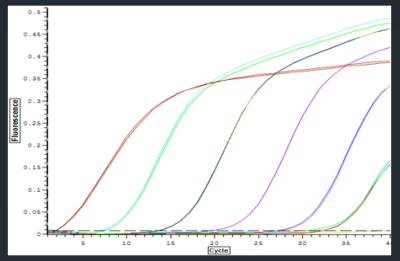

Real-Time PCR

PCR training course
June 13-17, 2016

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NATIONAL PUBLIC HEALTH CENTER
NATIONAL RESEARCH DIRECTORATE FOR RADIOBIOLOGY AND RADIOHYGIENE

Objectives

Today we'll talk about Real-Time PCR:

1. **What is real-time PCR used for?**
2. **How does real-time PCR work?**
3. **What chemicals and instruments are used to detect DNA?**
4. **What does real-time data look like?**
5. **How can we demonstrate real-time PCR in the lab?**

What is Real-Time PCR?

PCR, or the Polymerase Chain Reaction, is a process for the amplification of specific fragments of DNA.

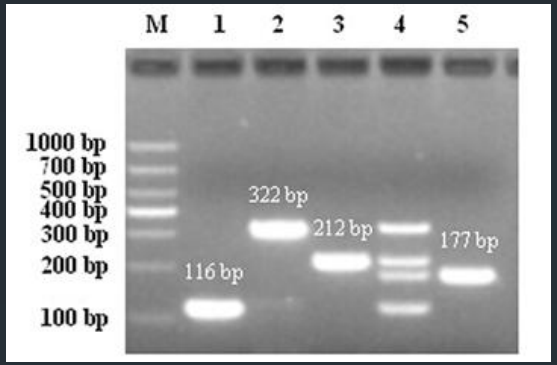
Real-Time PCR a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses.

As we will see, Real-Time PCR allows us to measure minute amounts of DNA sequences in a sample!

What is Real-Time PCR?

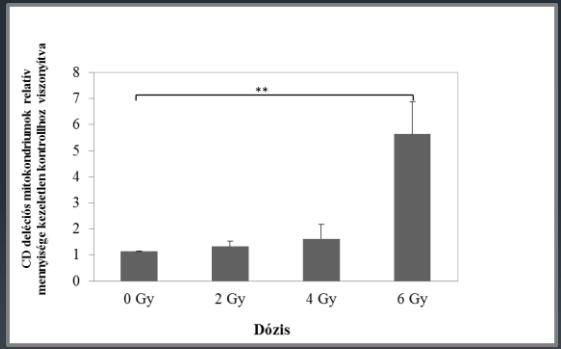
Conventional PCR

tells us “what”.



Real-Time PCR

tells us “how much”.



What is Real-Time PCR used for?

Real-Time PCR has become a cornerstone of molecular biology. Just some of the uses include:

- **Gene expression analysis**
  - Cancer and Drug research
- **Disease diagnosis and management**
  - Viral quantification
- **High resolution melting curve (HRM) analysis**
  - Identifying SNPs, novel mutations, and methylation patterns
- **Food testing**
  - Percent GMO food
- **Animal and plant breeding**
  - Gene copy number
- **Forensics**
  - Sample identification and quantification

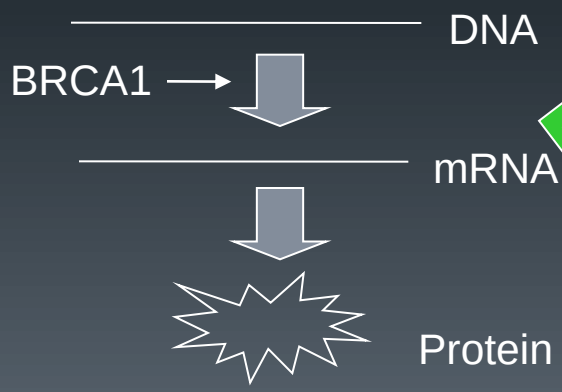
Real-Time PCR in Gene Expression Analysis

Example: BRCA1 Expression Profiling

BRCA1 is a gene involved in tumor suppression.

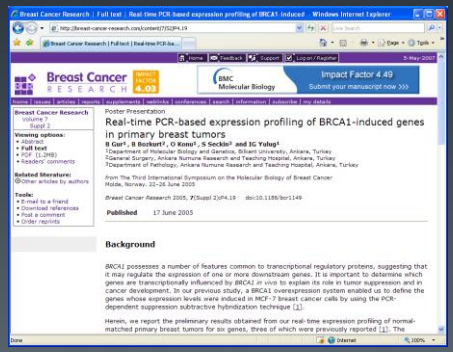
BRCA1 controls the expression of other genes.

In order to monitor level of expression of BRCA1, real-time PCR is used.



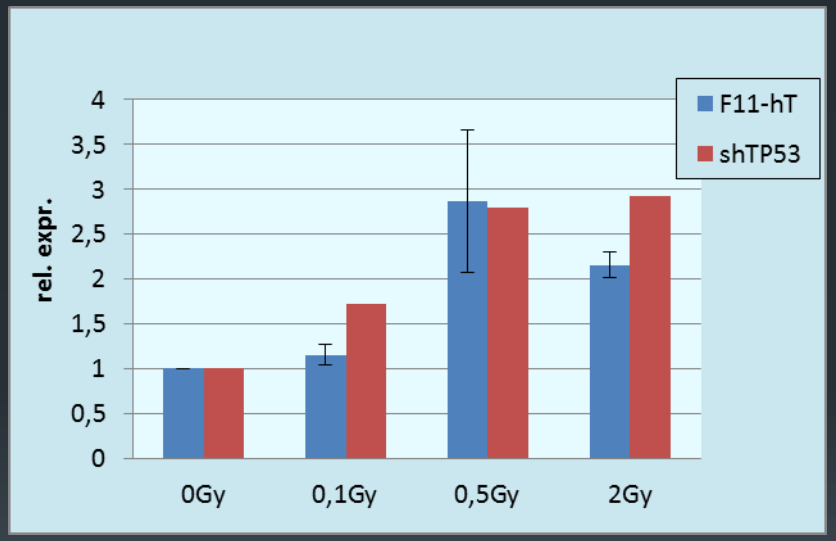
Real-Time PCR

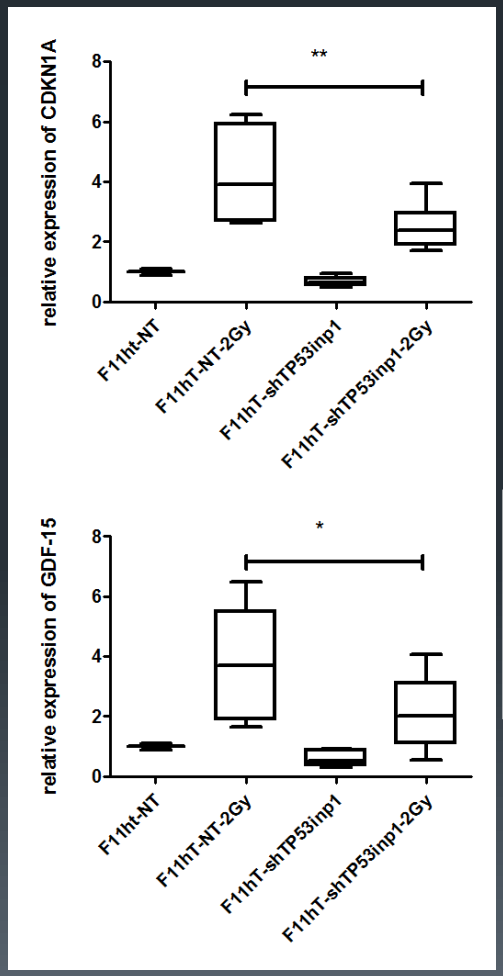
Determine gene expression and publish scientific paper!



Real-Time PCR in Radiobiology

Example: Determining low dose induced gene expression





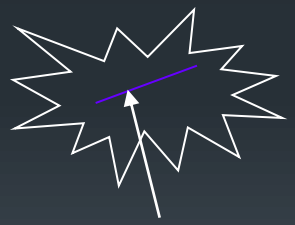
Determine gene expression and publish scientific paper!

Real-Time PCR in Disease Management

Example: HIV Treatment

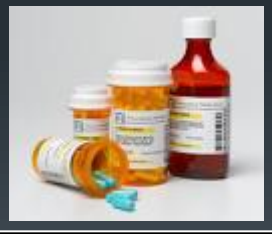
Drug treatment for HIV infection often depends on monitoring the "viral load".

Real-Time PCR allows for direct measurement of the amount of the virus RNA in the patient.



Viral RNA

Real-Time PCR



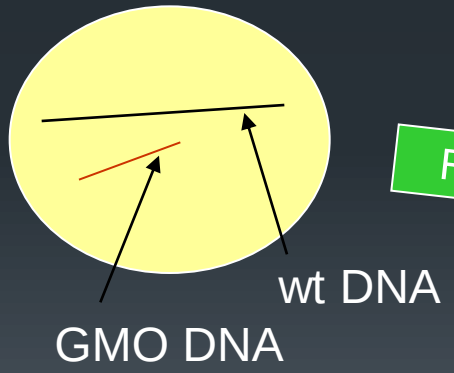
Measure amount of virus, adjust prescriptions.

Real-Time PCR in Food Testing

Example: Determining percentage of GMO food content

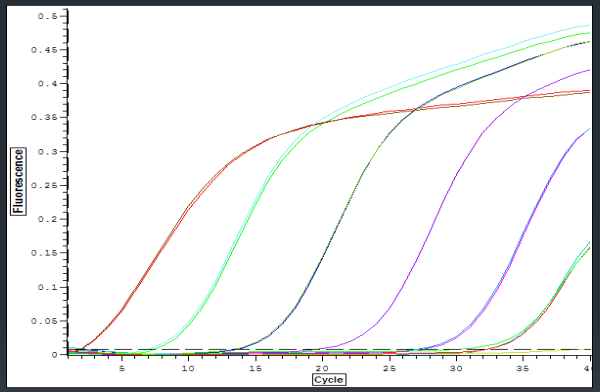
Determination of percent GMO food content important for import / export regulations.

Labs use Real-Time PCR to measure amount of transgenic versus wild-type DNA.



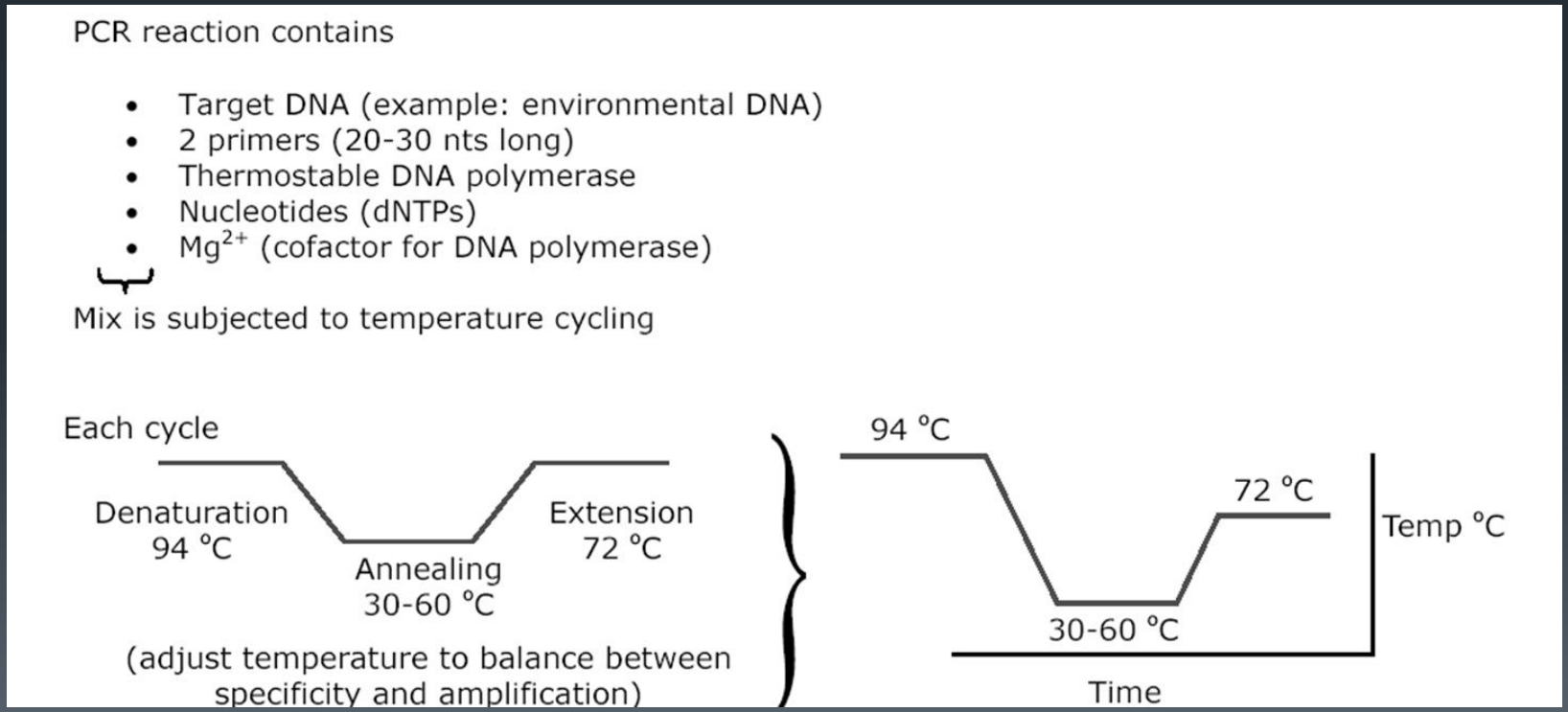
International shipments depend on results!

Part 2: How does Real-Time PCR work?

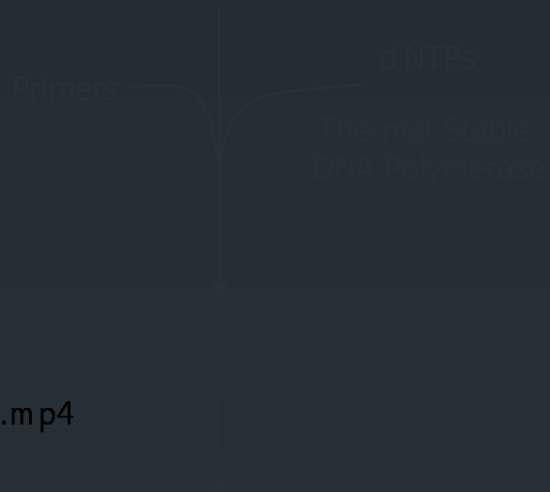


How does real-time PCR work?

To best understand what real-time PCR is, let's review how regular PCR works...



The Polymerase Chain Reaction How does PCR work??



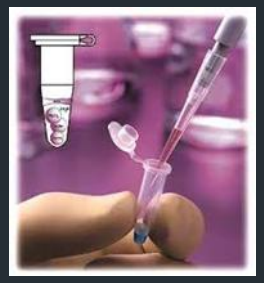
_polymerase_chain_reaction.mp4

How does Real-Time PCR work?

…So that's how traditional PCR is usually presented.

- **In order to understand real-time PCR, let's use a "thought experiment", and save all of the calculations and formulas until later…**
- **To understand real-time PCR, let's imagine ourselves in a PCR reaction tube at cycle number 25…**

Imagining Real-Time PCR



What's in our tube, at cycle number 25?

A soup of nucleotides, primers, template, amplicons *(the amplified DNA product)*, enzyme, etc.

1,000,000 copies of the amplicon right now.

Imagining Real-Time PCR

How did we get here?

What was it like last cycle, 24?

Almost exactly the same, except there were only 500,000 copies of the amplicon.

And the cycle before that, 23?

Almost the same, but only 250,000 copies of the amplicon.

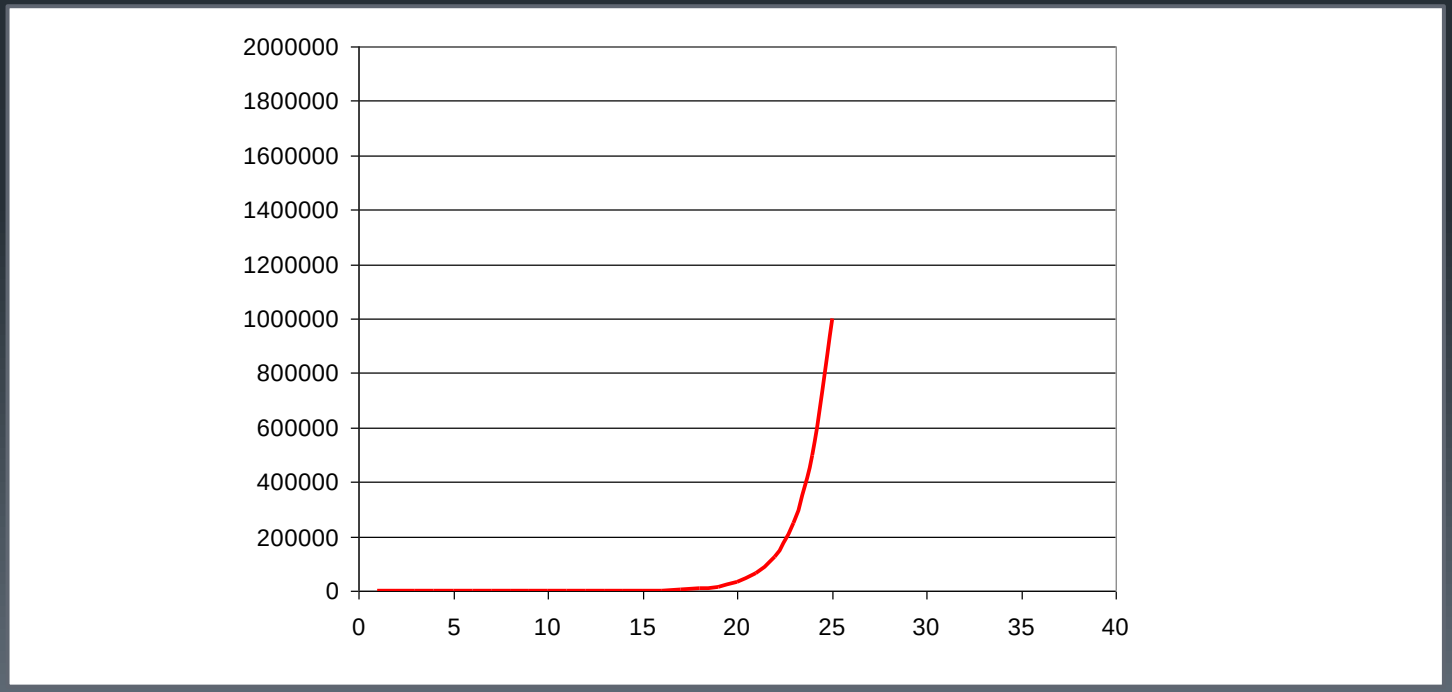
And what about cycle 22?

Not a whole lot different. 125,000 copies of the amplicon.

Imagining Real-Time PCR

How did we get here?

If we were to graph the amount of DNA in our tube, from the start until right now, at cycle 25, the graph would look like this:



Imagining Real-Time PCR

How did we get here?

- **So, right now we're at cycle 25 in a soup with 1,000,000 copies of the target.**
- **What's it going to be like after the next cycle, in cycle 26?**
- **Probably there will be 2,000,000 amplicons.**
- **And cycle 27?**
- **Maybe 4,000,000 amplicons.**

So where are we going?

Imagining Real-Time PCR

So where are we going?

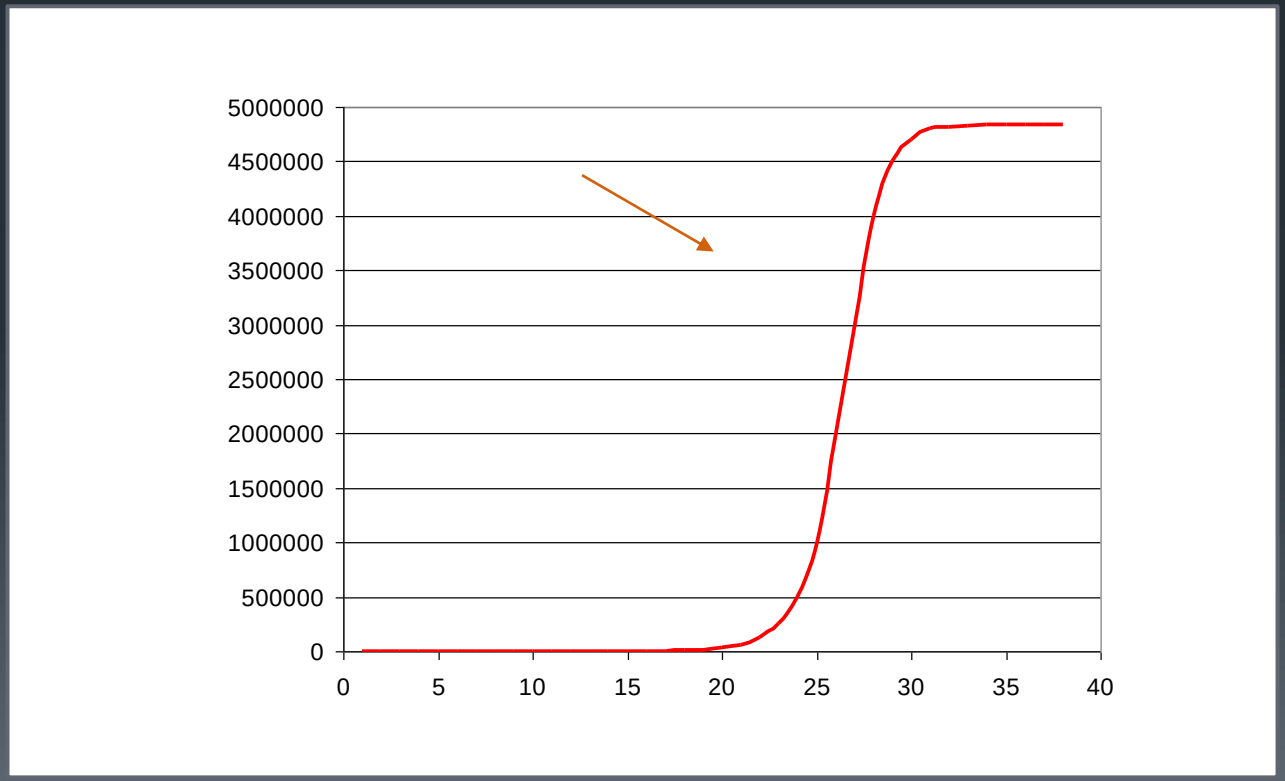
4,000,000 amplicons.

- **And at cycle 200?**
  - In theory, there would be 1,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000 amplicons…
  - Or 10^{35} tons of DNA… To put this in perspective, that would be equivalent to the weight of ten billion planets the size of Earth!!!!
  - A clump of DNA the size of ten billion planets won't quite fit in our PCR tube anymore!!!
  - Realistically, at the chain reaction progresses, it gets exponentially harder to find primers, and nucleotides. And the polymerase is wearing out.
  - So exponential growth does not go on forever!

Imagining Real-Time PCR

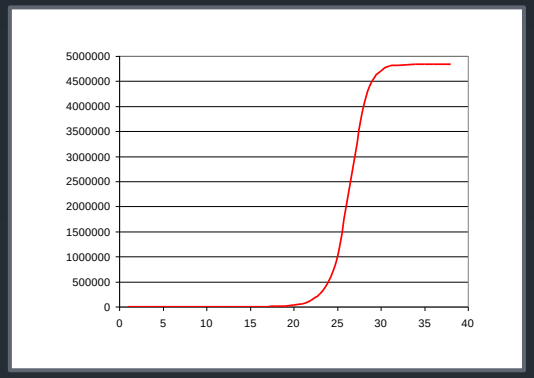
So where are we going?

If we plot the amount of DNA in our tube going forward from cycle 25, we see that it actually looks like this:



Measuring Quantities

How can all this be used to measure DNA quantities??



Let's imagine that you start with four times as much DNA as I do.

Picture our two tubes at cycle 25 and work backwards a few cycles.

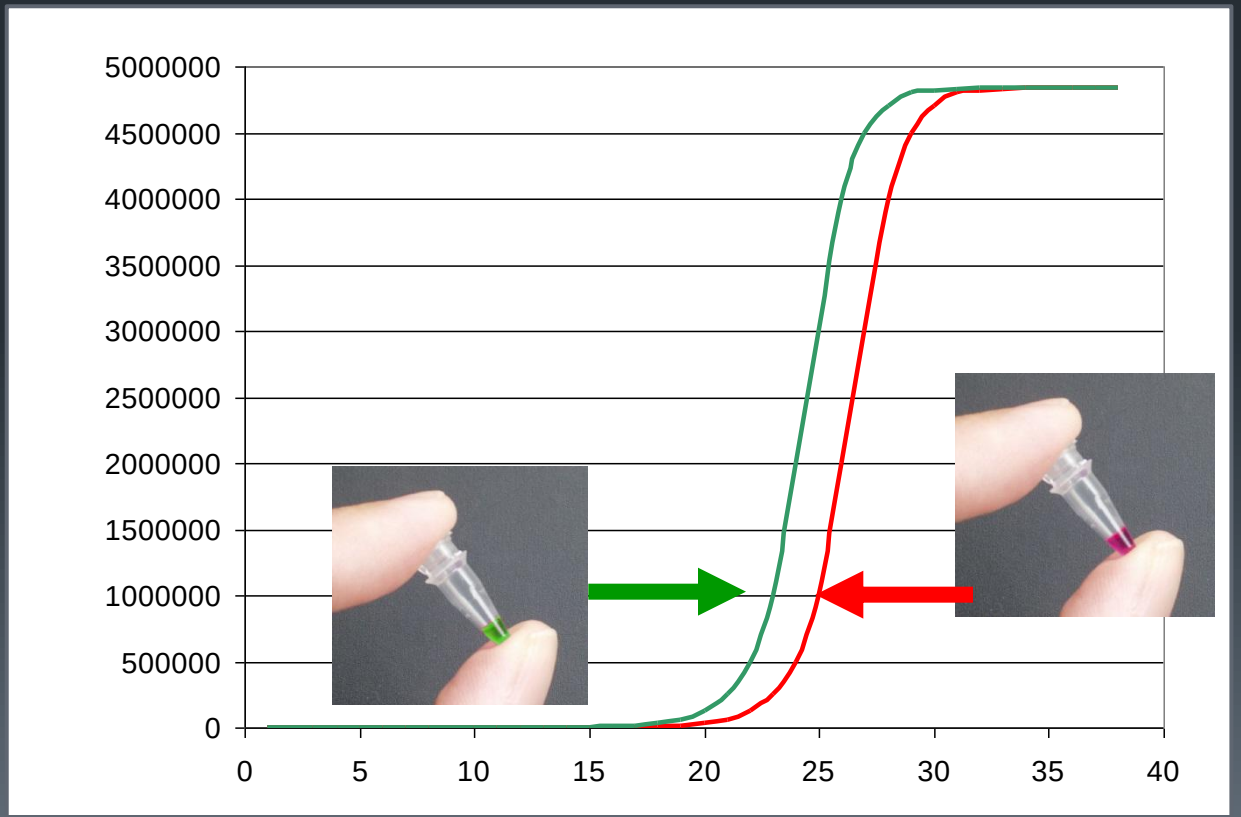
Cycle 25 →

Cycle	Me	You
25	1,000,000	4,000,000
24	500,000	2,000,000
23	250,000	1,000,000

Measuring Quantities

So, if YOU started with FOUR times as much DNA template as I did…

…Then you'd reach 1,000,000 copies exactly TWO cycles earlier than I would!



Measuring Quantities

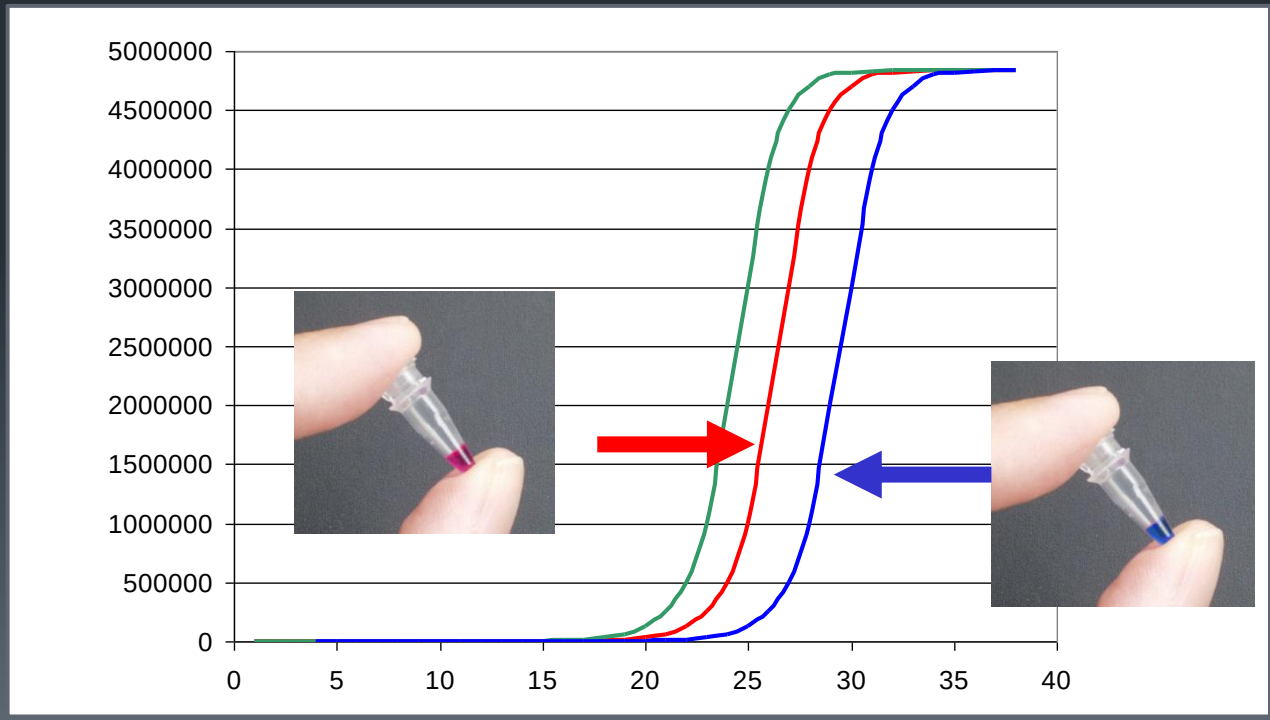
What if YOU started with EIGHT times LESS DNA template than I did?

Cycle 25 →

Cycle	Me	You
25	1,000,000	125,000
26	2,000,000	250,000
27	4,000,000	500,000
28	8,000,000	1,000,000

Measuring Quantities

- **What if YOU started with EIGHT times LESS DNA template than I did?**
- **You'd only have 125,000 copies right now at cycle 25…**
- **And you'd reach 1,000,000 copies exactly THREE cycles later than I would!**

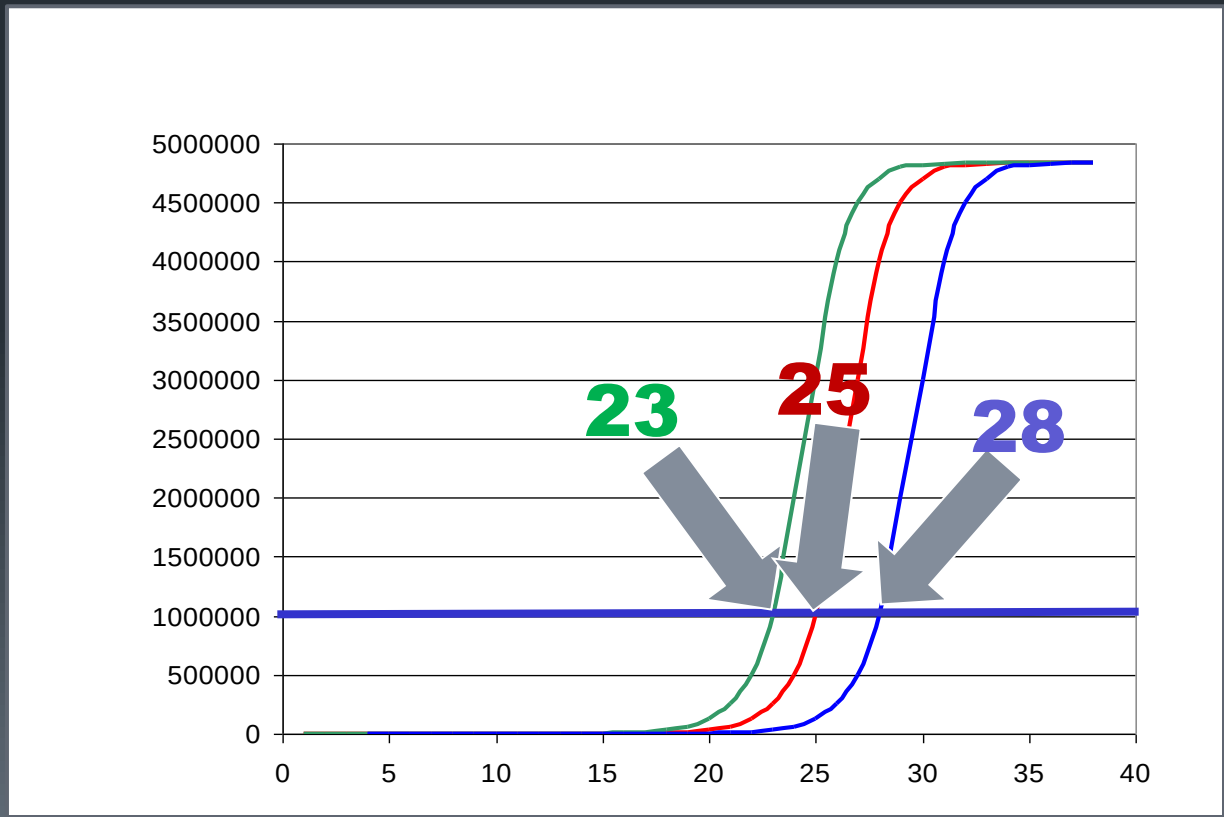


Measuring Quantities

We can easily see that the left-right shift in the curves is related to the starting quantity of DNA!

Ct (threshold cycle) values identify the curve positions, based on where they cross a threshold.

DNA Quantity and Ct value are related as: Quantity ~ 2^{Ct}



Measuring Quantities

Definitions:

Baseline

The baseline of the real-time PCR reaction refers to the signal level during the initial cycles of PCR, usually cycles 3 to 15, in which there is little change in fluorescent signal.

Threshold

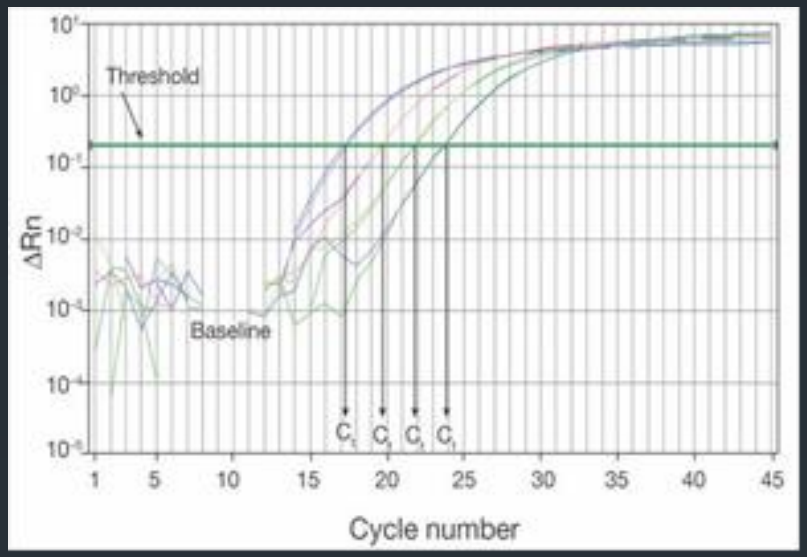
The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal

Threshold cycle

The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold

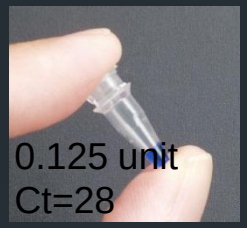
DNA quantity = ΔRn:

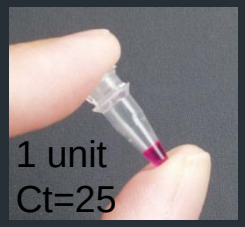
The magnitude of the fluorescence signal generated during the PCR at each time point.

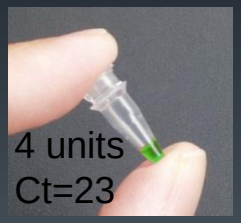


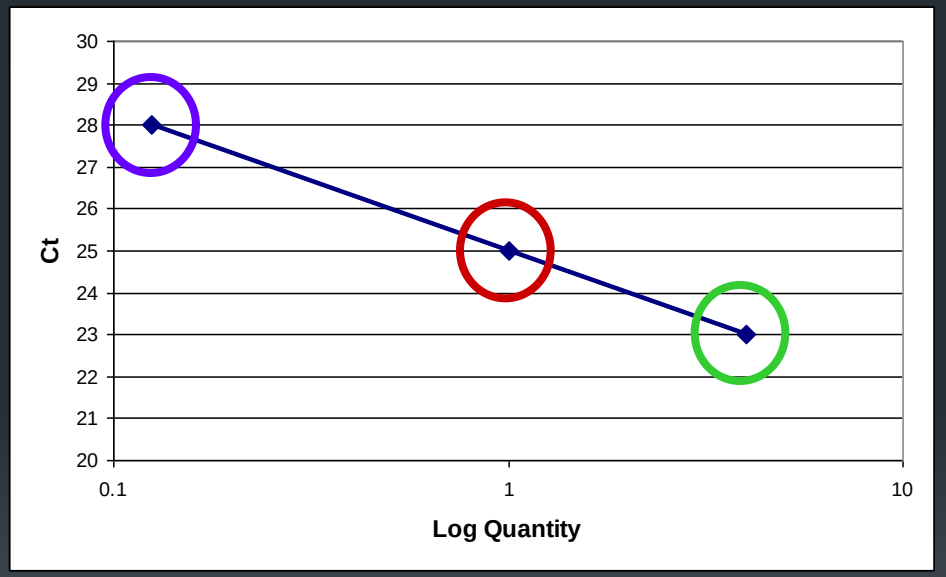
Measuring Quantities

We can plot the Ct value versus the Log Quantity on a graph…





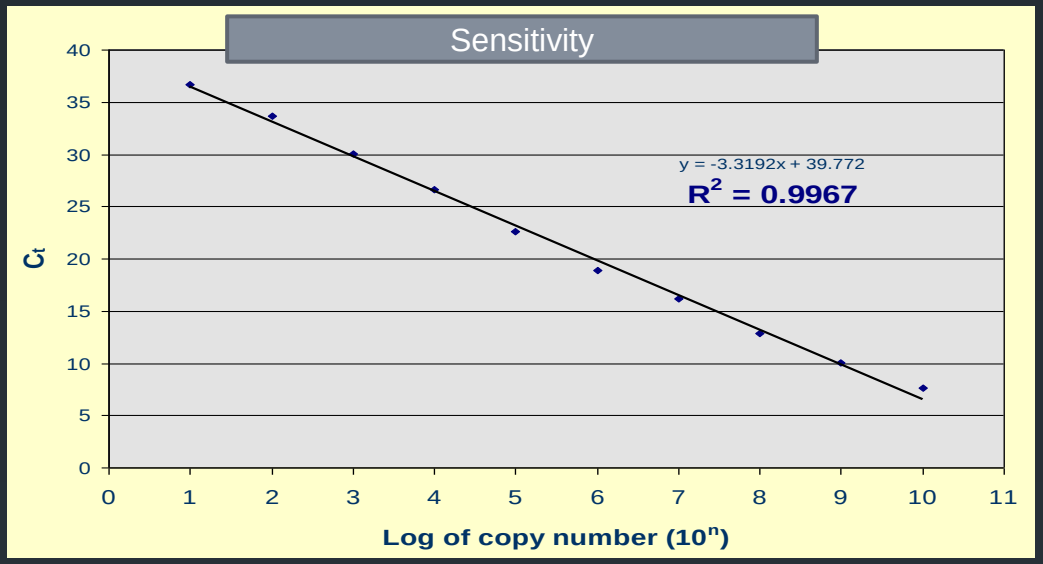




… and calculate the quantity of any 'unknown' right off of the line!!

Real-Time PCR Sensitivity

How sensitive is Real-Time PCR?

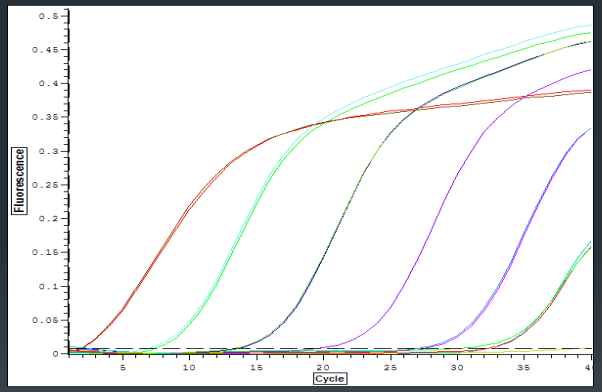


Ultimately, even a single copy can be measured!
In reality, typically about 100 copies is around the minimum amount.

One hundred copies of a 200-bp gene is:

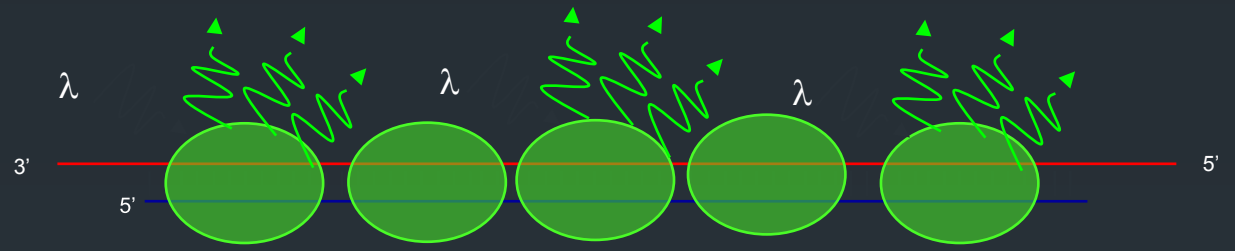
- **twenty attograms (2×10^{-17} g) of DNA!**
- **this is just 2/100ths of a *microliter* of blood!**

Part 3:
How do we detect and measure DNA?



How do We Measure DNA in a PCR Reaction?

We use reagents that fluoresce in the presence of amplified DNA!

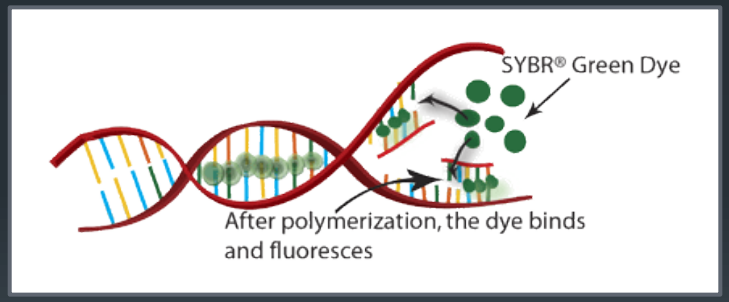


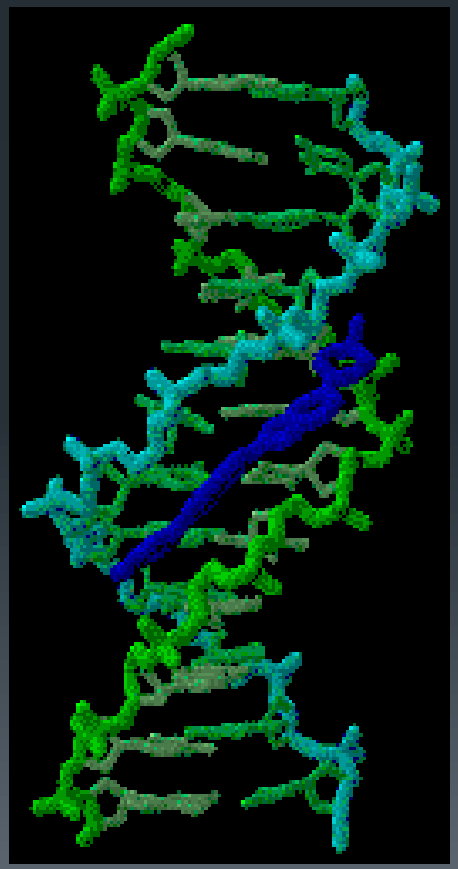
Measuring DNA: SYBR Green I

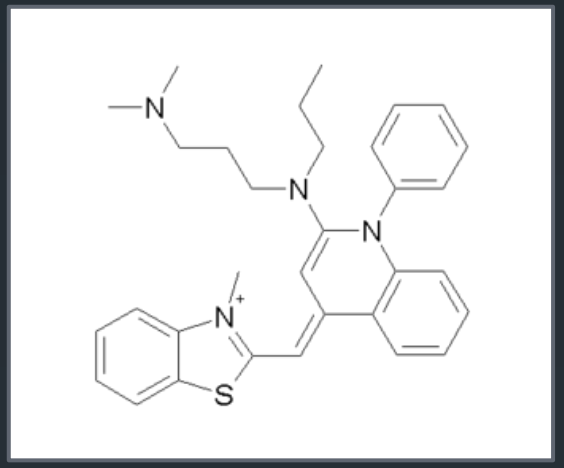
SYBR Green I

+ = Bright fluorescence!

+ = Low toxicity!





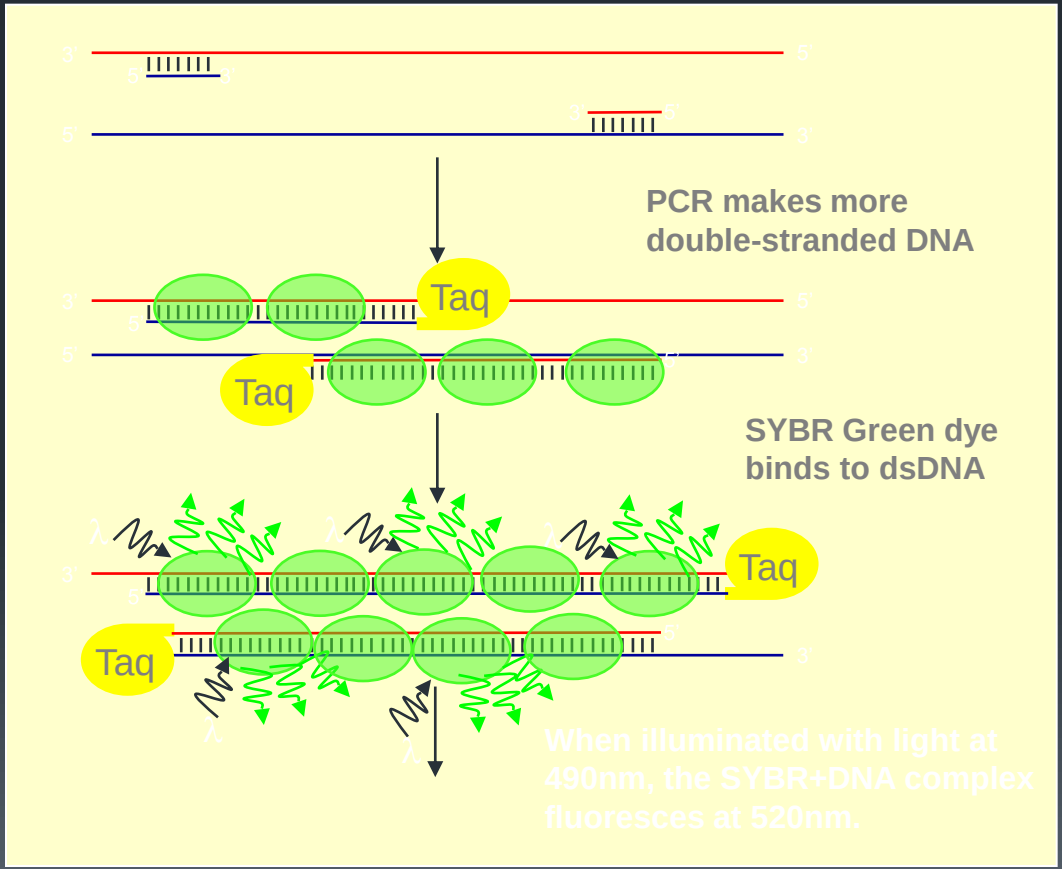


Fluorescent Dyes in PCR

Intercalating Dyes

SYBR® Green I dye is a fluorescent DNA-binding dye that binds to the minor groove of any double-stranded DNA.
Excitation of DNA-bound SYBR® Green dye produces a much stronger fluorescent signal compared to unbound dye.

SYBR Green in Action!

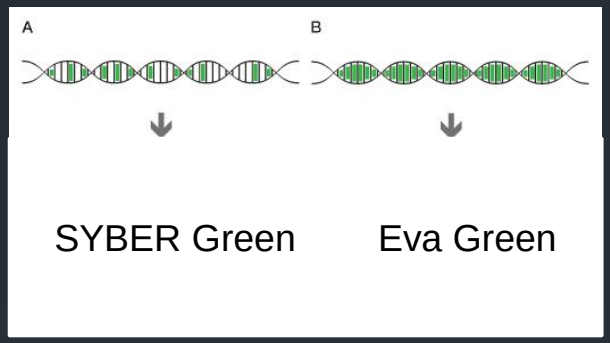


Fluorescent Dyes in PCR

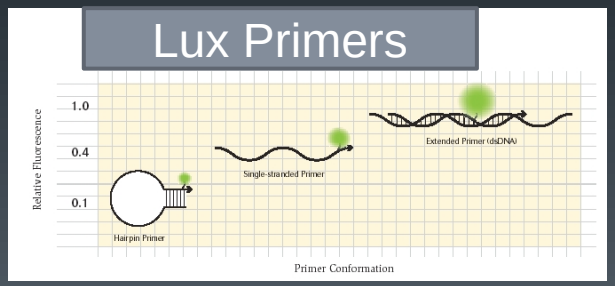
Other Options

Even more ways to detect PCR products:

EVAGREEN



Lux Primers



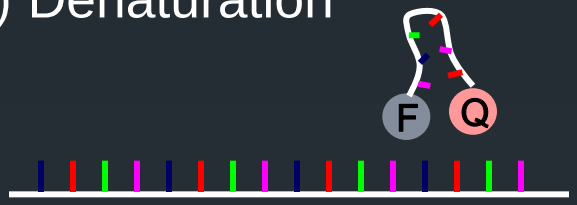
- Other Intercalating Dyes
  - Eva Green
- Probes
  - TaqMan Probes
- Primer/Probe Combinations
  - Scorpions
  - LUX Primers

TaqMan Probes

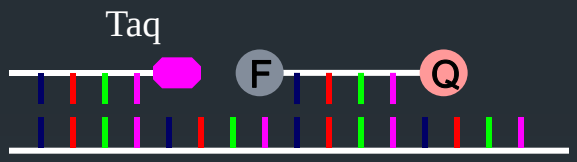
Even more ways to detect PCR products:

TaqMan® probe

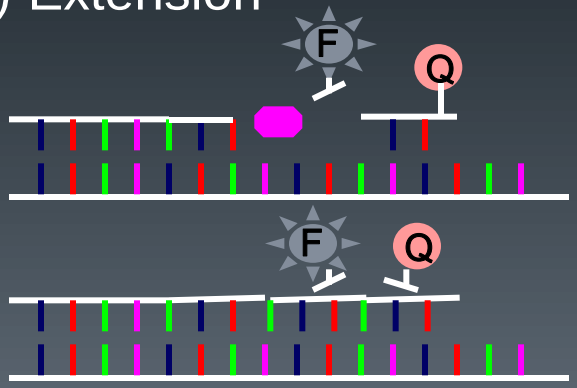
1) Denaturation



2) Annealing



3) Extension



1) Fluorescent reporter dye at the 5’ end is quenched by fluorescent quencher dye at the 3’ end.

2) When amplification occurs the TaqMan probe is degraded due to the 5'-->3' exonuclease activity of *Taq* DNA polymerase, thereby separating the quencher from the reporter during extension.

3) The TaqMan assay accumulates a fluorescence signal.

TaqMan Probes

Even more ways to detect PCR products:

TaqMan® probe

1. Advantages:

- Increased specificity
- Better capacity of multiplexing

2. Disadvantages:

- Little expensive (dual-labeled probe)
- Less effective and less flexible compared to other techniques in the real-time detection of specific mutation
- Require the design of probes

What Type of Instruments are used with Real-Time PCR?

What about the Instruments?

Real-time PCR systems consist of THREE main components:

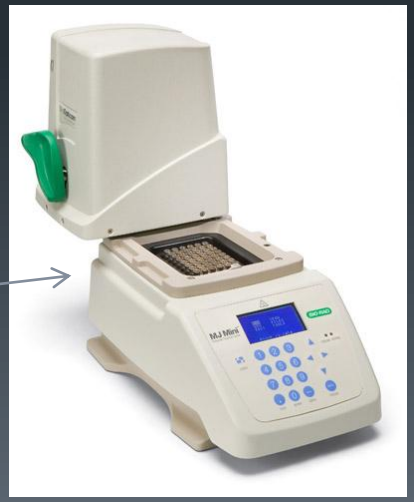
1. **Thermal Cycler (PCR machine)**
2. **Optical Module (to detect fluorescence in the tubes or plate during the run)**
3. **Computer (to translate the fluorescence data into meaningful results).**

What Type of Instruments are used with Real-Time PCR?

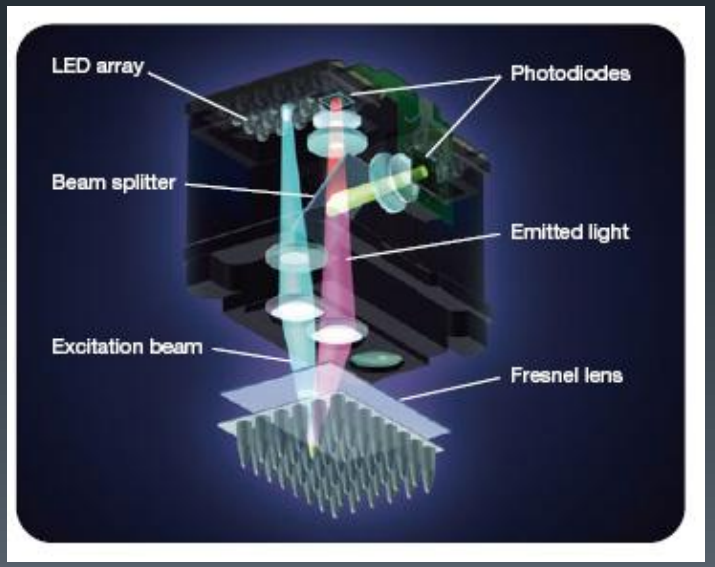
A good example is the MiniOpticon real-time instrument.

Optical Module

Thermal Cycler Base



plate



What Type of Instruments are used with Real-Time PCR?

One more example is the Rotor Gene

Optical Module
Thermal Cycler Base

Tubes

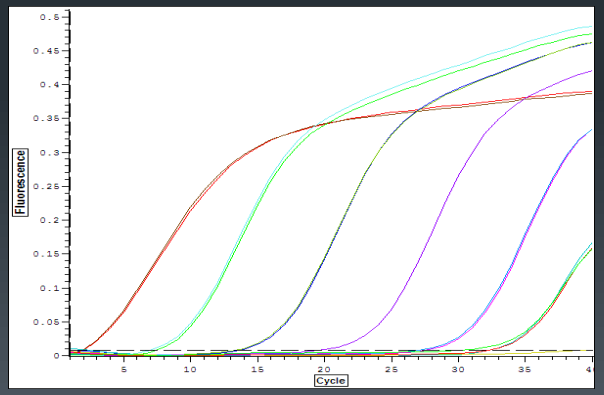
What Type of Software is used with Real-Time PCR?

The computer, running real-time software, converts the fluorescent signals in each well to meaningful data.

Workflow:

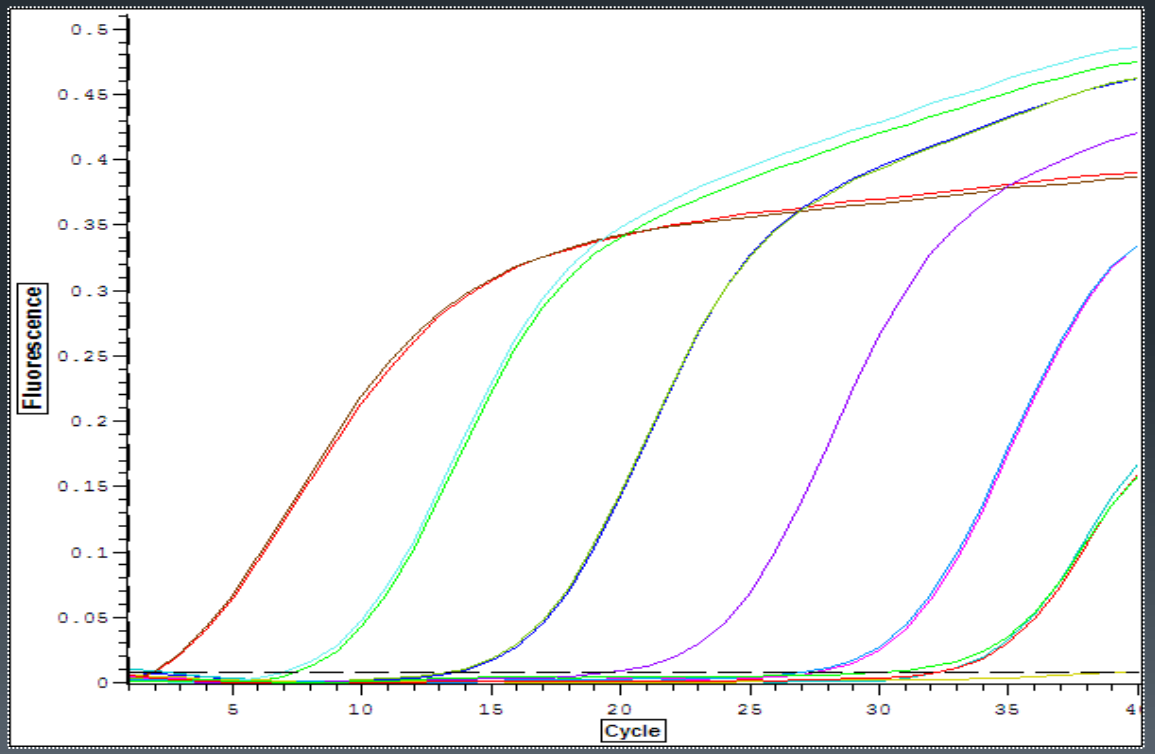
1. **Set up PCR protocol.**
2. **Set up plate/ tube layout.**
3. **Collect data.**
4. **Analyze data.**

Part 4:
What does actual real-time data look like, and what are melt curves?



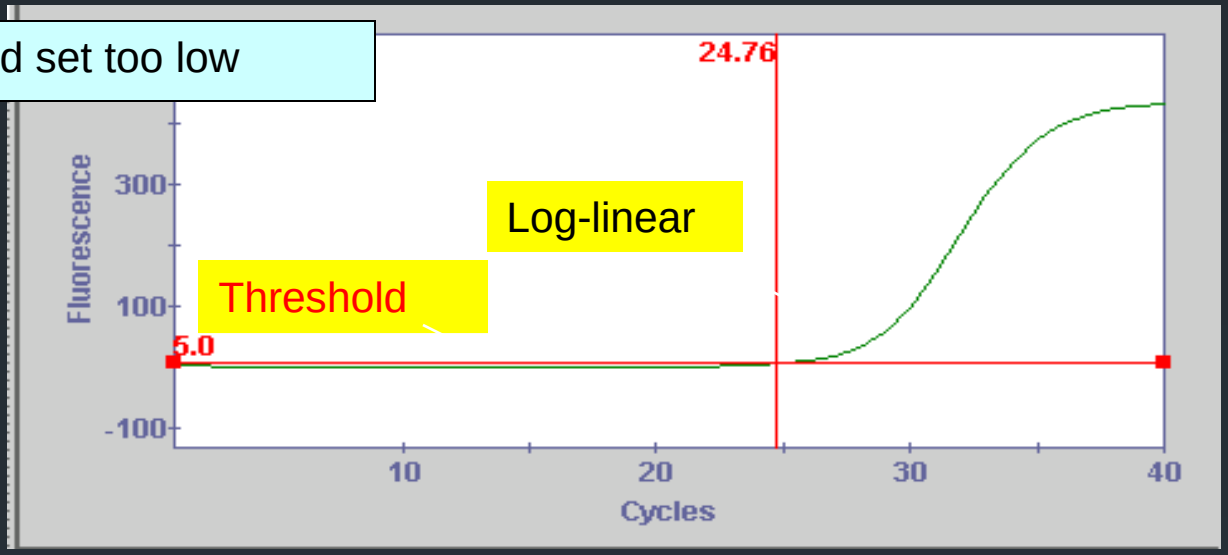
Real-Time PCR Actual Data

- **This is some actual data from a recent real-time PCR run.**
- **Data like this can easily be generated by preparing different amount of DNA.**

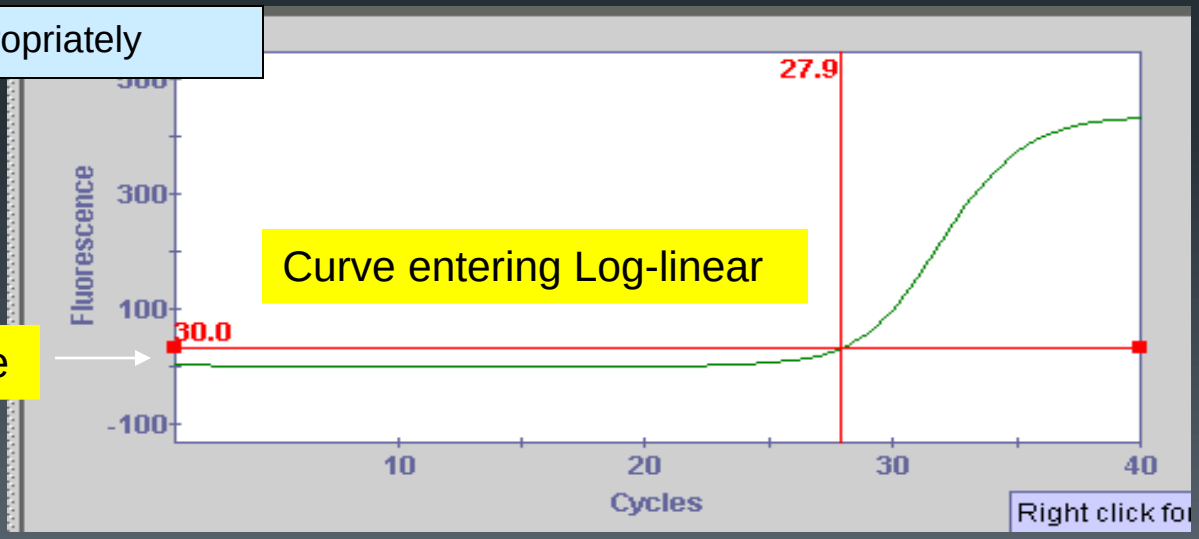


Evaluation of Growth Curve

Threshold set too low



Threshold set appropriately

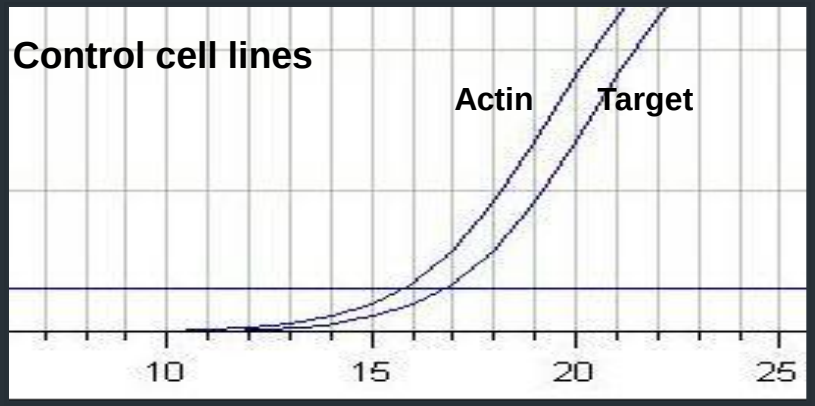


Results interpretation

- Following run evaluation
  - Valid positive and negative control
  - Specimen has a normal curve
- Record the cycle threshold (Ct) values
  - If a sample has no cycle threshold values (0.00) it is negative
- Determine if there are any suspect samples
  - Weak positives- Ct values >35

Real-Time PCR
Relative Quantification

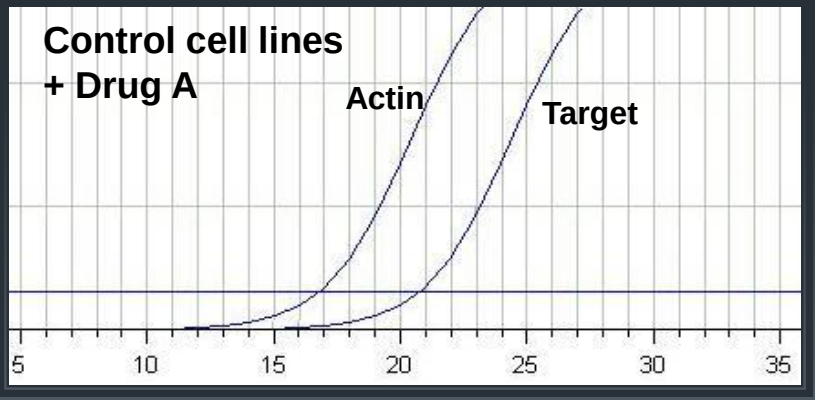
Relative Quantification (Drug-induced gene expression)



Actin : 16 Ct

Target : 17 Ct

=> Delta Ct = 1 Ct



Actin A : 17 Ct

Target B : 21 Ct

=> Delta Ct = 4 Ct

DeltaDelta CT= 4-1=3

Relative expression 2^{-3}= 0.125

Drug A treatment decreased target X gene expression

Real-Time PCR
Relative Quantification

Endogenous control (housekeeping gene)

- By using an endogenous control as an active reference, quantification of an gene of interest target can be normalized for differences in the amount of total DNA added to each reaction.
- Regardless of the gene that is chosen to act as the endogenous control, that gene must be tested under all of one's experimental conditions, to ensure that there is consistent expression of the control gene under all conditions.

Real-Time PCR
Relative Quantification

Endogenous control (housekeeping gene)

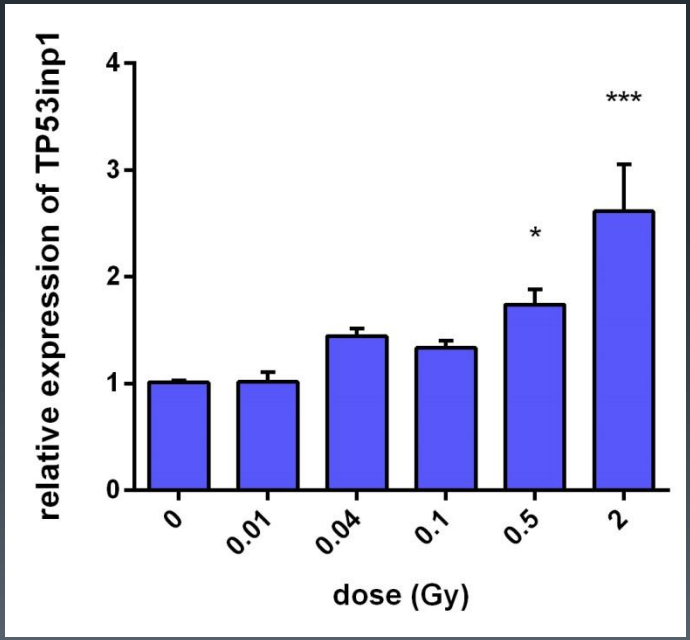
Common endogenous normalizers in real-time PCR include:

- β-actin (ACTB): cytoskeletal gene
- 18S ribosomal RNA (rRNA): ribosomal subunit
- Cyclophilin A (PPIA): serine-threonine phosphatase inhibitor
- Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): glycolysis pathway
- β-2-microglobulin (B2M): major histocompatibility complex
- β-glucuronidase (GUSB): exoglycosidase in lysosomes
- Hypoxanthine ribosyltransferase (HPRT1): purine salvage pathway

Comparative C_T ($\Delta\Delta$ C_T) Method

- C_T (target gene, control) – C_T (endog. refer. gene, control) = $\Delta C_{T,cont}$ (control tissue)
- C_T (target gene, exp.) – C_T (endog. refer. gene, exp.) = $\Delta C_{T,exp}$ (experimental tissue)
- $\Delta C_{T,exp}$ - $\Delta C_{T,cont}$ = $\Delta\Delta C_T$

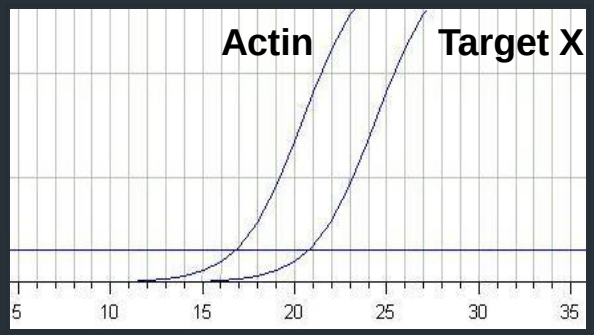
Target gene exp = $2^{-(\Delta\Delta C_T)}$



Real-Time PCR
Relative Quantification

Relative Quantification (Tissue specific gene expression)

- **Brain tissue**



Actin : 17 Ct

Target : 21 Ct

=> Delta Ct = 4 Ct

- **Liver tissue**

Actin : 22 Ct

Target : 26 Ct

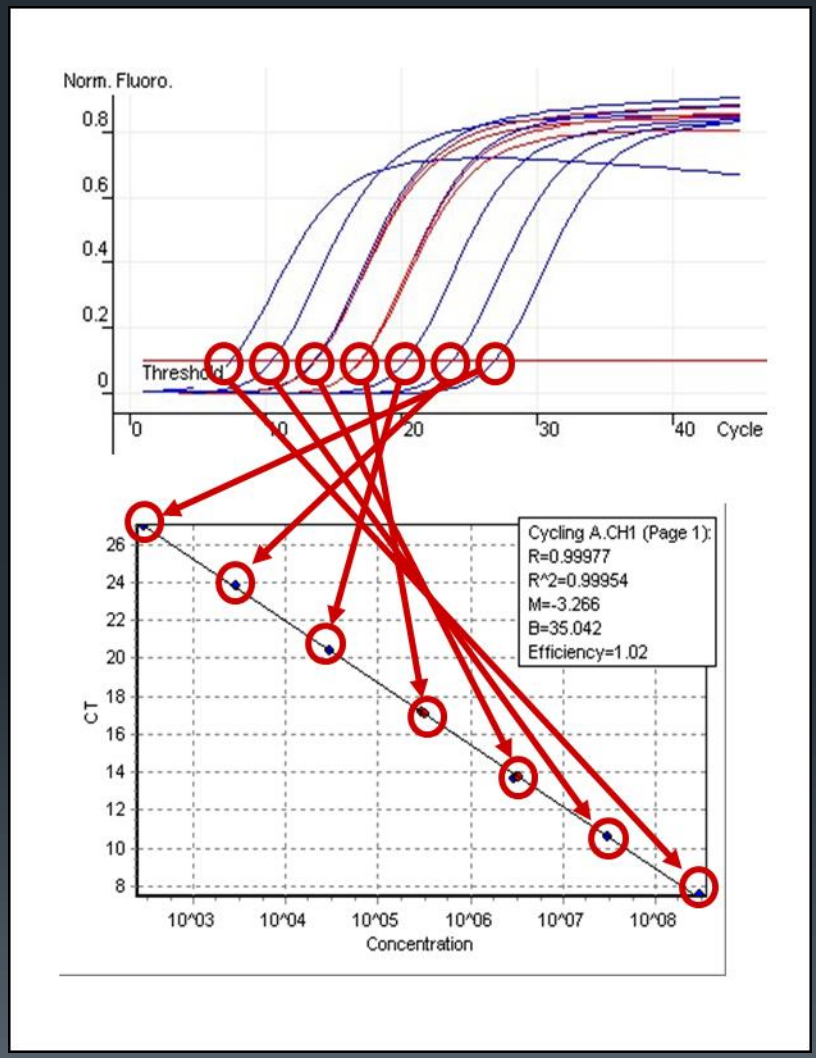
=> Delta Ct = 4 Ct

Target gene expression level between brain and liver tissue is same

Real-Time PCR
Absolute Quantification

Absolute quantification describes a real-time PCR experiment in which samples of known quantity are serially diluted and then amplified to generate a standard curve.

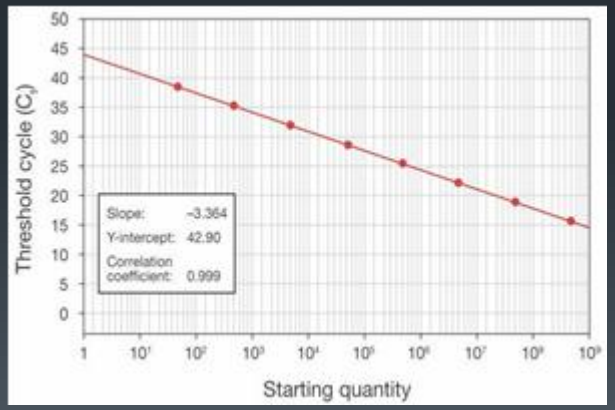
Unknown samples are then quantified by comparison with this curve.



Real-Time PCR
Absolute Quantification

Standard curve

A dilution series of known template concentrations can be used to establish a standard curve for determining the initial starting amount of the target template in experimental samples or for assessing the reaction efficiency.
The log of each known concentration in the dilution series (x-axis) is plotted against the Ct value for that concentration (y-axis).



Example of a standard curve of real-time PCR data. A standard curve shows threshold cycle (Ct) on the y-axis and the starting quantity of RNA or DNA target on the x-axis. Slope, y-intercept, and correlation coefficient values are used to provide information about the performance of the reaction.

Real-Time PCR
Absolute Quantification

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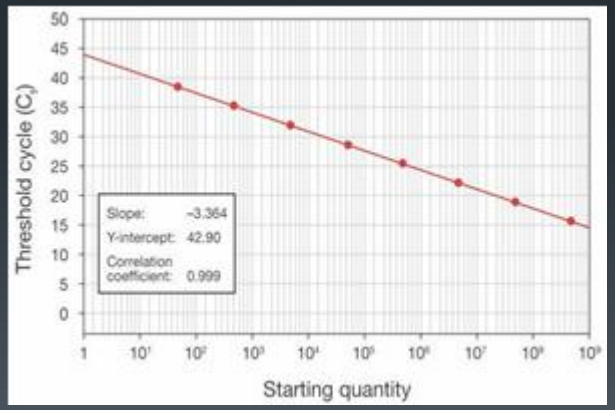


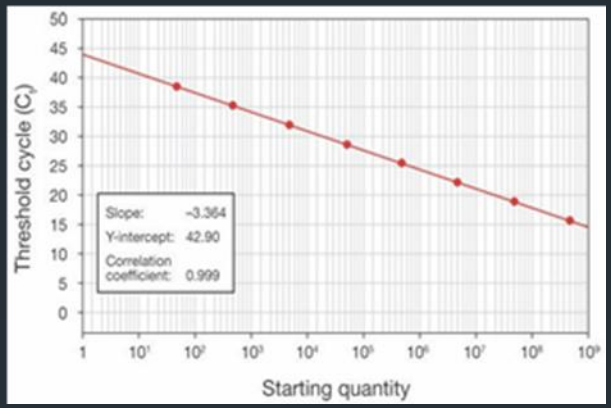
Example of a standard curve of real-time PCR data. A standard curve shows threshold cycle (Ct) on the y-axis and the starting quantity of RNA or DNA target on the x-axis. Slope, y-intercept, and correlation coefficient values are used to provide information about the performance of the reaction.

Real-Time PCR
Absolute Quantification

Definitions:

Slope

The slope of the log-linear phase of the amplification reaction is a measure of reaction efficiency. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of –3.32



Y-intercept

The y-intercept corresponds to the theoretical limit of detection of the reaction

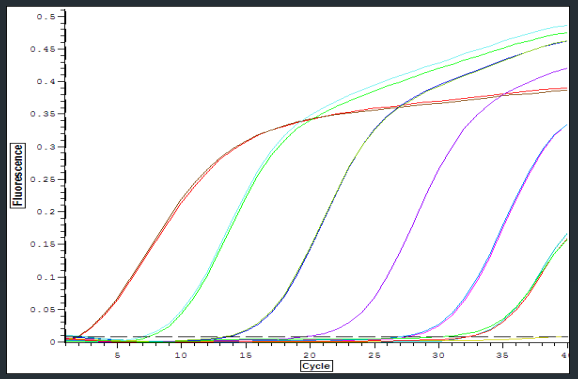
Correlation coefficient R^2

The correlation coefficient is a measure of how well the data fit the standard curve. The R^2 value reflects the linearity of the standard curve. Ideally, R^2 = 1, although 0.999 is generally the maximum value.

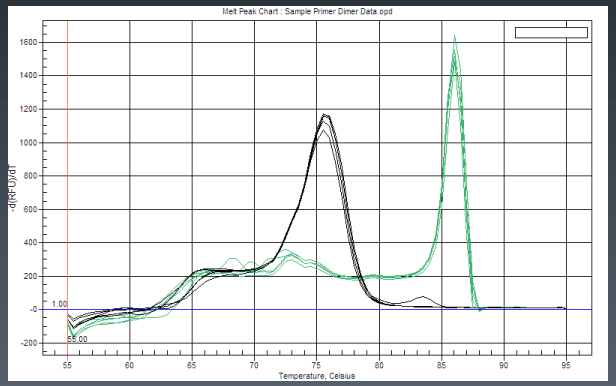
Melt Curve Analysis

Real-Time PCR Melt Curves

- **The fluorescence data collected during PCR tells us “how much” …**



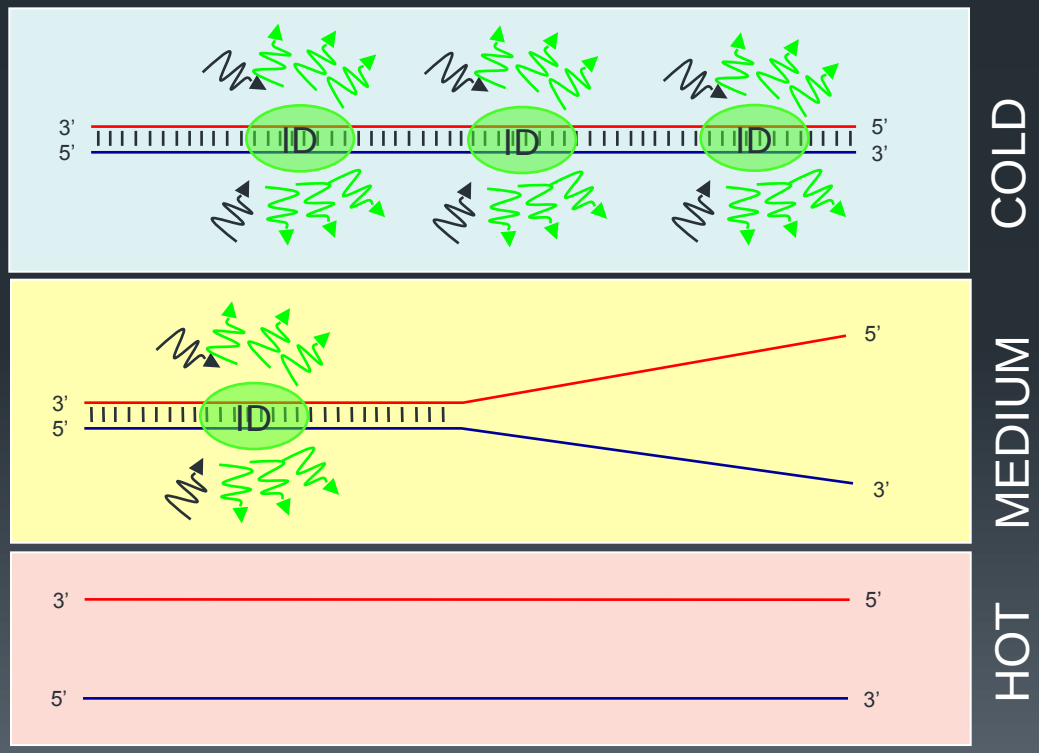
…. but there is another type of analysis we can do that tells us “what”!



Melt Curves Basics

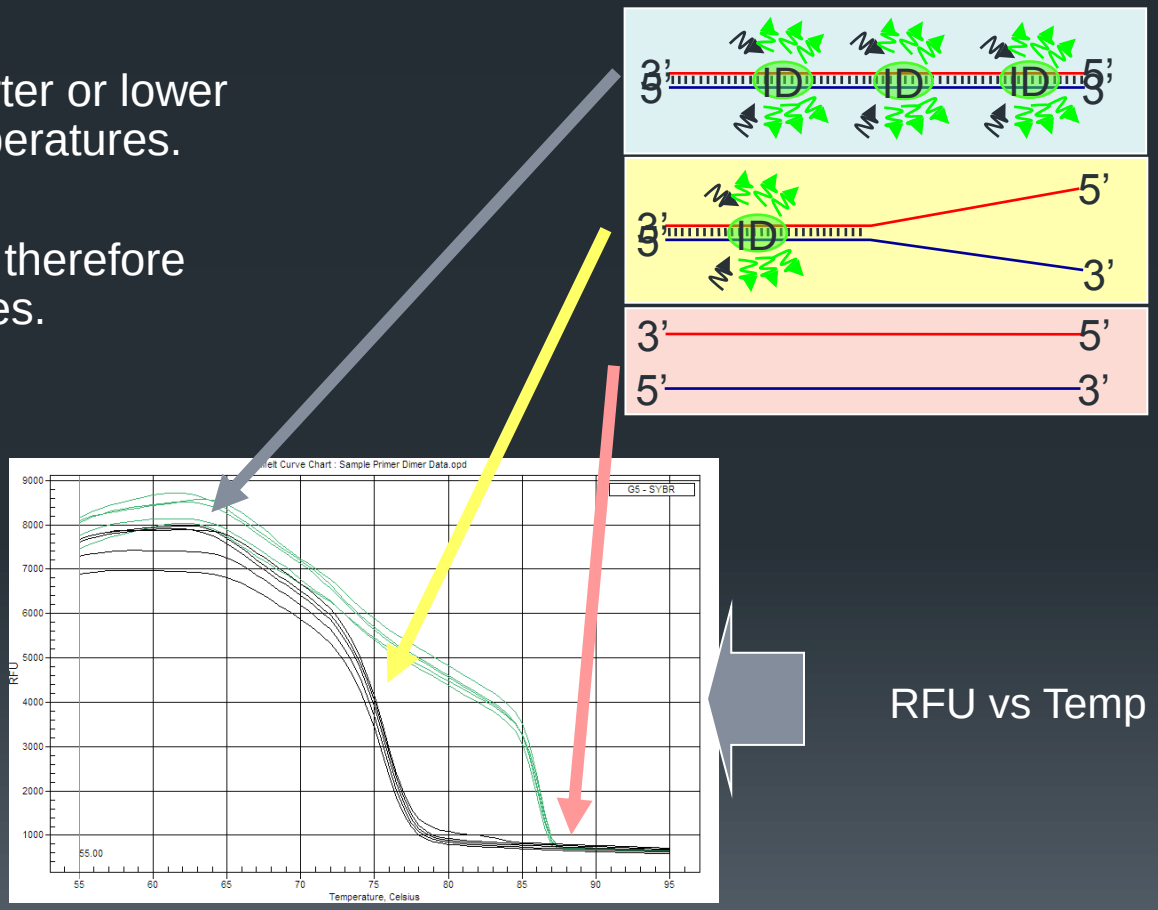
Melt curves can tell us what products are in a reaction.

- The principle of melt curves is that as DNA melts (becomes single stranded), DNA-binding dyes will no longer bind and fluoresce.



Melt Curves Basics

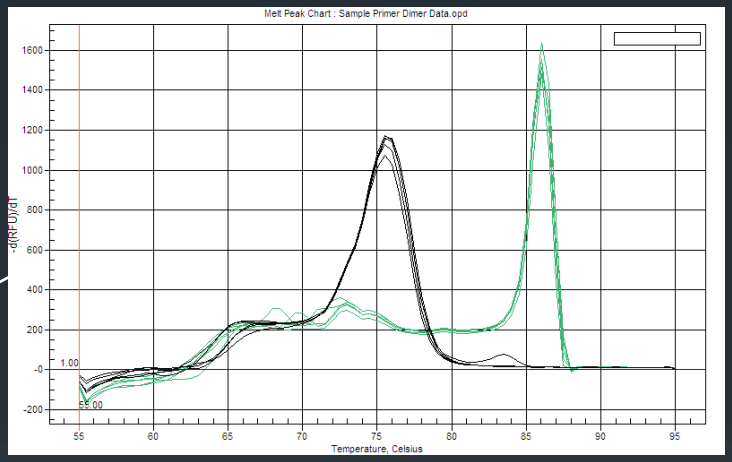
- Melt curves can tell us what products are in a reaction.
- PCR products that are shorter or lower G+C will melt at lower temperatures.
- Different PCR products will therefore have different shaped curves.



RFU vs Temp

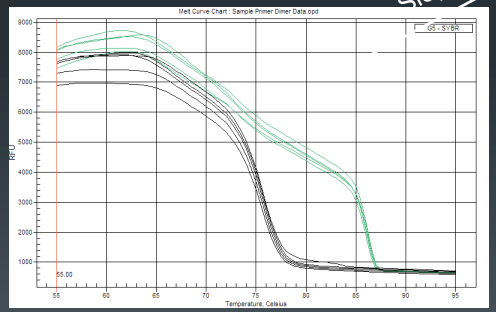
Melt Curves
Typical Data

- 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.



dRFU/dT

Slope vs. Temp



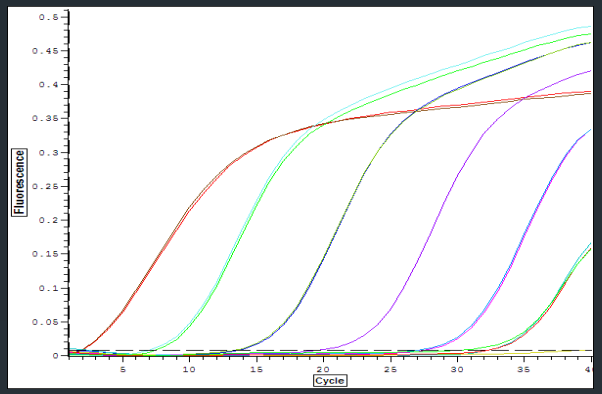
Different DNA sequences will “melt” at different temperatures! Talk about base-pairing, secondary structure, energy levels, etc!

Conclusions

We've covered the following topics today:

1. **What is real-time PCR used for?**
2. **How does real-time PCR work?**
3. **What chemicals and instruments are used to detect DNA?**
4. **What does real-time data look like?**

Part 5:
How do we optimize Real-Time PCR and troubleshoot problems?



Optimization

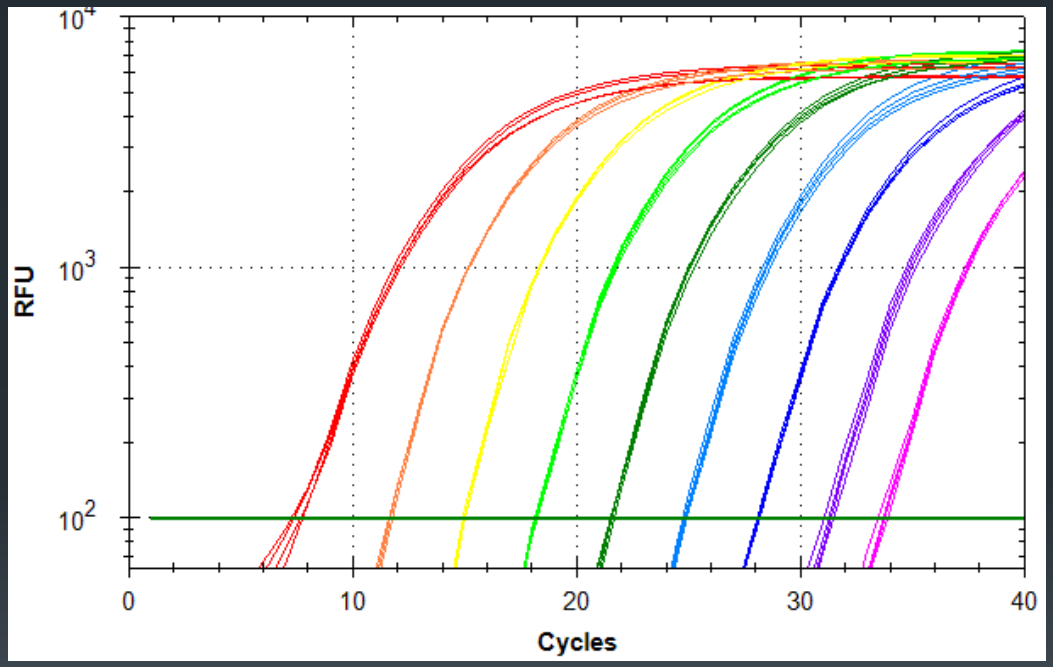
Why?

- Optimization of real-time PCR reactions is important:
  - Since real-time PCR calculations are based on a doubling of product every cycle, if the reaction isn't optimized, this doubling will not occur.

Optimization

Example

- A well-optimized reaction will have evenly spaced standard curves with tight replicates:



- At 100% efficiency, 10-fold serial dilutions will be spaced 3.3 cycles apart from each other.

Optimization

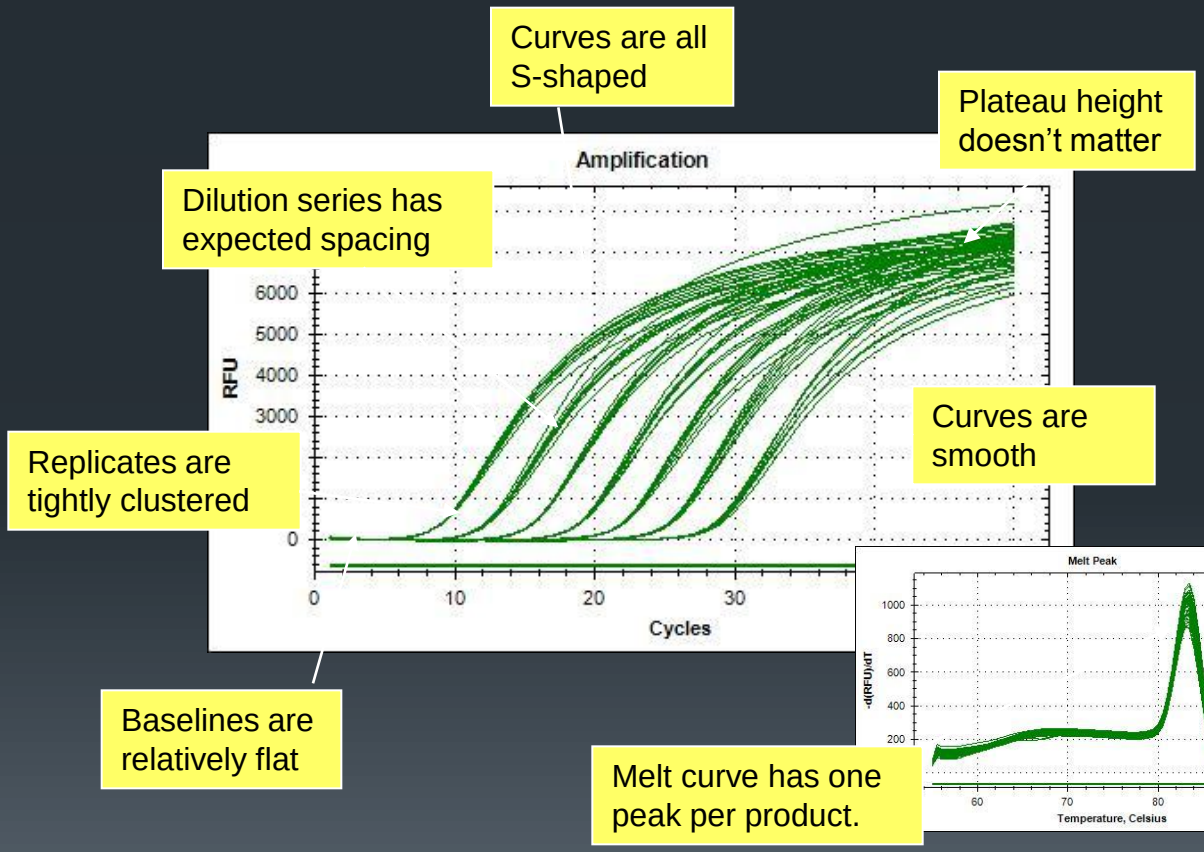
Basics

- Optimization is normally done as follows:

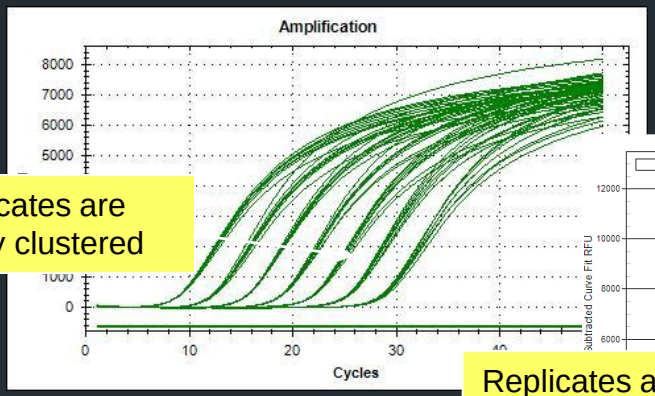
1. Design multiple primer sets.
2. Empirically test each primer set with a standard curve.
3. Select best primer set, then run a temperature gradient experiment to determine best annealing temperature.
4. Standard curves are ideal for assessing optimization.

Trouble-Shooting

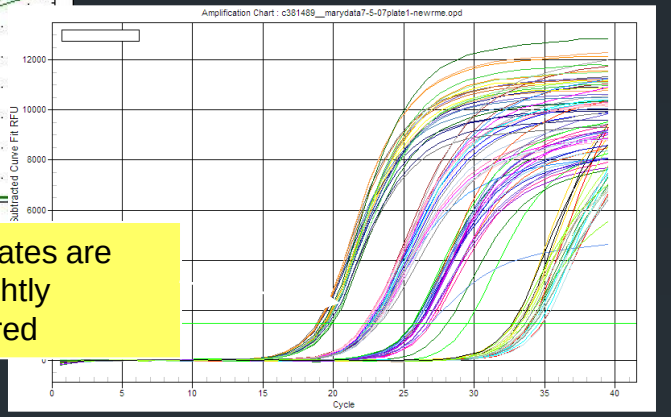
- A successful real-time PCR experiment will have the following characteristics:



Trouble-Shooting Replicates



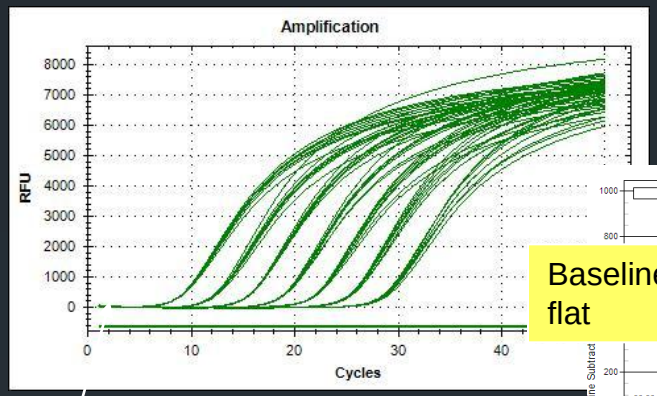
Replicates are tightly clustered



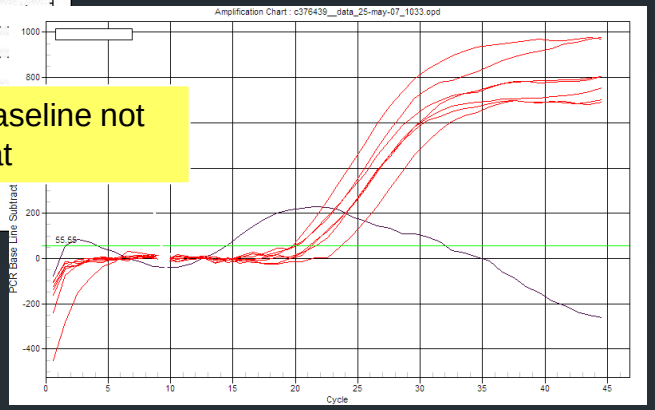
Replicates are **not** tightly clustered

- If replicates aren't tightly clustered, suspect:
  - Pipetting error
  - Poorly optimized PCR reactions
  - Sample degradation
  - Unknowns outside of range of detection
  - Instrument calibration

Trouble-Shooting Baselines



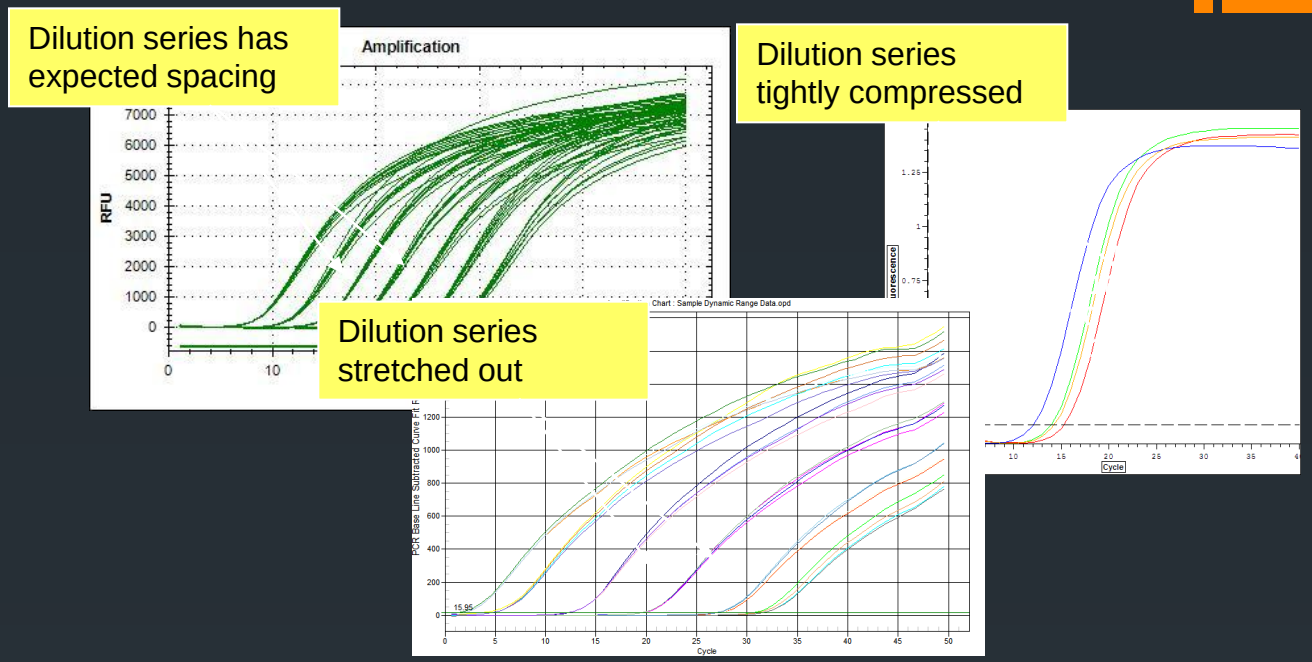
Baselines are relatively flat



Baseline not flat

- If baselines aren't flat, suspect:
  - Sample evaporation
  - Bubbles
  - Reagents not thoroughly mixed
  - Baseline "window" not properly set

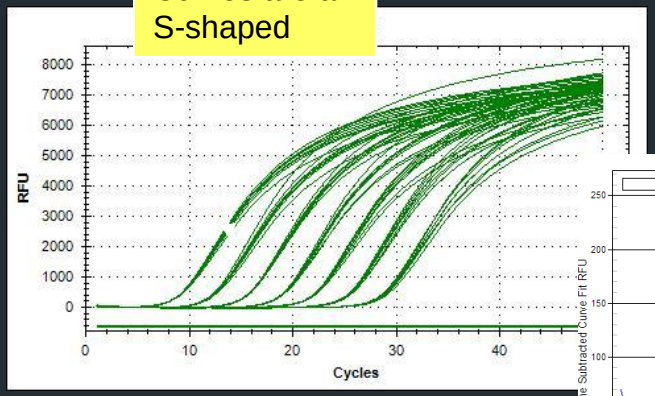
Trouble-Shooting Dilutions



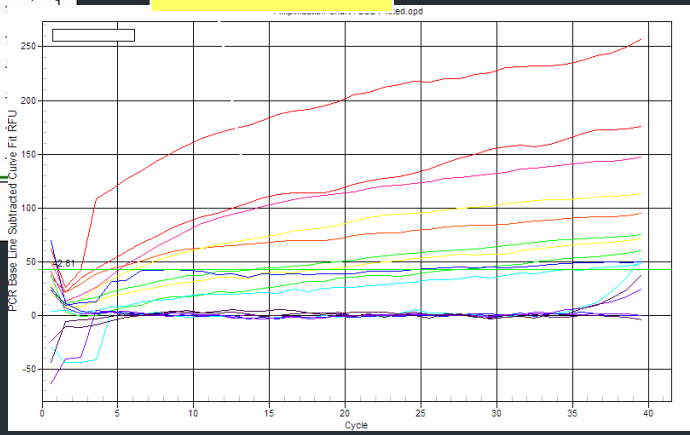
- If the dilution series comes out “compressed” or “stretched”, suspect:
  - Pipetting
  - Too much DNA (for your assay)
  - PCR inhibitors
  - Too little DNA (for your assay)
  - Poor PCR efficiency

Trouble-Shooting Curve Shape

Curves are all S-shaped

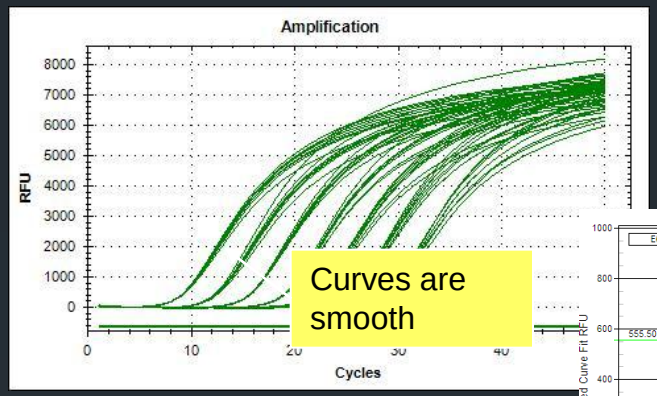


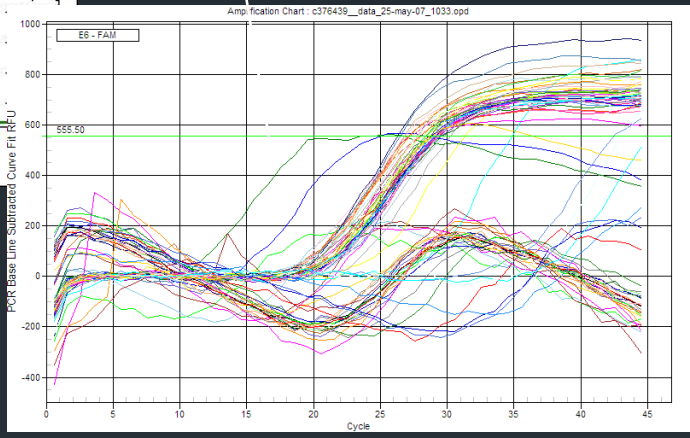
Curves are not S-shaped



- If curves are not S-shaped, suspect:
  - Curves are not actual PCR products!
  - Sample degradation
  - Fluorescence drift in unamplified samples
  - Something seriously wrong with assay

Trouble-Shooting Curve Shape

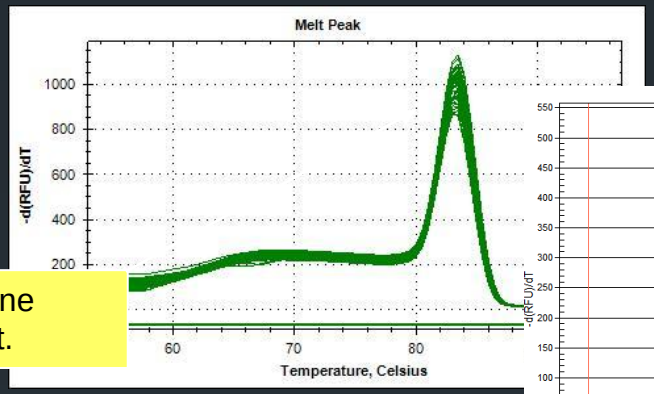




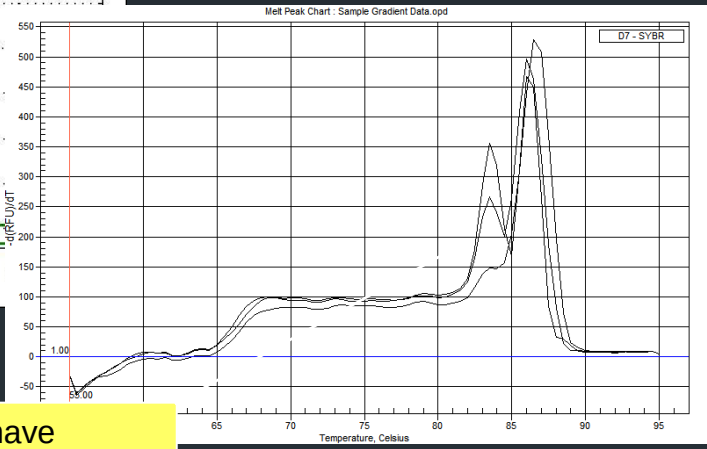
- If curves are not smooth, suspect:
  - Poor pipetting (bubbles)
  - Sample degradation
  - Poor assay (low fluorescence reagents)
  - System malfunction (line noise)

Trouble-Shooting

Melt Peaks



Melt curve has one peak per product.

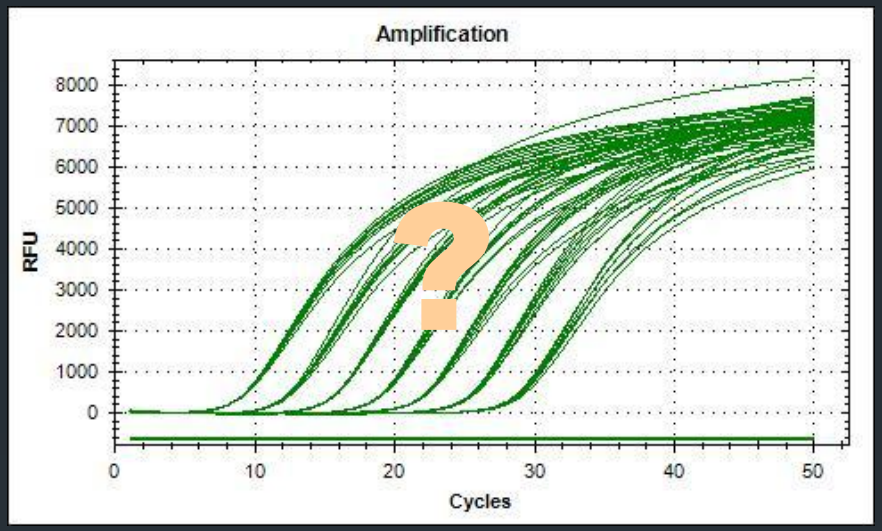


Melt curves have multiple peaks.

- If melt curves have more than one peak:
  - More than one product
  - Possible normal primer-dimers
  - Using too low an annealing temperature
  - Primers need to be redesigned

Trouble-Shooting

Common themes in troubleshooting:



Care in pipetting.

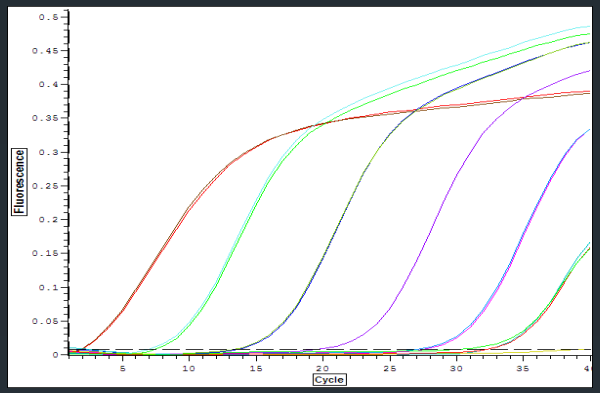
Care in choice of plastics.

Care in experimental design.

Use of Positive and Negative Controls.

The importance of properly designed experiments !!

Questions?



Thank You for your attention!

Dr.Hargita Hegyesi