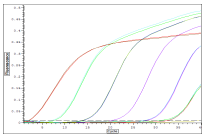


Part 1:

What is Real-Time PCR and what is it used for?

What is Real-Time PCR?

The Polymerase Chain Reaction (PCR) 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 used for?

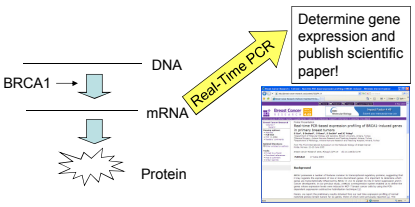
Real-Time PCR has become a cornerstone of molecular biology. Just some of the uses include:

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

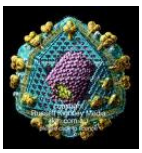
Real-Time PCR in Gene Expression Analysis

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 in Disease Management



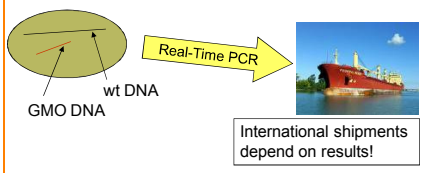
Example: HIV Treatment

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.

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.



Real-Time PCR 2009
Contra Costa College

Imagining Real-Time PCR

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

A soup of nucleotides, primers, template, amplicons, enzyme, etc.

1,000,000 copies of the amplicon *(the amplified DNA product)* right now.

Real-Time PCR 2009
Contra Costa College

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.

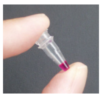
And the cycle before that, 23?
- Almost the same, but only 250,000 copies of the amplicon.

And what about cycle 22?
- Not a whole lot different. 125,000 copies of the amplicon.

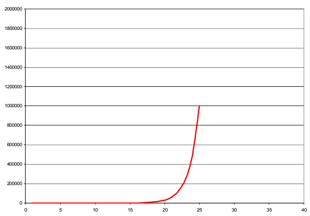
Real-Time PCR 2009
Contra Costa College

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:



Real-Time PCR 2009
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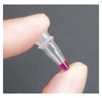
Imagining Real-Time PCR

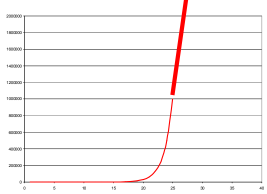
How did we get here?



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?

?



Real-Time PCR 2009
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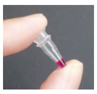
Imagining Real-Time PCR

So where are we going?



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.

And at cycle 200?
- In theory, there would be 1,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000,000 amplicons…
- Or 10^35 tonnes of DNA…
- To put this in perspective, that would be equivalent to the weight of ten billion planets the size of Earth!!!!

Real-Time PCR 2009
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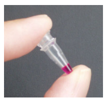
Imagining Real-Time PCR

So where are we going?



A clump of DNA the size of ten billion planets won't quite fit in our PCR tube anymore.

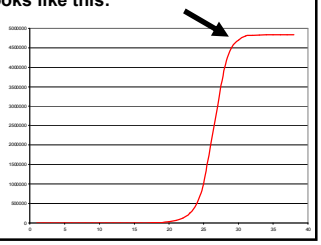
Realistically, at the chain reaction progresses, it gets exponentially harder to find primers, and nucleotides. And the polymerase is wearing out.

So exponential growth does not go on forever!

Imagining Real-Time PCR

So where are we going?

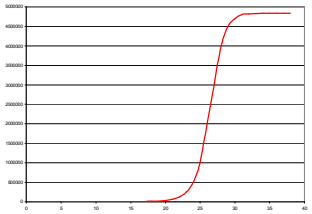
If we plot the amount of DNA in our tube going forward from cycle 25, we see that it actually looks like this:



Imagining Real-Time PCR

Measuring Quantities

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



Imagining Real-Time PCR

Measuring 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 →

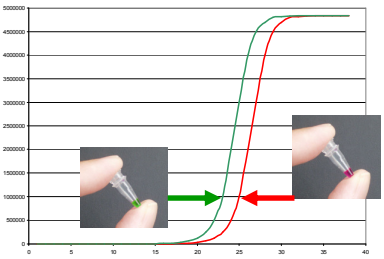
Cycle	Me	You
23	250,000	1,000,000
24	500,000	2,000,000
25	1,000,000	4,000,000

Imagining Real-Time PCR

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!



Imagining Real-Time PCR

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

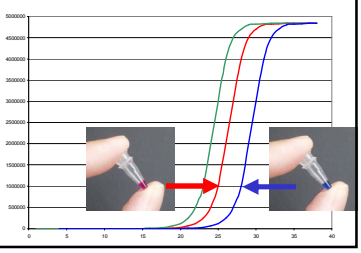
Imagining Real-Time PCR

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!



Imagining Real-Time PCR

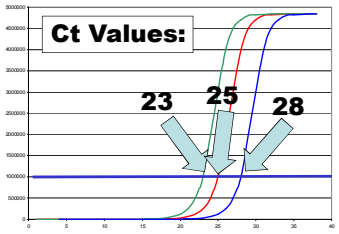
Measuring Quantities

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

This is called the "Ct Value".

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

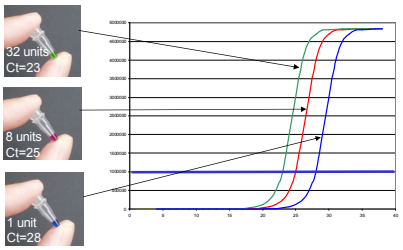
Quantity = $2^{\wedge Ct}$



Imagining Real-Time PCR

Measuring Quantities

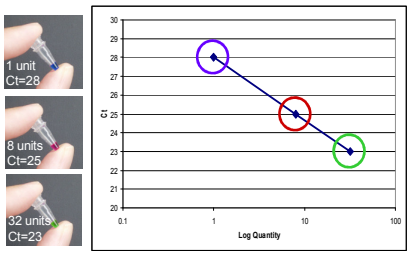
Let's recap…



Imagining Real-Time PCR

Measuring Quantities

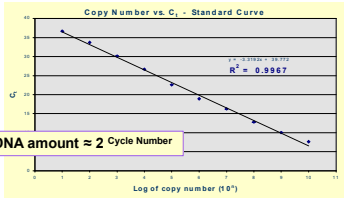
We can plot the Ct value versus the Log Quantity on a graph…



Real-Time PCR

Measuring Quantities

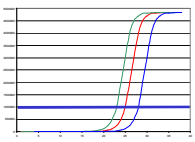
- In reality, there is DIRECT relationship between the starting amount of DNA, and the cycle number that you'll reach an arbitrary number of DNA copies (Ct value).
- This allows us to plot a "standard curve" for all of our known DNA samples. We can then use the standard curve to find the starting concentrations in our unknowns!



DNA amount ≈ 2 Cycle Number

Real-Time PCR

Quiz



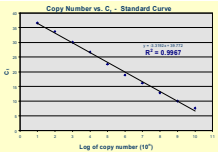
The table below shows typical real-time PCR results. There are three standards. Can you estimate the starting quantity of DNA in the unknown wells?

Well Number	Type	Ct	Starting Quantity
A1	Standard	20	1600
A2	Standard	22	400
A3	Standard	24	100
B1	Unknown	20	1600
B2	Unknown	21	800
B3	Unknown	25	50

Real-Time PCR

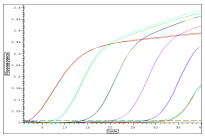
Sensitivity

How <u>sensitive</u> 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 equivalent to just twenty attograms (2×10^{-17} g) of DNA!



Part 3:
How do we actually measure DNA?

How do We Measure DNA in a PCR Reaction?

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

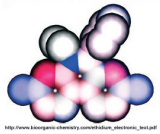
Ethidium bromide and SYBR Green I dye are two such reagents.

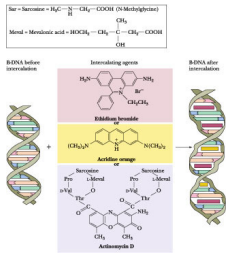
They bind to double-stranded DNA and emit light when illuminated with a specific wavelength.

SYBR Green I dye fluoresces much more brightly than ethidium.

Measuring DNA: Ethidium Bromide

Ethidium Bromide

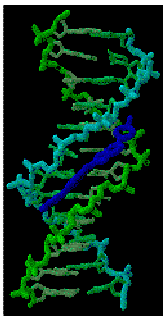


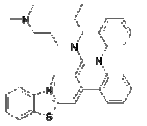


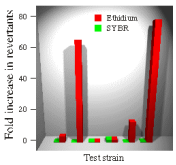
http://www.web.virginia.edu/Heidi/chapter12/chp12.htm

Measuring DNA: SYBR Green I

SYBR Green I





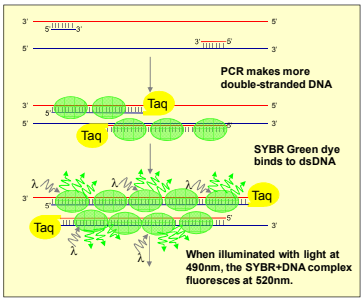


Ames test results from Molecular Probes
Singer et al., Mutat. Res. 1999, 439: 37- 47

Fluorescent Dyes in PCR

Intercalating Dyes

SYBR Green in Action!



Fluorescent Dyes in PCR

Probes

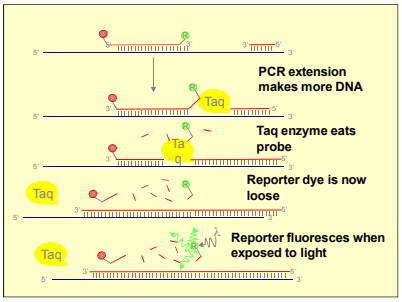
- Fluorescent probes come in many varieties, the most common is "TaqMan".
- The probes work by having a fluorescent molecule (called a reporter) linked to a non-fluorescent molecule (called a quencher).
- As long as reporter and quencher are close to each other, the probe itself will not fluoresce.
- As the PCR reaction goes on, the reporter is separated from the quencher, and will now fluoresce.

The main advantage of fluorescent probes is that they can be designed in different colors or wavelengths.

This allows several different colored probes to detect several different PCR targets in the same PCR tube – "multiplexing".

Fluorescent Dyes in PCR Probes

TaqMan Probes in Action!



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

Real-time PCR instruments consist of THREE main components:

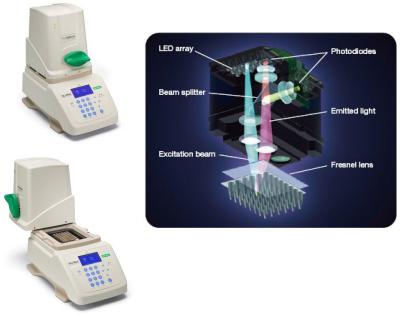
1. Thermal Cycler (PCR machine)
2. Optical Module (to detect fluorescence in the tubes during the run)
3. Computer (to translate the fluorescence data into meaningful results)

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

An example of such an instrument is the Bio-Rad iQ5 real-time PCR instrument.

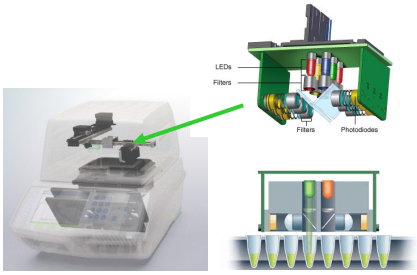
What Type of Instruments are used with Real-Time PCR?

Another example is the MiniOpticon real-time instrument.



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

One more example is the Bio-Rad CFX real-time PCR instrument.

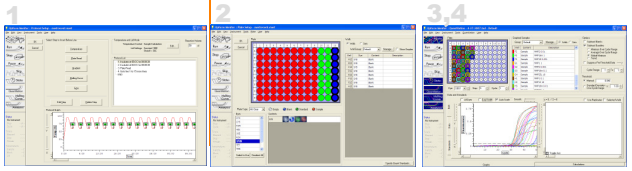


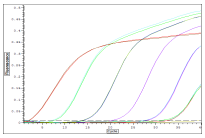
The CFX scans the PCR plate with LEDs and records fluorescence in each well at each PCR cycle.

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

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

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





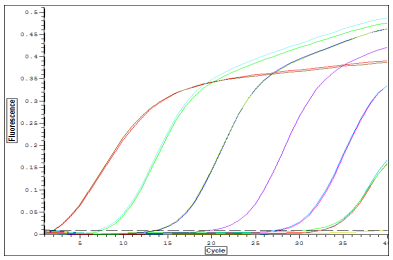
Part 4:

What does real-time data look like?

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 a dilution series of DNA.



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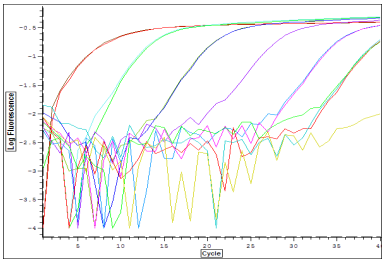
Real-Time PCR

Actual Data

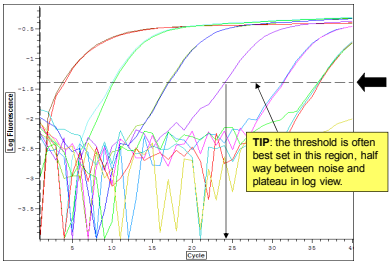
- The same data set in log view



Real-Time PCR

Setting Thresholds

- Once threshold is set, Ct values can be calculated automatically by software.



- Ct values can then be used to calculate quantities of template DNA.

Real-Time PCR

Final Product

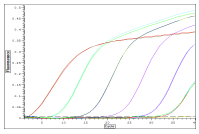
- The final product of real-time PCR is a table of Ct values, from which amounts of DNA can be determined.

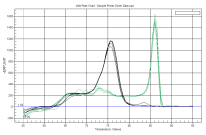
Well	*Fluor*	*Content*	*Threshold Cycle (C(t))*
A03	SYBR	Std-1	8.90
A04	SYBR	Std-2	12.20
A05	SYBR	Std-3	15.34
A06	SYBR	Std-4	18.77
A07	SYBR	Std-5	21.84
A08	SYBR	Std-6	25.24
A09	SYBR	Std-7	28.82
B03	SYBR	Std-1	8.85
B04	SYBR	Std-2	12.12
B05	SYBR	Std-3	15.31
B06	SYBR	Std-4	18.69
B07	SYBR	Std-5	21.76
B08	SYBR	Std-6	25.24

Real-Time PCR

Actual Data

- The fluorescence data collected during PCR tells us "how much" … but there is another type of analysis we can do that tells us "what"!





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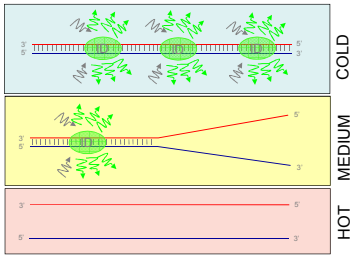
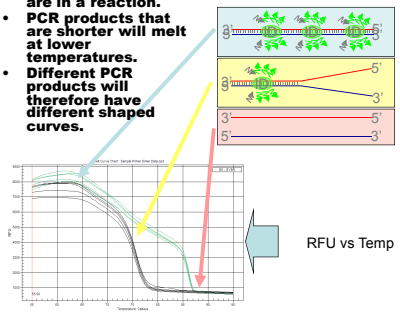
Real-Time PCR – the Concept of MELT CURVES…

- Melt curves can tell us what products are in a reaction.
- Based on the principle that as DNA melts (becomes single stranded), DNA-binding dyes will no longer bind and fluoresce.



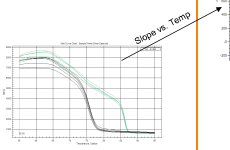
Real-Time PCR – the Concept of MELT CURVES…

- 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.

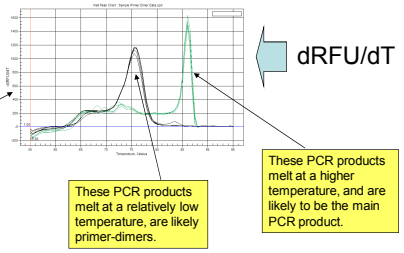


Real-Time PCR

The Concept of MELT CURVES



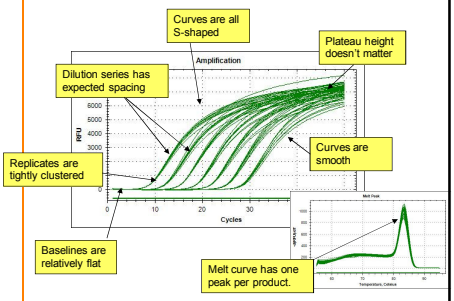
- For convenience, we typically view the derivative *(slope)* of the actual melt curve data.
- The resulting graph looks like a chromatogram, with peaks that represent different PCR products.

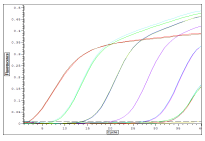


Real-Time PCR

Putting it all Together

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

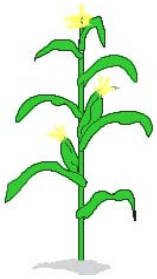




Part 5:

How do we use mathematics to convert real-time results to useful numbers?

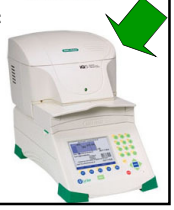
GMO Investigator Kit in Real-Time



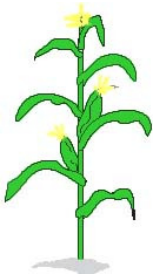
- To run the GMO Kit in real-time, only two additional items are needed…

- iQ SYBR Green Supermix

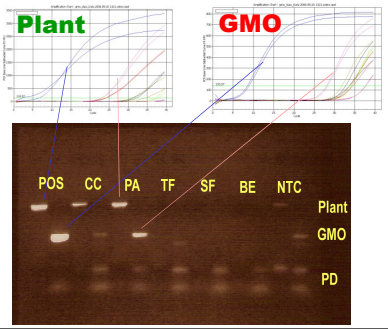
- A real-time PCR instrument



GMO Investigator Kit in Real-Time



Classroom Results, Contra Costa College May 2006



Real-Time PCR 2009
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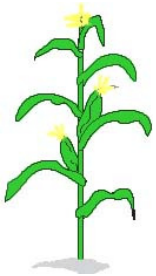
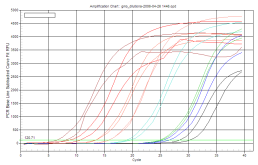
GMO Investigator Kit





Quantification and Normalization

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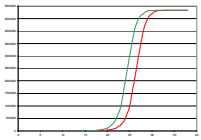
Quantification and Normalization



- How can we determine the GMO content of a food?
- We can do this simply by comparing the amount of the GMO "target gene" to the amount of the plant "reference gene"!
- By comparing the two amounts, we then have a basis to compare one food with another!
- This process is called "normalization".

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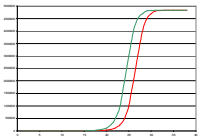
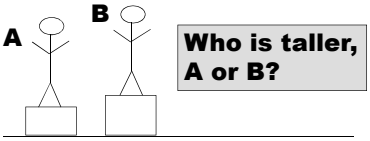
Normalization, what is it?



- An analogy to demonstrate the concept of "normalization"…



- Height of person A = (total height of A) – (height of box A)
- Height of person B = (total height of B) – (height of box B)
- In qPCR, the "box" may be the Ct value of a standard sample type (ie. 100% gmo food), or may be the Ct value of a reference gene compared to an unknown gene.

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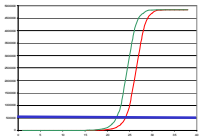
Quantification and Normalization



- Example: Ct values for the plant gene and GMO gene from different food samples.

Food	Plant Ct	GMO Ct
100% GMO	25	28
Non-GMO	26	36
Unknown 1	20	23
Unknown 2	27	31

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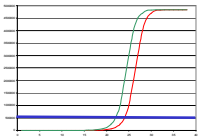
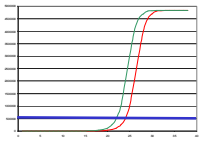
Quantification and Normalization



- First we'll compare relative amounts of plant DNA … relative to the 100% GMO control.
- Using the formula that relates relative quantity to 2^(Cta-Ctb), we can calculate amounts of DNA relative to a single sample.

Food	Ct Value	Delta Ct	Relative Quantity
100% GMO	25	0	1
Non-GMO	26	-1	0.5
Unknown 1	20	5	32
Unknown 2	27	-2	0.25

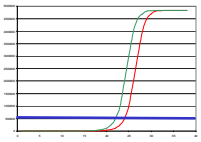
Quantification and Normalization



- Second we'll determine relative amounts of GMO DNA … again, relative to the 100% control.
- We can do the exact same calculations for the Ct values from the GMO genes of the same food samples.

Food	Ct Value	Delta Ct	Relative Quantity
100% GMO	28	0	1
Non-GMO	36	-8	0.004
Unknown 1	23	5	32
Unknown 2	31	-3	0.125

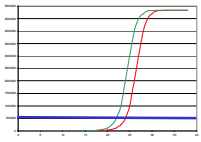
Quantification and Normalization



- Now we compare the relative amounts of plant to GMO DNA in each sample…
- …This gives us our relative GMO content.
- This is called delta-delta-Ct analysis, and is the basis of real-time quantification analysis.

Food	Plant DNA	GMO DNA	Relative GMO Content
100% GMO	1	1	1 (100%)
Non-GMO	0.5	0.004	0.008 (0.8%)
Unknown 1	32	32	1 (100%)
Unknown 2	0.25	0.125	0.5 (50%)

Quantification and Normalization



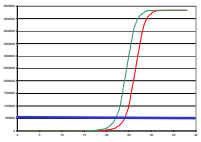
Summary

- First we determined how much plant DNA is in each sample,
- Second we determined how much GMO DNA is in each sample,
- Finally we corrected / normalized the GMO DNA against the plant DNA to determine relative amount of GMO for each sample.

Normalization

Food	Relative GMO Content
100% GMO	100%
Non-GMO	0.8%
Unknown 1	100%
Unknown 2	50%

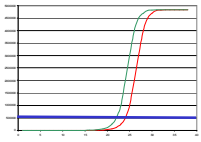
Quantification and Normalization



- Practice! Try this example.
- What is the GMO content of product A and product B??

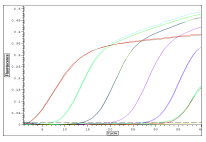
Food	Plant Ct	GMO Ct
100% GMO	23	27
Non-GMO	24	39
Product A	22	30
Product B	25	29

Quantification and Normalization



- Results…
- What is the GMO content of product A and product B??

Food	Plant Ct	GMO Ct	Delta Ct Plant	Delta Ct GMO	GMO Plant ddCt	Ratio 2^{-ddCt}	Ratio %
100% GMO	23	27	0	0	0	**1**	**100**
Non-GMO	24	39	-1	-12	-11	**0.0004**	**0.04**
Product A	22	30	1	-3	-4	**.0625**	**6.3**
Product B	25	29	-2	-2	0	**1**	**100**



Part 6:
Real-Time PCR Demonstration

Today's Experiment: An Overview

- **Today we'll use the DNA in the Crime Scene Kit to make some dilutions for our real-time experiment!**
- **Each workgroup will prepare four real-time PCR reactions:**
  - Unknown DNA (replicate 1)
  - Unknown DNA (replicate 2)
  - Unknown DNA diluted 1:100 (replicate 1)
  - Unknown DNA diluted 1:100 (replicate 2)
- **Each workgroup will have DNA from the Crime Scene kit that has been diluted 1:10, 1:100, 1:1000, 1:10000, or undiluted.**
- **If all goes well, you'll be able to tell from the Ct values:**
  - Which unknown DNA you started with,
  - How accurate your pipetting is,
  - Whether your mini-dilution series demonstrates high-efficiency PCR.

Today's Experiment: Setup

Initial Setup (already completed)…

Supplies Needed

Chromo4 Real-Time System
Low-Profile strip tubes (white)
Optical flat caps
Pipettors, etc.
SYBR Green Supermix
CSI Kit Refill baggie.
Hard shell plates (to hold strip tubes)
Screw-cap microfuge tubes.

Before Workshop (assuming 10 workgroups)

Spike 1200ul SYBR Green Supermix with 12ul of CSI primers.
Aliquot 100ul of spiked supermix to 10 x 1.5ml screw-cap microfuge tubes.
Dilute DNA samples (CS DNA) undiluted, 1:10, 1:100, 1:1000, 1:10000. Label 1-5 respectively.
Aliquot 100ul of above to 2 x 1.5ml screw-cap microfuge tubes.
Aliquot 100ul of wawter to 10 x 1.5ml screw-cap microfuge tubes.

Needed at each workgroup (assuming 10 workgroups)

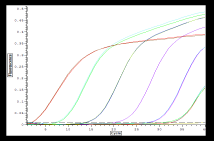
1 hardshell plate
1 low profile strip tube
1 strip of optical flat caps
1 tube of spiked SYBR Green Supermix (4 rxns = 100ul)
1 tube of 100ul Crime Scene DNA, random dilution (tube 1,2,3,4, or 5)
1 tube of 99ul water.
Pipette tips
20ul pipettor

Today's Experiment: Step-By-Step

- **Step 1:**
  - Make your DNA dilutions (screw-cap tubes).
  - Dilute your "unknown" DNA 1:100
  - 1 ul of your DNA into 99 ul of water.
- **Step 2:**
  - Prepare your PCR tubes.
  - Add 20 ul of the spiked SYBR Green Supermix (contains 0.2 ul of Crime Scene Primers) to your four PCR tubes.
- **Step 3:**
  - Complete your PCR reactions.
  - Add 20 ul of your DNA samples to each PCR tube.
    - Two tubes undiluted, two tubes 1:100.
  - Mix gently, avoiding bubbles!
- **Step 4:**
  - Place your reactions in the real-time PCR machine.

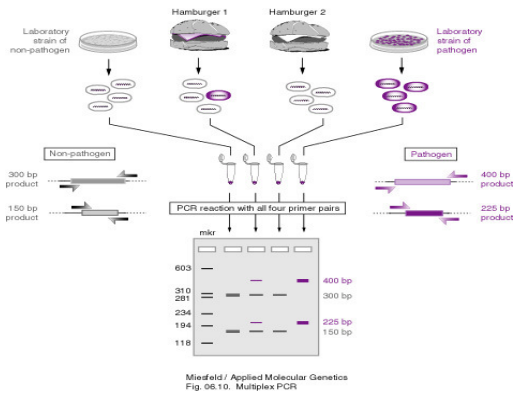
Today's Experiment: PCR Protocol

- **Our PCR protocol will look like this:**
  - 1. 95C for 3 min (activates Taq)
  - 2. 95C for 10 sec (denatures)
  - 3. 55C for 30 sec (extend / anneal)
  - 4. Plate read (captures fluorescence data)
  - 5. Goto Step 2 for 39 more times



Real-Time PCR

David A. Palmer, Ph.D.
Technical Support, Bio-Rad Laboratories
Adjunct Professor, Contra Costa College

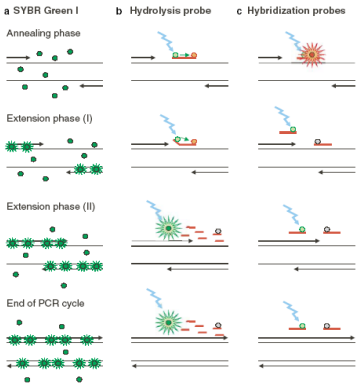


Miesfeld / Applied Molecular Genetics
Fig. 06.10. Multiplex PCR

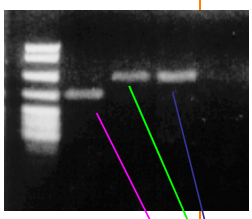
- Absolute quantitation
  - Standard curve
  - Standards must be accurately quantitated
  - Best used for viral load determination
- Relative quantitation
  - Standard curve
  - Standards are serial dilutions of a calibrator template
  - Best used for gene expression studies
- Comparative quantitation
  - Mathematical determination
  - Calibrator sample used as a 1x standard
  - Best used when particular ratios are expected or to verify trends

Ambion THE RNA COMPANY

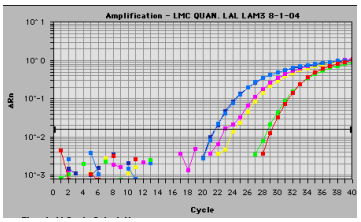
(www)



PCR Tradicional



PCR a tiempo real

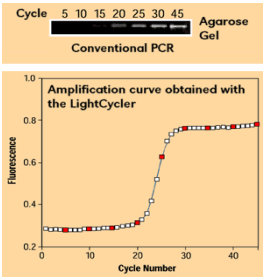


7000 copias

100 copias

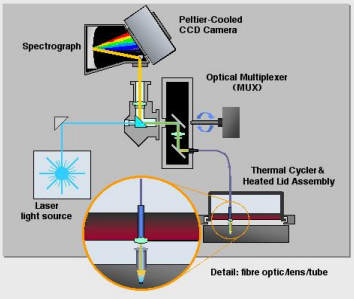
2500 copias

LIGHT CYCLER



ABI Prism™ 7700 Sequence Detection System

optics of the virtual filter system



PE Biosystems