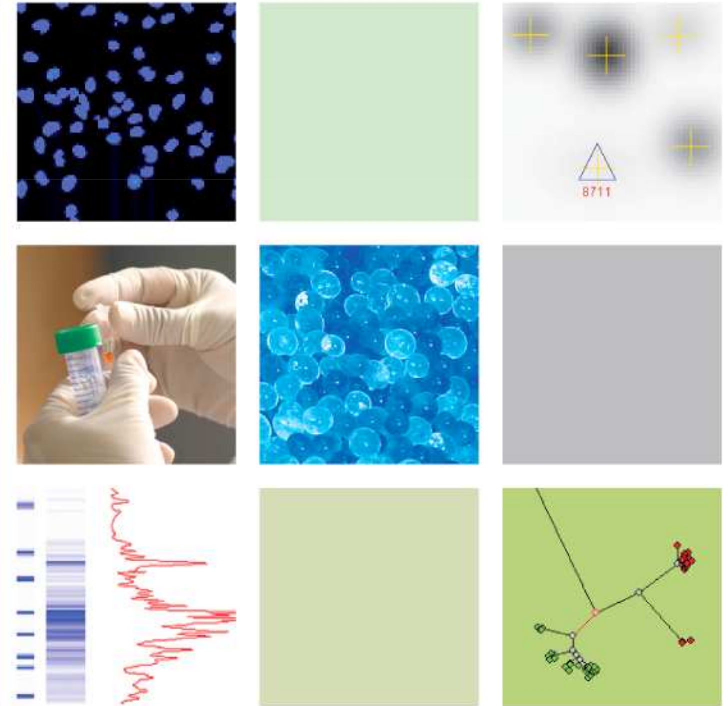
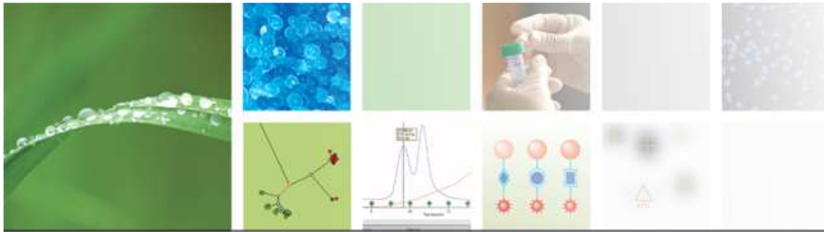
An Introduction to Real-Time PCR

賴智偉

技術服務部 技術服務主任

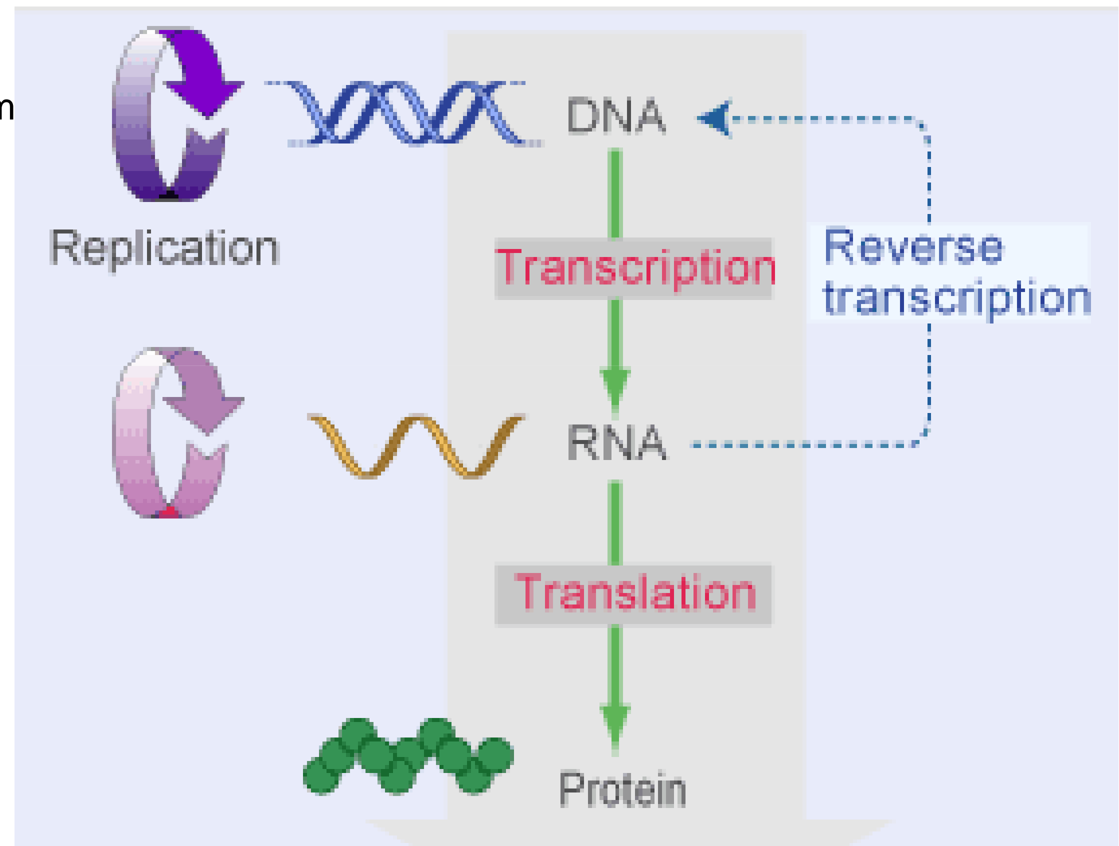
Bio-Rad Laboratories 台灣分公司



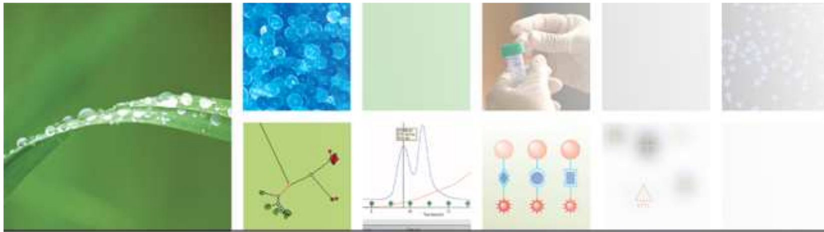


“中心法則 Central Dogma”

Central dogma describes information flow from DNA→RNA→protein

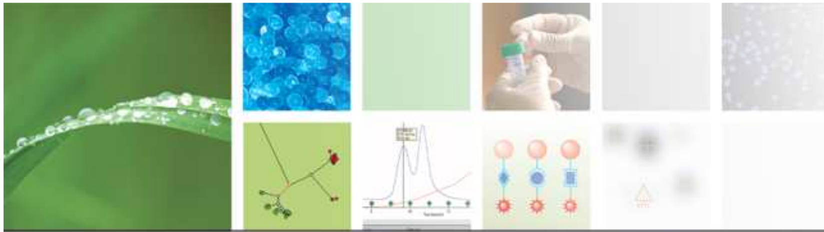


Protein considered the functional unit within the cell

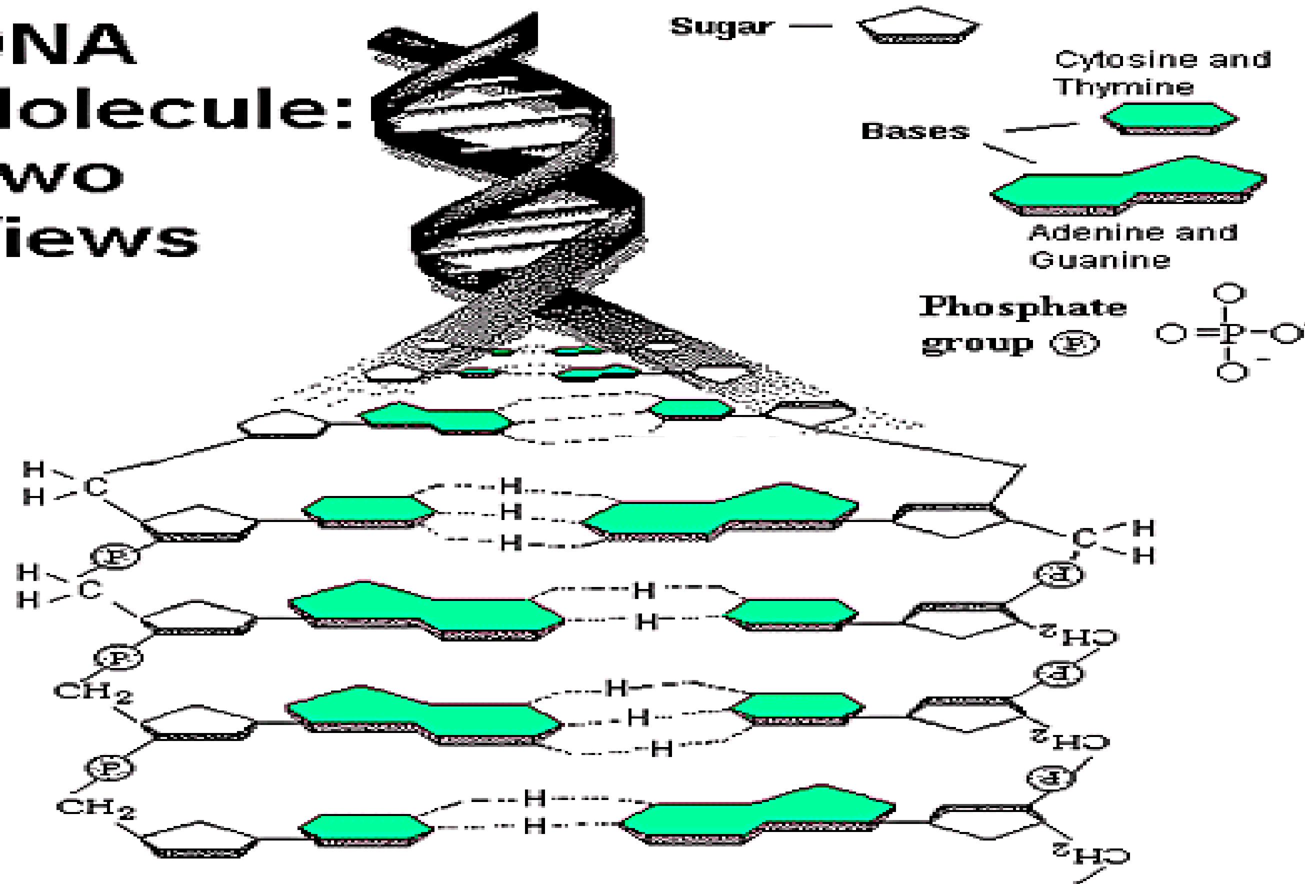


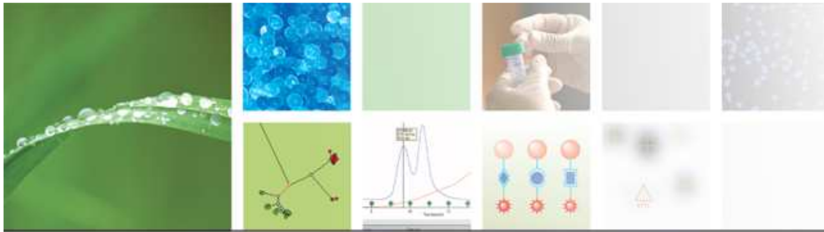
What is DNA (去氧核糖核酸)?

- Huge 1D molecule - two strands of nucleotides – double helix
- Information for life
- Cell > DNA > Nucleotide (核甘酸) > Base pair
- Nucleotide bases(鹼基)
  - –Guanine (G)，Adenine (A), Cytosine (C) Thymine (T)

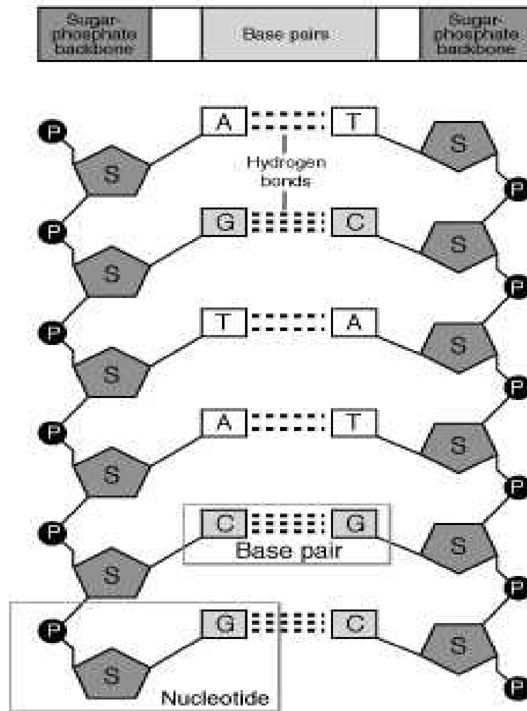


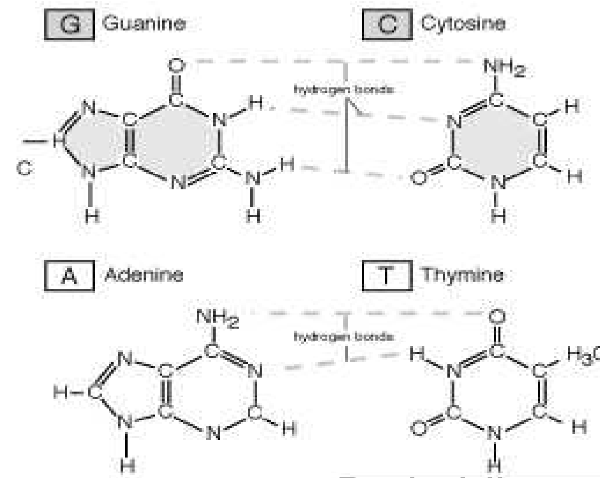
Structure of DNA



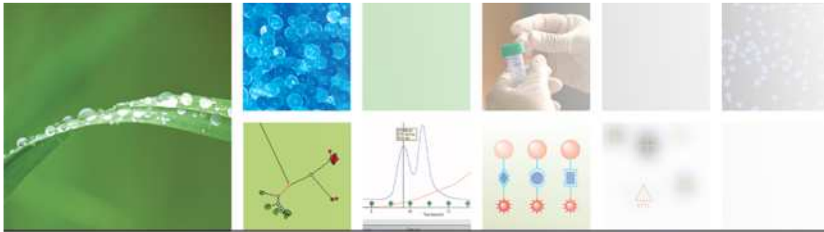


Nucleotides





- Pyrimidine 嘧啶（鹼基）
  - –T (Thymine) 胸腺嘧啶
  - –C (Cytosine) 胞嘧啶
- Purine 漂呤（鹼基）
  - –A (Adenine) 胺漂呤
  - –G (Guanine) 鳥漂呤



Polymerase Chain Reaction

Targeted DNA replication using thermostable DNA polymerase

Taq polymerase synthesizes DNA complementary to template in 5` to 3` direction

The use of two primers allows targeting of specific sequences

Primers are complementary to opposite strands of target region but not complementary to any other sequences

94°C

DENATURE

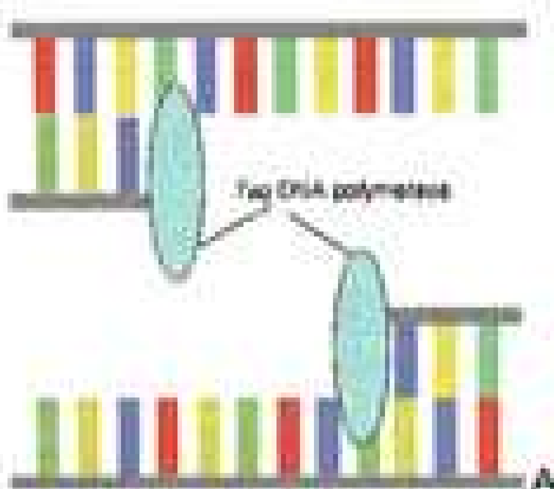
50-65°C

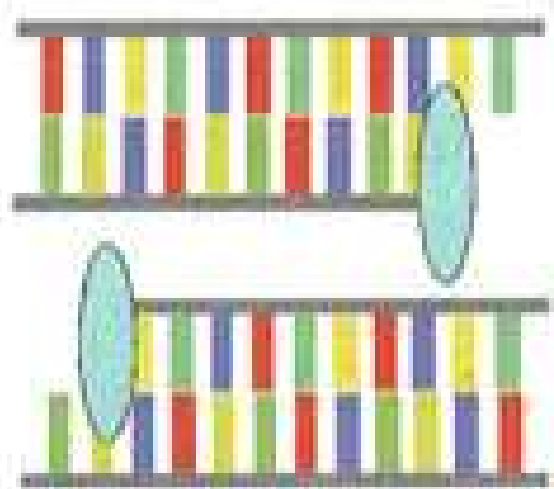
ANNEAL PRIMERS

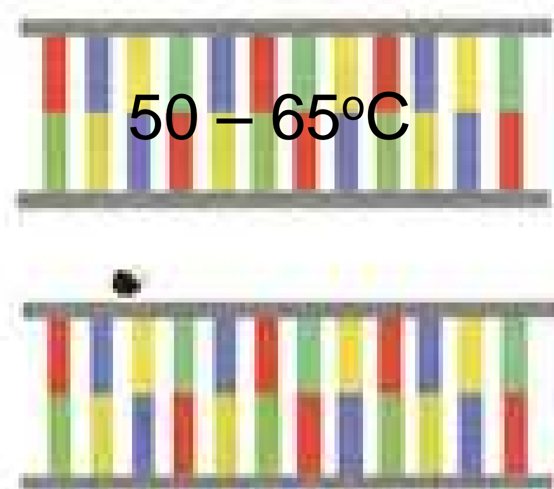
72°C

EXTEND STRANDS

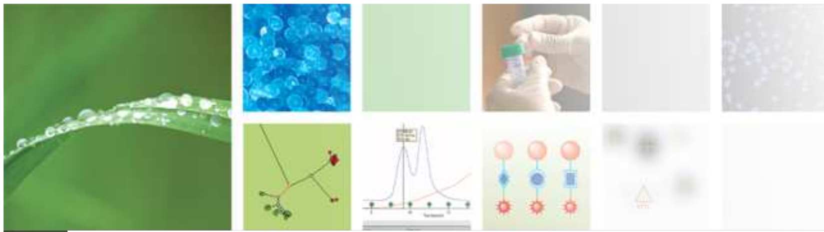
72°C

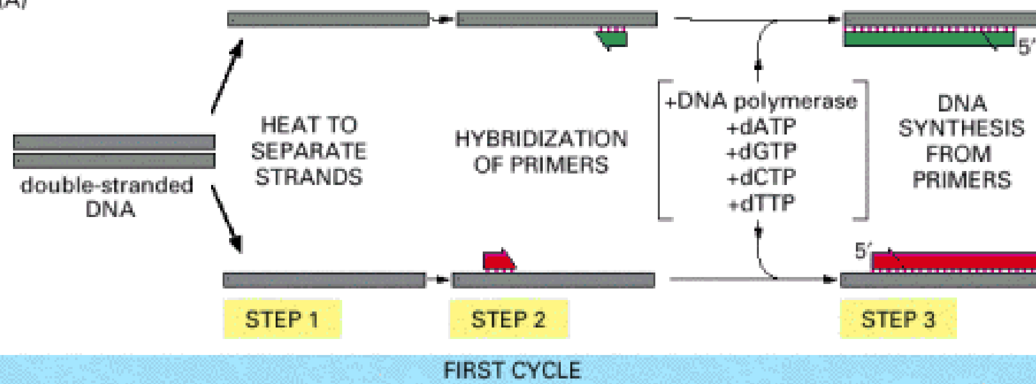






50 – 65°C





(B)

separate DNA strands and add primer

DNA synthesis

separate DNA strands and anneal primer

DNA synthesis

separate DNA strands and anneal primer

DNA synthesis

DNA oligonucleotide primers

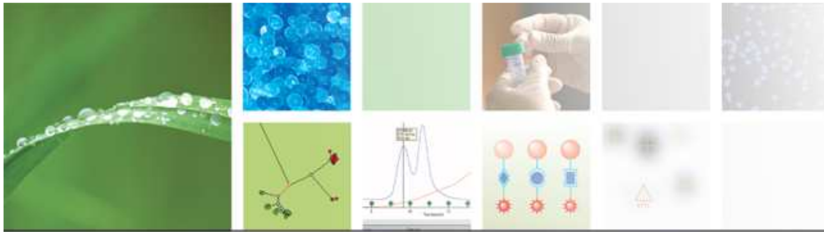
region of double-stranded chromosomal DNA to be amplified

etc.

FIRST CYCLE (producing two double-stranded DNA molecules)

SECOND CYCLE (producing four double-stranded DNA molecules)

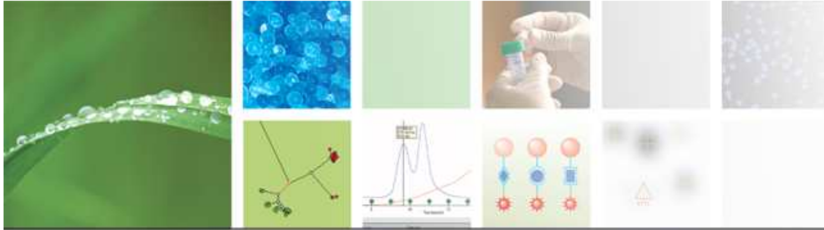
THIRD CYCLE (producing eight double-stranded DNA molecules)



What is Real-Time PCR?

Simply - fluorescent molecules are used to monitor the reaction while amplification is taking place.

You are able to view this occurring in real-time on your instrument.

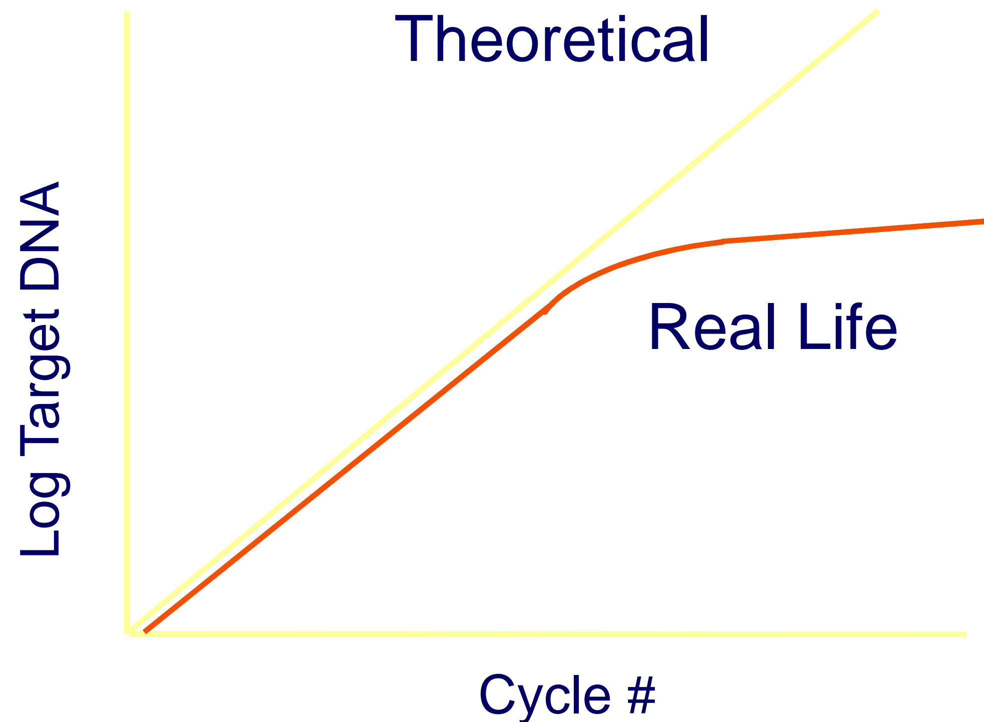


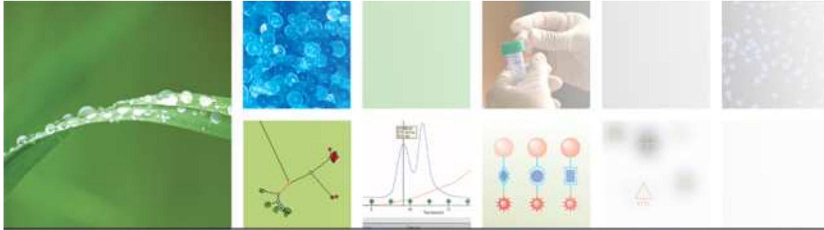
Reality vs. Theory

Amplification is exponential, but the exponential increase is limited:

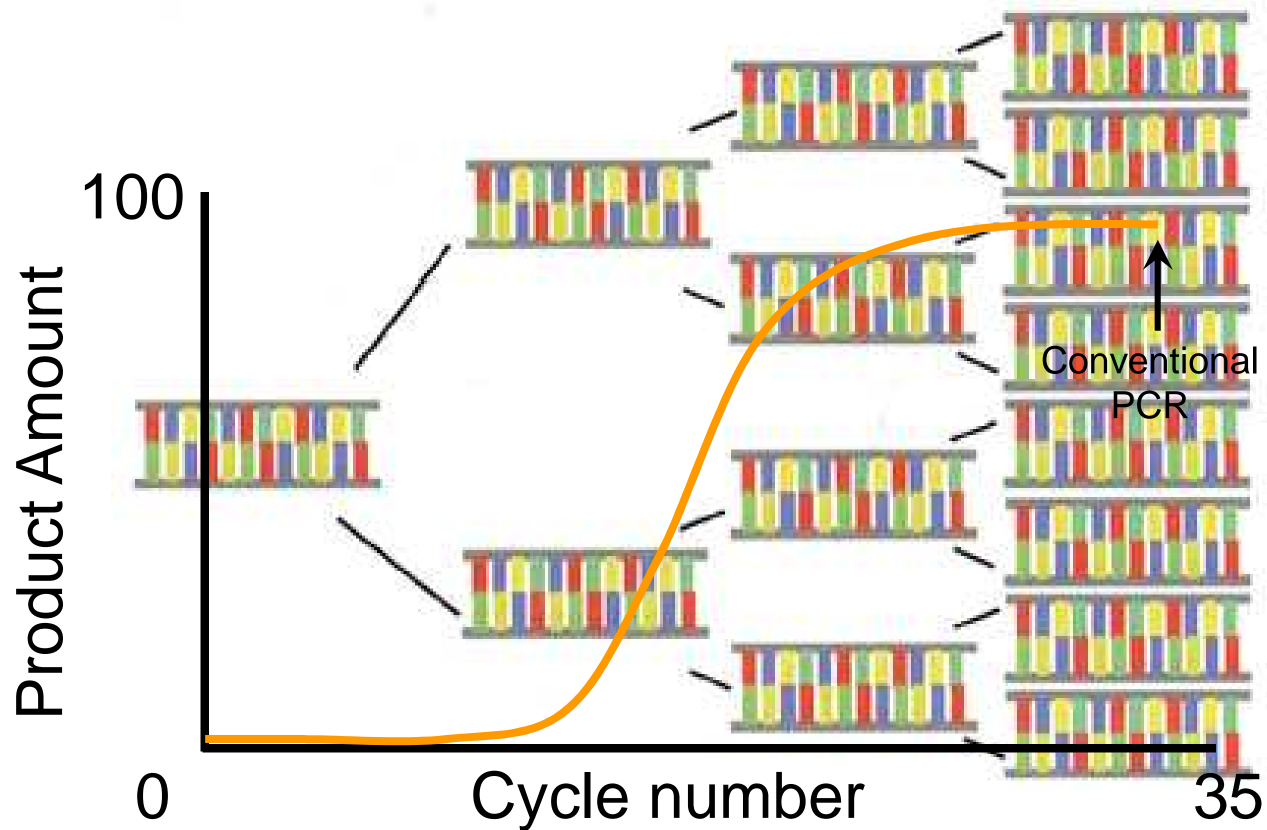
- **A linear increase follows exponential**
- **Eventually plateaus**

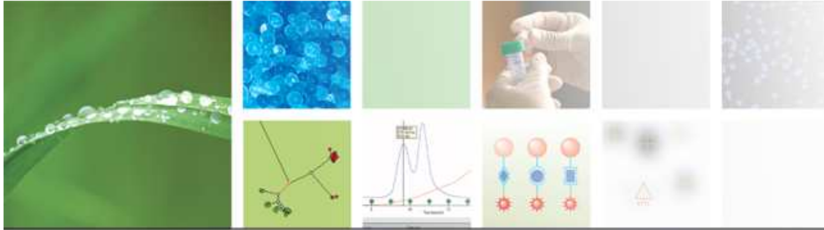
Real-Time PCR allows us to ‘see’ the exponential phase so we can calculate how much we started with.





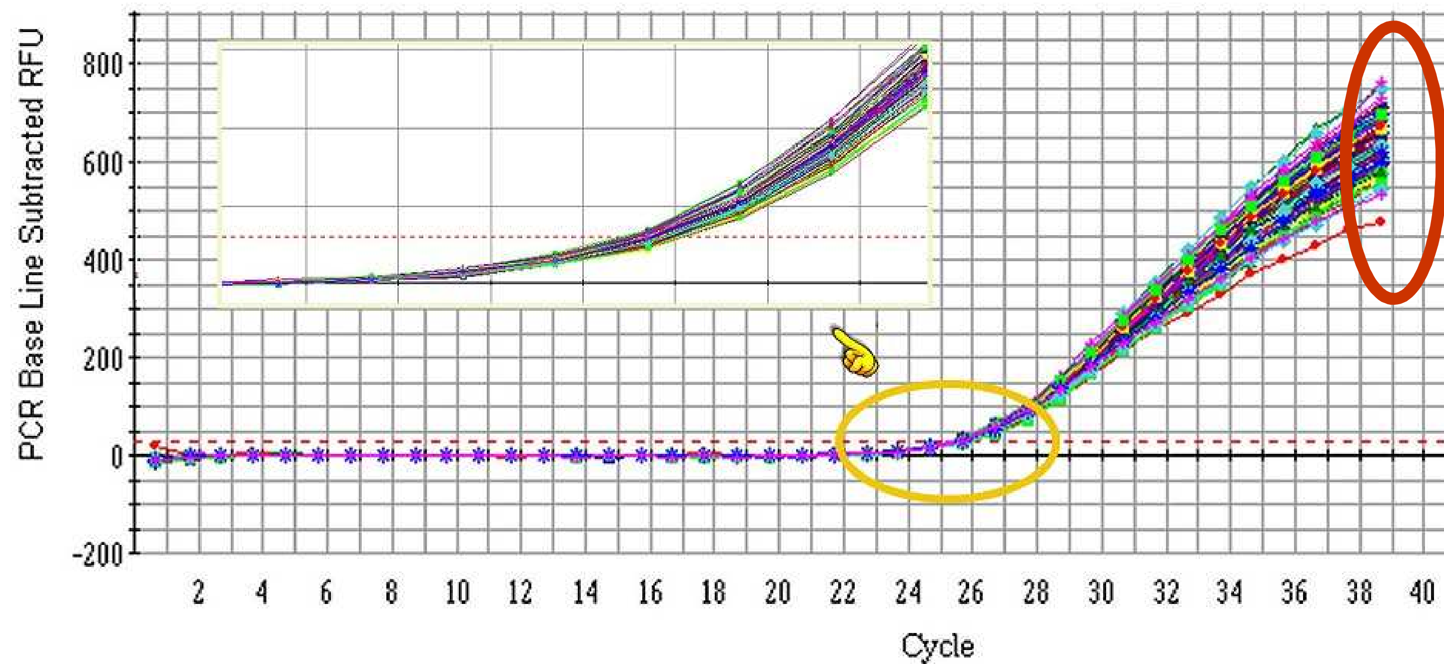
Real-time qPCR enables assessment of the reaction after each cycle

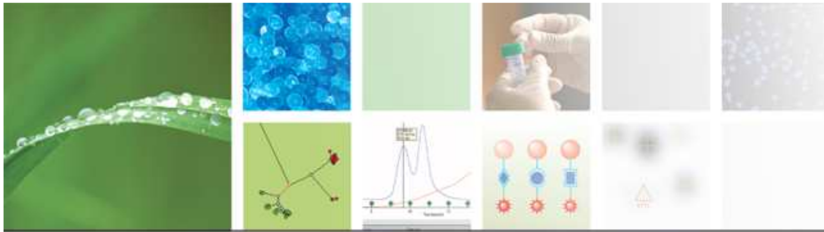




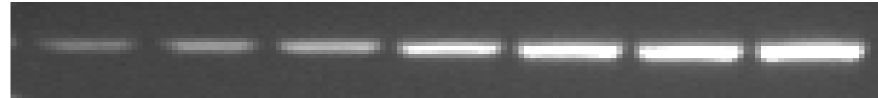
End Point Measurements

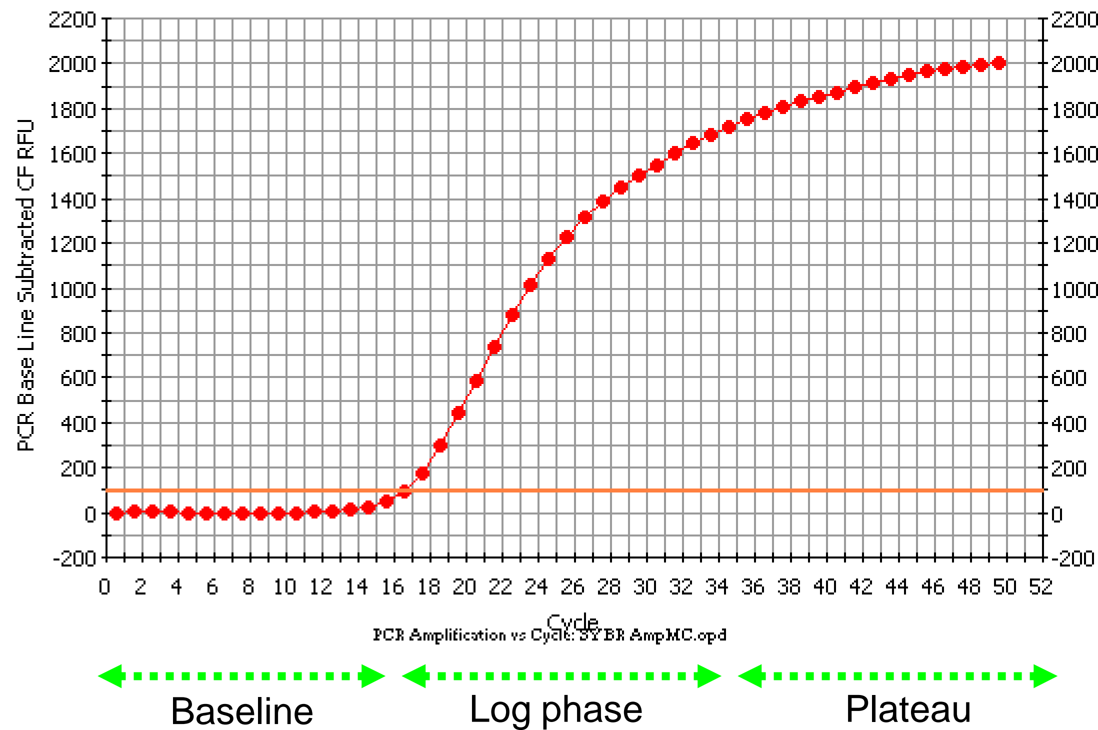
96 replicates of an identical reaction can have very different final amounts of fluorescence



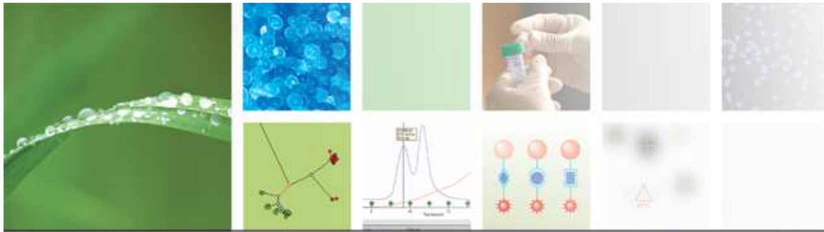


Real Time PCR 原理



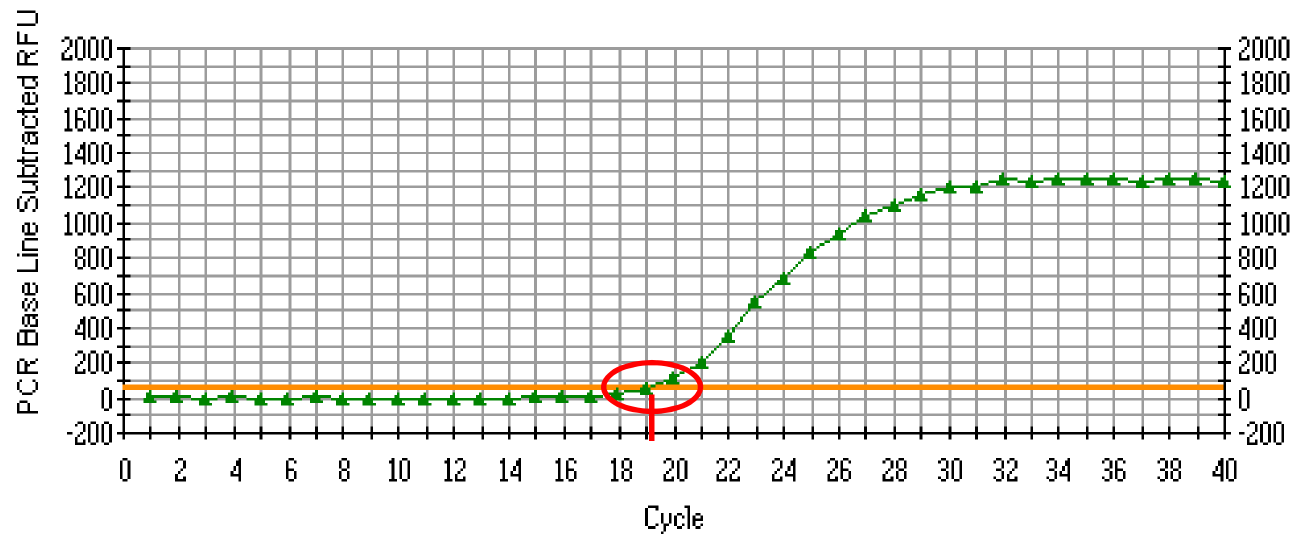


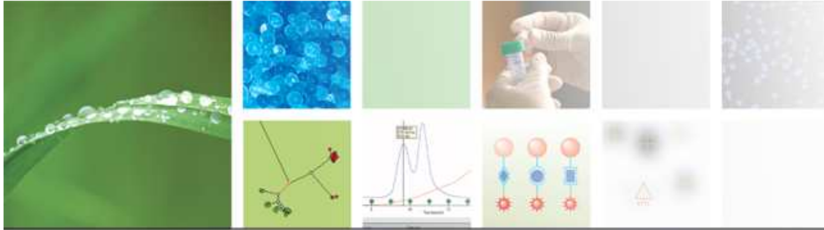
- **Plateau**
  螢光強度
- **Log phase**
  切線斜率
- Threshold 閾值



Threshold Cycle, C_T

The point at which the fluorescence rises appreciably above background





Quantitative PCR

[DNA]

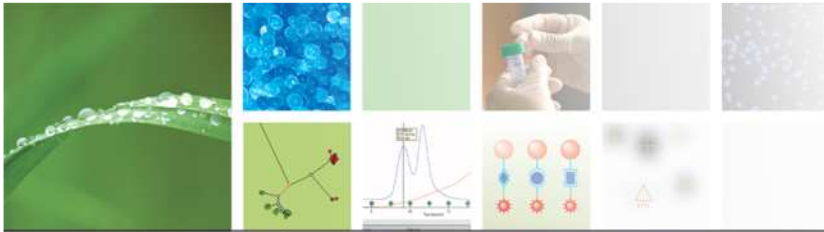
Limit of detection

Ct

Ct

Ct

Cycle #

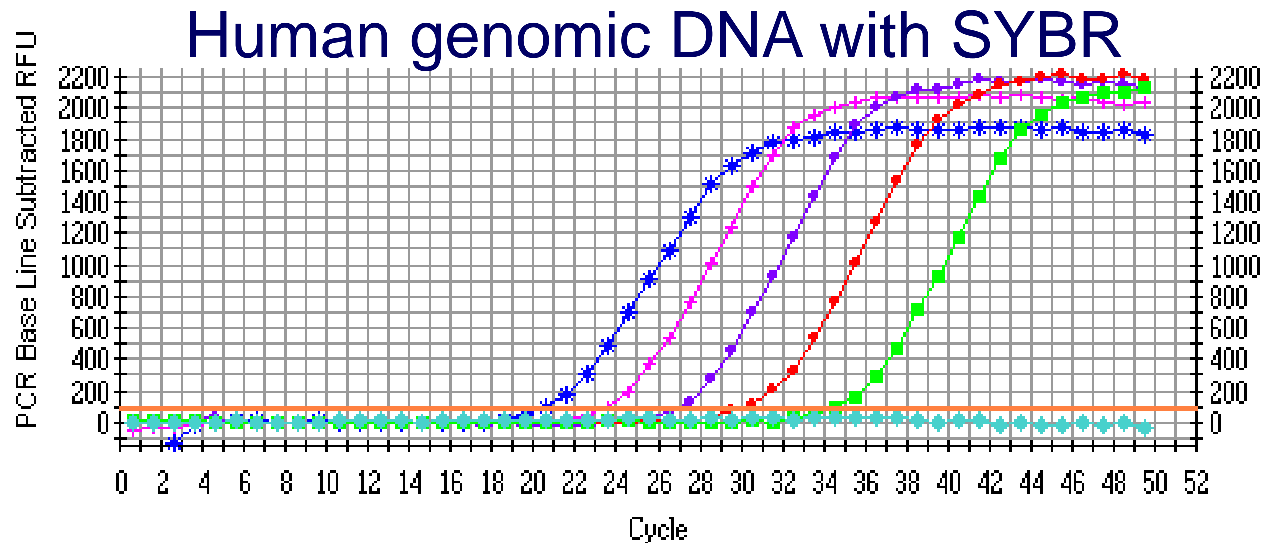


Threshold Cycle, C_t

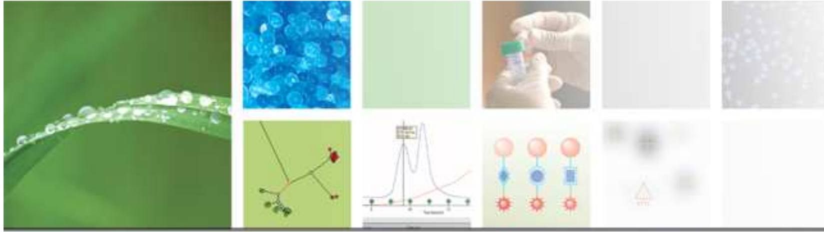
- Correlates strongly with the starting copy number
  - If you have twice the template, you get to C_t one cycle earlier
  - If you have half the template, you reach C_t one cycle later
- Is linear with the log of starting copy number over six or more orders

-

Human genomic DNA with SYBR



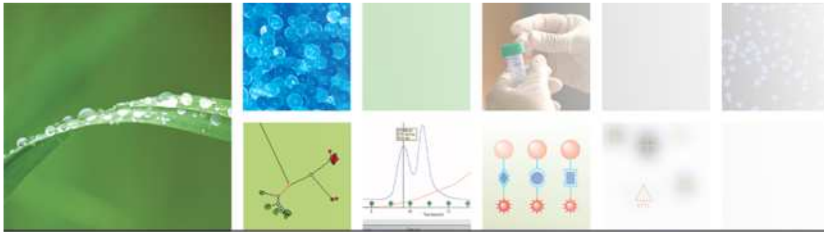
50-0.005 ng of template- FV Leiden primers



C_T 値 v.s. 濃度

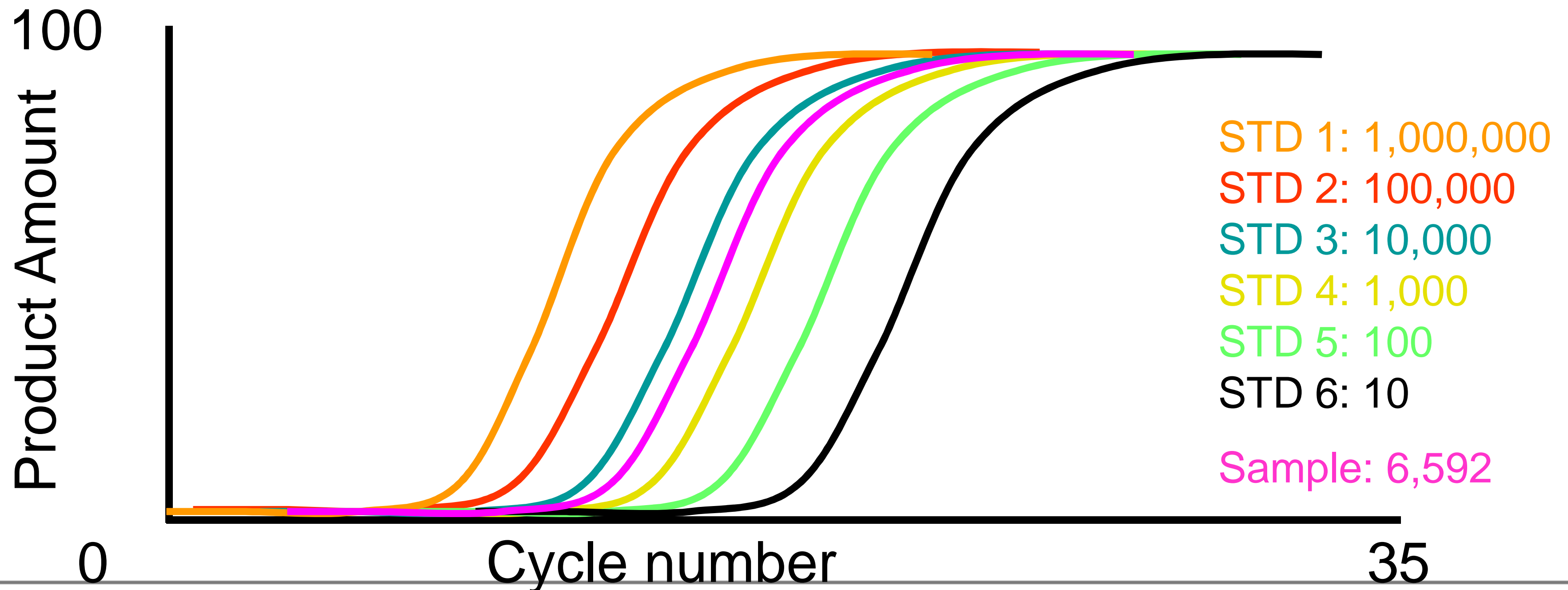
- 1 cycle = 2 fold difference
- 3.32 cycles ≅ 10 fold difference
- Assumes 100% efficiency

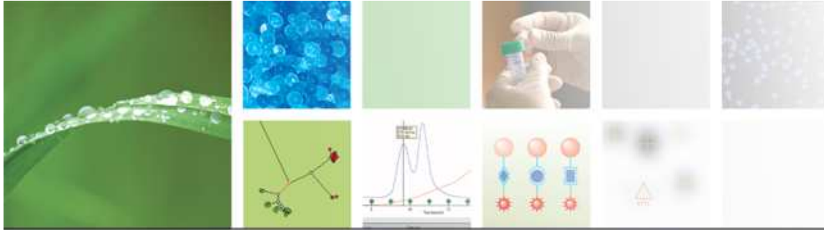
$$Y = N_0\, 2^n \longrightarrow Y = N_0\, (1+E)^n$$



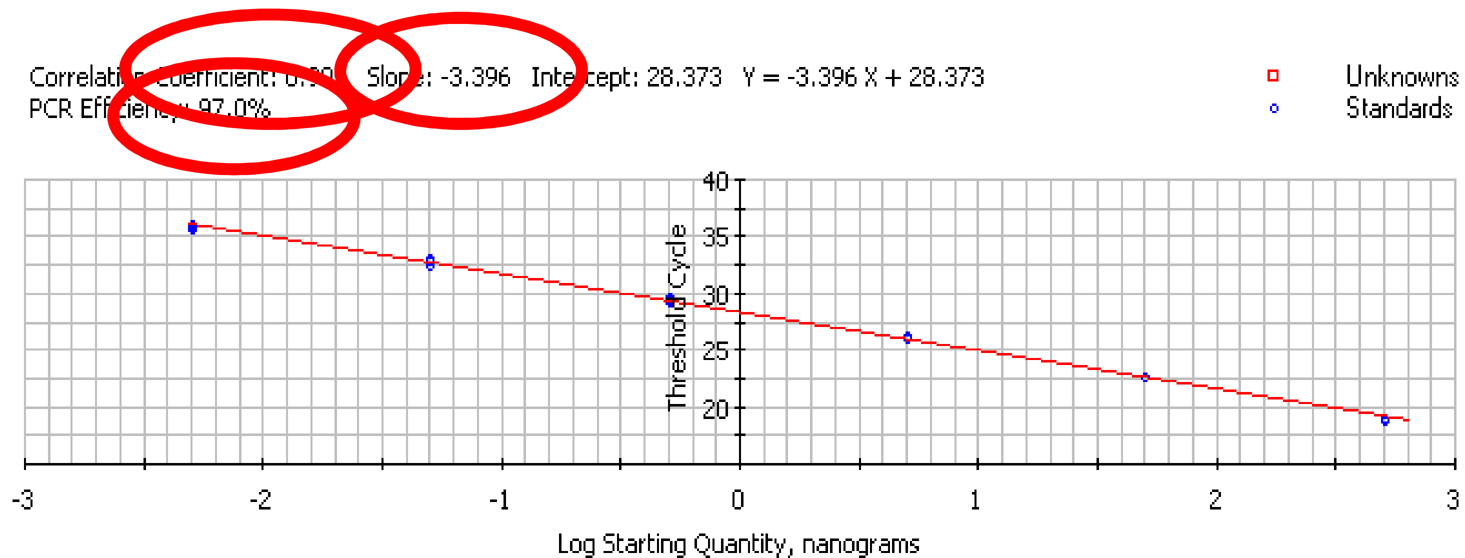
Create a standard curve with 10-fold serial dilutions of PCR product – assign arbitrary values

Compare values from standards with values for unknown sample





C_T is linear with the log of starting copy number



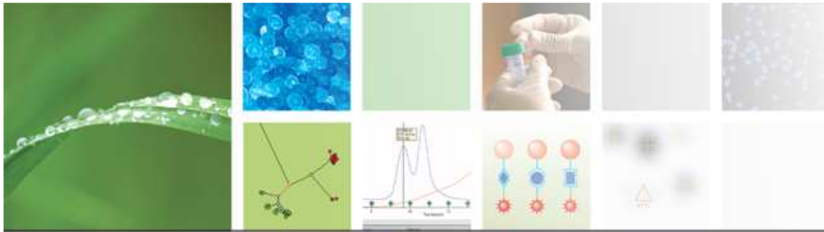
The **slope** of the standard curve can be directly correlated to the **efficiency** of the reactions:

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

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

when slope = -3.32, Efficiency = 100%

Aim for R value (Correlation Coefficient) of >0.98



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

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

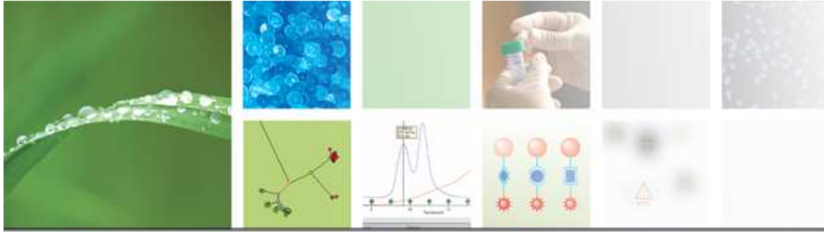
$$E = 10^{[-1/\text{slope}]} - 1$$

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:

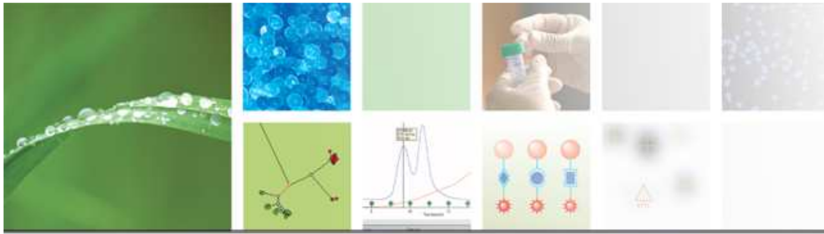
Good = 90 – 110%

Fantastic = 95 – 105%

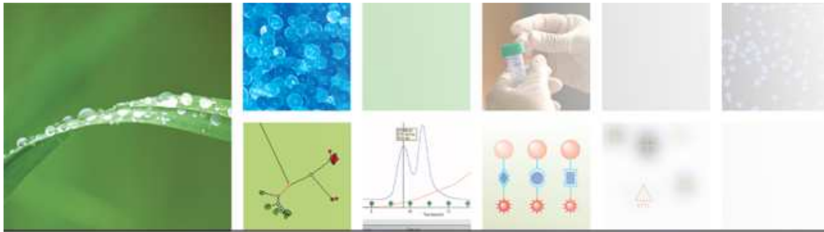


Points to Remember

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

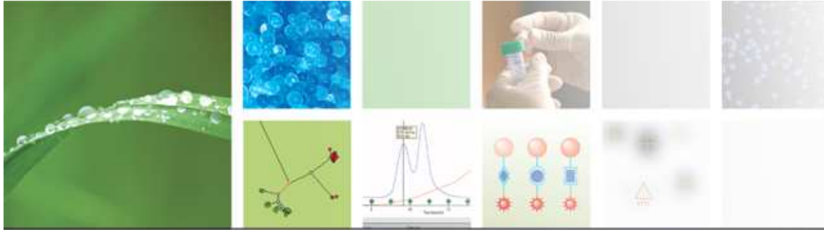


What are the most common detection strategies used for Real-Time PCR?



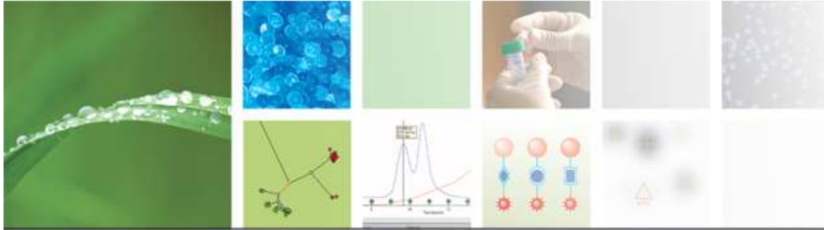
Real-Time PCR

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



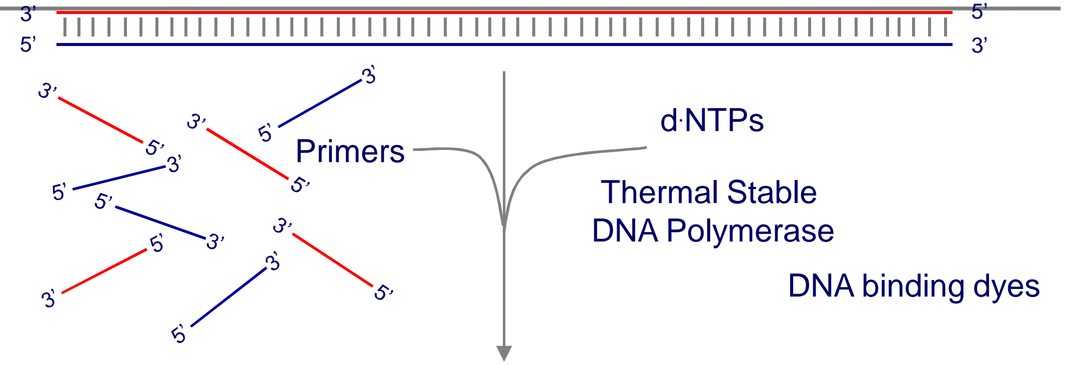
DNA binding dyes

- DNA binding dyes are inexpensive compared to hybridization probes.
- A dye based strategy allows one to get a general confirmation of amplification.
- Higuchi demonstrated the key principle of real-time PCR using Ethidium Bromide -
  - EtBr fluoresces 25 times more brightly when bound to dsDNA
- SYBR® Green I, a more sensitive DNA binding dye, is an even more powerful approach
  - SYBR Green I fluoresces 200 times more brightly when bound to dsDNA



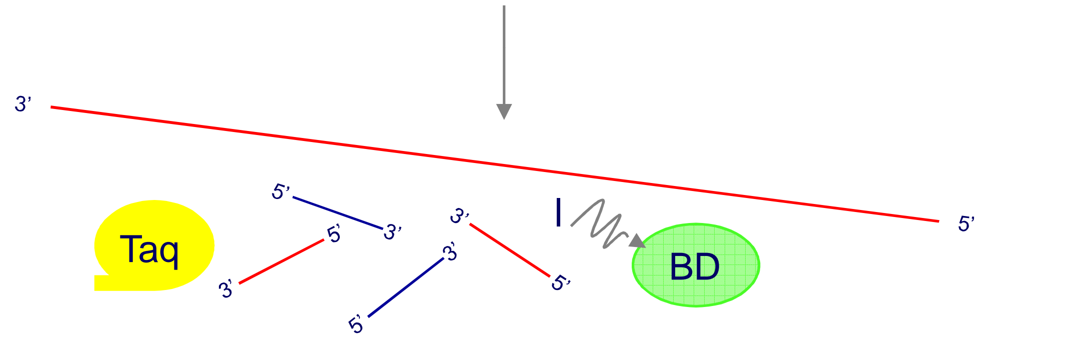
DNA binding dyes

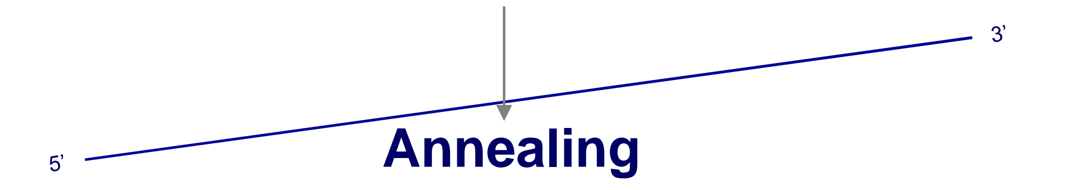
Add Master Mix & Sample



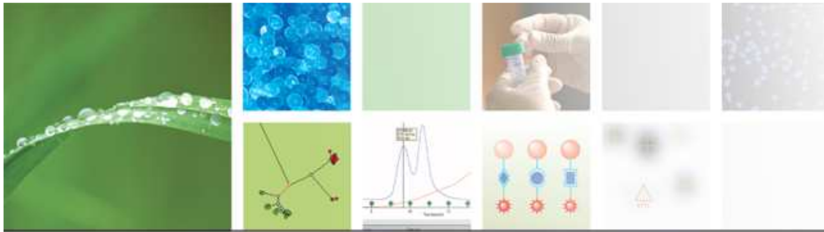
Reaction Tube

Denaturation

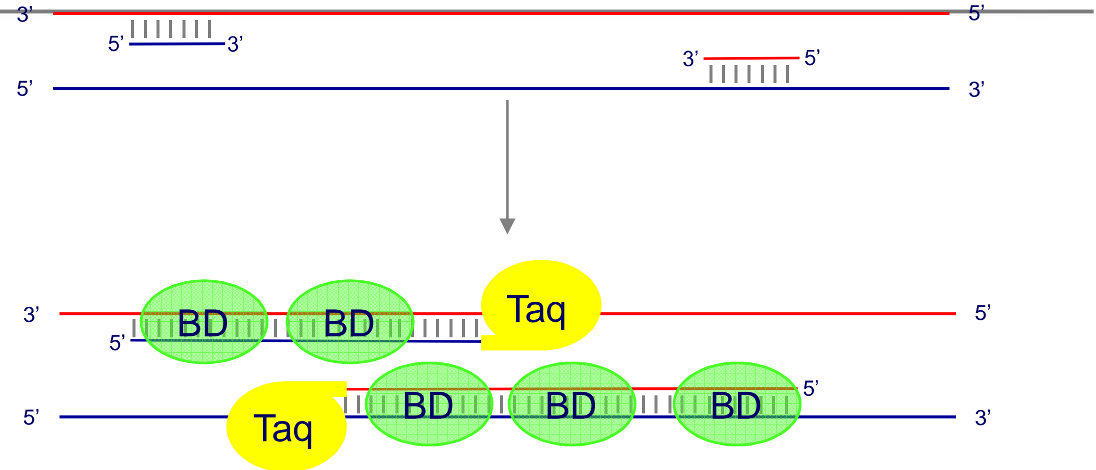




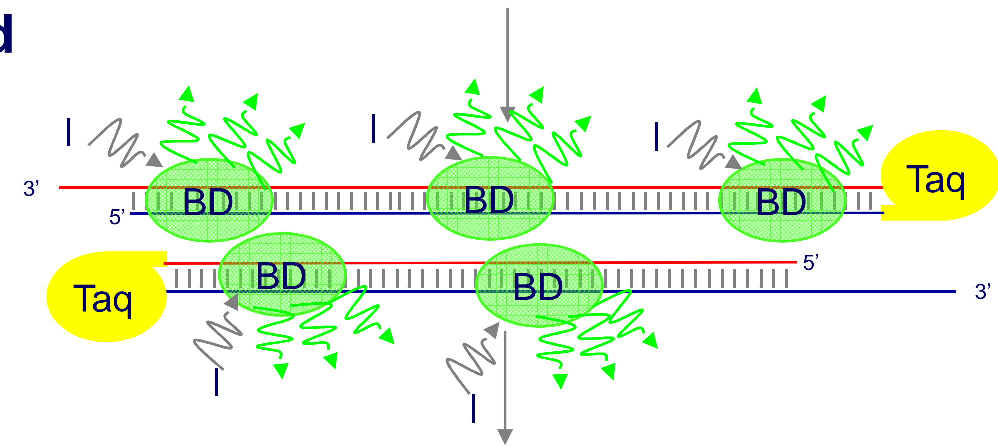
Annealing



DNA binding dyes



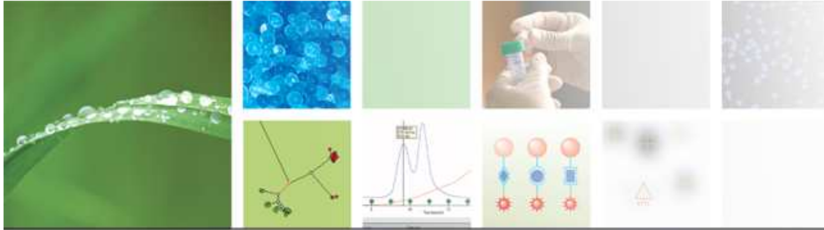
Extension



Extension Continued
Apply Excitation Wavelength

Repeat

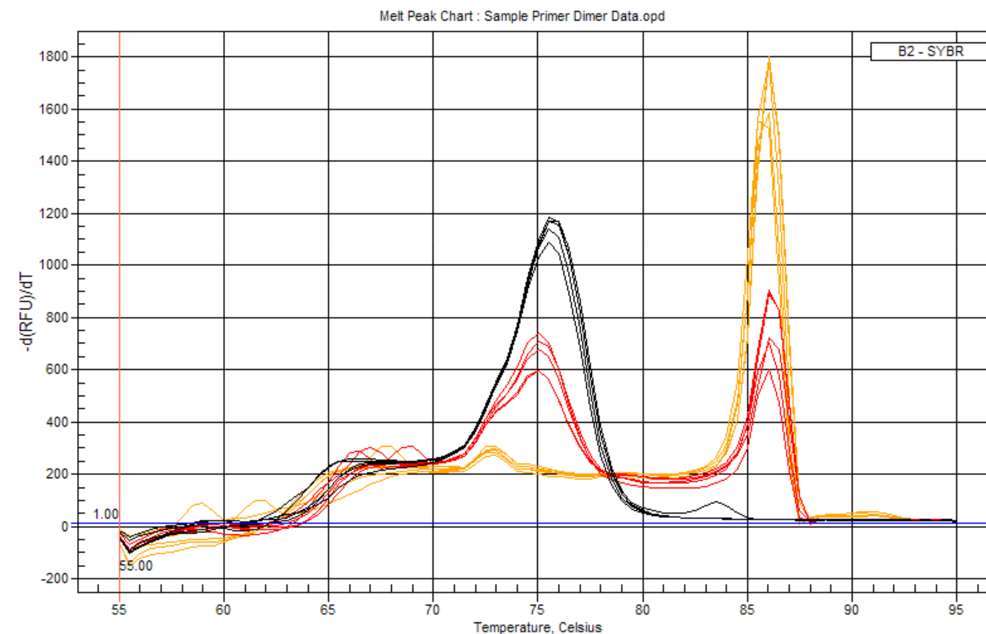
BIO-RAD



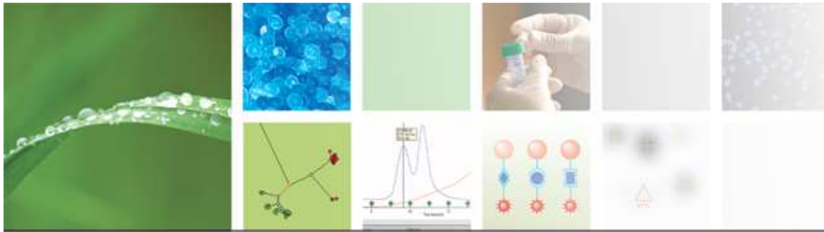
SYBR Green I

- Inexpensive
- Accurate
- Large dynamic range

- Primer specificity
- Confirmatory assay
- Single target



BIO-RAD



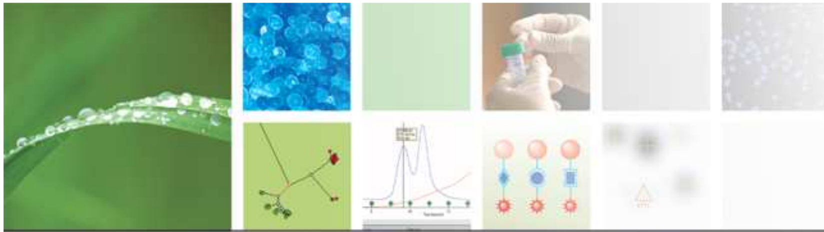
Melt Curve Analysis

Melt Curve Chart : SYBR AmpMCPrimerDimer.opd

RFU: 0, 1000, 2000, 3000, 4000, 5000, 6000, 7000

55.00

Temperature, Celsius: 55, 60, 65, 70, 75, 80, 85, 90, 95



Melt Curve Analysis

Melt Peak Chart : SYBR AmpMCPrimerDimer.opd

B7 - SYBR

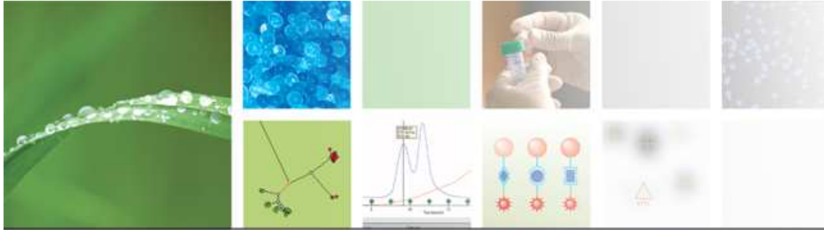
-d(RFU)/dT

1400
1200
1000
800
600
400
200
0

1.00
55.00

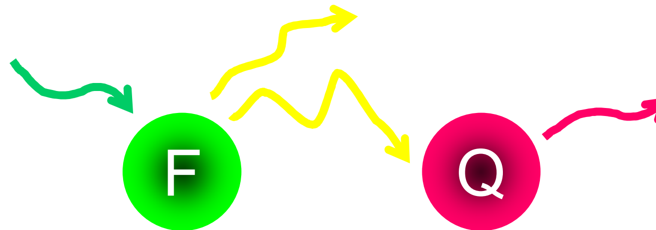
55 60 65 70 75 80 85 90 95

Temperature, Celsius

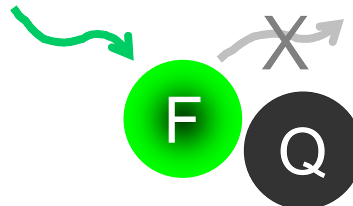


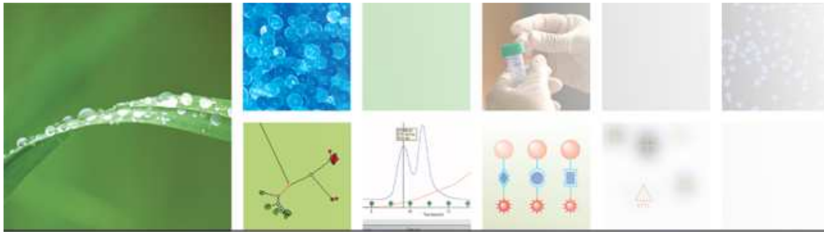
Dyes and Quenchers

FRET: Fluorescence (Förster) Resonance Energy Transfer



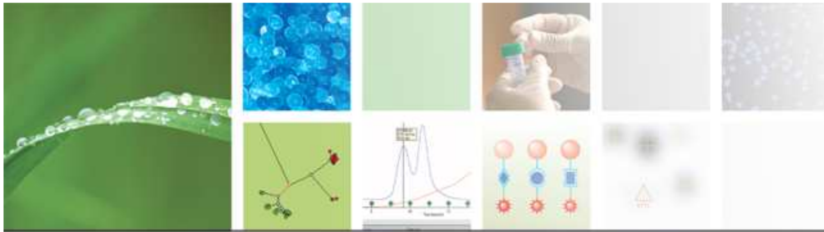
Dark Quenchers : Ground state or static quenching
Energy is dissipated as heat
Molecular interactions inhibit fluorescence





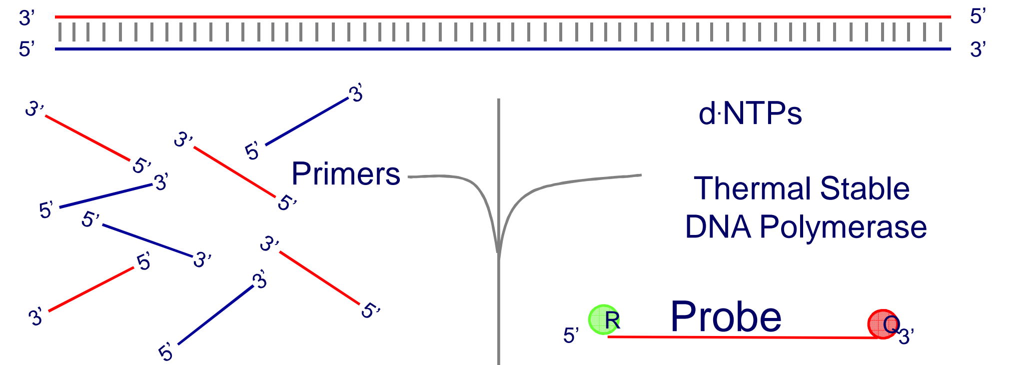
Hybridization Probes

- Cleavage-based assay
  - –TaqMan Assays
- Displaceable probe assays
  - –molecular beacons
  - –Dual oligo FRET probes
- Probes incorporated directly into the primers
  - –Amplifluor
  - –Scorpions



Cleavage-based assay: TaqMan

Add Master Mix & Sample



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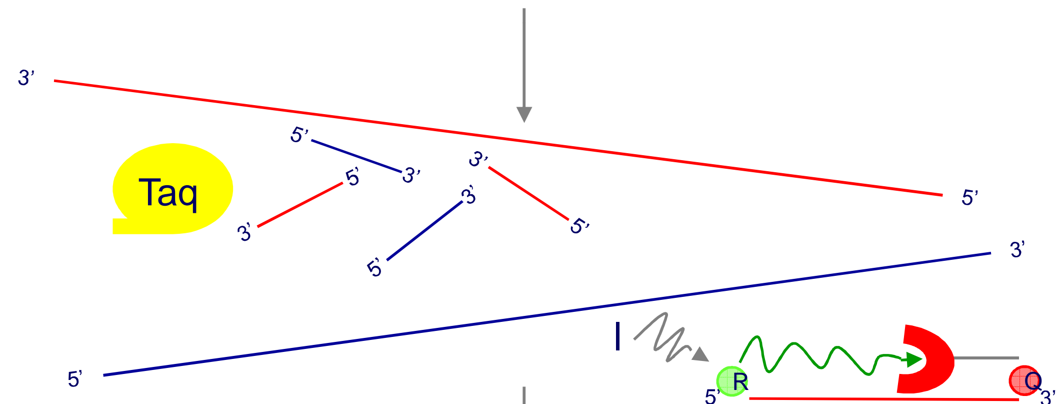
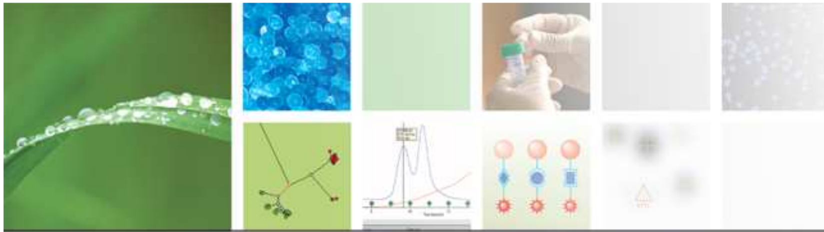
Denaturation



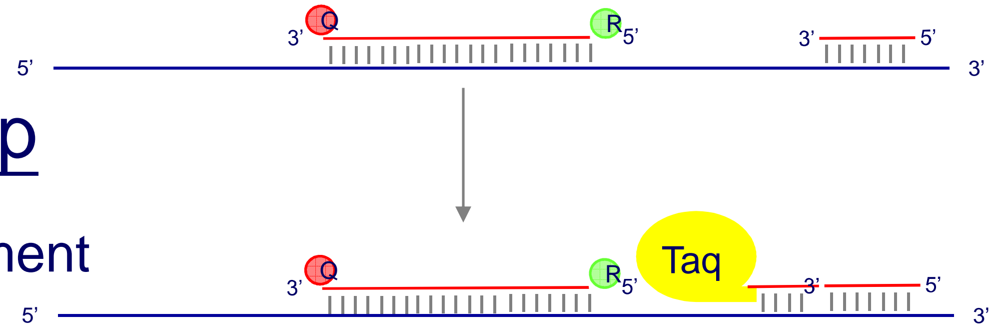
Annealing



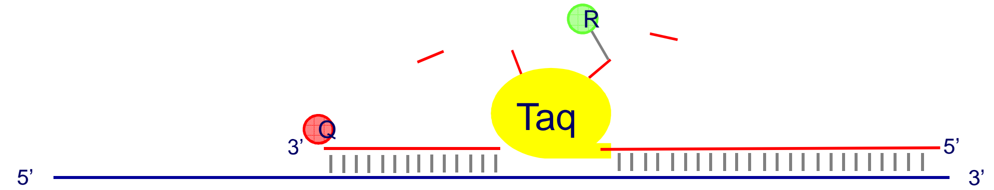
Cleavage-based assay: TaqMan

Extension Step

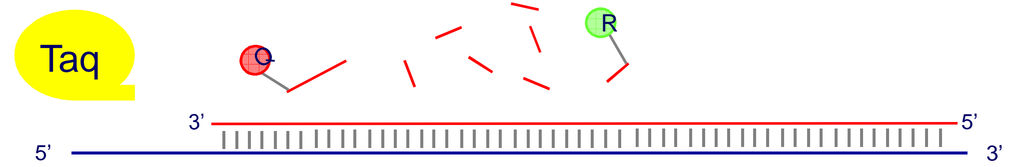
1. Strand Displacement



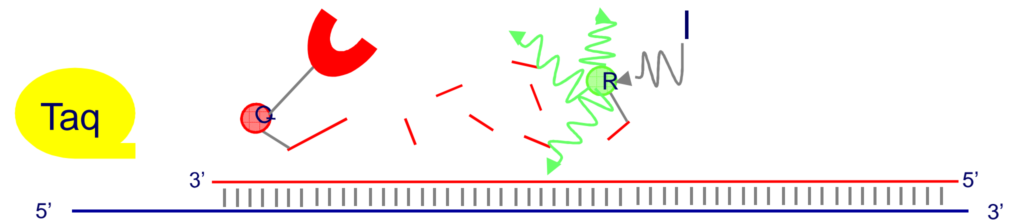
2. Cleavage

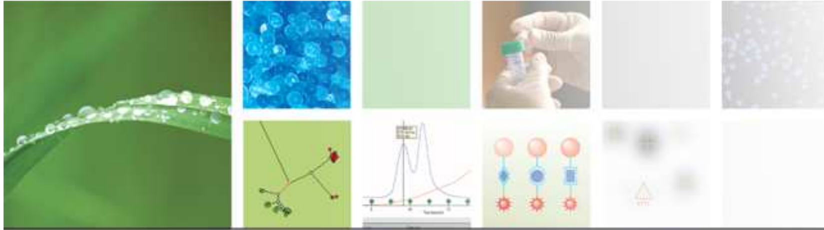


3. Polymerization Complete



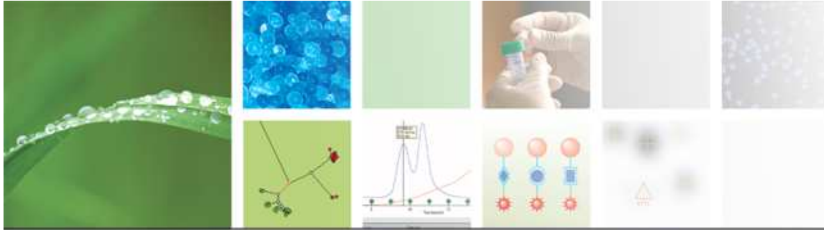
4. Detection





TaqMan Assay Strengths

- More specific than SYBR Green I
- Multiplex able using different reporter dye
- SNP genotyping application
- More high cost than SYBR green I
- Data collected in extension stage
- The TaqMan assay generates a robust cumulative fluorescence signal -
  - If you start with x copies of template, TaqMan liberates x reporters in the first cycle repeat.
  - In the second cycle repeat TaqMan results in a total of 3x reporters in solution
  - In the 25th cycle repeat, TaqMan results in a total of 33,554,431x reporters in solution
- The TaqMan assay approach is the predominant assay represented in Real-Time PCR literature

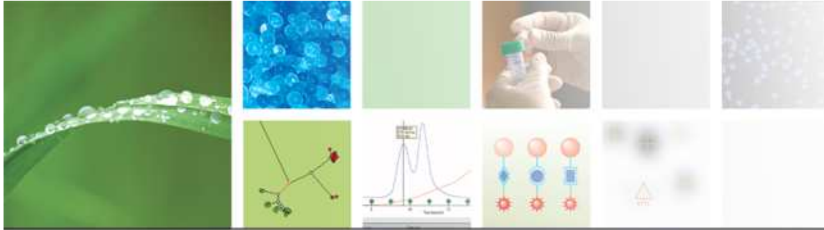


TaqMan Weaknesses

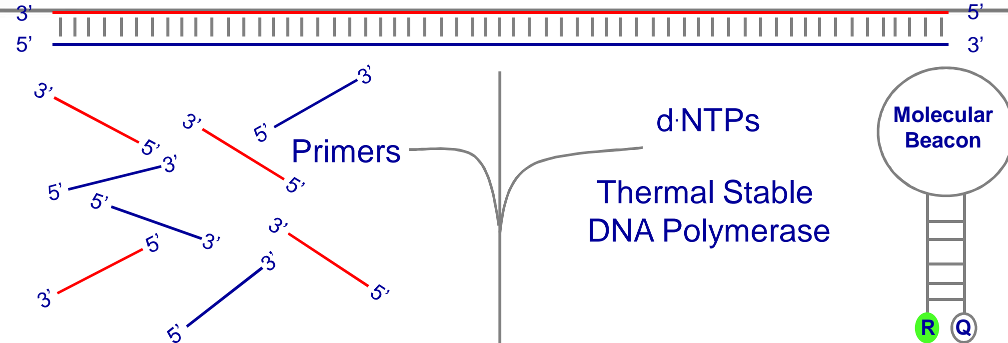
- Old probes used TAMRA as a quencher which you used to monitor. Now use black hole quenchers.

- Sometimes *taq* ‘knocks’ the probe off the template rather than cleaving it.

- TaqMan-MGB probes are proprietary to Applied Biosystems

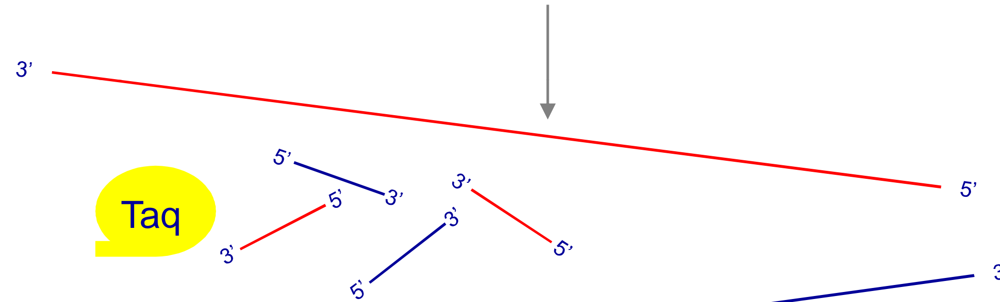


Molecular Beacons



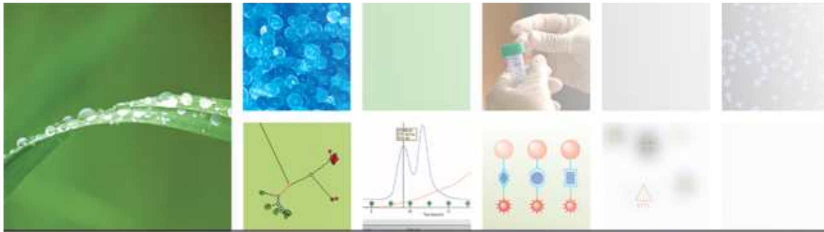
Add Master Mix & Sample

Reaction Tube



Denaturation

Annealing



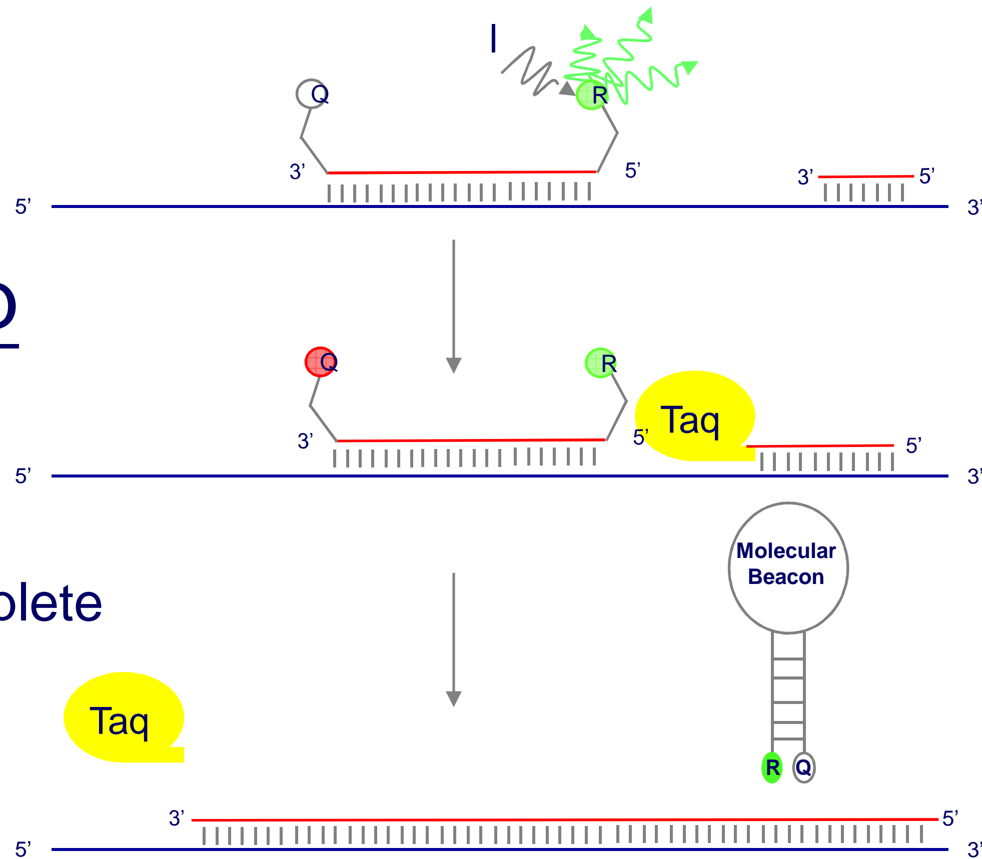
Molecular Beacons

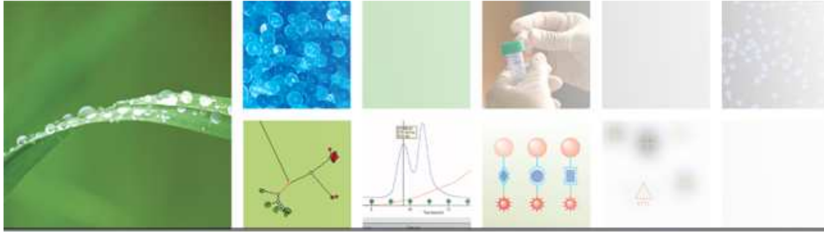
Detection

Extension Step

1. Strand Displacement

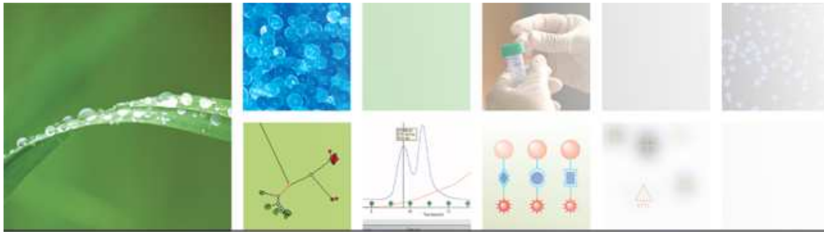
2. Polymerization Complete
   Probe Silent





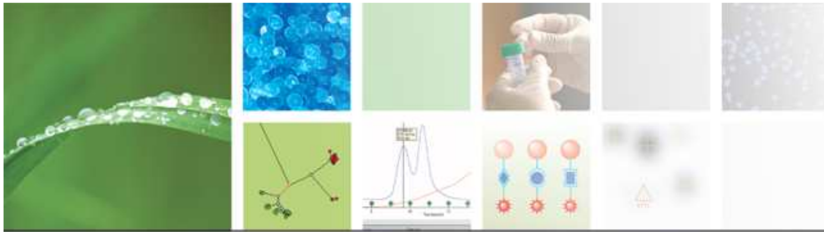
Molecular Beacon Strengths

- The hairpin loop structure is strongly favored over the the extended structure.
- Therefore molecular beacons will only bind to PERFECT matches making them optimally suited for applications such as:
  - –Screening for known mutations
  - –SNP characterization
- Beacons also feature a “dark” quencher. Instead of using a fluorogenic quencher, this quencher dissipates the energy transferred as heat. Advantages of this:
  - –Demonstrated functionality with several different reporters - do not require that reporters stimulate quencher fluorescence.



Molecular Beacon Weaknesses

- The Loop structure of the probe requires more optimization than other probes in design of sequence:
  - Care must be taken with regard to the annealing temperature of the probe to target compared to the stem unwinding temperature
  - The specificity of the molecular beacons may be a drawback if single mismatches are not relevant to the assay of interest
- Generally lower signal inherent in displacement probe assays (compared to a cleavage assay like TaqMan) - this will not affect quantitative results



Real-time PCR Sample Preparation

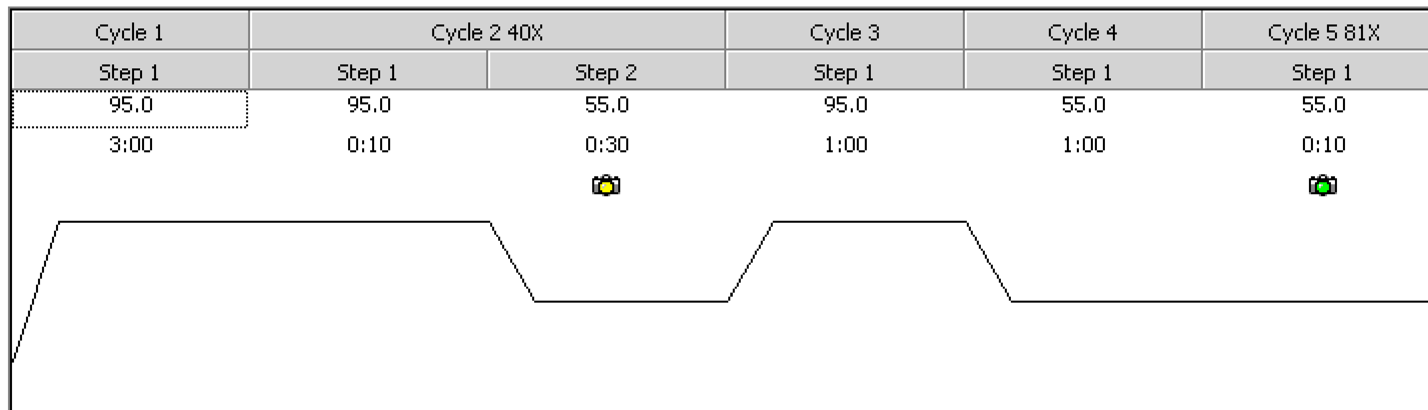
SYBR Green Chemistry

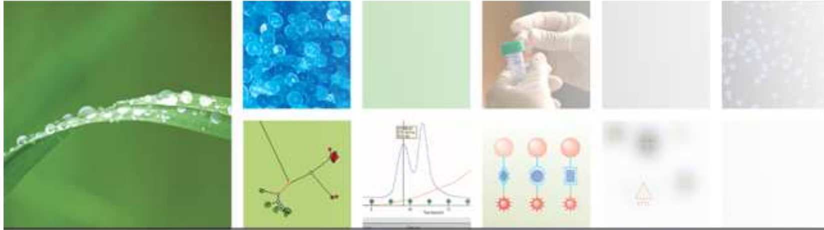
Component	Volume per reaction	Final concentration
iQ SYBR Green Supermix	25 µl	1X
Primer 1	x µl	100 nM–500 nM
Primer 2	x µl	100 nM–500 nM
Sterile water	x µl	
DNA template	x µl	
Total Volume	50 µl	

Probe Chemistry

Component	Volume per reaction	Final concentration
iQ Supermix	25 µl	1X
Primer 1	x µl	100 nM–500 nM
Primer 2	x µl	100 nM–500 nM
Probe	x µl	100 nM–500 nM
Sterile water	x µl	
DNA template	x µl	
Total Volume	50 µl	

Hot Start　PCR　Melting Curve

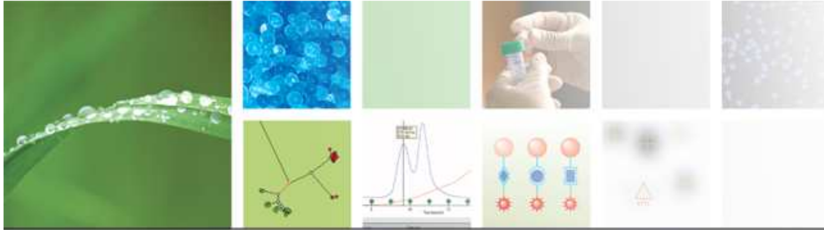




- Nucleic Acid quality:
  - Genomic DNA
  - Plasmid DNA
  - cDNA

- 絕對定量 (AQ) or 相對定量 (RQ)
  - Standard selection: Genomic DNA or plasmid
  - Serial dilution
  - Housekeeping gene selection: stable

- Target position:
  - Amplicon Size: 75~150 bps
  - 50% < GC% < 60%
  - Avoid >4 repeats of single bases
  - Analyze 2nd structure (hand out)



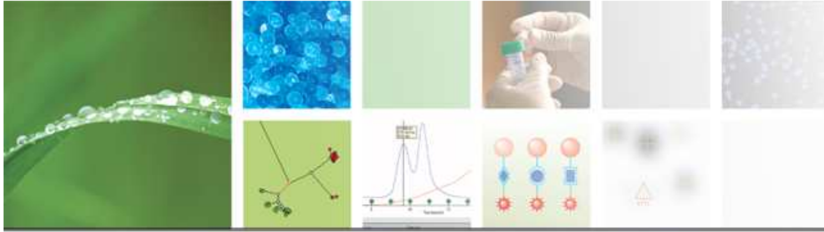
General QPCR Working Process
-- 上機前, User 該注意的事是…(II)

- Primer Design:
  - Annealing temp. > all 2nd structure melting temp.
  - Avoid repeats of G or C long than 3 bases
  - Place G or C on end of primers
  - GC% < 50% in the 5 base of 3' end of primer
  - No any primer-dimmer

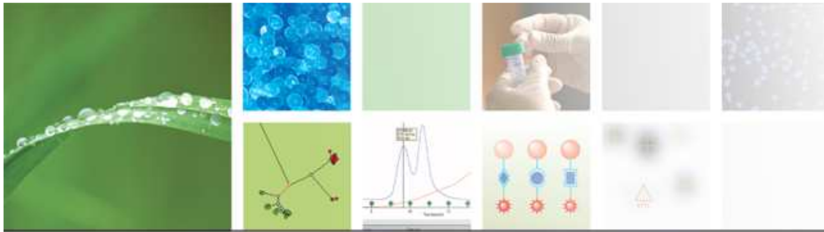
- Reaction Component:
  - [MgCl2]: 3~6 mM
  - [dNTP]:200~600uM each, increasing [dNTP] → increasing [MgCl2]
  - Enz: 1.25 ~ 4.5 U/50 ul
  - [F.Primer] = [R. Primer] may be helpful

- PCR condition:
  - Annealing temperature: 50~65 degree

- PCR efficiency:
  - You need serial dilution
  - You need gradient
  - Linier phase slop

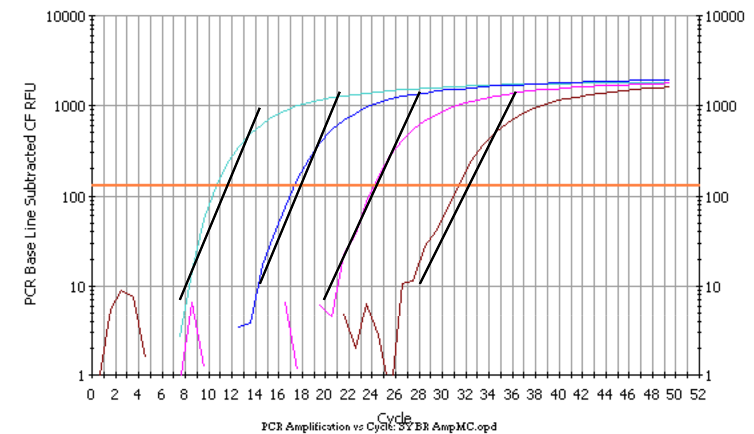


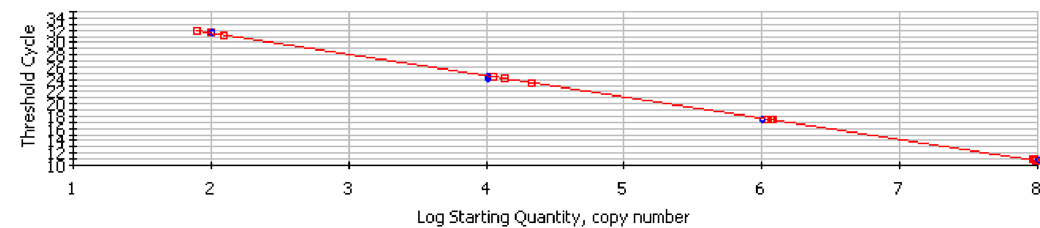
- Reagent
  - –Never vortex master mix
- Standard
  - –Vortex well before use
- Reaction content
  - –Template volume > 5 ul
- Pipetting
  - –Never " 杯壁下流"



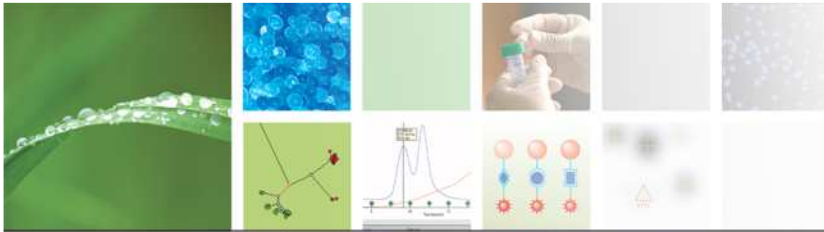
General QPCR Working Process -- Data analysis

- Amplification plot
  - Reproducibility? So you need duplication or triplication….
  - Determination Ct Value? Threshold
- Absolute Q., Relative Q. or SNP
  - ΔCt or ΔΔCt
- PCR efficiency from std. Curve
  - <100%
  - >100%
  - Dynamic range
- Reproducibility
  - Duplication or triplication
- Melting curve analysis
  - Primer dimer
  - Non specific production





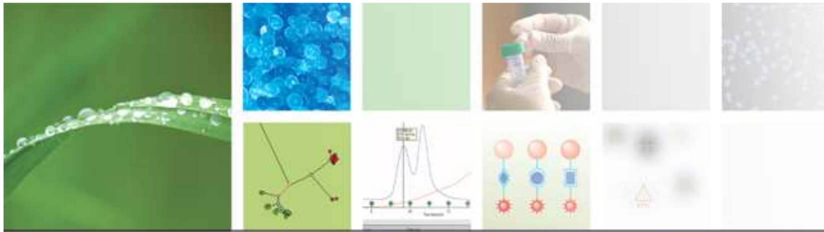
PCR Standard Curve: SYBR AmpMC.opd



Remember...

Real-Time PCR is not ‘cookbook chemistry’ - a real-time instrument will not optimize your experiments for you

However, once you do **optimize your reactions**, you will get **reproducible, accurate results**



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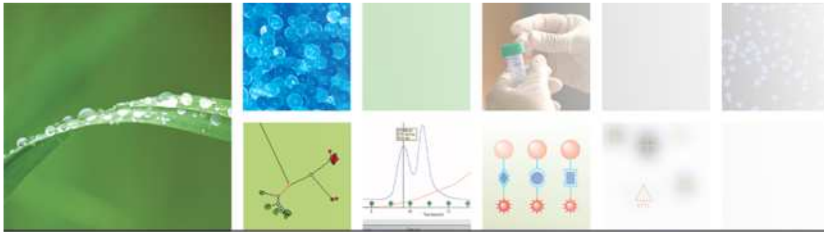

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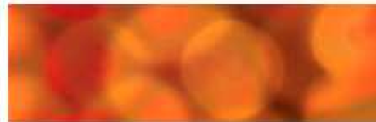
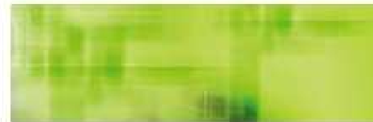
Glossary



PCR Doctor

Cure your data ailments with this interactive troubleshooting tool for PCR and real-time PCR assays.

- Real-Time PCR Doctor
- Conventional PCR Doctor
- Bio-Rad Technical Support



Tutorials

Learn the key principles of conventional and real-time PCR.

- Real-Time PCR Fundamentals
- Real-Time PCR Chemistries
- Downloadable Presentations

Assay Design

Access design considerations for conventional and real-time PCR assays.

- Primer and PCR Product Design
- Assay Validation and Optimization Overview
- Quantitative PCR Analysis

Top 20 Citations

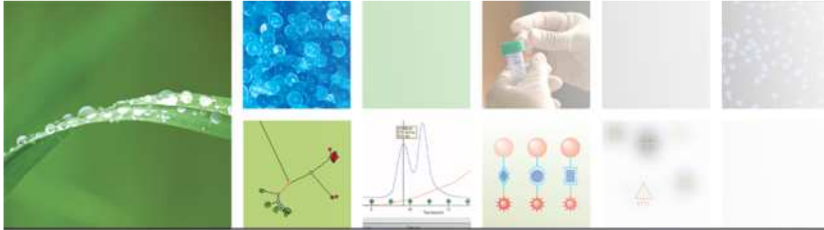
Recommended articles featuring the most popular amplification-related applications. **learn more**

RNAi Solutions

From design to detection, Bio-Rad offers an extensive set of tools for effective gene silencing and analysis. **learn more**

Tip of the Week

To avoid genomic DNA amplification when using cDNA as the starting template, it is helpful to design primers at splice junctions.



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

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