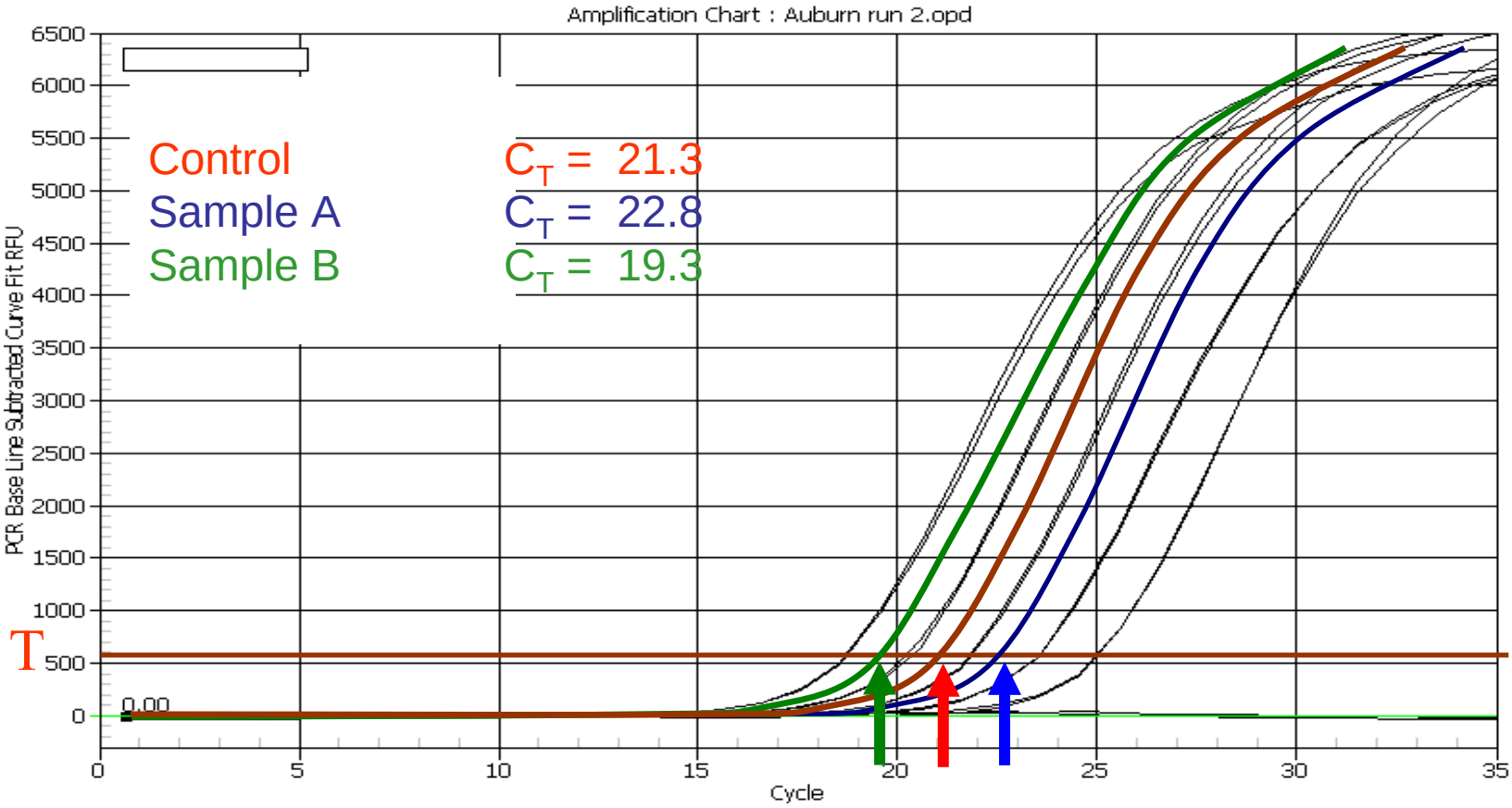
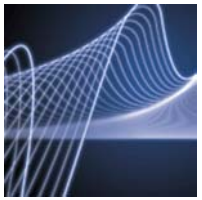
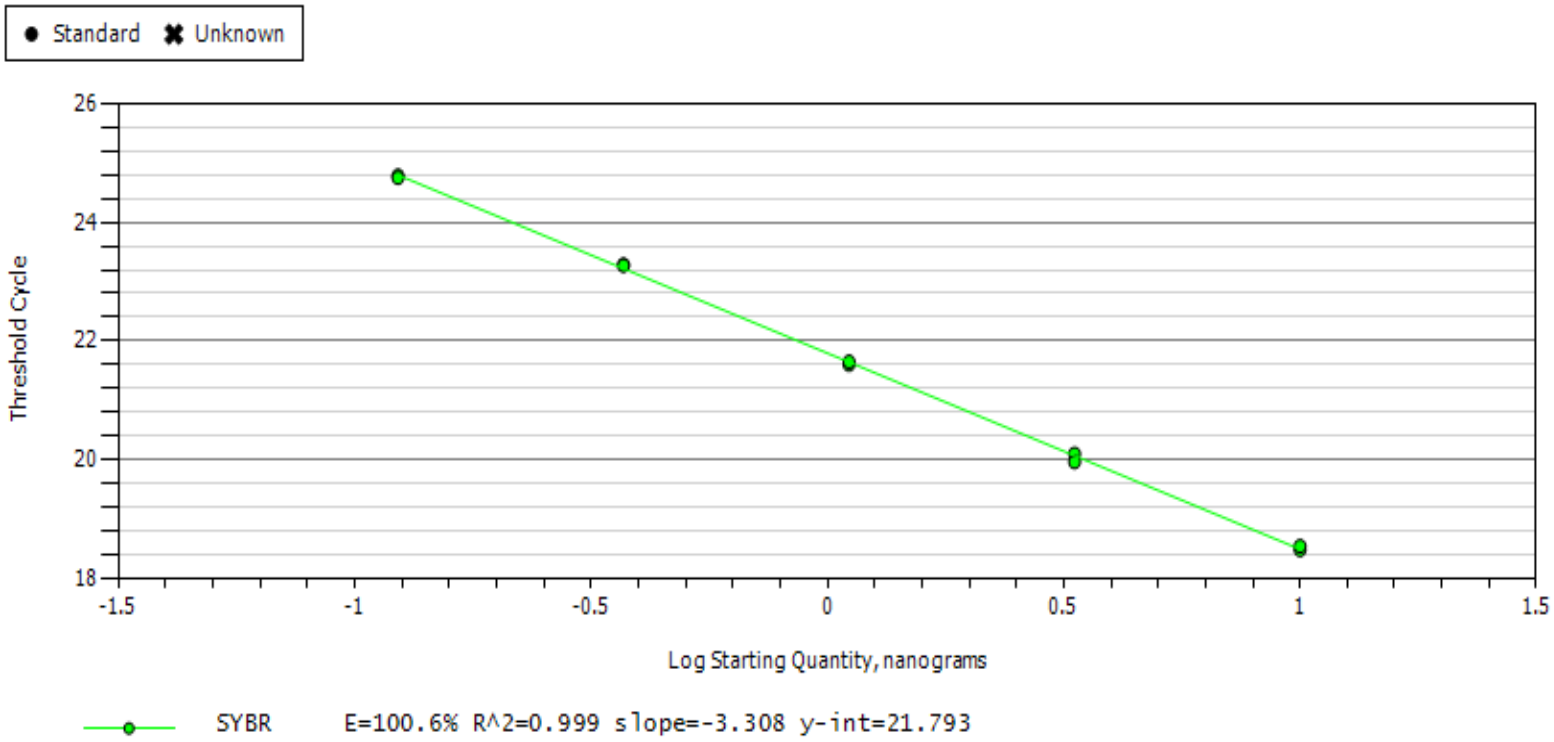


Relative quantification





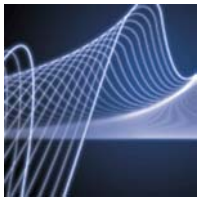
Relative quantification



Control C_T = 21.3 = 1.5ng / tube

Sample A C_T = 22.8 = 0.5ng / tube

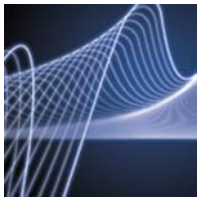
Sample B C_T = 19.3 = 6.0ng / tube



Gene Expression Normalization - Comparative C_T

- Relative Quantity (△ CT)
  - – Not normalized
  - – Normalization accomplished via equal loading of samples
  - – Post analysis normalization

- Normalized Expression (△△ CT)
  - – Accounts for loading differences
  - – Usually normalize to reference gene
  - – Relative quantity of GOI is normalized by the relative quantity of the reference genes

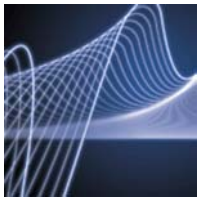


Normalized Expression

- 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

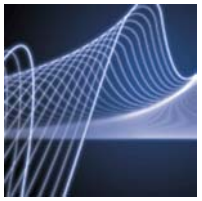
Complex



Relative Quantification - ΔCt

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

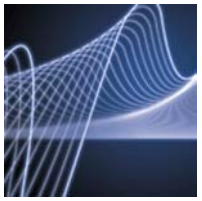
Fold induction = 2^2 = 4



Comparative Ct Method (2-ΔΔCt)

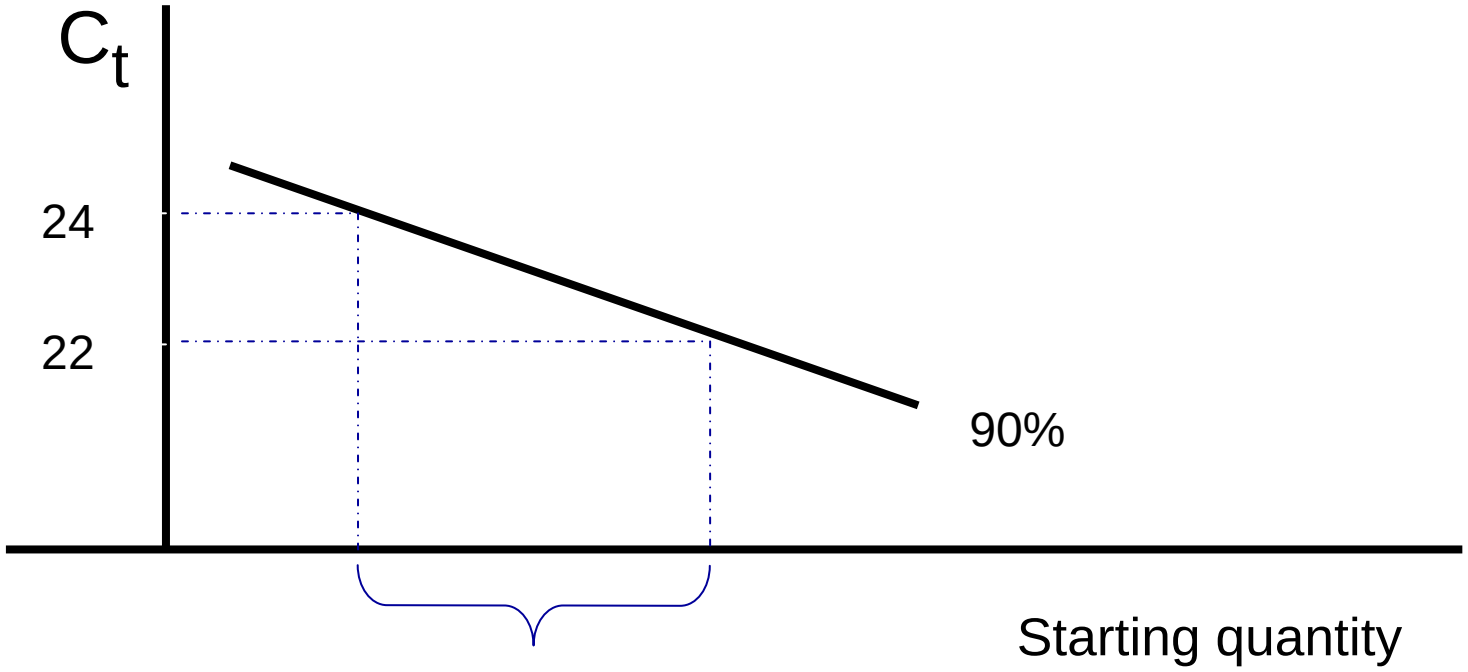
		Reference	GOI
	Tissue #1:	21	22
	Tissue #2:	20	24
1st Delta	Delta Ct #1:		22-21 = 1
	Delta Ct #2:		24-20 = 4
2nd Delta	Delta Ct:		4-1 = **3**

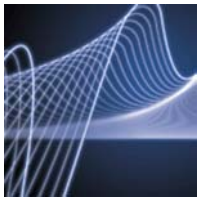
Fold induction = 2^3 = 8



Relative Quantification

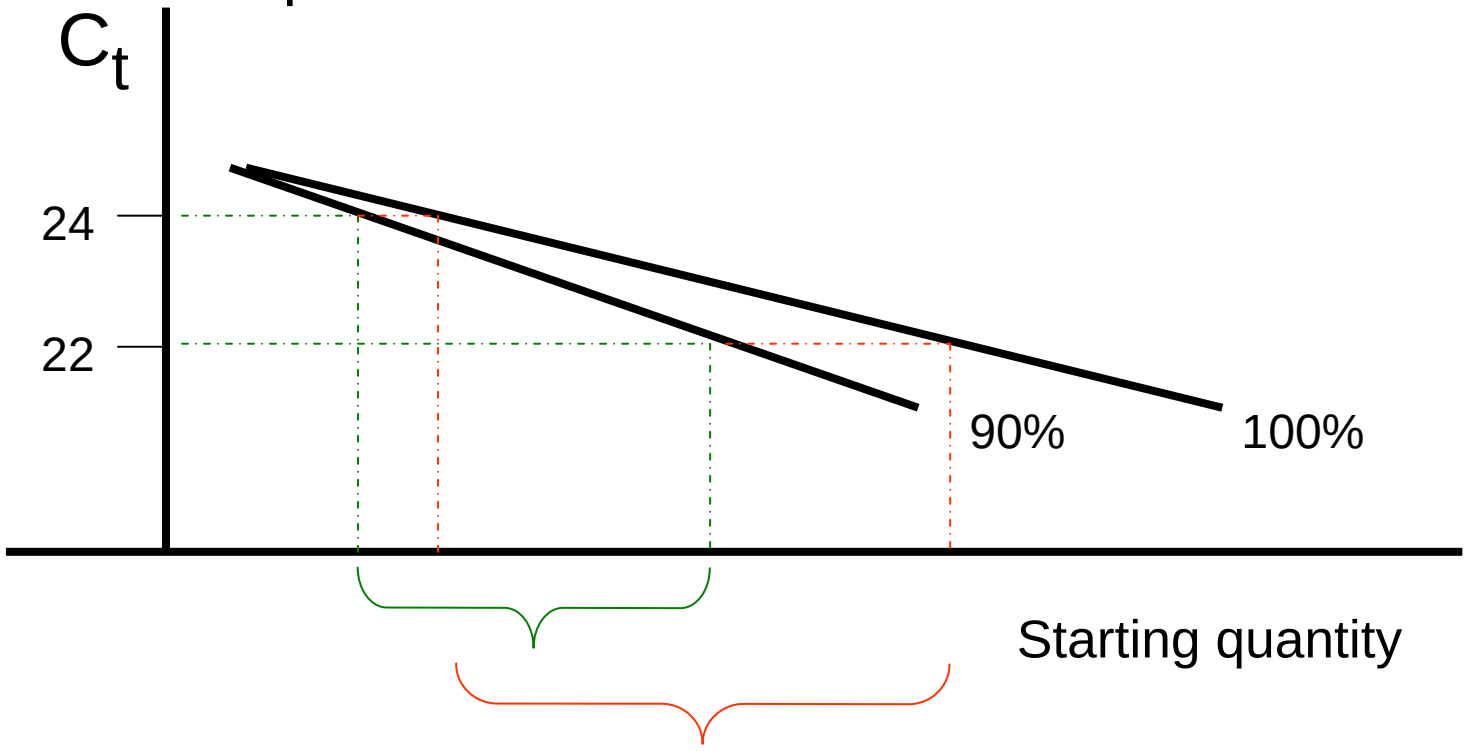
- Problem with the ΔΔCT

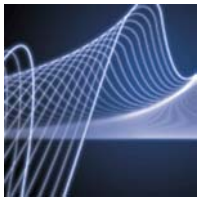




Relative Quantification

- Problem with the ΔΔCT
- Slopes are not parallel



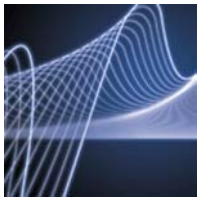


Relative Quantification

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

(Pfaffl, 2001; Nucleic Acid Research)

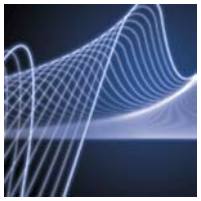
$$\text{Efficiency} = 10^{-1/\text{slope}}$$



Relative Quantification –Pfaffl Modification

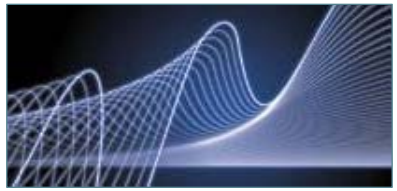
		Primer set #1Reference	Primer set #2 GOI
	Tissue #1:	21	22
	Tissue #2:	20	24
(From Standard curve)	Efficiency:	90% = 1.9	100% = 2
	Delta Ct:	20-21 = -1	24-22 = 2

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



Methods Comparison

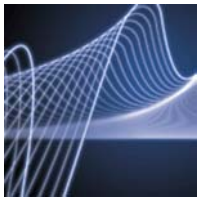
- Δ Ct method: (no reference gene)
  - Fold induction : 4
- ΔΔ Ct method: (reference gene)
  - Fold induction : 8
- Pfaffl modification: (reference gene and efficiency)
  - Fold induction : 7.5



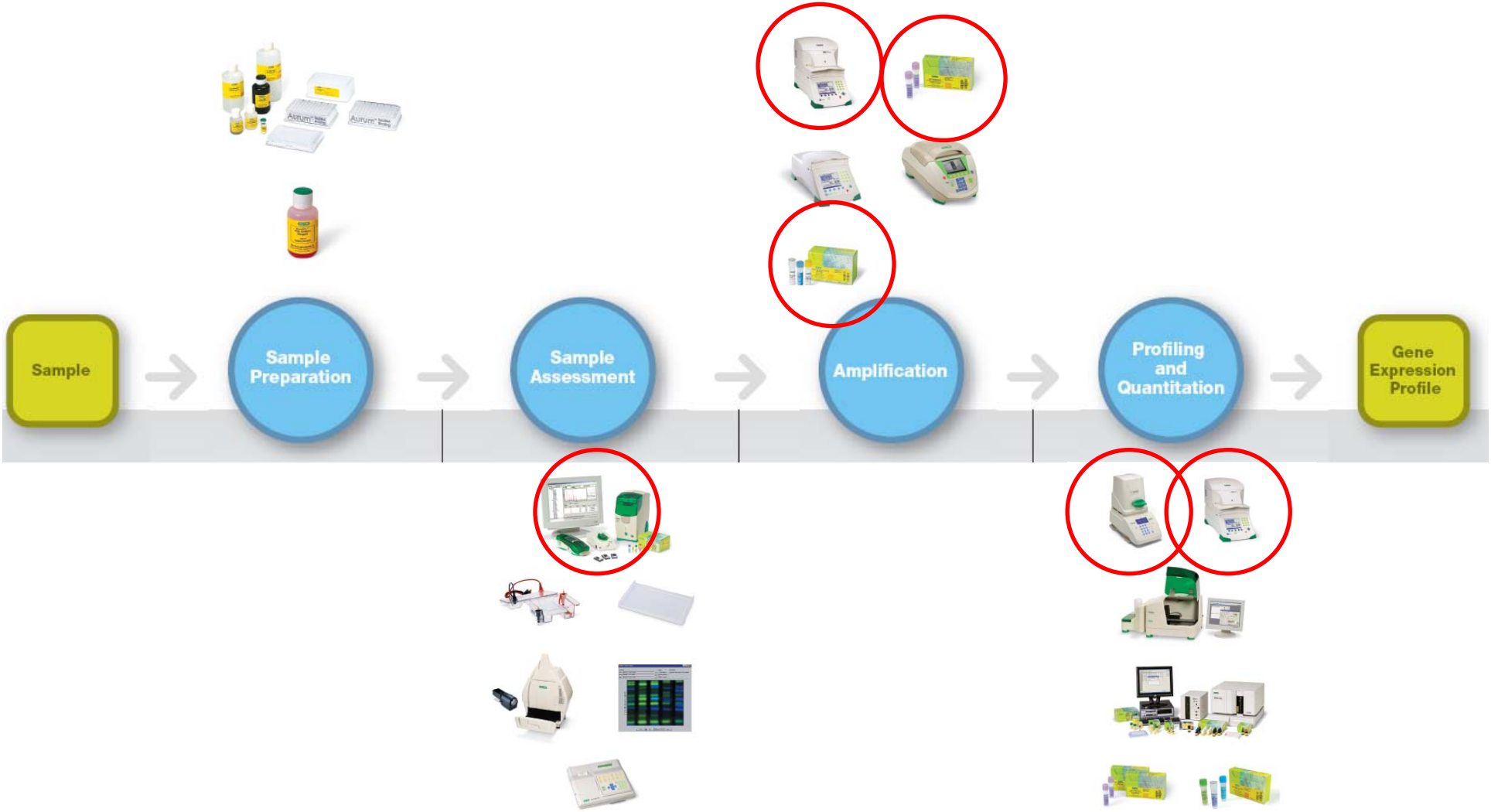
How to design Real Time PCR experiment

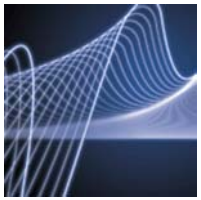
Version 1.0

AMPLIFICATION



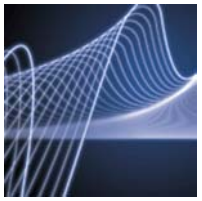
Gene Expression Workflow





Assay Considerations

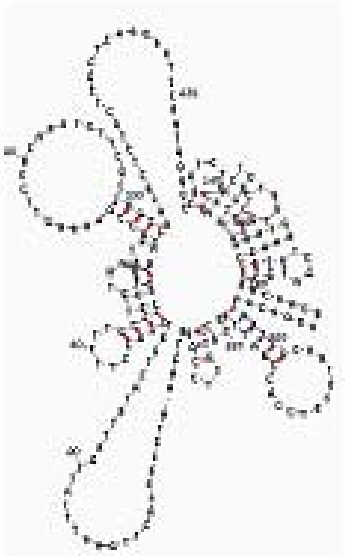
- ✓ *Gene structure*
- ✓ *Primer design*
- ✓ *Quality of RNA*
- ✓ *Reagent quality*
- ✓ *Pipetting*
- ✓ *Good lab practices*



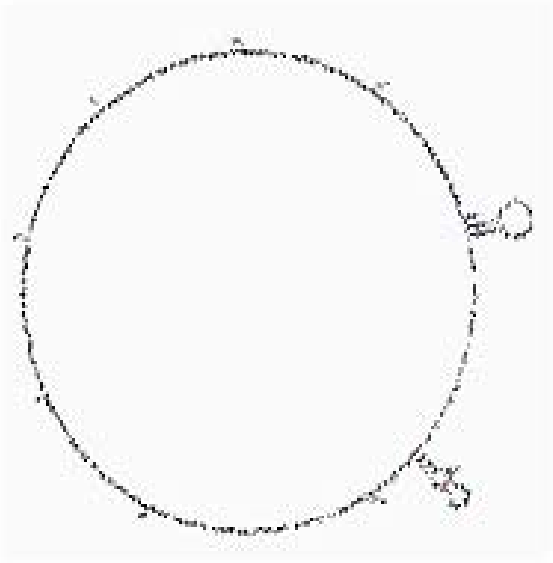
Amplicon Secondary Structures

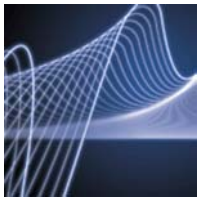
http://mfold.rna.albany.edu/?q=DINAMelt/Quickfold

- Bad location for primers



- Good location for primers

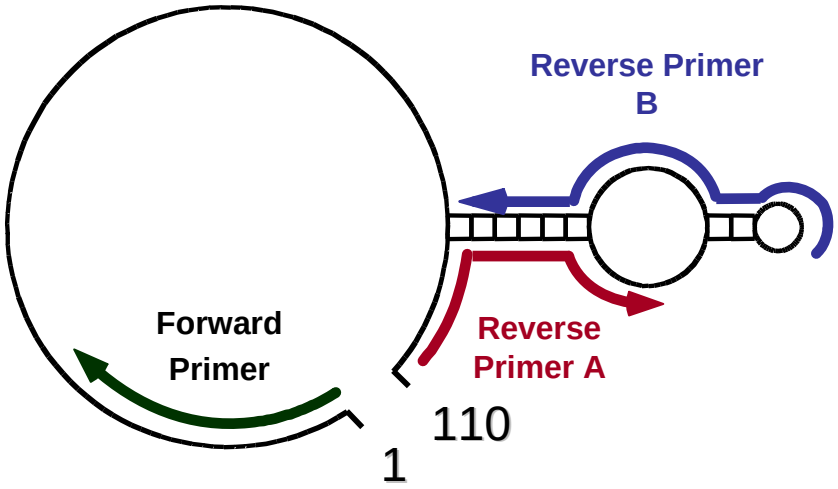


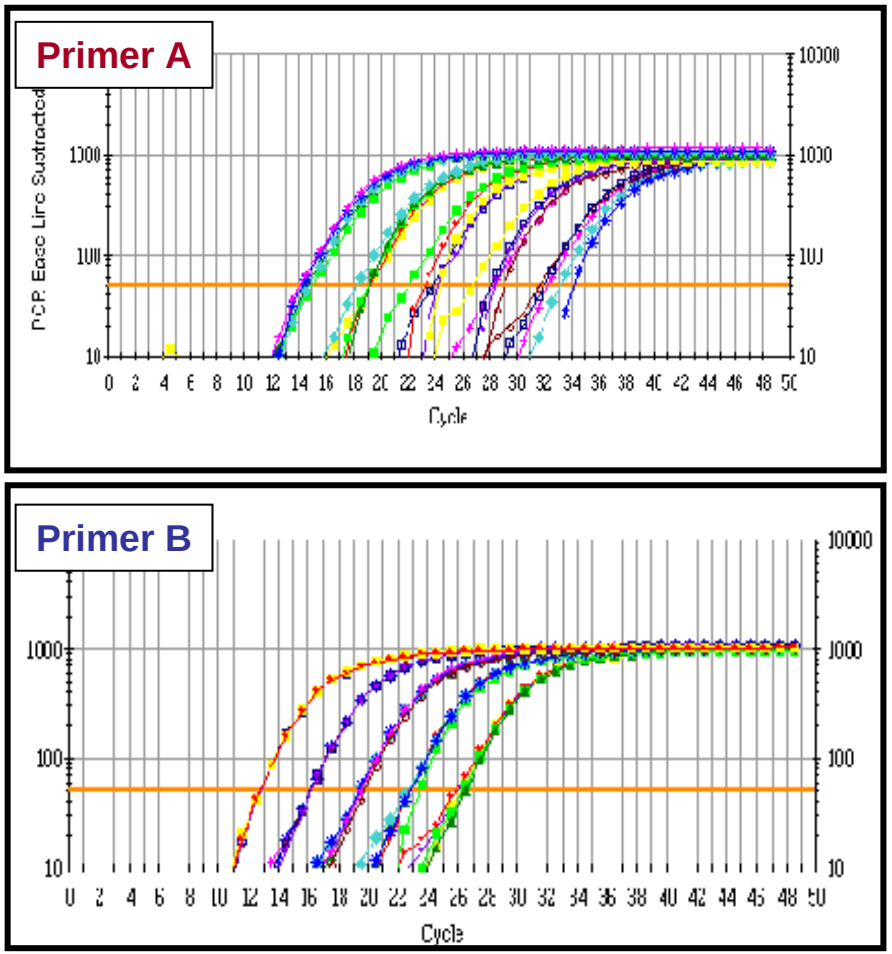


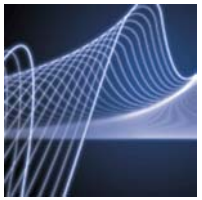
Effect of primer location

Primer A: η = 66.3 %

Primer B: η = 95.8 %

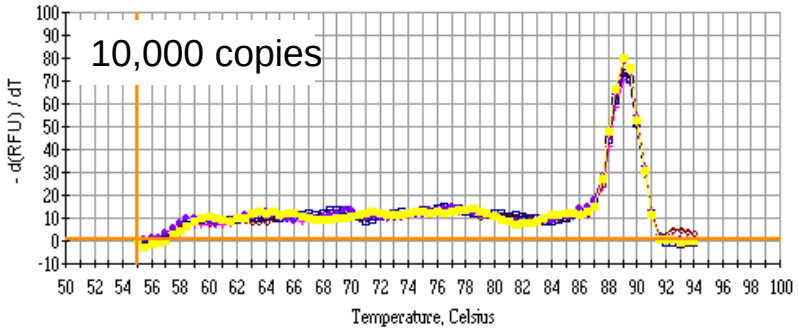




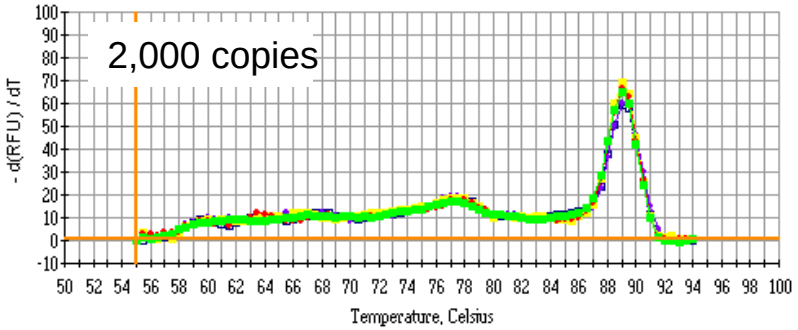


Non validated primers

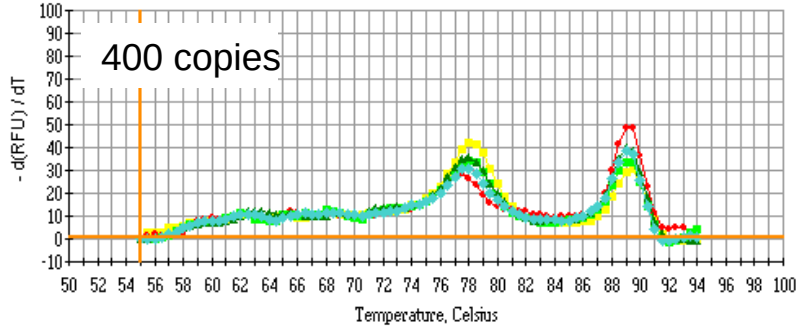
Fluorescence vs Temperature | -dF/dT vs Temperature



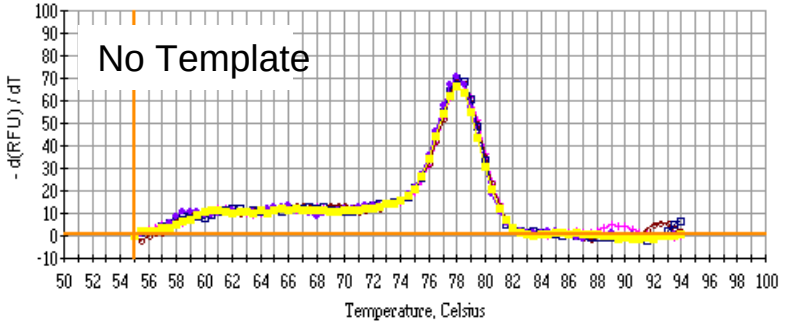
Fluorescence vs Temperature | -dF/dT vs Temperature

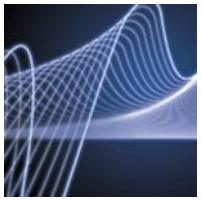


Fluorescence vs Temperature | -dF/dT vs Temperature

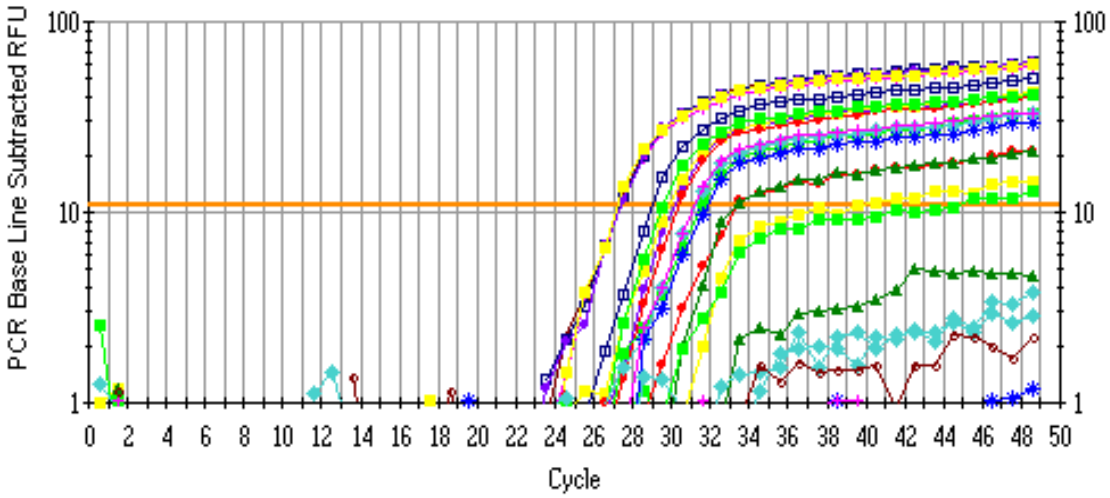


Fluorescence vs Temperature | -dF/dT vs Temperature

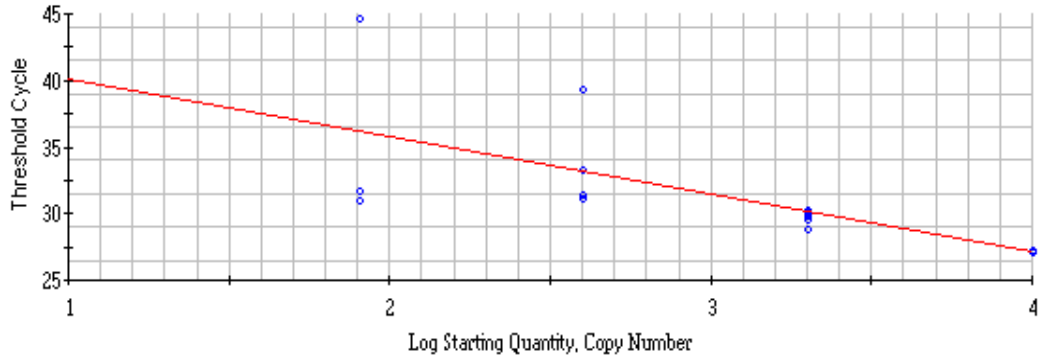




Non validated primers



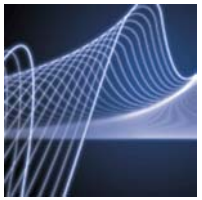
Same primers with a specific probe



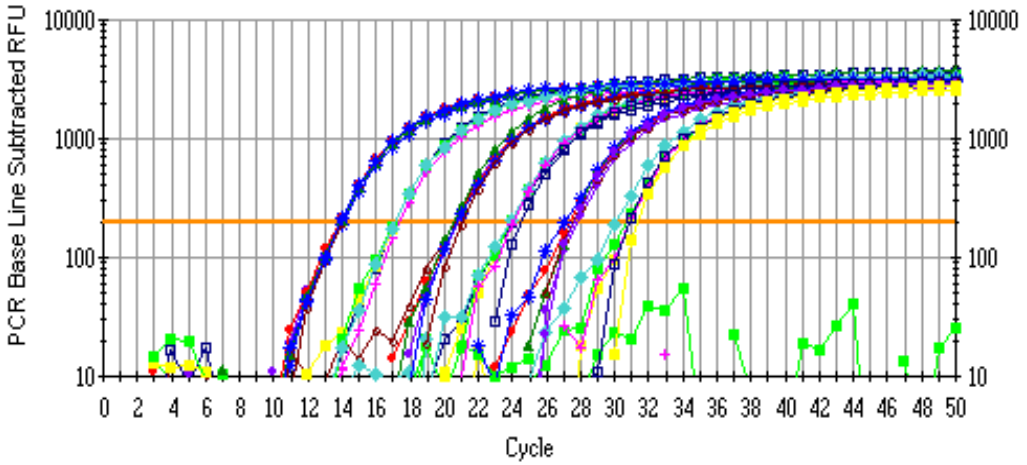
Poor resolution

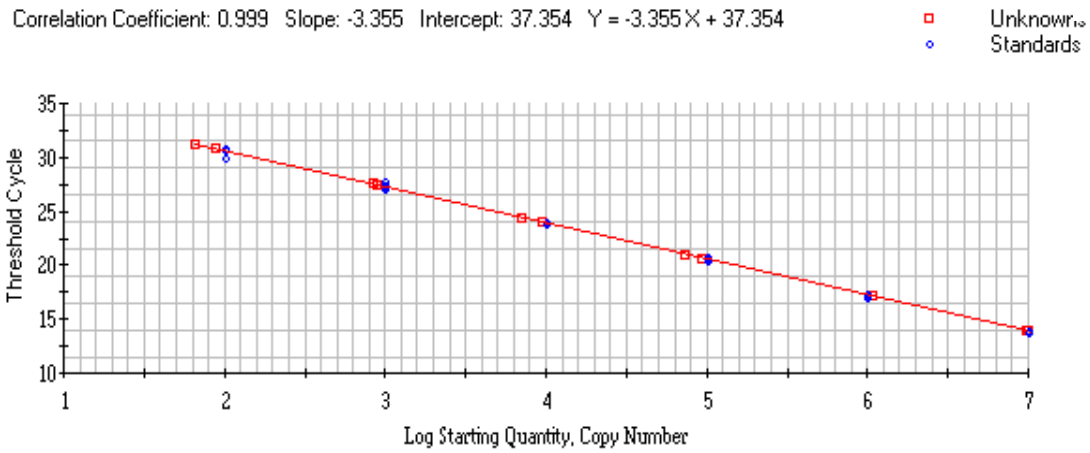
Poor replicates

η = 71%



Validated primers

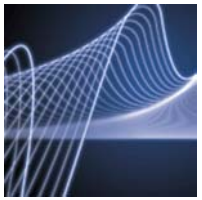




After primer re-design to eliminate primer-dimers

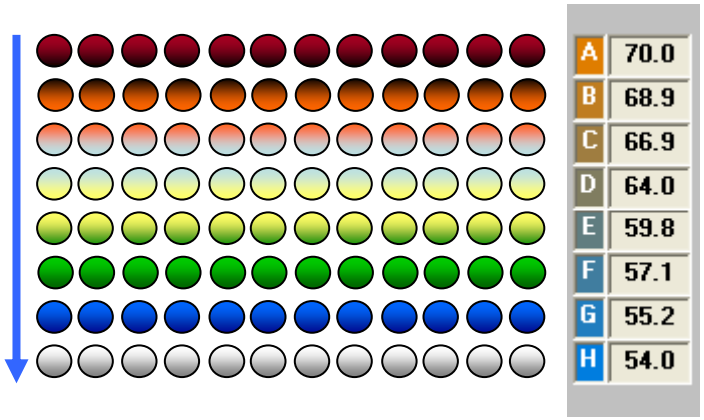
r = 0.999

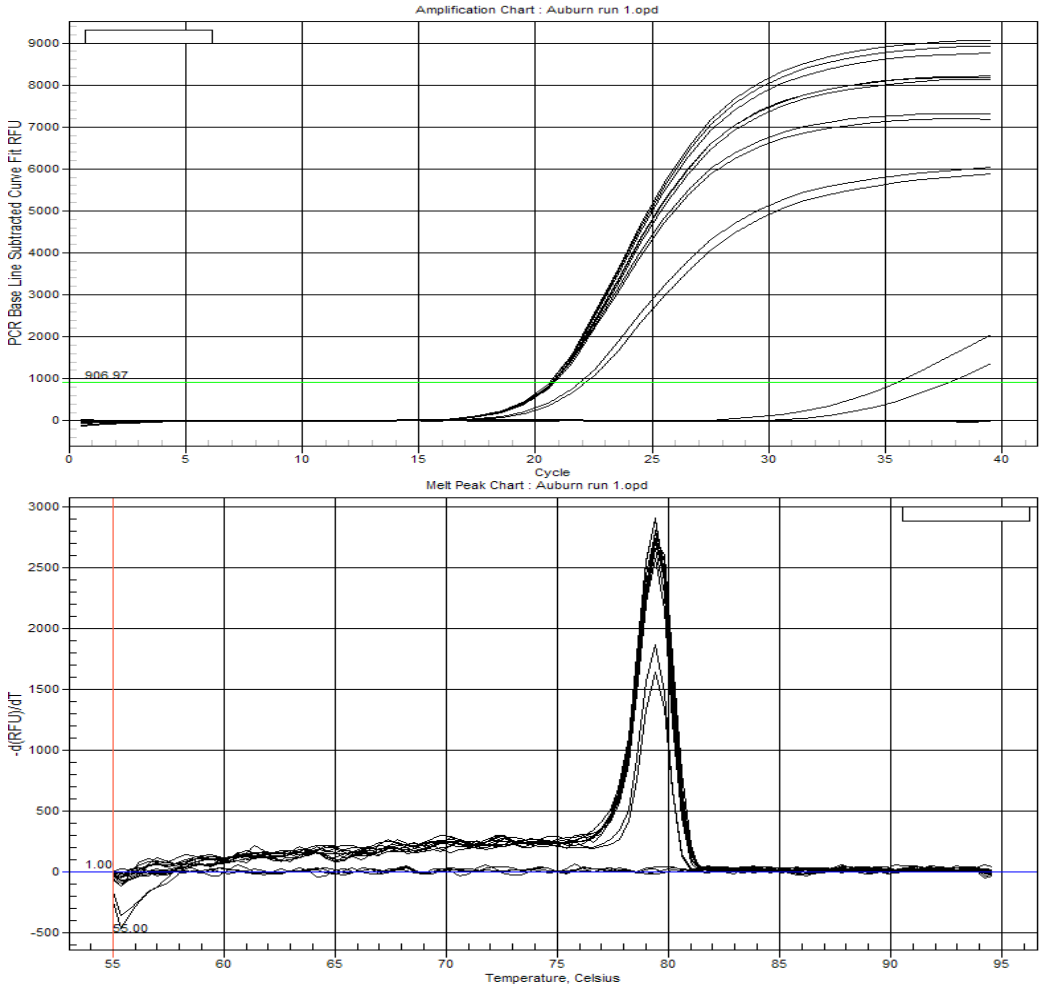
η = 91.3%

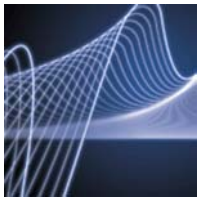


Fast Assay optimization

dynamic thermal gradient

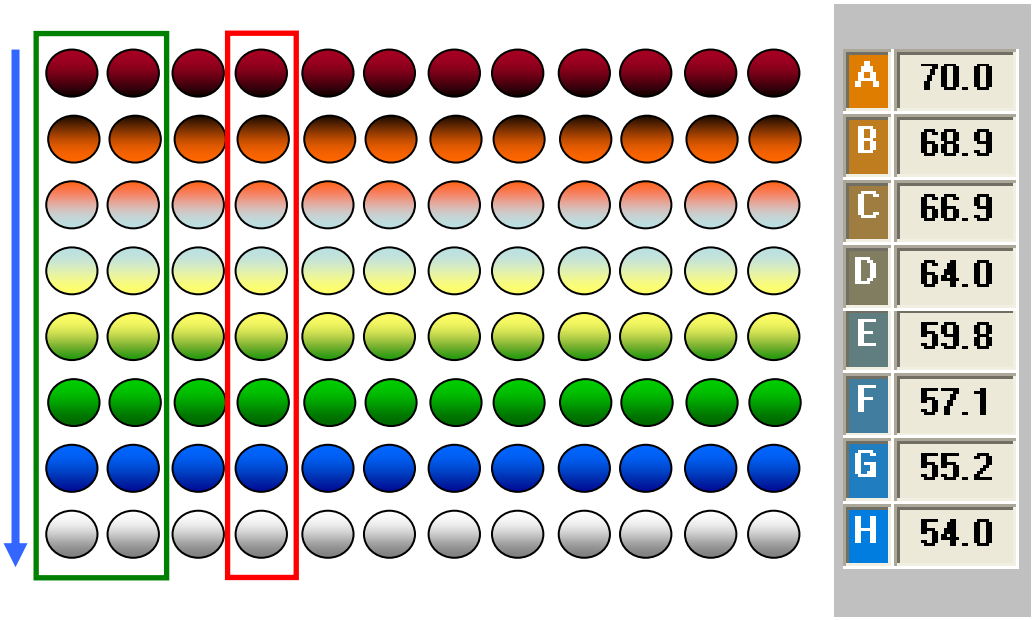






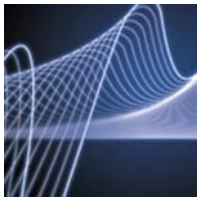
Fast Assay optimization

dynamic thermal gradient

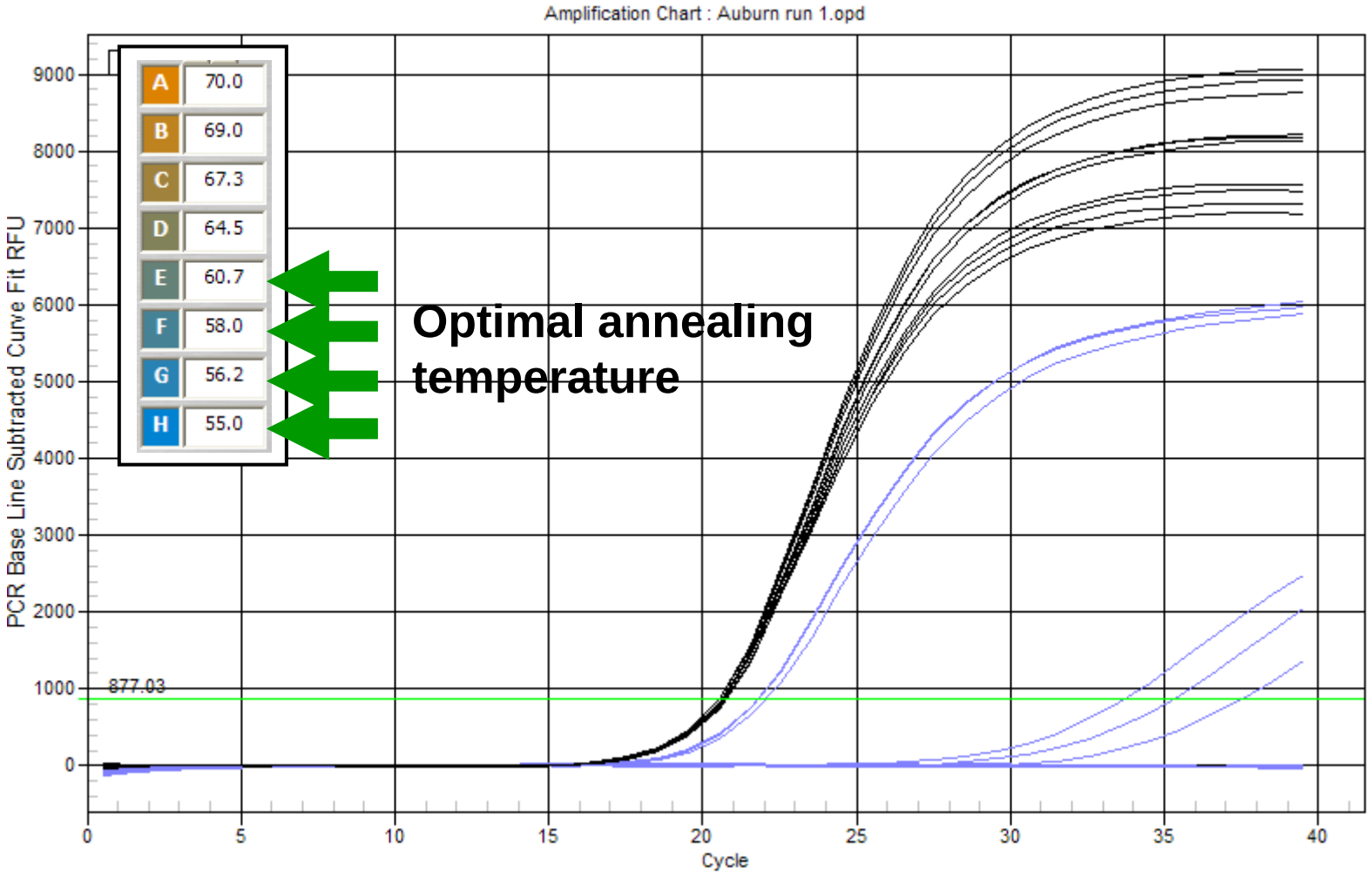


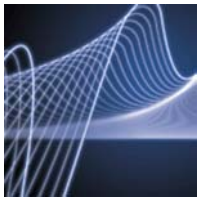
10°C Above

6°C Below



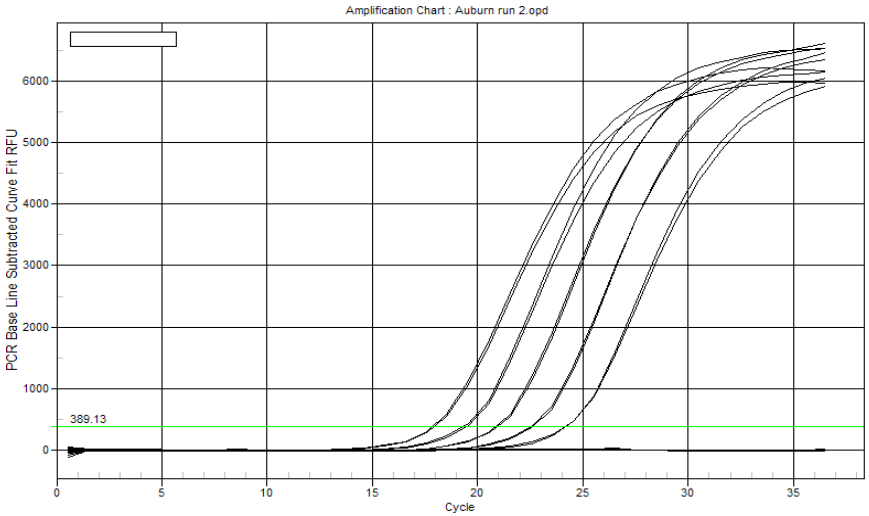
Gradient optimization

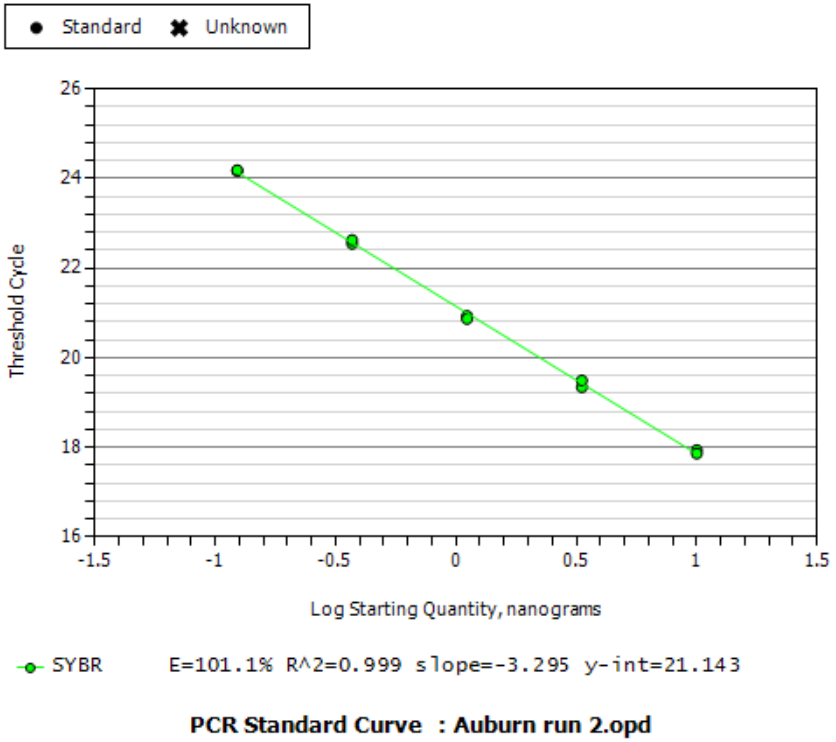


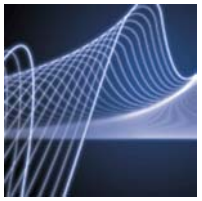


Fast Assay Optimization

Test dynamic range

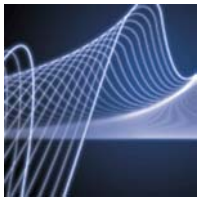






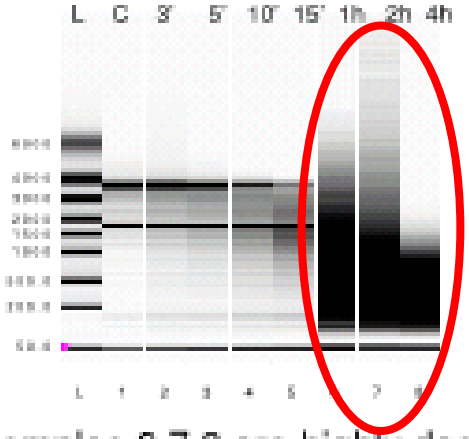
Sample and Template Preparation

- Extract and analyze RNA
- Careful quantification is necessary
  - RiboGreen Assay - Quantification
  - Implen nanophotometer – Quantification and purity
  - Experion™- Quality and quantification



Analysis of RNA purity and integrity

Experion Virtual Gel



Samples 6,7,8 are highly degraded.

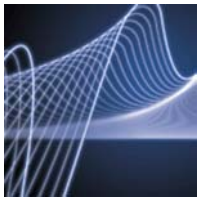
Corresponding Nanodrop Readings

Sample	Conc (ng/ul)	A260/280*	A260/230*
Control-no heat	115	1.90	2.44
3 min @ 90C	114	1.93	2.40
5 min @ 90C	115	2.06	2.37
10 min @ 90C	115	2.03	2.37
15 min @ 90C	116	2.02	2.31
1 hr @ 90C	109	1.99	2.16
2hr @ 90C	117	2.00	2.32
4 hr @ 90C	118	1.89	2.23

*Generally accepted ratios (A260/280 and A260/230) for good quality RNA are > 1.8.

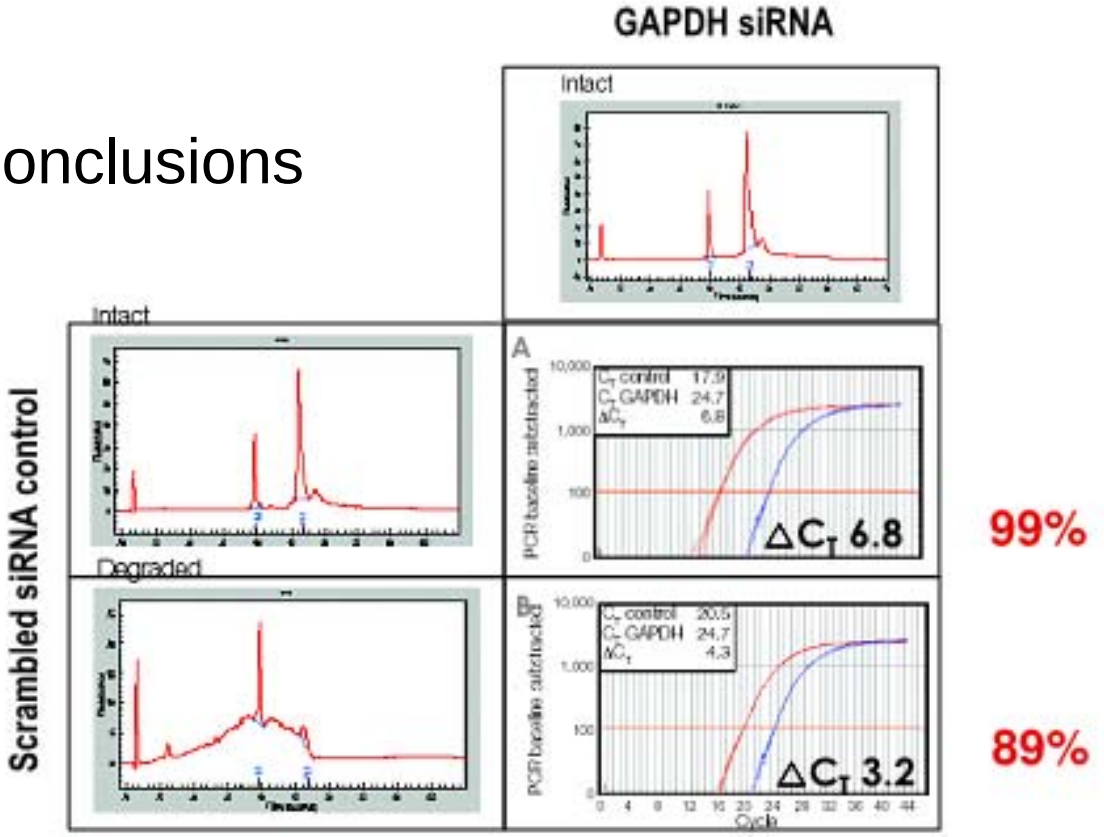
RQI Value & Color Coded Classification

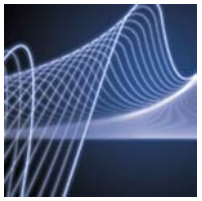
Well ID	Sample Name	RNA Area	RNA Concentration (ng/µl)	Ratio [28S/18S]	RQI	RQI Classification	RQI Alert
1	Control - no heat	278.23	102.60	1.60	9.8		
2	3 min @ 90C	317.12	116.94	1.23	9.2		
3	5 min @90C	306.89	113.17	0.89	8.1		
4	10 min @ 90C	257.56	94.98	0.50	6.5		
5	15 min @ 90C	247.31	91.20	0.15	5.5		
6	1 hour @ 90C	200.94	74.10	0.46	2.2		
7	2 hour @ 90C	252.37	93.07	0.81	2.0		
8	4 hour @ 90C	274.16	101.10	0.00	1.8		



Experion™ Analysis of RNA

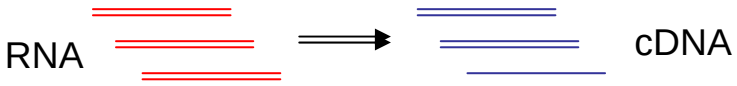
- Experiment: Evaluate siRNA-mediated gene silencing
- Prevent faulty conclusions





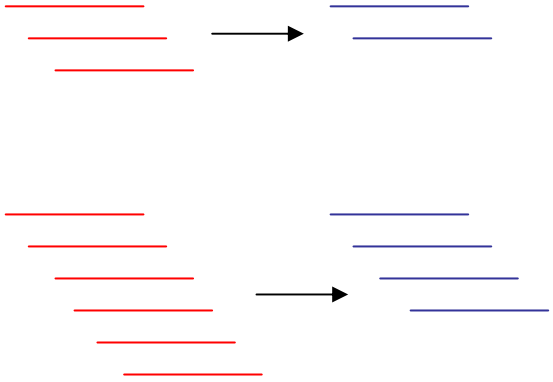
Assay Considerations

Reverse Transcription Efficiency

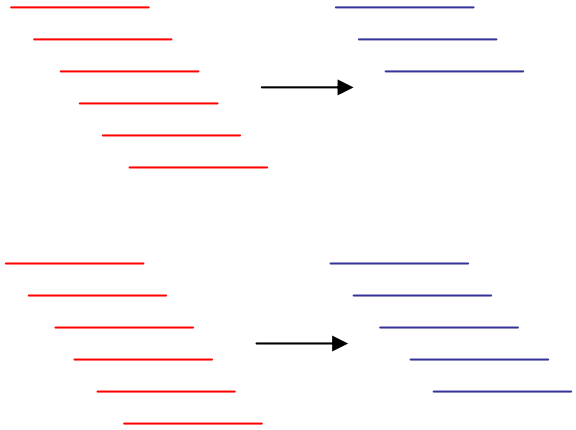


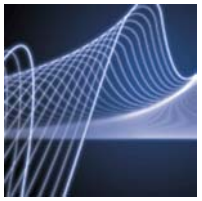
Ideal Reality ?

Reproducible Data



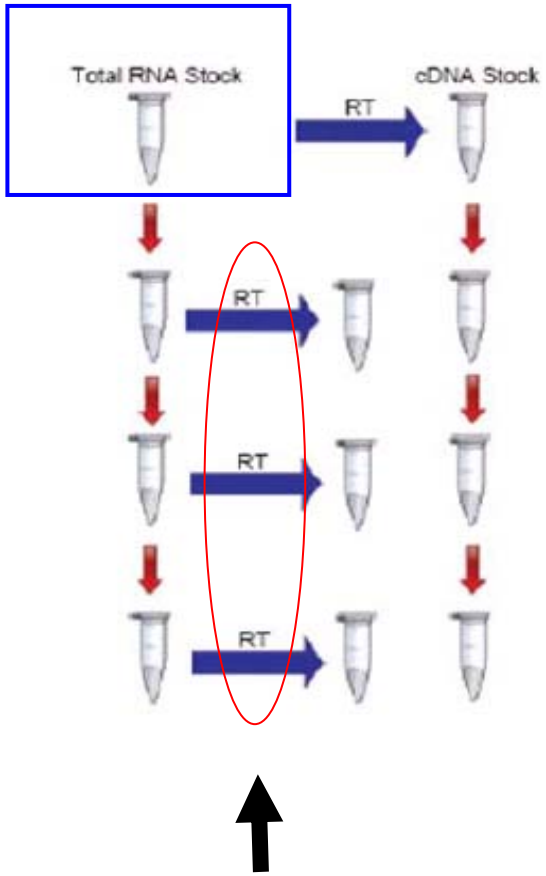
Not Reproducible





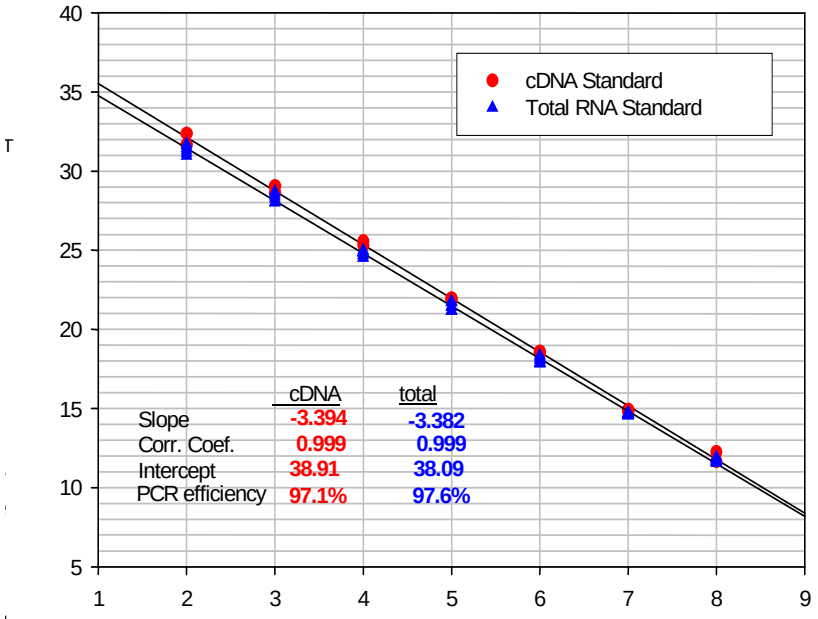
cDNA Synthesis

- Serial diluted RNA & cDNA

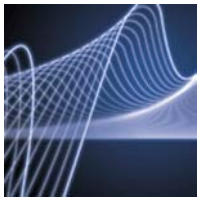


iScript qRT-PCR Standard Curve Comparison:

cDNA serial dilution vs. total RNA serial dilution



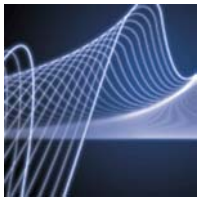
Note: 1/10th of cDNA reaction used for PCR



RNA to cDNA

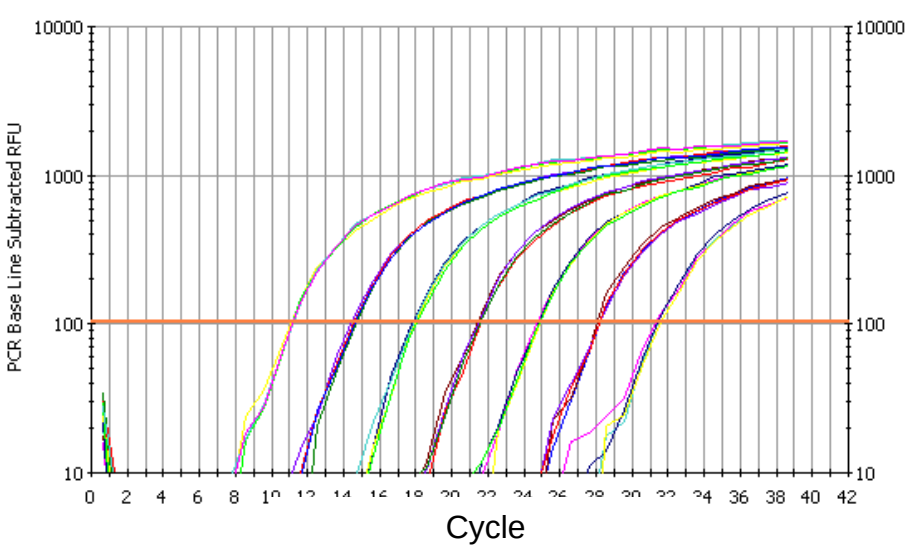
– Prior to RT: Treat RNA with RNase-free DNase

– After RT: use enzyme that has RNaseH activity to digest away RNA from RNA:DNA hybrid

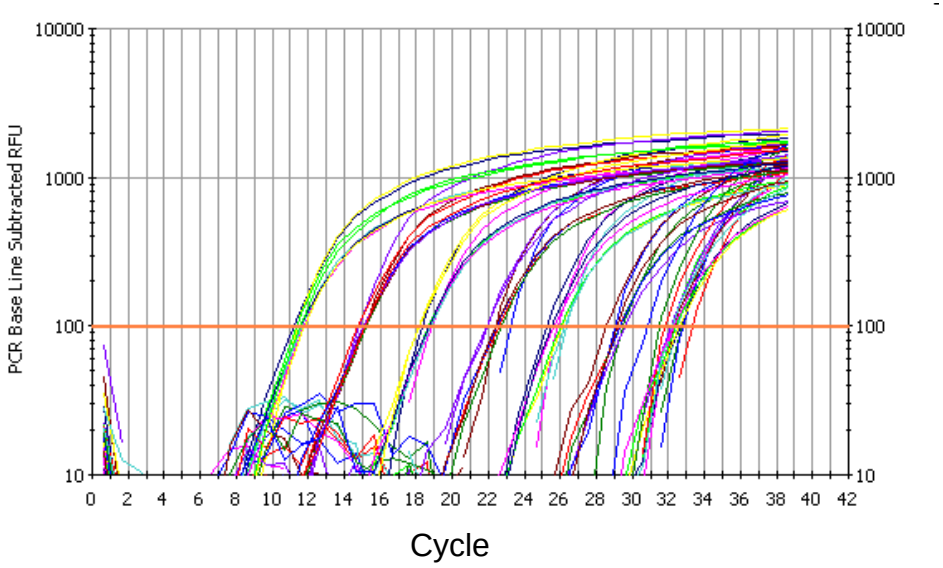


Assay Considerations

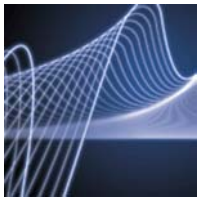
Same Reagents, Different Hands



Good Technique



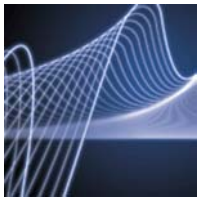
Poor Technique



Assay Considerations

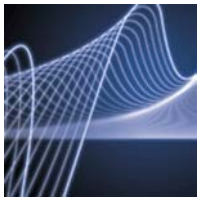
Improving Reproducibility

- Laboratory Techniques
  - Use clean bench (hood)
  - Use tips with filters
  - Use calibrated micropipettors
  - Use large volumes (3µL and up)
  - Minimal pipetting into each reaction well



實驗配方 for iQ SYBR green

• 2X iQ SYBR Green Supermix	12.5 ul
• Primer F (5 uM)	2 ul
• Primer R (5 uM)	2 ul
• ddH_2O	3.5 ul
• Template	5 ul
• Total	25 ul

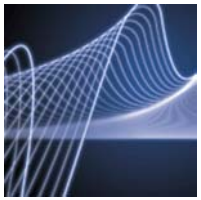


Conclusions

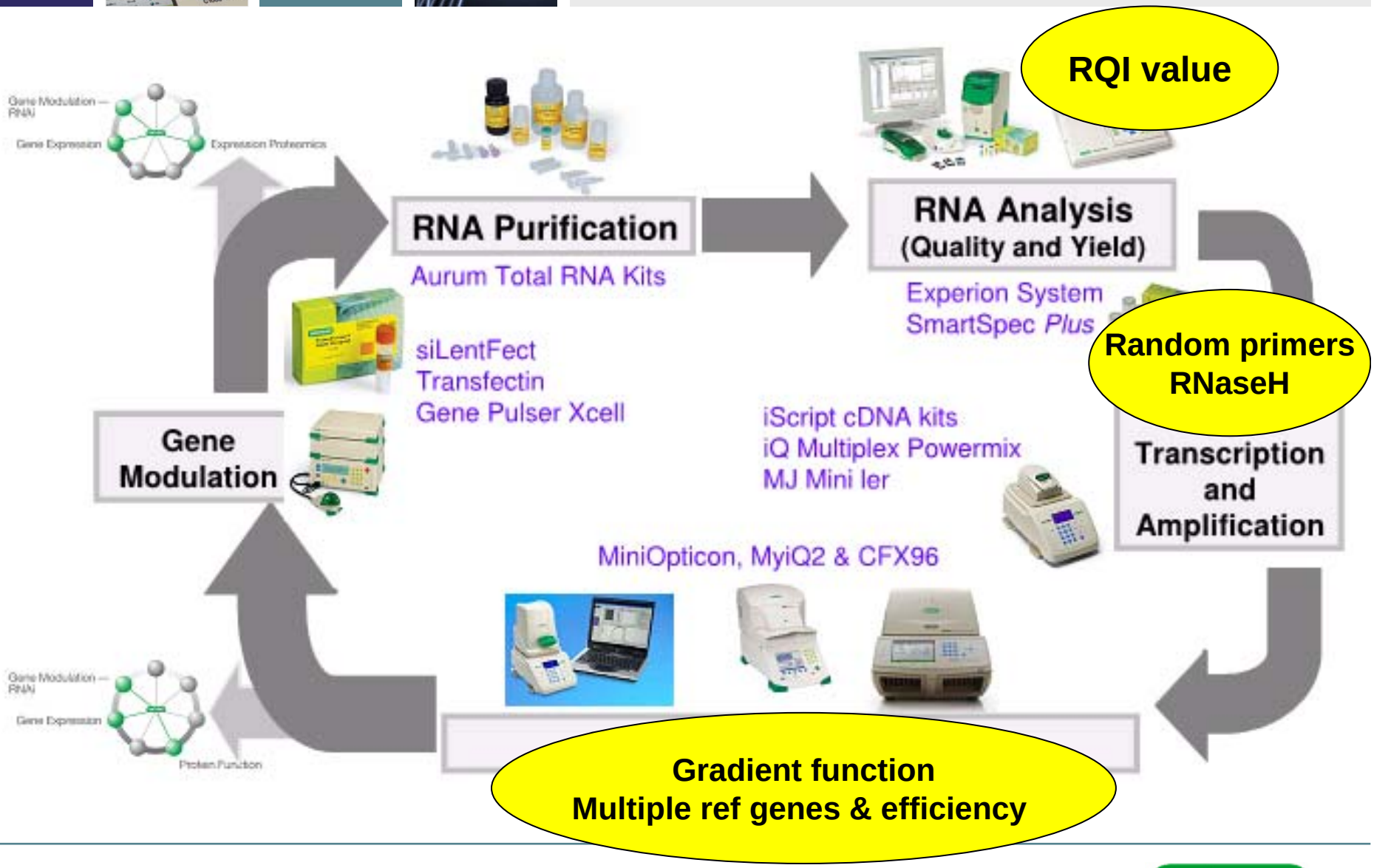
- Real-time PCR is a powerful technique that permits quantitative analyses to be performed
- Many detection strategies exist for qPCR, each requiring specific excitation and detection parameters
- Bio-Rad has the complete solution for all aspects of real-time PCR

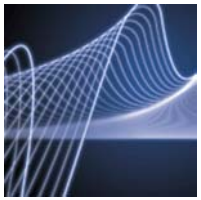
Gene Expression Gateway

www.bio-rad.com/genomics/



Complete solutions





THANK YOU