# Troubleshooting of Real Time PCR

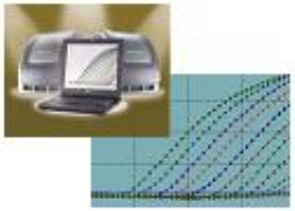
**Ameer Effat M. Elfarash**

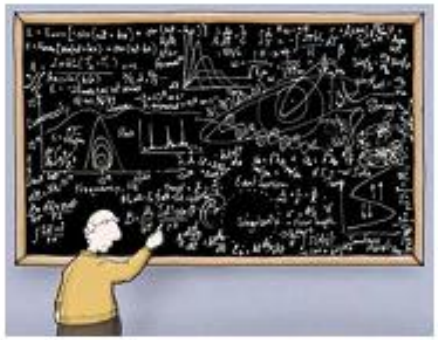
Dept. of Genetics
Fac. of Agriculture, Assiut Univ.
aelfarash@aun.edu.eg

# What is Real-Time PCR used for?

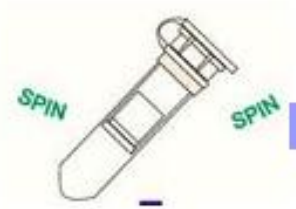
- **Gene expression analysis**
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

# Steps towards successful Real-time QPCR experiments





1.Experimental Design



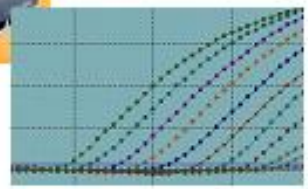

2.Sample preparation and purification

3. RNA/DNA quantification and quality

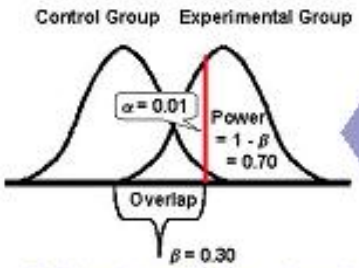
cDNA


Total RNA

4.Reverse Transcription



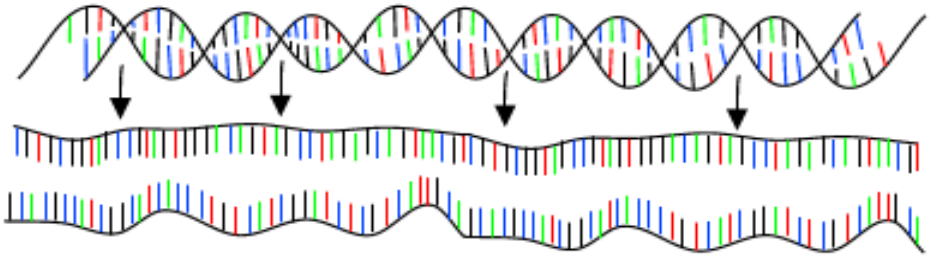
5.Real time QPCR




6.Post-run Analysis

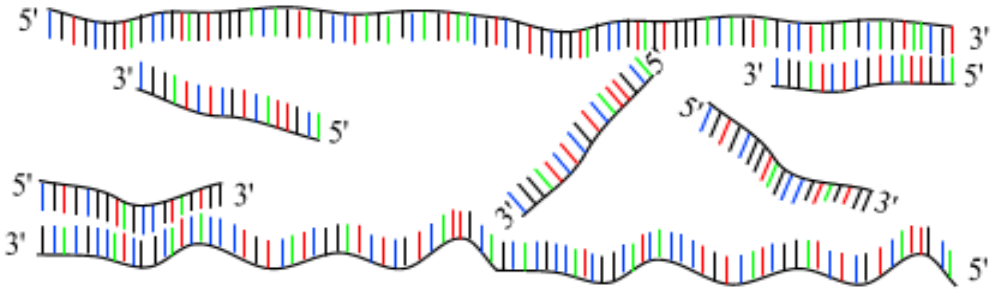
# PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



**Step 1 : denaturation**

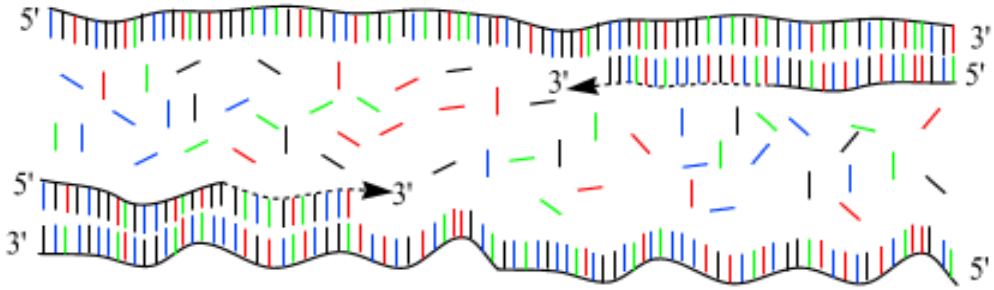
1 minut 94 °C




**Step 2 : annealing**

45 seconds 54 °C

forward and reverse primers !!!

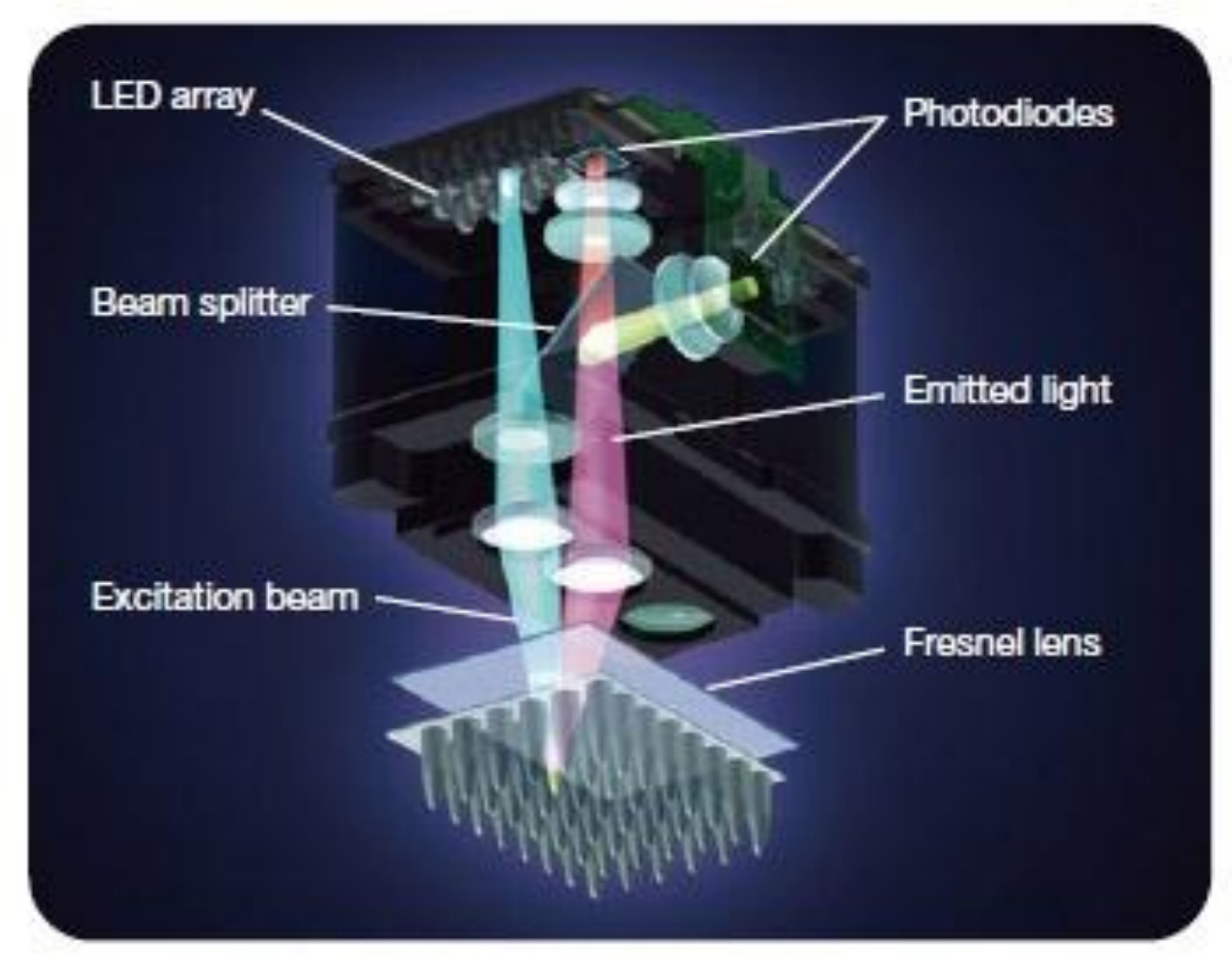


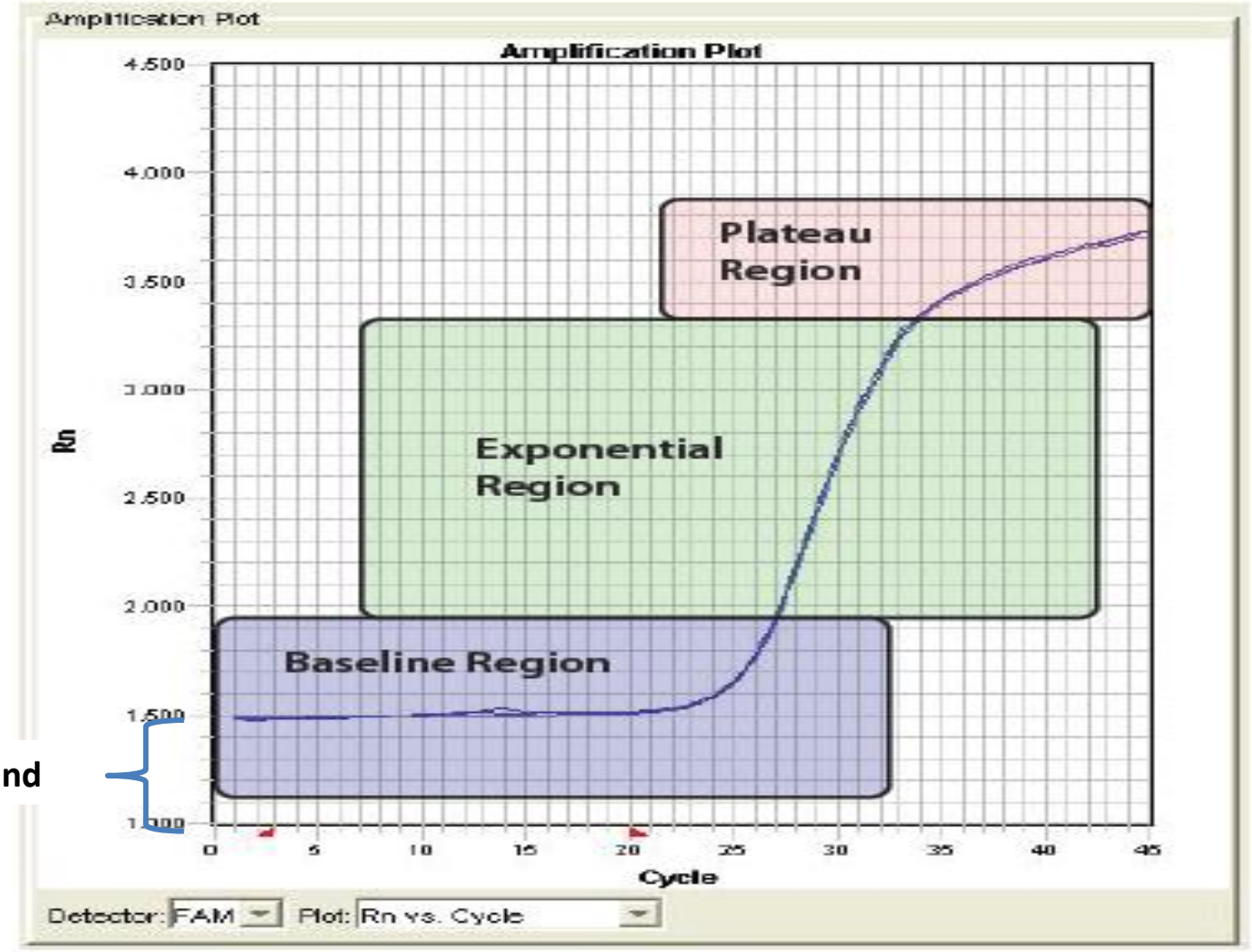

**Step 3 : extension**

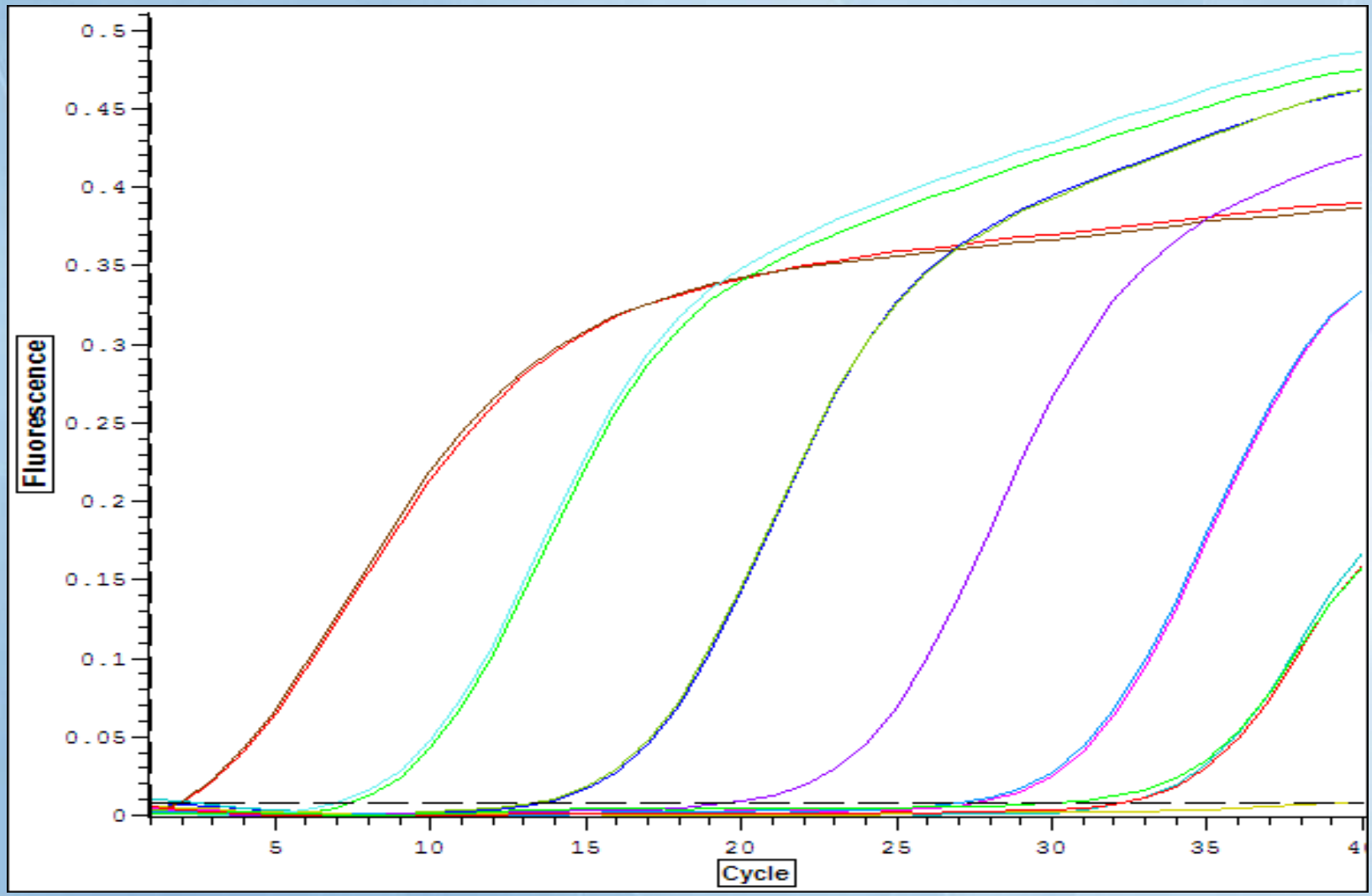
2 minutes 72 °C

only dNTP's

(Andy Vierstraete 1999)

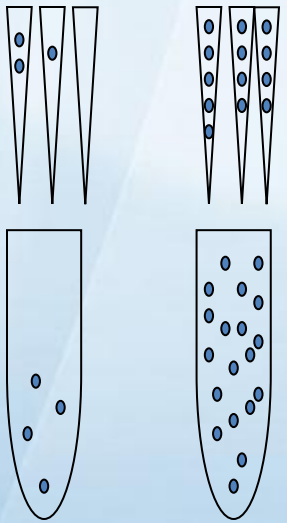

LED array
Photodiodes
Beam splitter
Emitted light
Excitation beam
Fresnel lens


Amplification Plot
Amplification Plot
Rn
Cycle
Plateau Region
Exponential Region
Baseline Region
Background
Detector: FAM
Plot: Rn vs. Cycle


Fluorescence
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
5
10
15
20
25
30
35
Cycle

# PCR itself as a problem

- The PCR reaction
  - Template concentration
  - Inhibitors
  - Optimization
- The operator
  - Pipetting errors
  - Setting up reactions
  - Wrong PCR programs

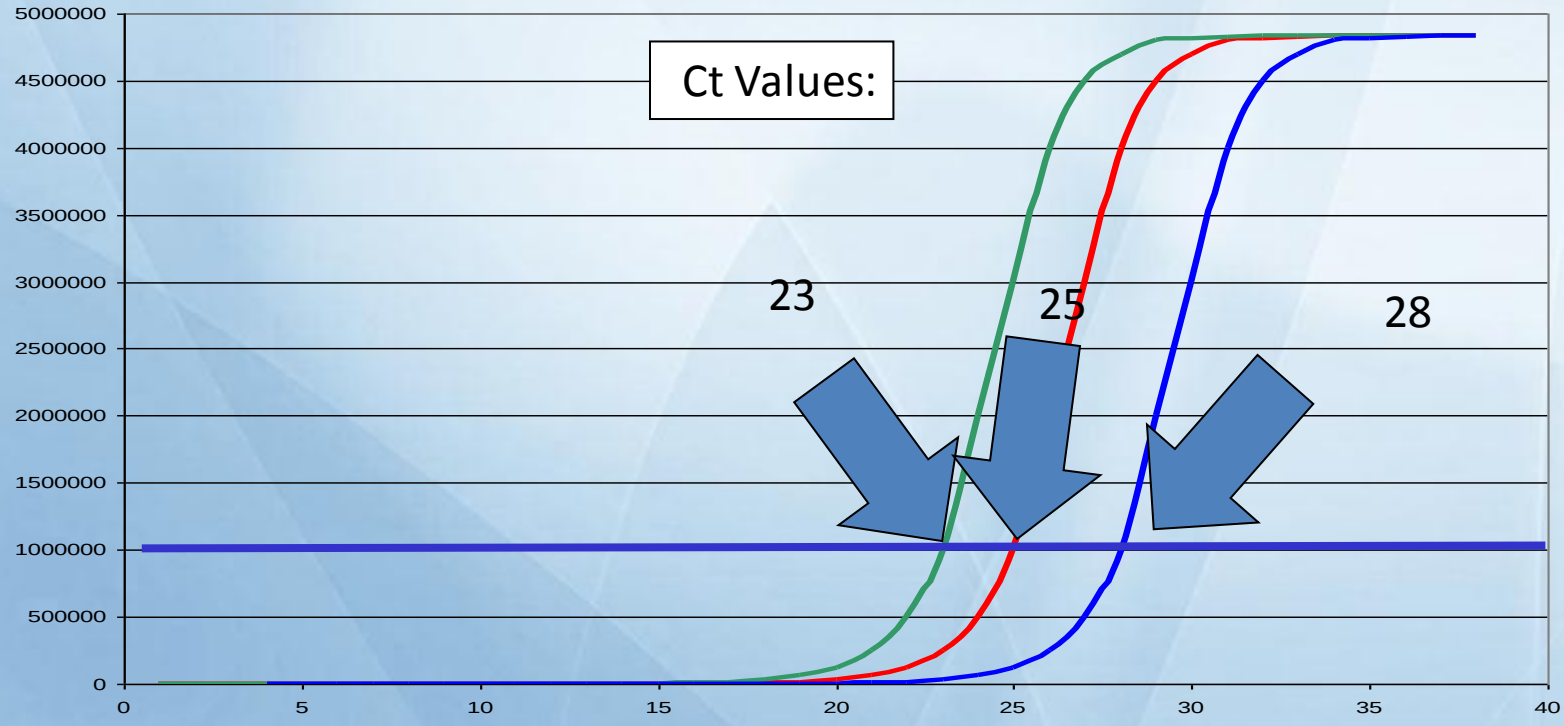


We describe the position of the lines with a value that represents the cycle number where the trace crosses a threshold.

This is called the cycle threshold "Ct Value".

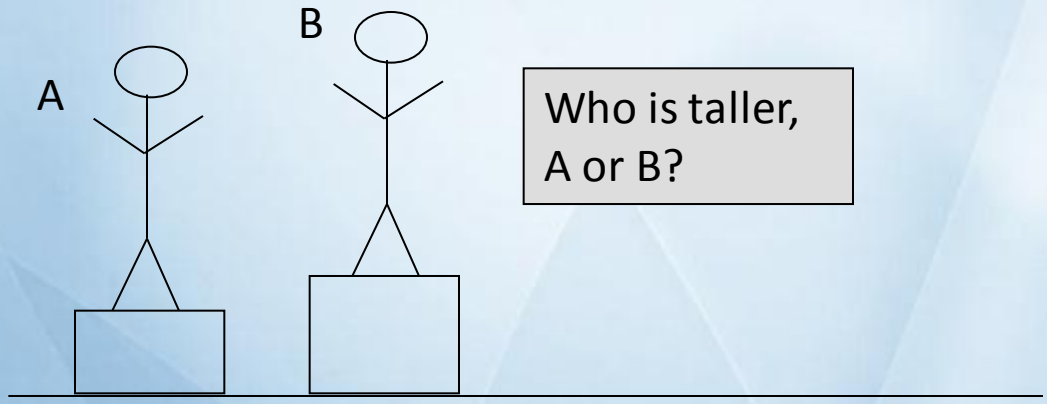
Ct values are directly related to the starting quantity of DNA, by way of the formula:

$$\text{Quantity} = 2^{\wedge Ct}$$

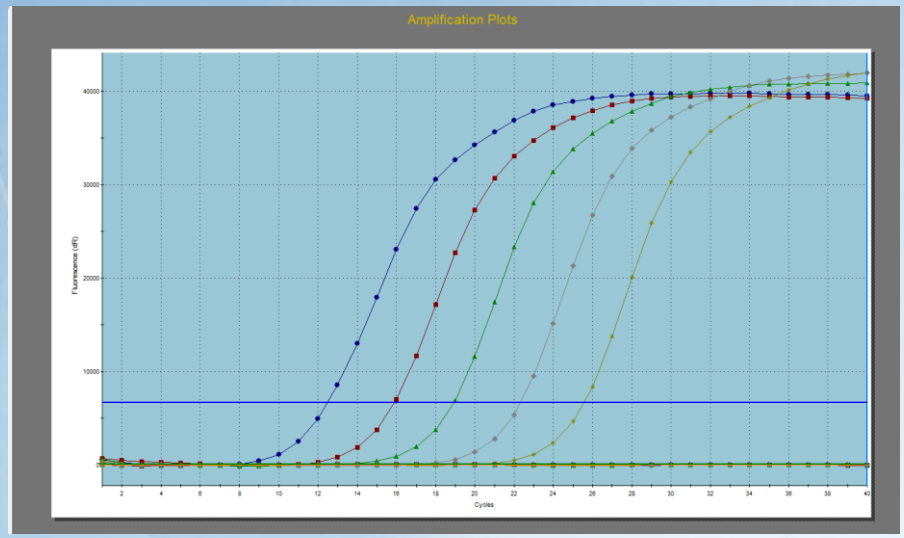


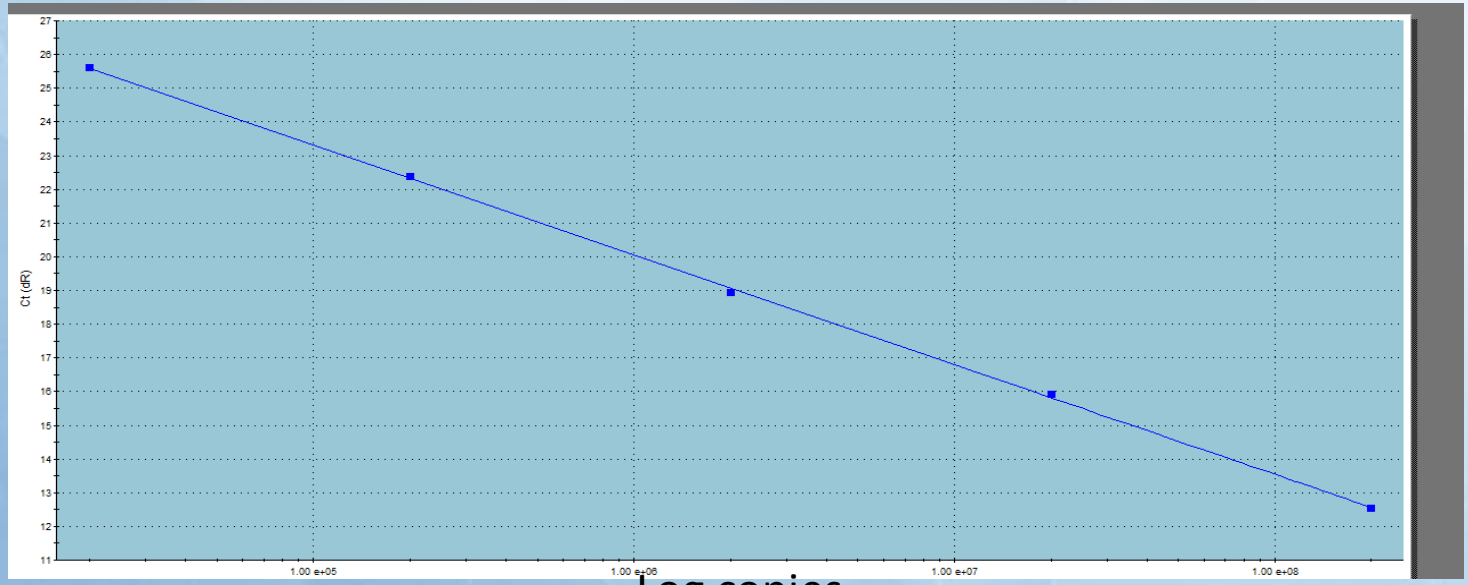
# Standard curves

- Serial dilutions of known sequences used for 'metering' of unknown concentrations



# Ideal Standard Curves



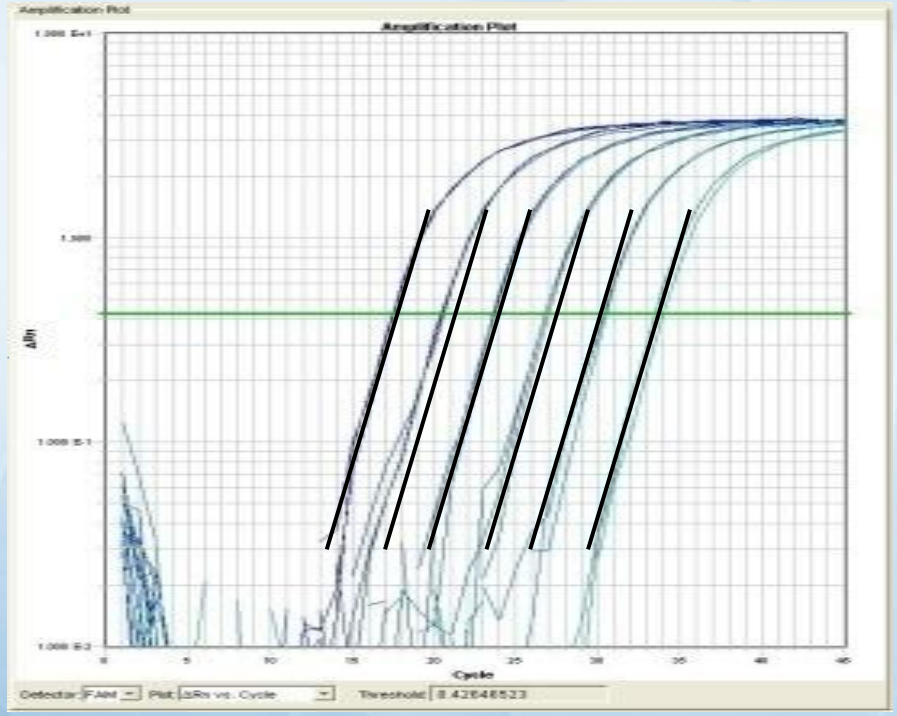

Ct

Log copies

# PCR Efficiency

| CYCLE NUMBER | AMOUNT OF DNA |
|---|---|
| 0 | 1 |
| 1 | 2 |
| 2 | 4 |
| 3 | 8 |
| 4 | 16 |
| 5 | 32 |
| 6 | 64 |
| 7 | 128 |
| 8 | 256 |
| 9 | 512 |
| 10 | 1,024 |
| 11 | 2,048 |
| 12 | 4,096 |
| 13 | 8,192 |
| 14 | 16,384 |
| 15 | 32,768 |
| 16 | 65,536 |
| 17 | 131,072 |
| 18 | 262,144 |
| 19 | 524,288 |
| 20 | 1,048,576 |
| 21 | 2,097,152 |
| 22 | 4,194,304 |
| 23 | 8,388,608 |
| 24 | 16,777,216 |
| 25 | 33,554,432 |
| 26 | 67,108,864 |
| 27 | 134,217,728 |
| 28 | 268,435,456 |
| 29 | 536,870,912 |
| 30 | 1,073,741,824 |
| 31 | 1,400,000,000 |
| 32 | 1,500,000,000 |
| 33 | 1,550,000,000 |
| 34 | 1,580,000,000 |

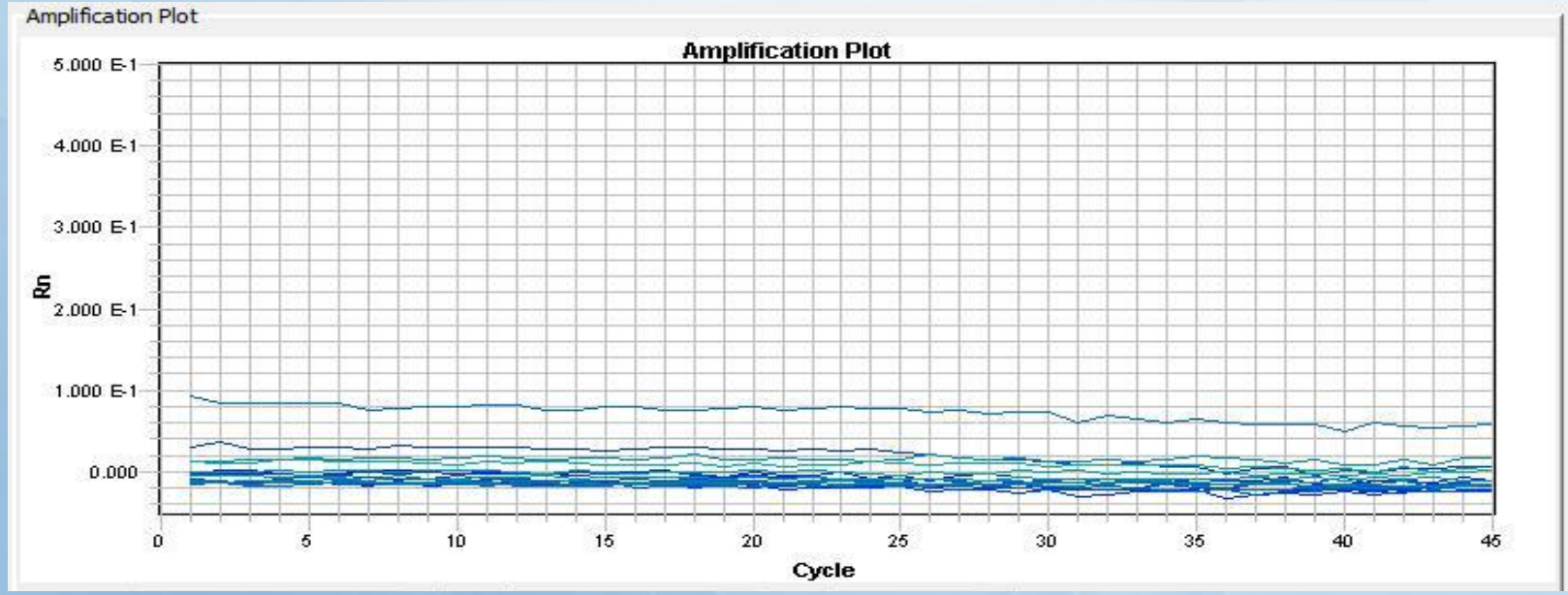
- Efficiency reflects whether DNA doubled every cycle
- It takes 3.32 cycles for DNA to be amplified 10 fold
- If samples have been correctly diluted, every 10-fold dilution should be 3.32 cycles



- **Problematic qRT-PCR**
  - No amplification
  - Unexpected efficiency
  - Delayed Ct
  - Scattered replicates
  - Unusual curves

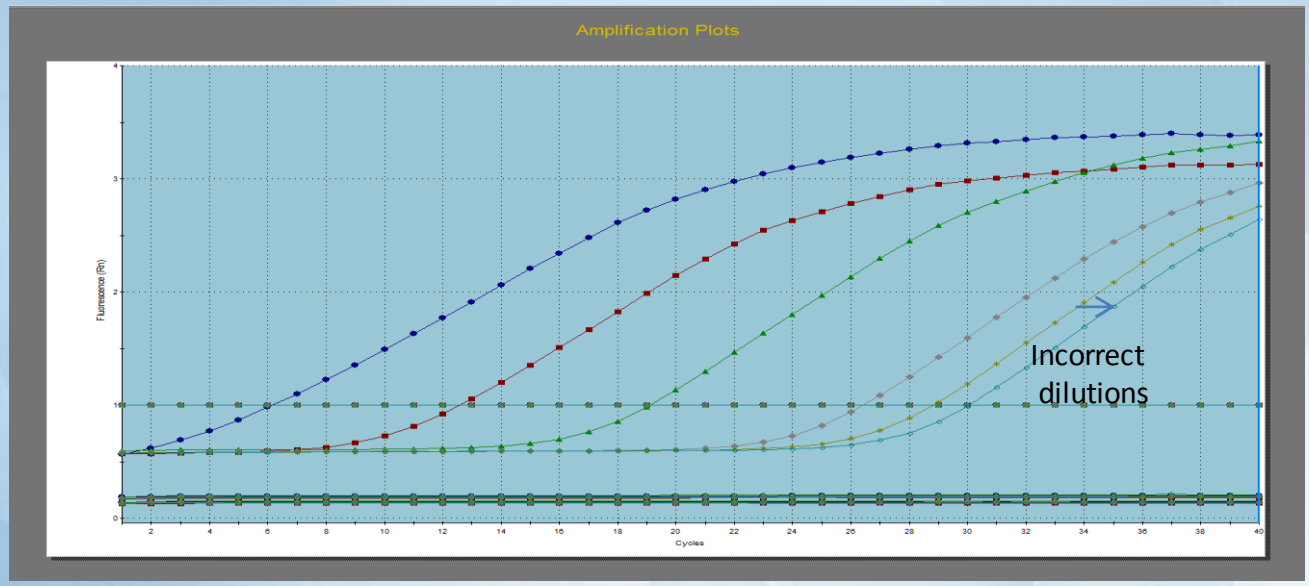
# No Amplification

- **Missing a master mix component(*Repeat the experiment*)**
- **Sample degradation (*Do a different cDNA prep - Test a positive control*)**
- **Machine not calibrated for dye (*Calibrate the instrument)***



# Unexpected PCR Efficiency

- **Lower efficiency (<85%)**
  - Incorrect dilutions causing errors in standard curve




  - L**ower fluorescence of** dye
  - Instrument not calibrated for dye
  - Sample inhibition

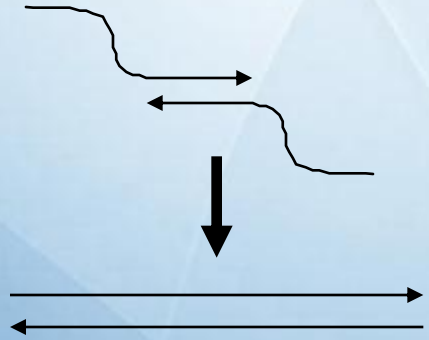
# Unexpected PCR Efficiency

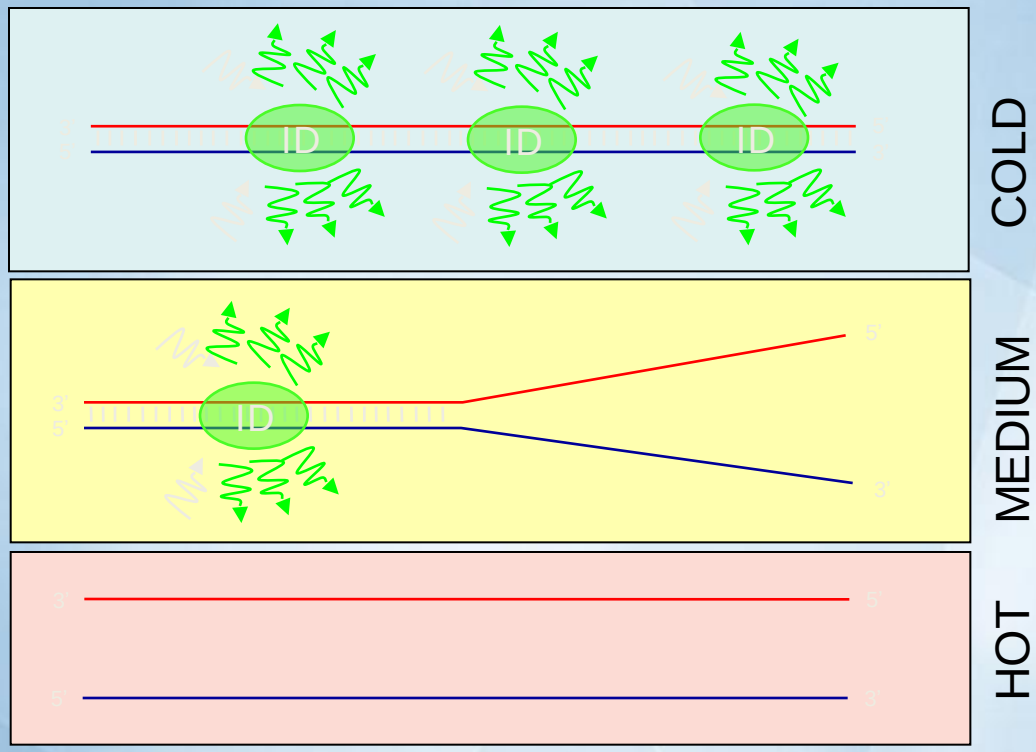
- Higher efficiency (>110%)
  - Incorrect dilutions causing errors in standard curve
  - Genomic DNA contamination
  - Incomplete DNase treatment
  - Primers Dimer

# primer dimers

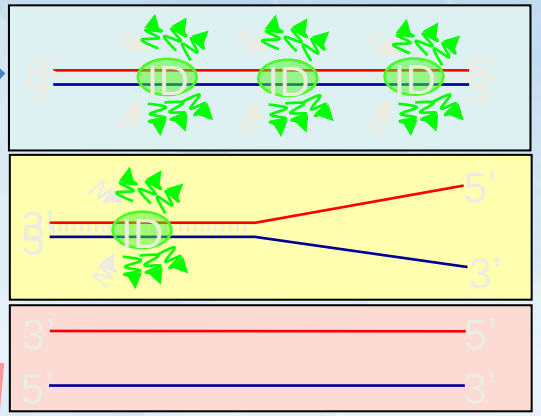
- Primer-dimers interferes with quantitation

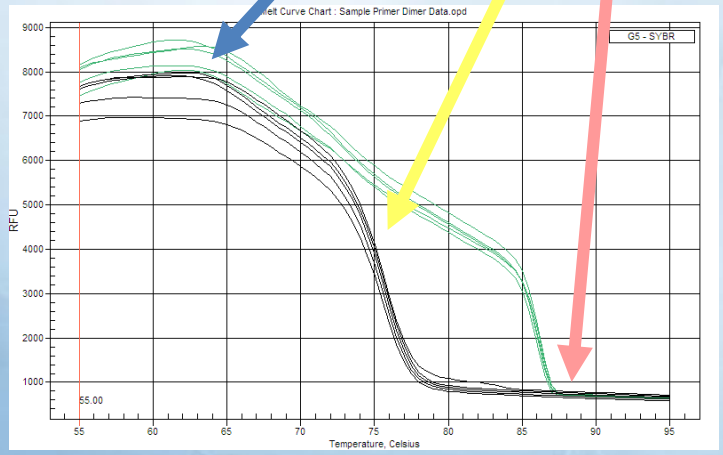
- Identifying primer-dimers: melting curve analysis
  - Pure, homogenous PCR products produce a single, sharply defined melting curve with a narrow peak. Primer dimers melt at relatively low temperatures and have broader peak



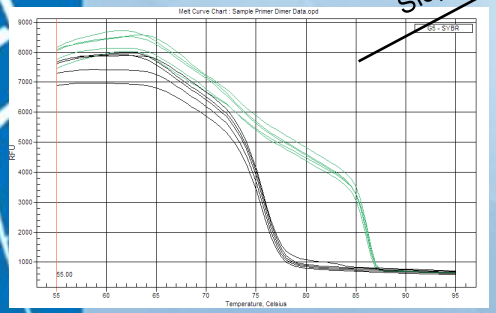

ID
ID
ID
ID
COLD
MEDIUM
HOT

- **Melt curves can tell us what products are in a reaction.**
- PCR products that are shorter will melt at lower temperatures.
- Different PCR products will therefore have different shaped curves.

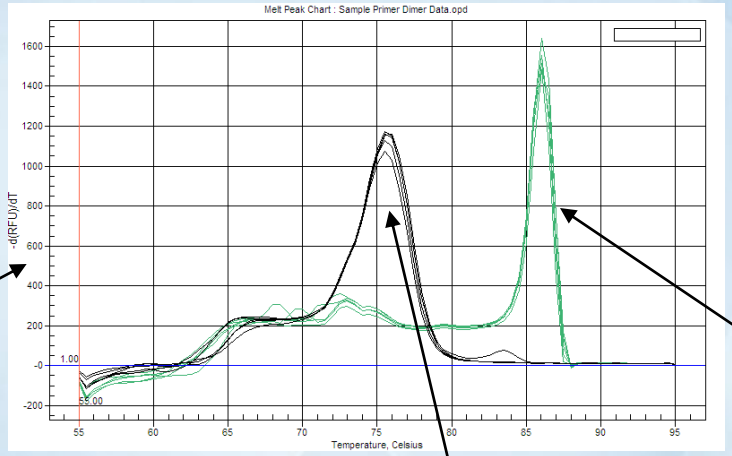




- For convenience, we typically view the derivative *(slope)* of the actual melt curve data.
- The resulting graph looks like a chromatogram, with peaks that represent different PCR products.




Slope vs. Temp



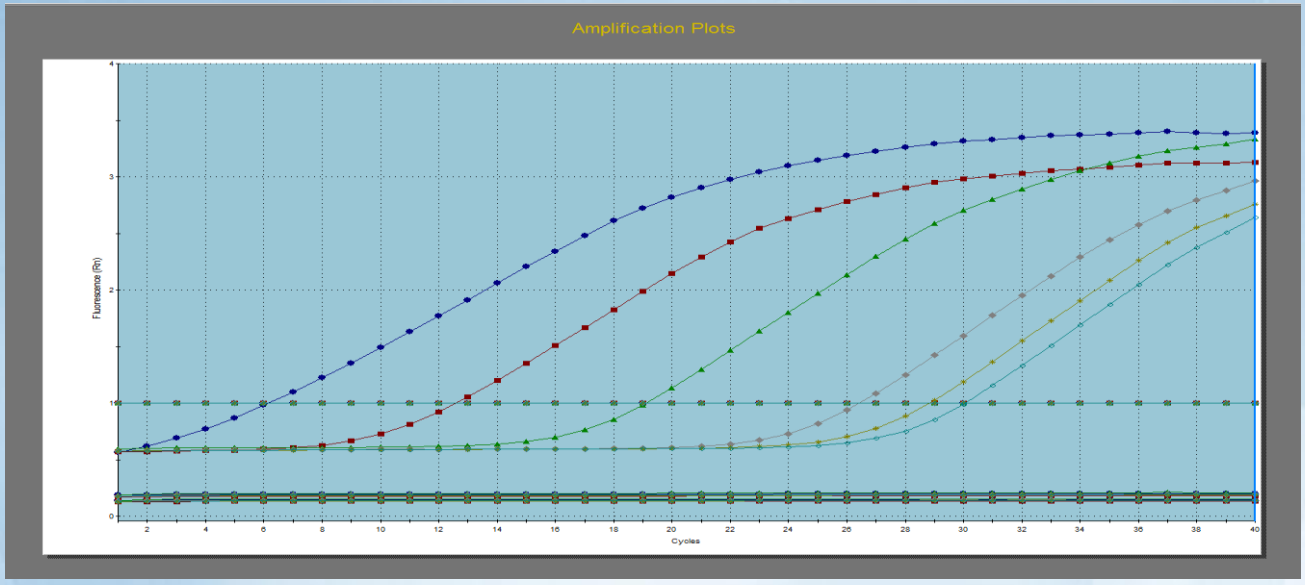

These PCR products melt at a relatively low temperature, are likely primer-dimers.

These PCR products melt at a higher temperature, and are likely to be the main PCR product.

# How to avoid primer/dimers?

- General approaches
  - Reduce delays in workflow
  - Optimize primer
  - Increase the annealing temperature Reduce annealing time to 1-5 sec.
  - Use hot start
  - Reduce number of cycles, e.g. to 40

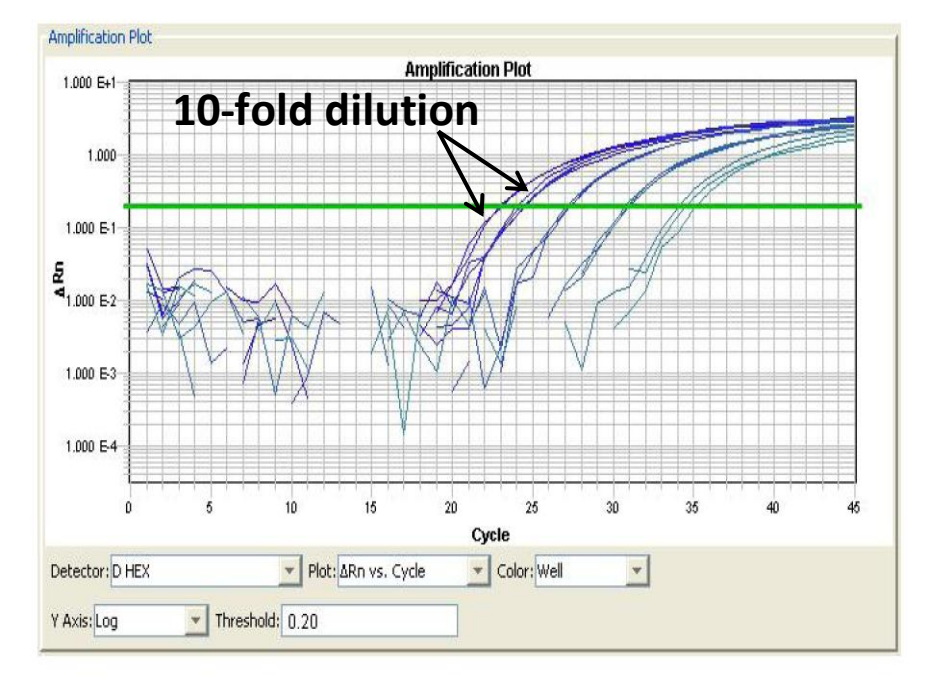
# Delayed Ct




- Low expression

# Delayed Ct……Sample inhibition

- The concentration of inhibitors is maximum in the least dilute sample
- As the sample is diluted, the inhibitory effect decreases
  - Make a new cDNA prep

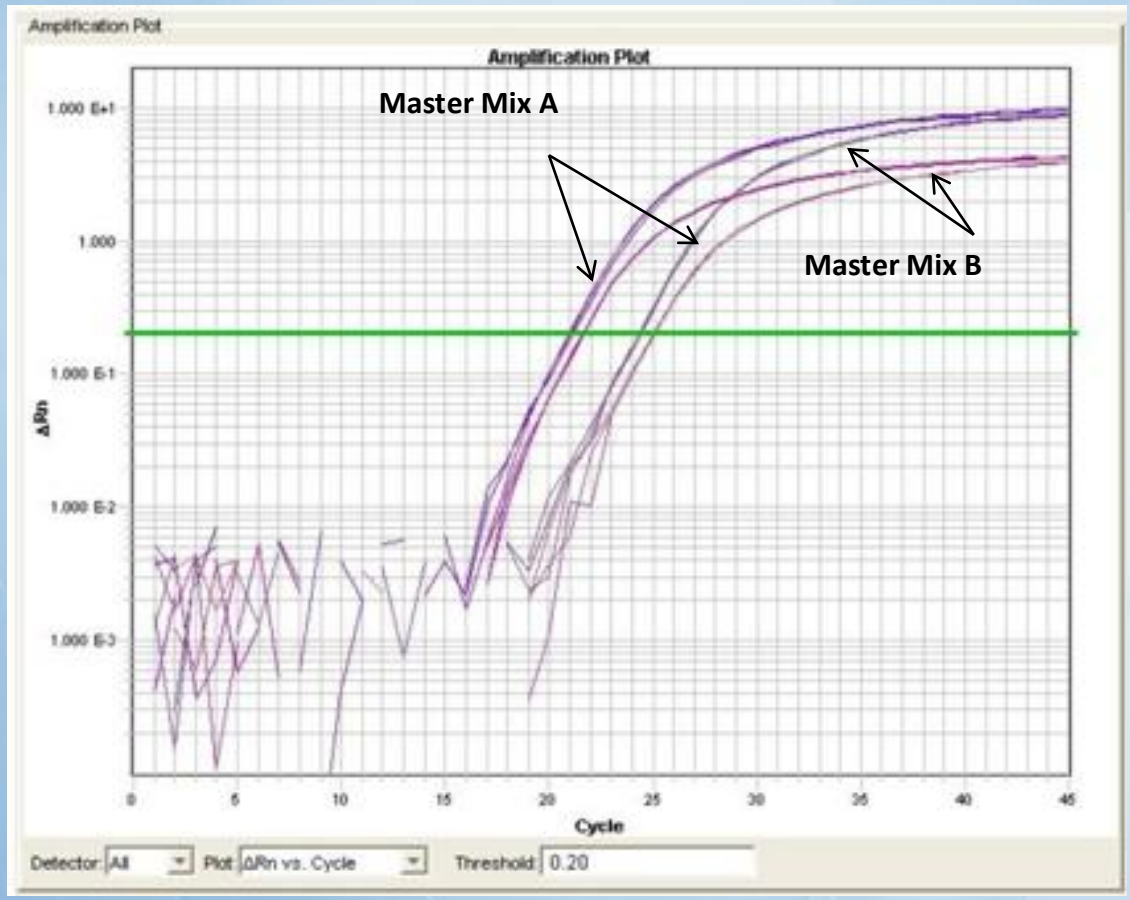


# Inhibitors

- Re-extraction, ethanol precipitation, and/or centrifugal ultrafiltration may correct the problem
  - chloroform
  - SDS concentrations of as low as 0.01% are inhibitory
  - phenol
  - heparin
  - bromphenol blue

# Delayed Ct……

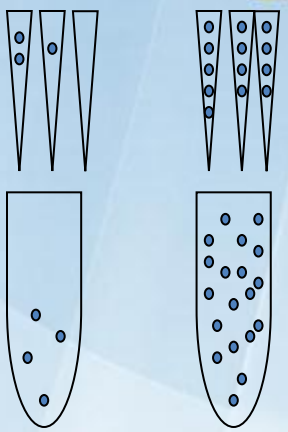
**Master mixes can make a difference**

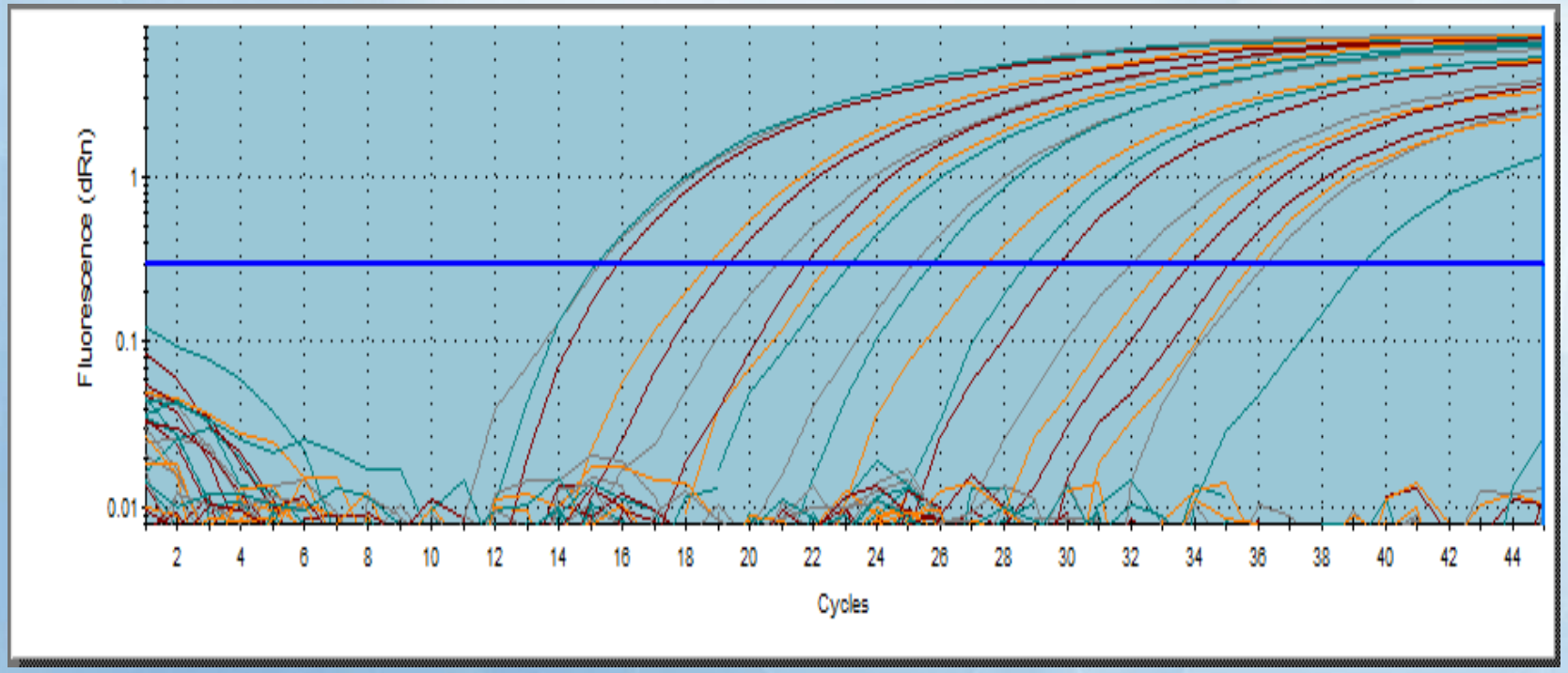


# Scattered Replicates

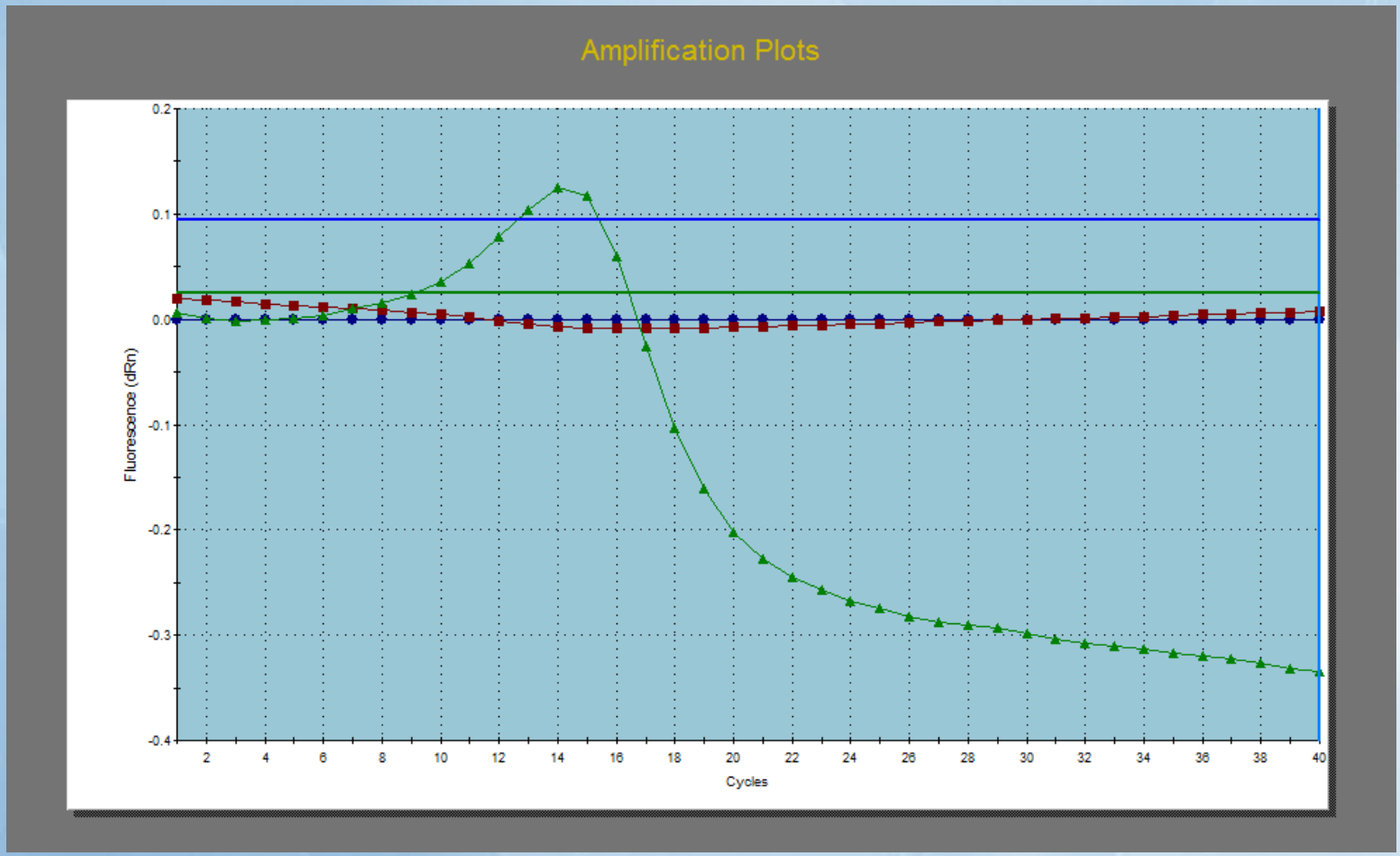
- Pipetting errors
- Incorrectly set baseline

*Replicates ideally should not be more than 0.5 Ct apart*



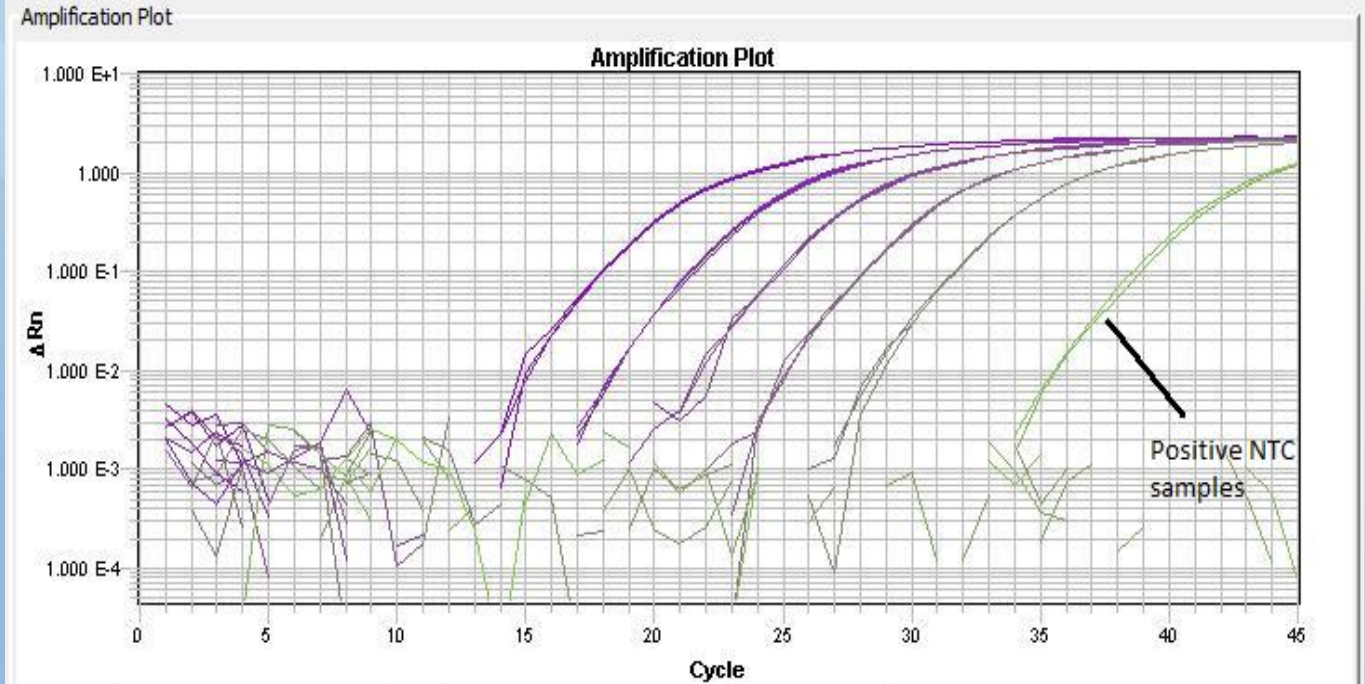


# Unusual curves.....Sample evaporation



# Unexpected Signal…

- Positive NTC: maybe master mix got contaminated
  - Assay design
- Positive –RT -> gDNA contamination
  - Incomplete DNase treatment



- A successful real-time PCR experiment will have the following characteristics: