Troubleshooting of Real Time PCR

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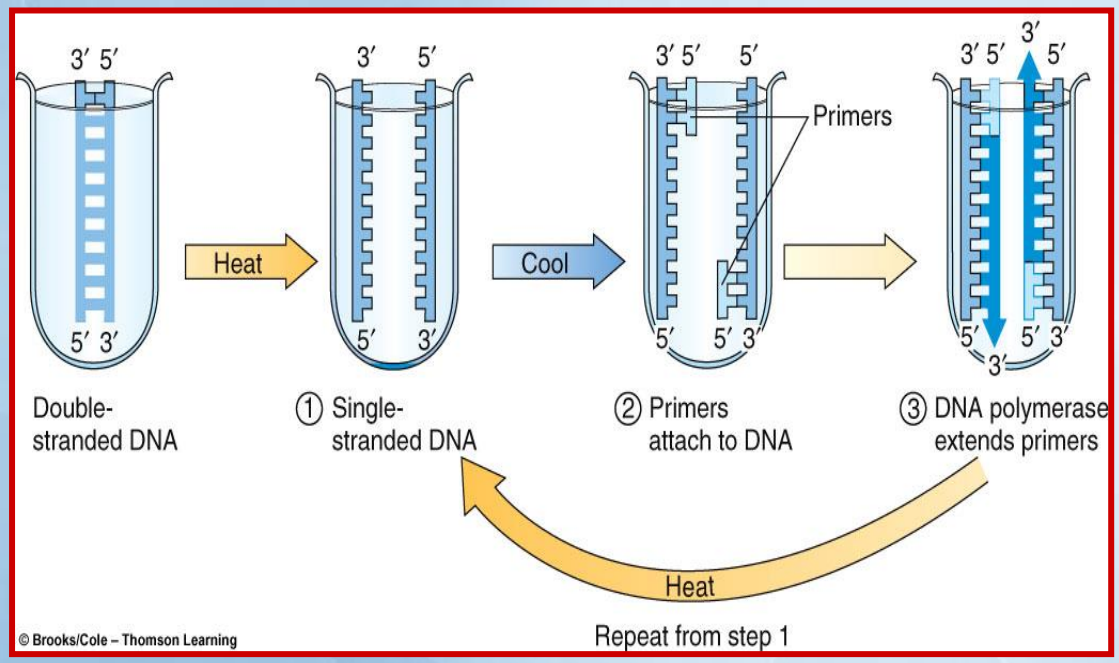
3′ 5′
5′ 3′
Heat
3′ 5′
5′ 3′
Cool
3′ 5′ 5′
Primers
5′ 5′ 3′
3′
3′ 5′ 5′
5′ 5′ 3′
3′
Double-stranded DNA
① Single-stranded DNA
② Primers attach to DNA
③ DNA polymerase extends primers
Heat
Repeat from step 1
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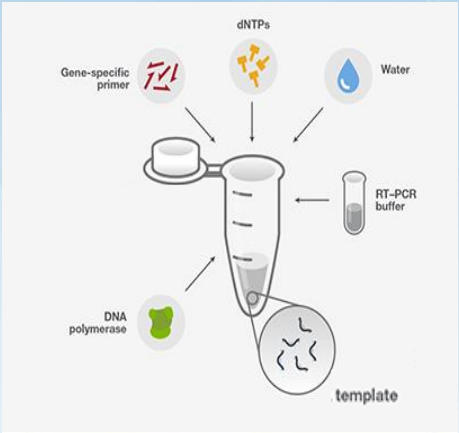
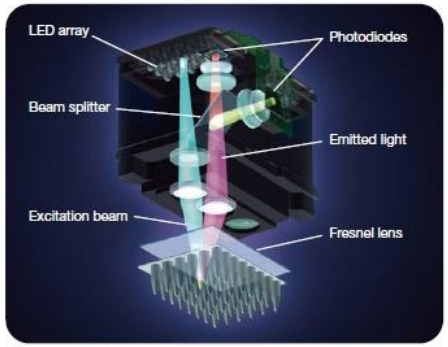
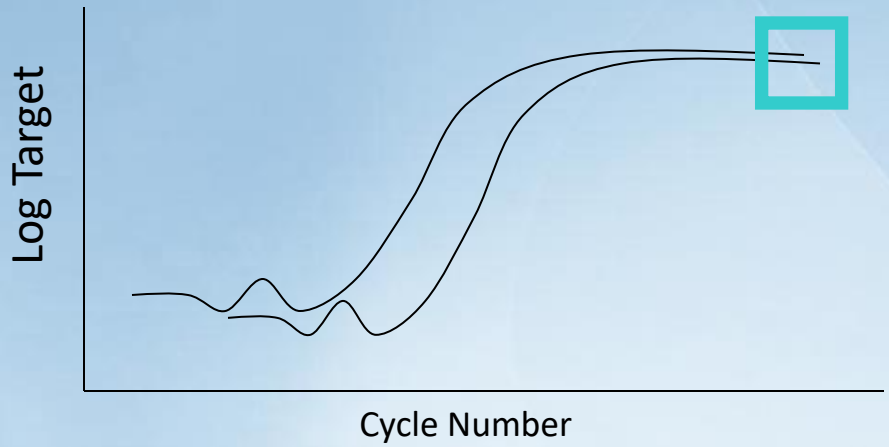
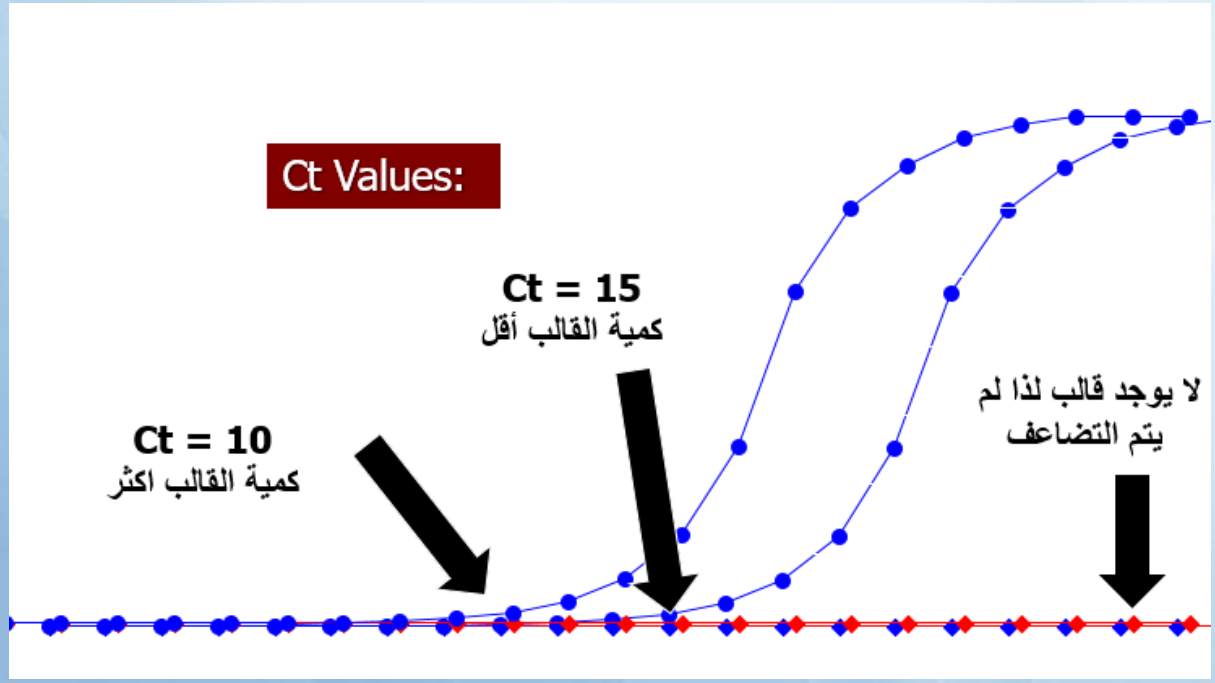

dNTPs
Gene-specific primer
Water
RT-PCR buffer
DNA polymerase
template


LED array
Photodiodes
Beam splitter
Emitted light
Excitation beam
Fresnel lens

Agarose gel electrophoresis following PCR

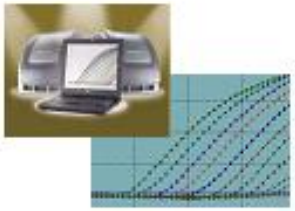


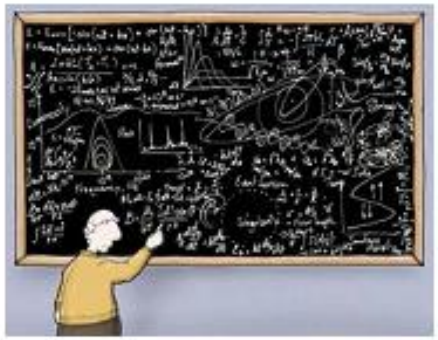


What is Real-Time PCR used for?

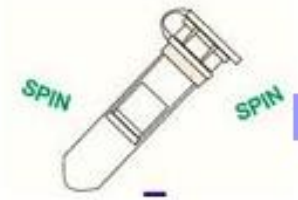
- **Gene expression analysis**
- **Disease diagnosis and management**
  - Viral quantification
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number

Steps towards successful Real-time QPCR experiments





1.Experimental Design



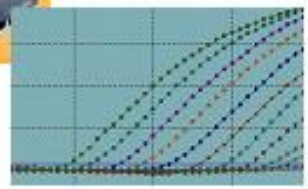
2.Sample preparation and purification

3. RNA/DNA quantification and quality

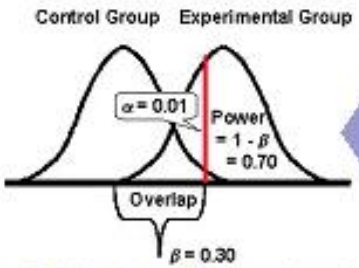
cDNA

Total RNA

4.Reverse Transcription



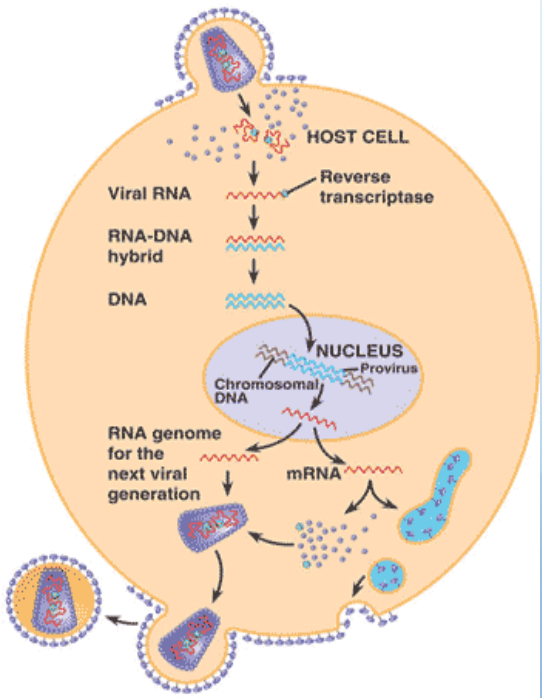
5.Real time QPCR

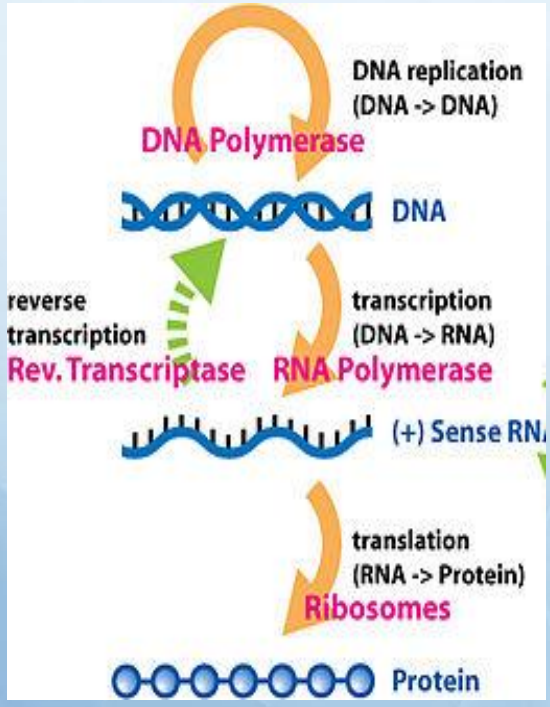


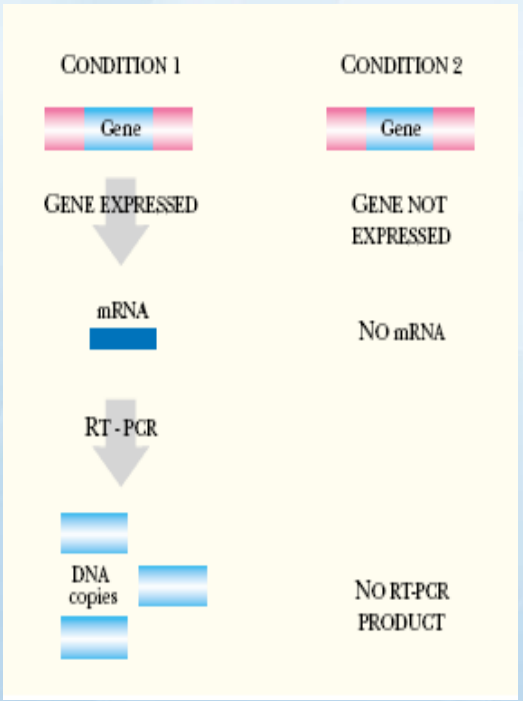
6.Post-run Analysis

Types of PCR

Technique	Abbreviation	Quantitative	Template
Polymerase chain reaction	PCR	No	DNA
Reverse transcriptase polymerase chain reaction	RT-PCR	No	RNA
Real-time polymerase chain reaction	qPCR	Yes	DNA
RT-PCR / qPCR combined technique	qRT-PCR	Yes	RNA







Avoiding Contamination

- Sample preparation, reaction mixture assemblage should be performed in separate areas.
- A Laminar Flow Cabinet with a UV lamp is recommended for preparing the reaction mixture.
- New gloves should be used for DNA/RNA purification.
- The use of tips with filters for both sample and reaction mixture preparation
- Autoclaving of all buffers is recommended.

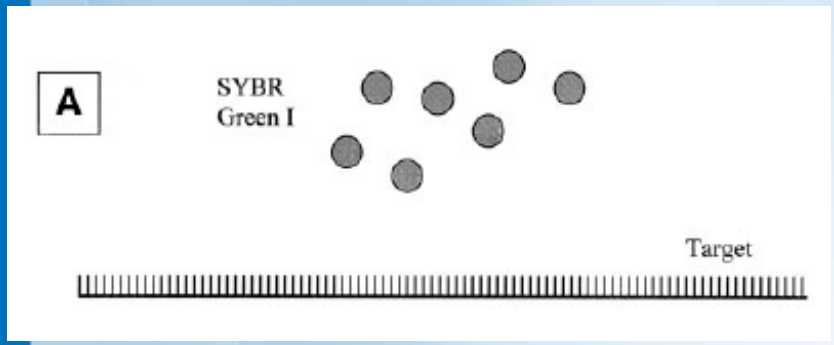
How to avoid problems with genomic DNA?

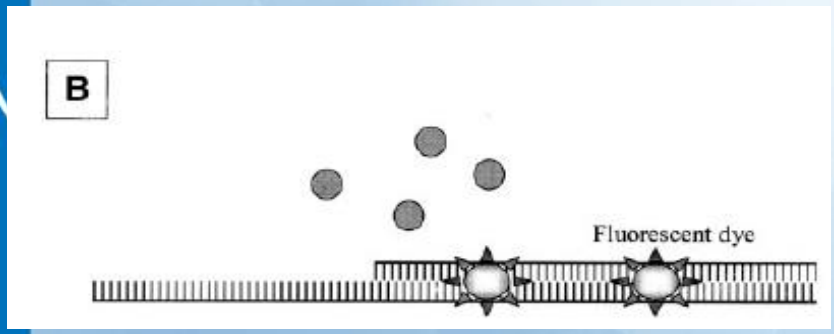
- Always include a DNase I purification step in the RNA isolation procedure
  - Included in the kit used here

Experiment design

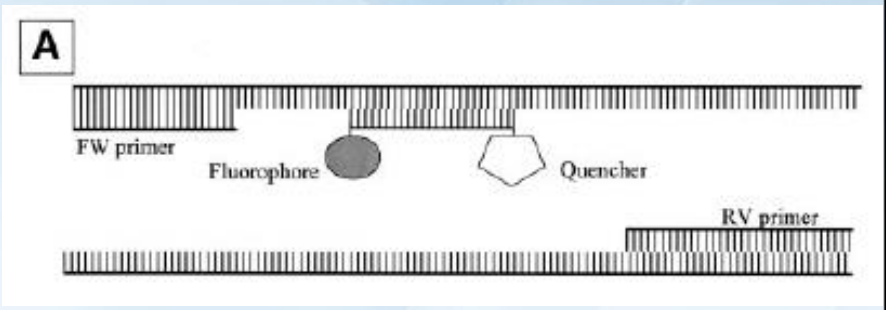
- **Blank reaction**
  - Controls for contamination
  - Contains all reagents except DNA template
- **Negative control reaction**
  - Controls for specificity of the amplification reaction
  - Contains all reagents and a DNA template lacking the target sequence
- **Positive control reaction**
  - Controls for sensitivity
  - Contains all reagents and a known target-containing DNA template
- **HKG (House keeping gene)**
- **Make at least 3 replicates**

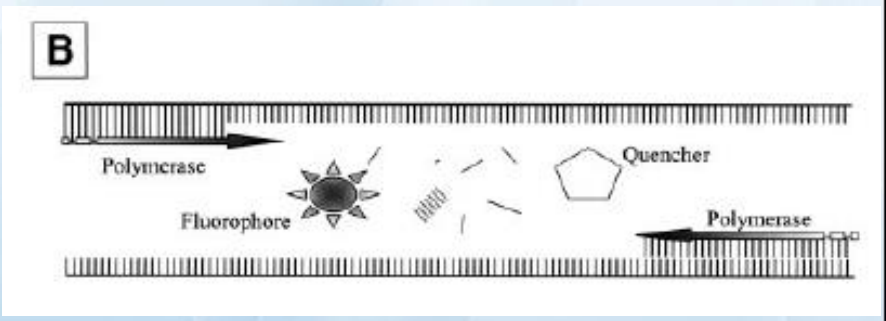
SYBR Green

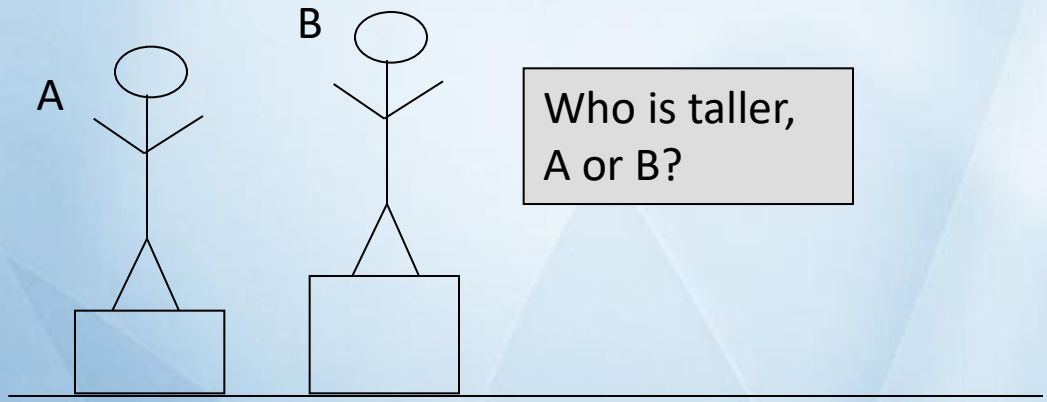




TaqMan







Who is taller,
A or B?

Sample 1

Sample 2

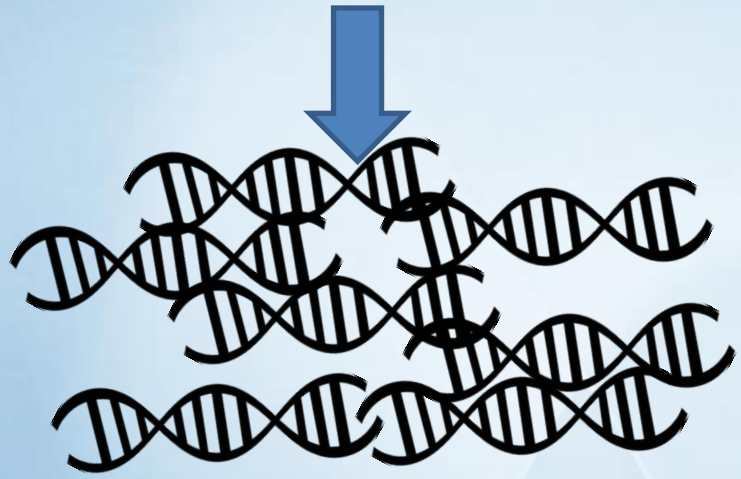
Housekeeping Genes

Highly conserved genes that must be continually expressed in all tissues of organisms to maintain essential cellular functions.

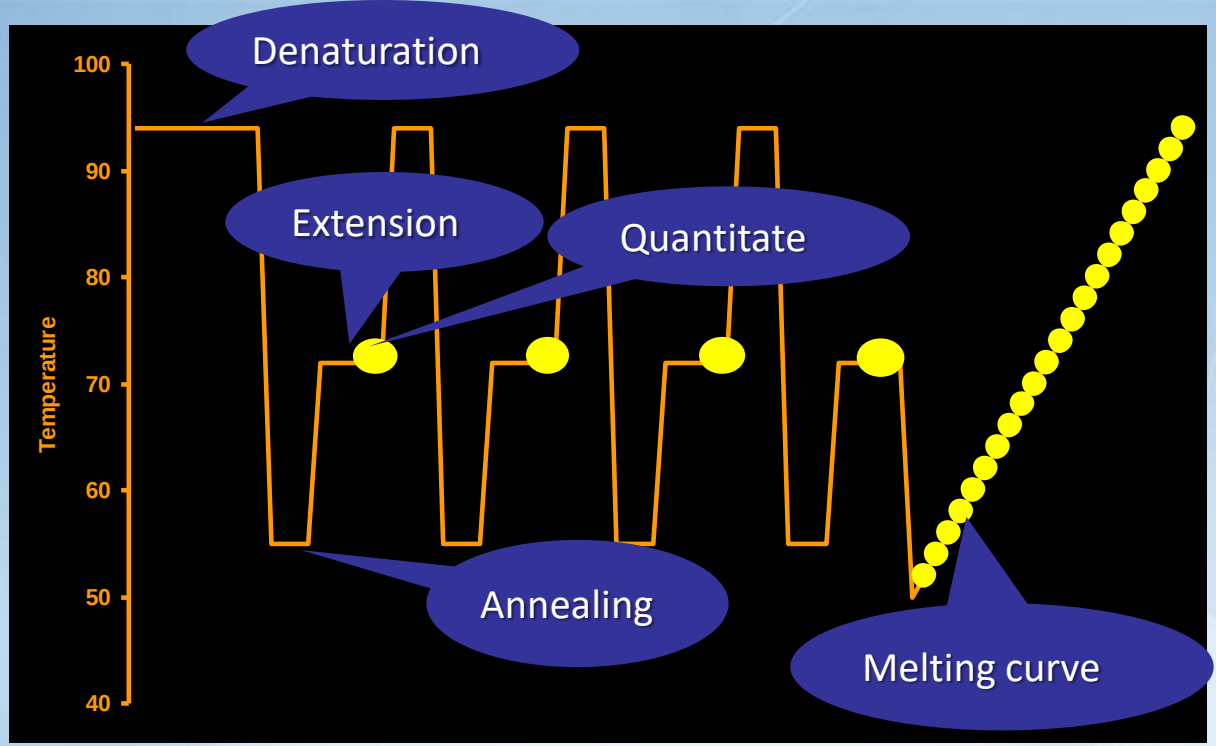
Gene	Genomic structure / pseudogenes	Regulation e.g.
ß-actin	multigene family; > 20 genes; 1 active locus 20 pseudogenes	↑: hormones of tyroid gland ↑: stomach tumor
γ-actin	multigene family; pseudogenes	
GAPDH	multigene family; 10-30 genes; > 200 in mouse mostly pseudogenes	↑: lung, pancreatic, colon cancer ↑: insulin, EGF
5.8S,18S, 28S RNA	pseudogenes	
ß2-microglobulin	no pseudogenes	↑: Non-Hodgkin lymhoma abnormal expression in tumors
G6PDH	no pseudogenes	↑: kidney, stomach tumor ↑: hormones, oxidant stress, growth factors
PBGD	no pseudogenes	
aldolase	pseudogenes	
HPRT	pseudogenes	
U3, U8, ...	Pseudogenes	
ornithin decarboxylase		↑: tumors
...		

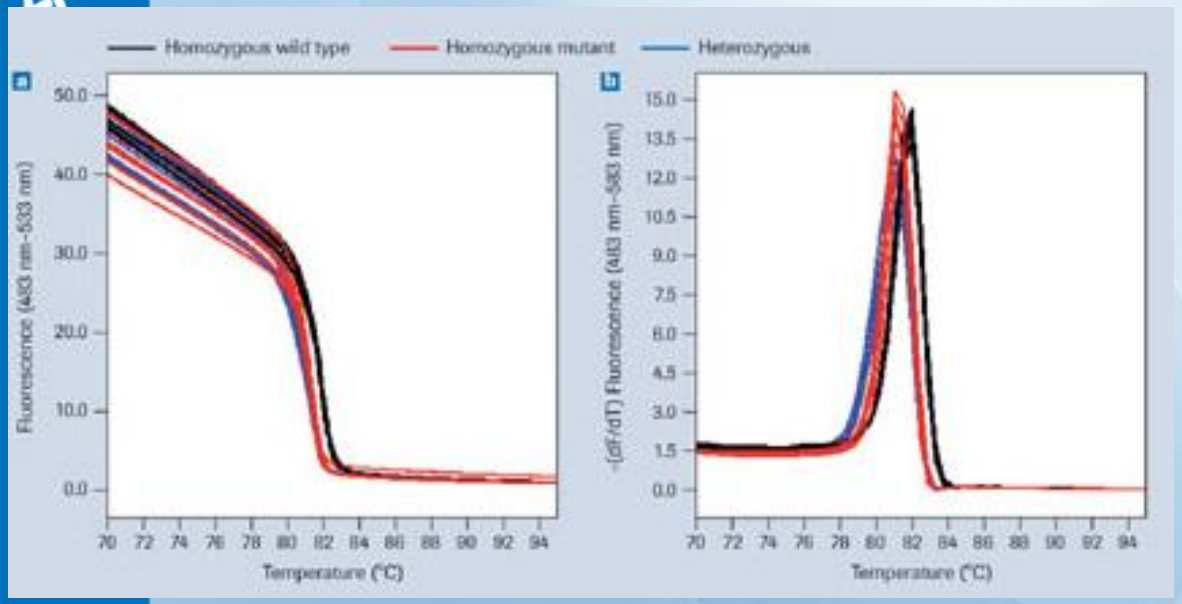
Sample 1

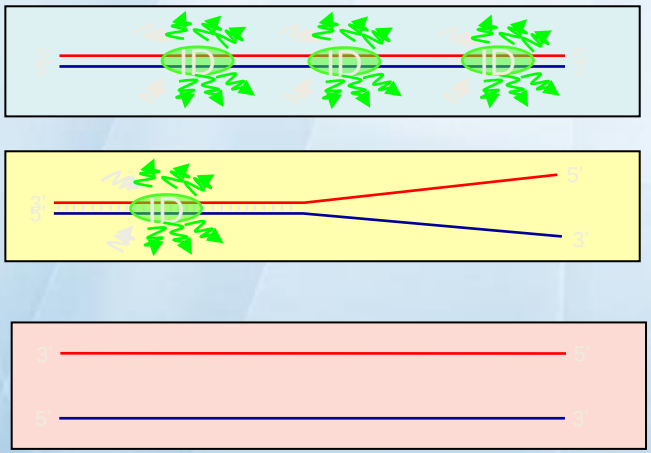
Sample 2



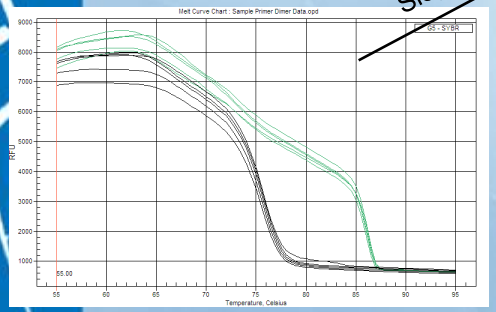
	Sample 1	Sample 2
HKG	5	10
Target Gene	20	20


Denaturation
Extension
Quantitate
Annealing
Melting curve
Temperature
100
90
80
70
60
50
40

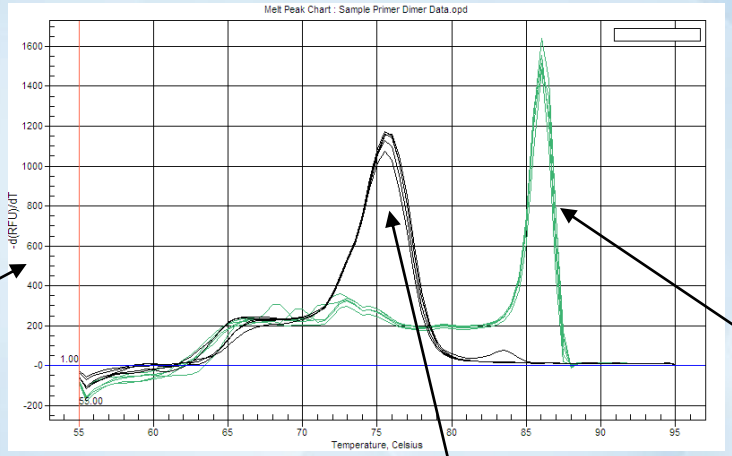

Homozygous wild type
Homozygous mutant
Heterozygous
Fluorescence (483 nm-533 nm)
-(dF/dT) Fluorescence (483 nm-533 nm)
Temperature (°C)
Temperature (°C)



- 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.

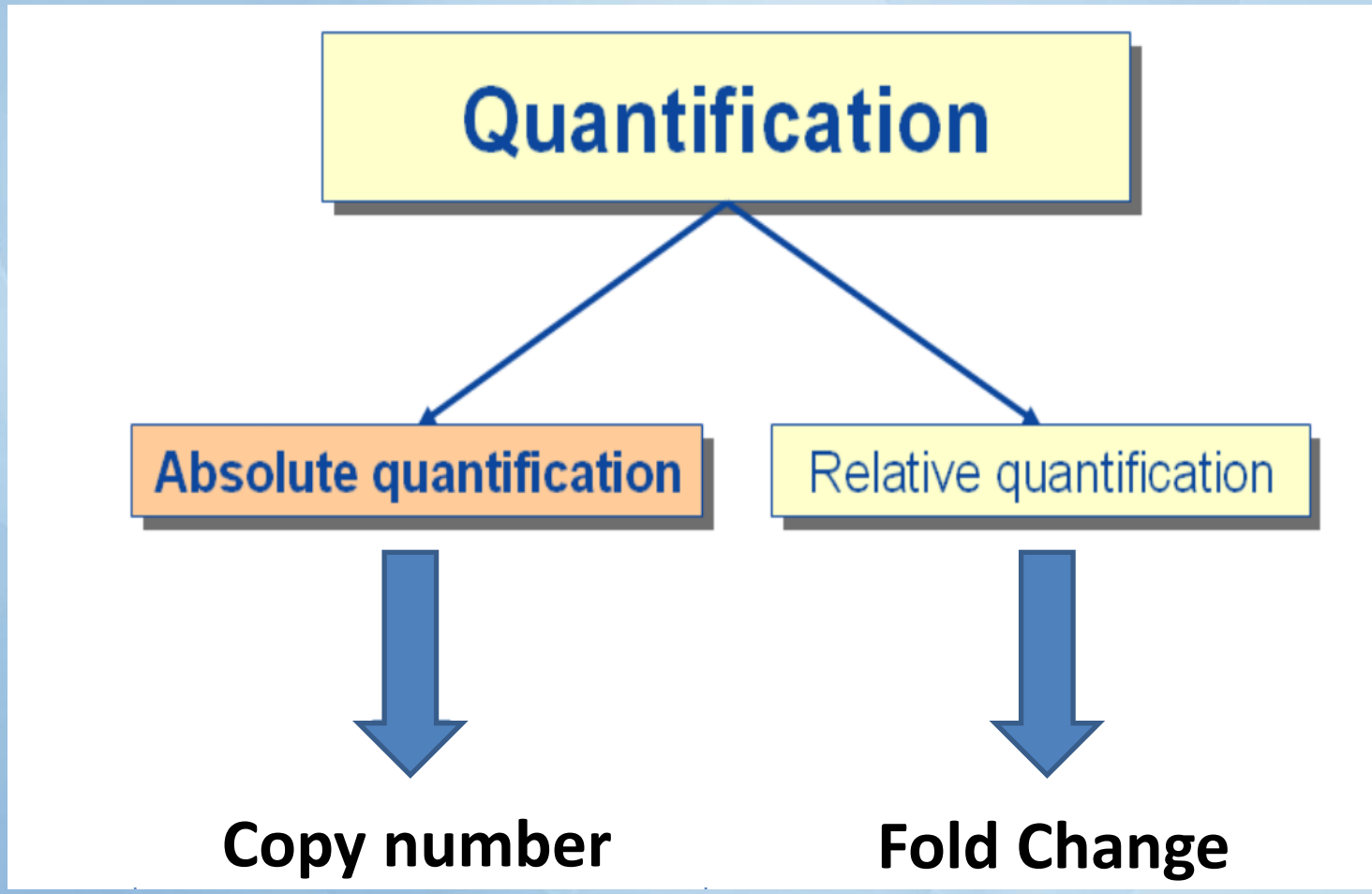


Slope vs. Temp



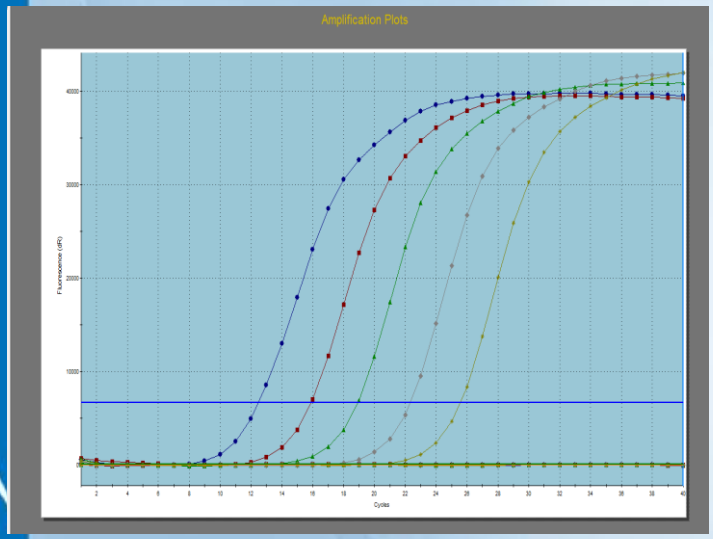
These PCR products melt at a relatively low temperature, are likely primer-dimers.

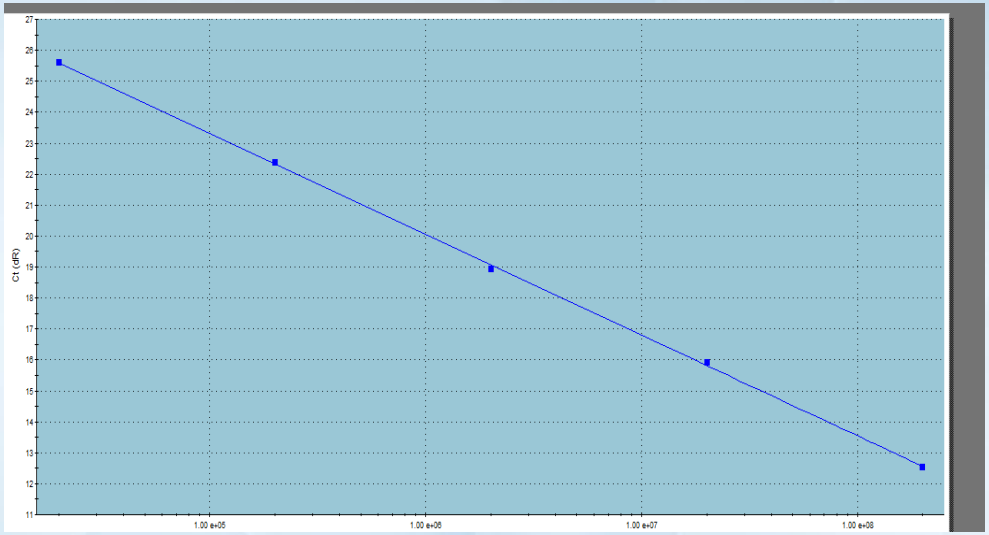
These PCR products melt at a higher temperature, and are likely to be the main PCR product.


Quantification
Absolute quantification
Relative quantification
Copy number
Fold Change

Standard curves

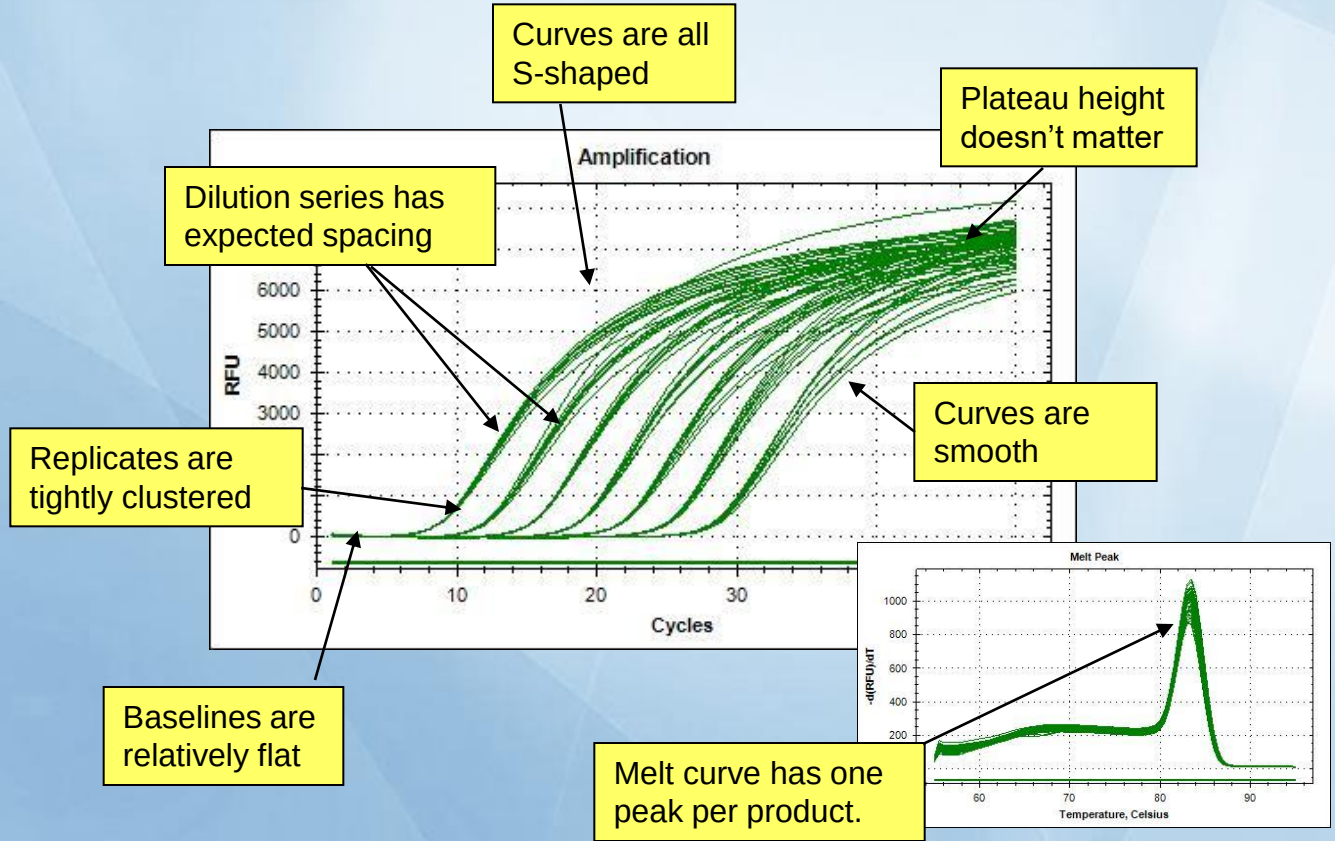
Serial dilutions of known sequences used for 'metering' of unknown concentrations





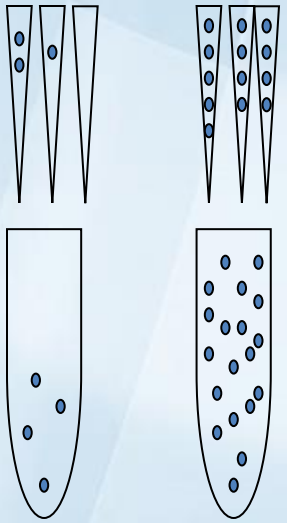
- **Use at least 5 concentrations for a standard curve**

- **Use serial dilutions that are one order of magnitude apart _ 1:10, 1:100, 1:1000,...**



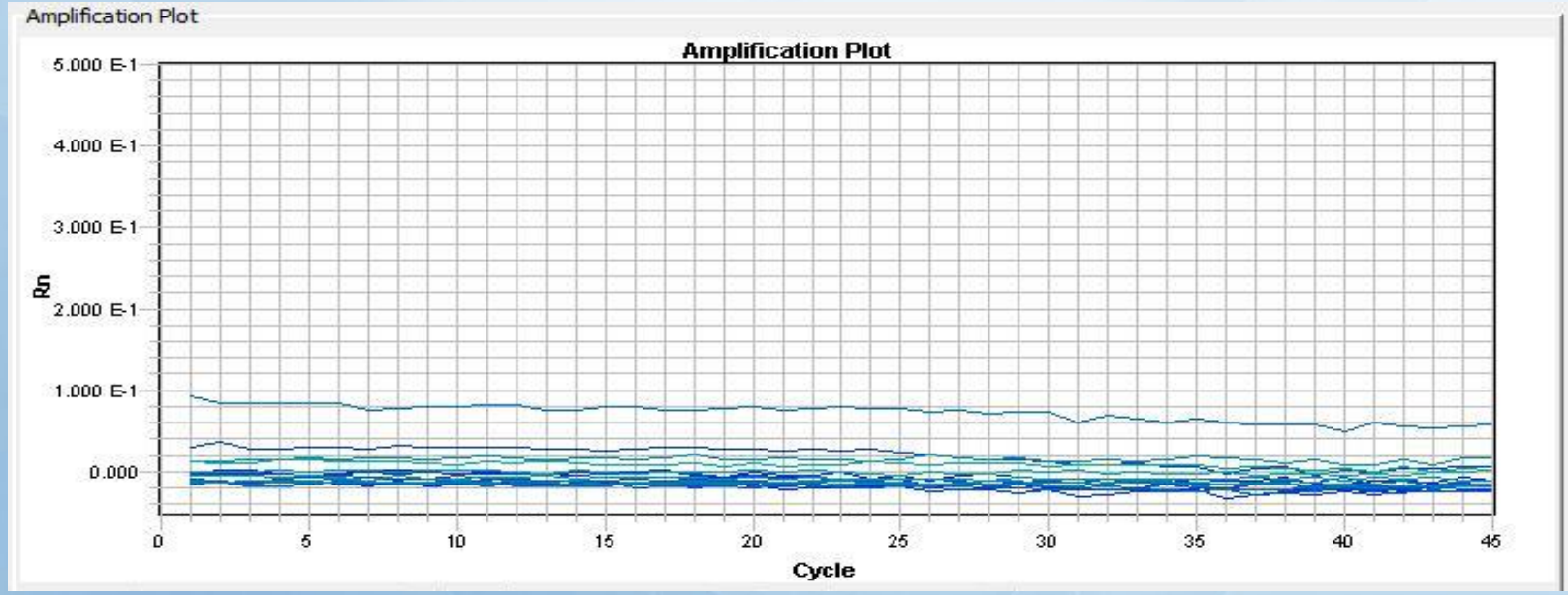
PCR itself as a problem

- The PCR reaction
  - Template
  - Conditions
- The operator
  - Pipetting errors
  - Setting up reactions
  - Wrong PCR programs
- The Thermal cycler



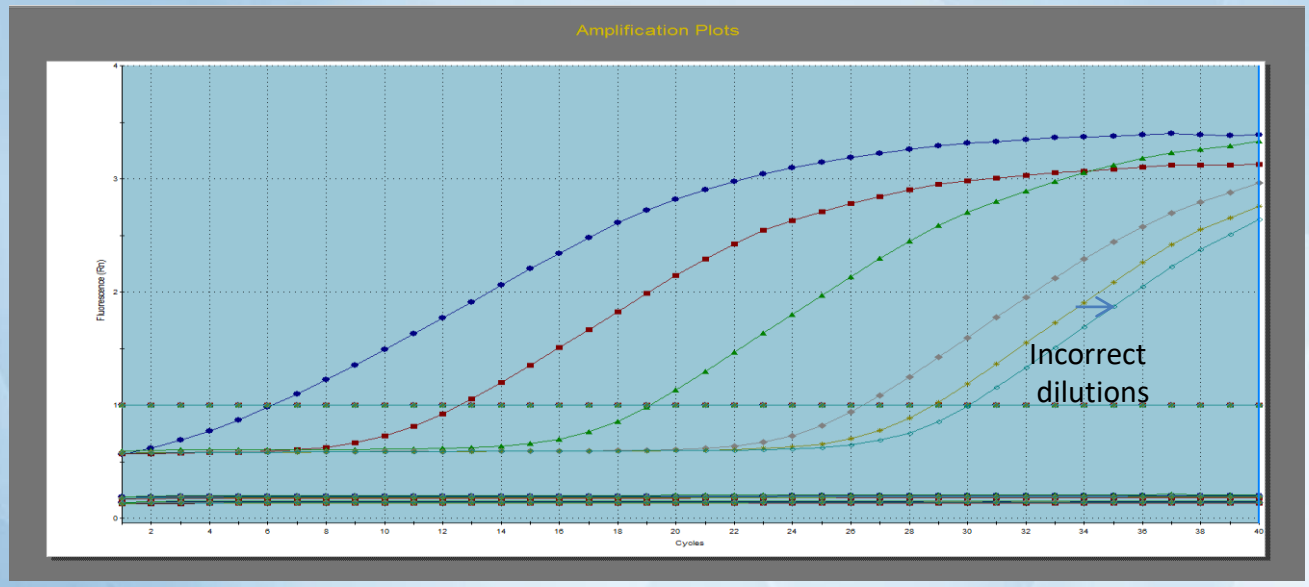
No Amplification

- **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



  - Sample inhibition

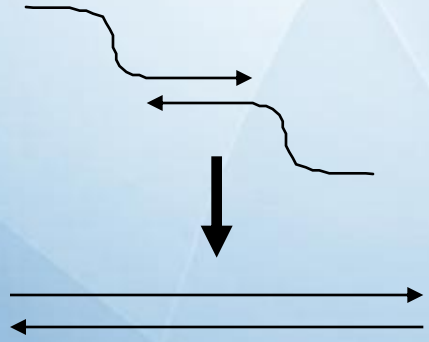
Unexpected PCR Efficiency

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

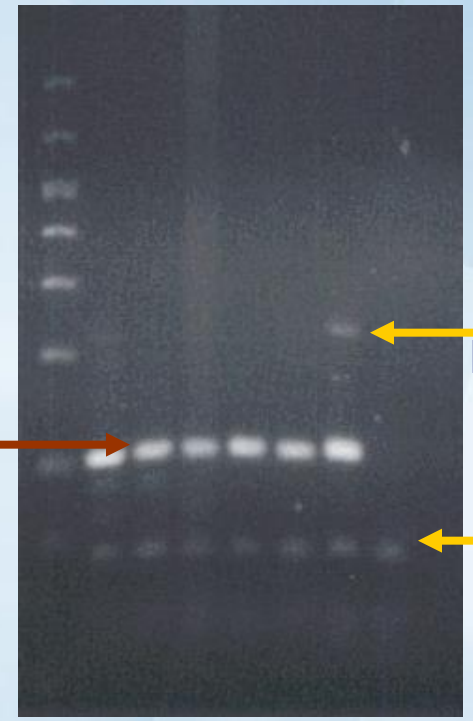
primer dimers

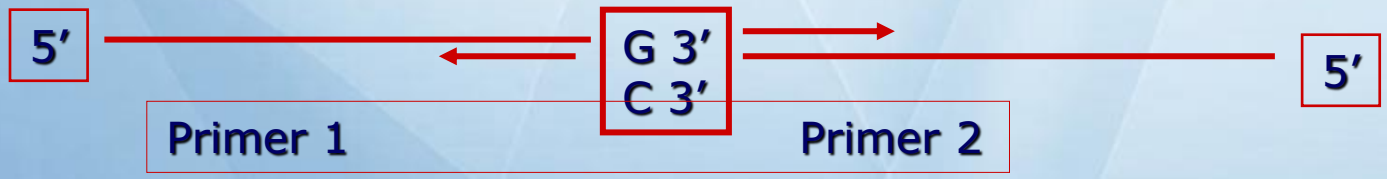
- Primer-dimers interferes with quantitation

- 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



- **Primer dimers and misprime:**
  - Annealing temp. too low (dimers) or too high (misprime).
  - excess primers
  - Design primers carefully
  - Size is the sum of two primer lengths.



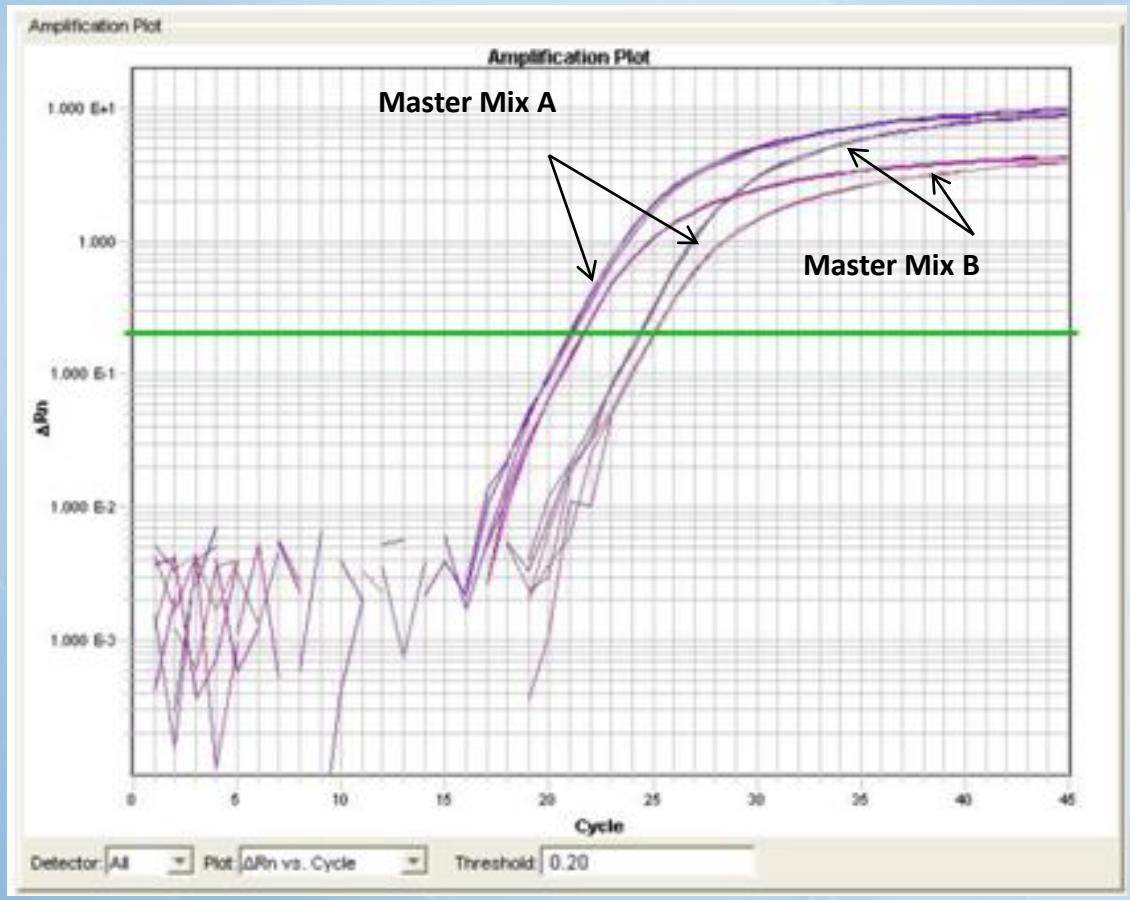


How to avoid primer/dimers?

- 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……

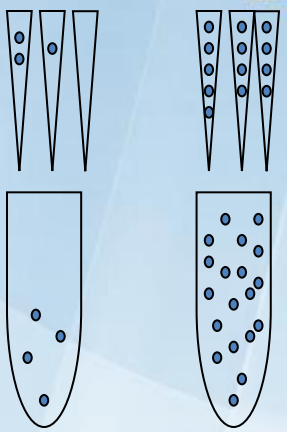
Master mixes can make a difference

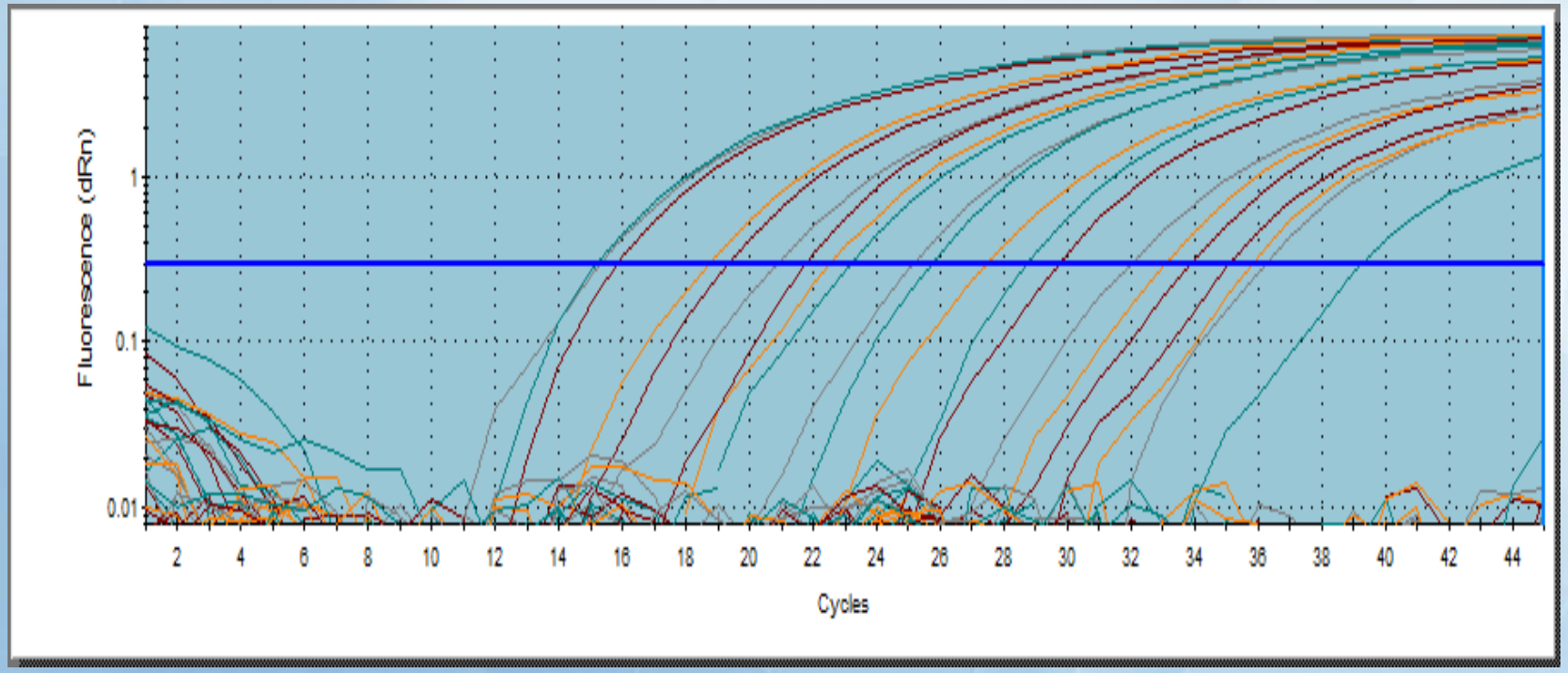


Scattered Replicates

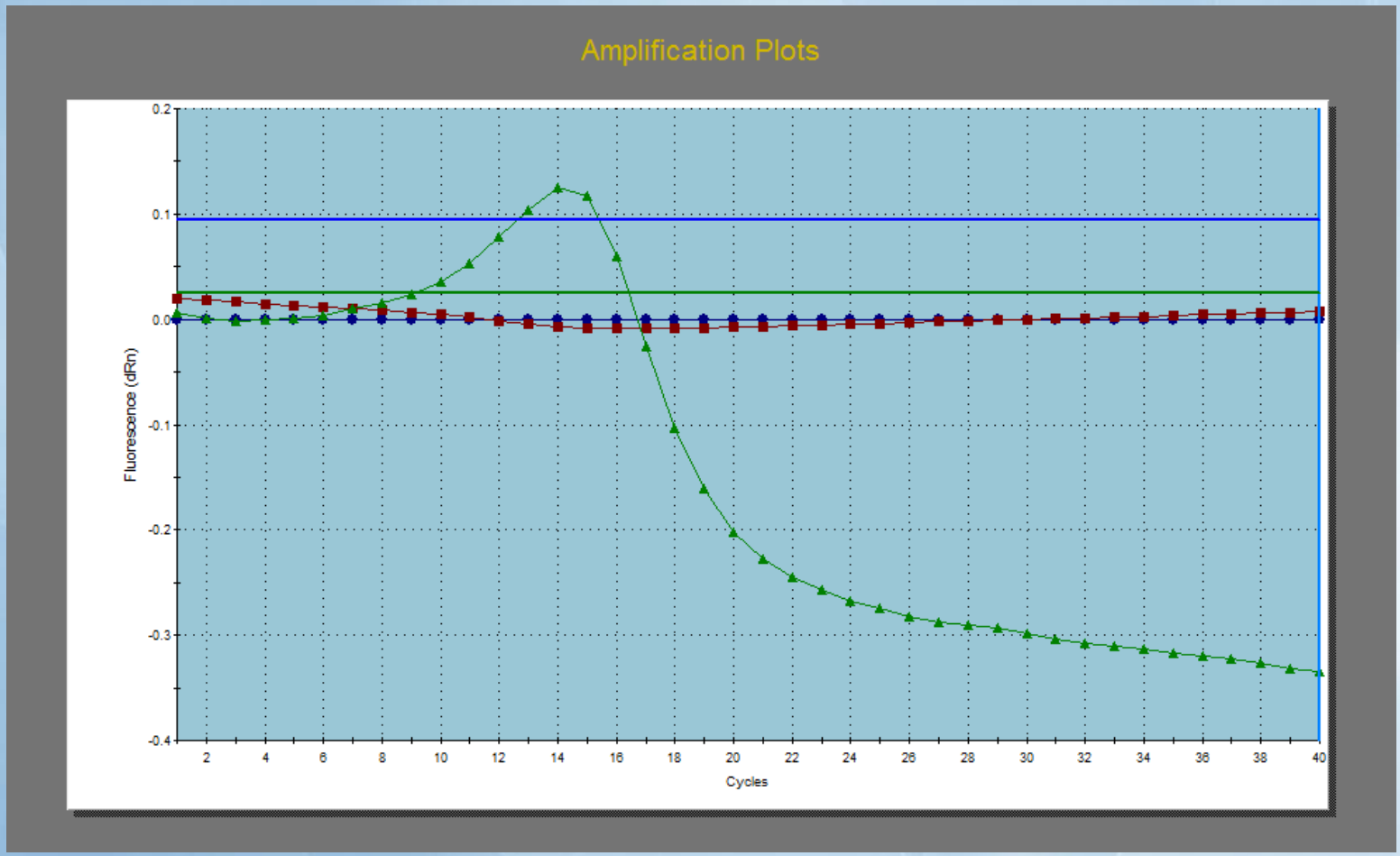
- Pipetting errors
- Incorrectly set baseline

Replicates ideally should not be more than 0.5 Ct apart



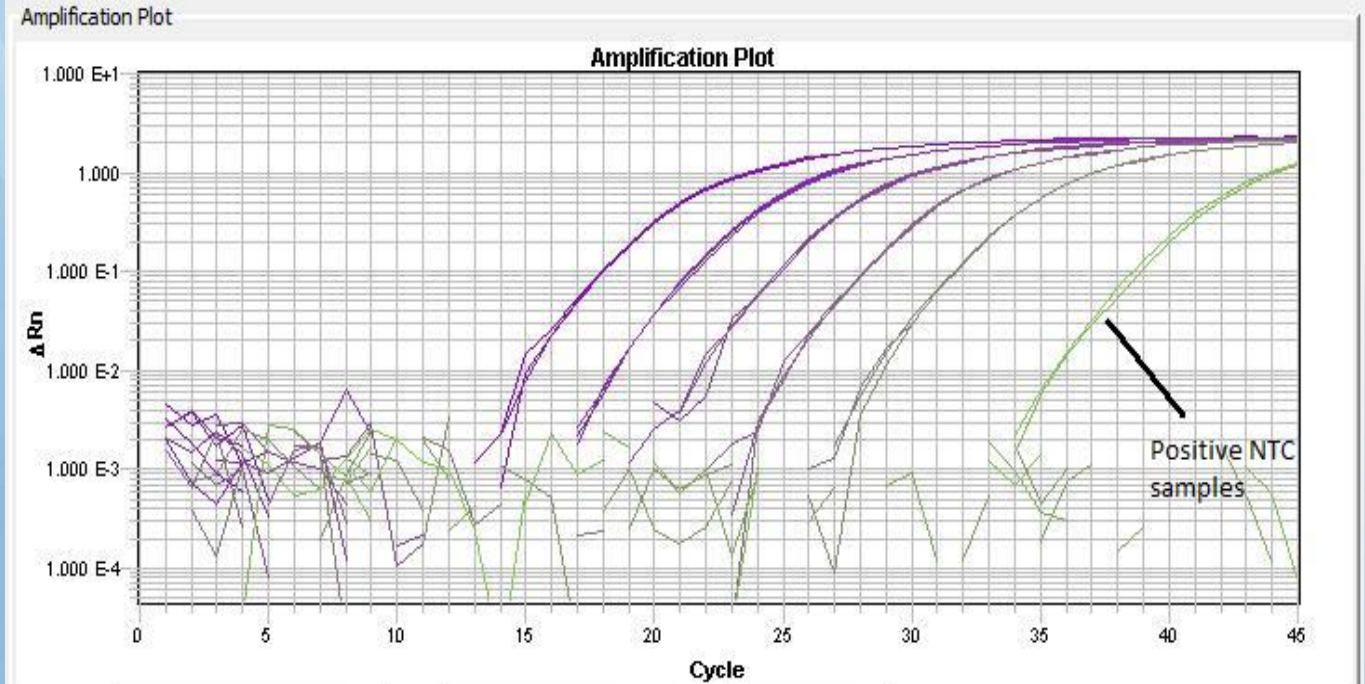


Unusual curves.....Sample evaporation



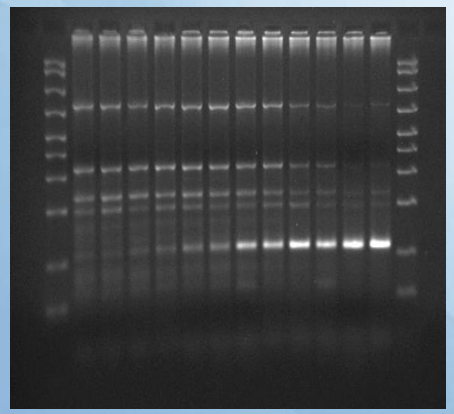
Unexpected Signal…

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



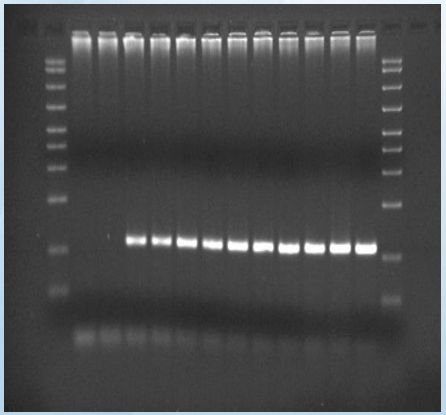
Cycle parameters

- **Annealing temperature**
  - starting approximately 5°C below calculated T_m
- **Extension time**
  - every 1kb of amplicon: 1 minute (Is it always true?)
- **Number of cycles**
  - 25-40 cycles



Not optimized

Tm effect



Well optimized