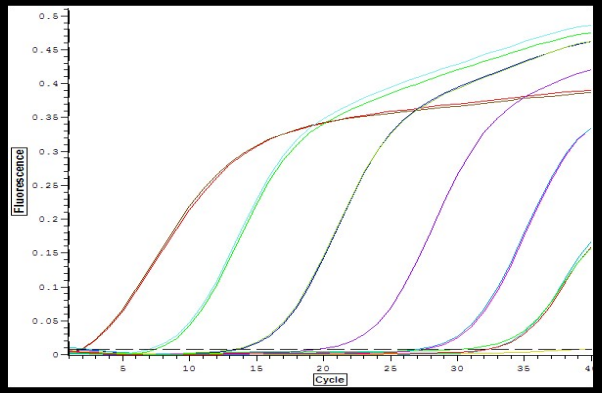

BIO-RAD
Professional Development

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD



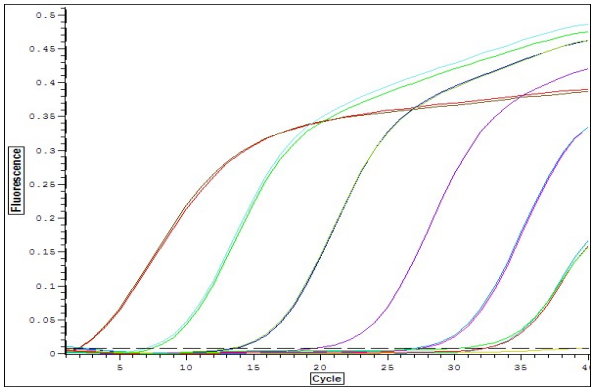
Real-Time PCR

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

Objectives

This presentation will cover the following topics:

- **What is real-time PCR used for?**
- **How does real-time PCR work?**
- **What instruments are used?**
- **What does real-time data look like?**
- **How can the Crime Scene Invesigator kit be used in a real-time setting?**



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:

- **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

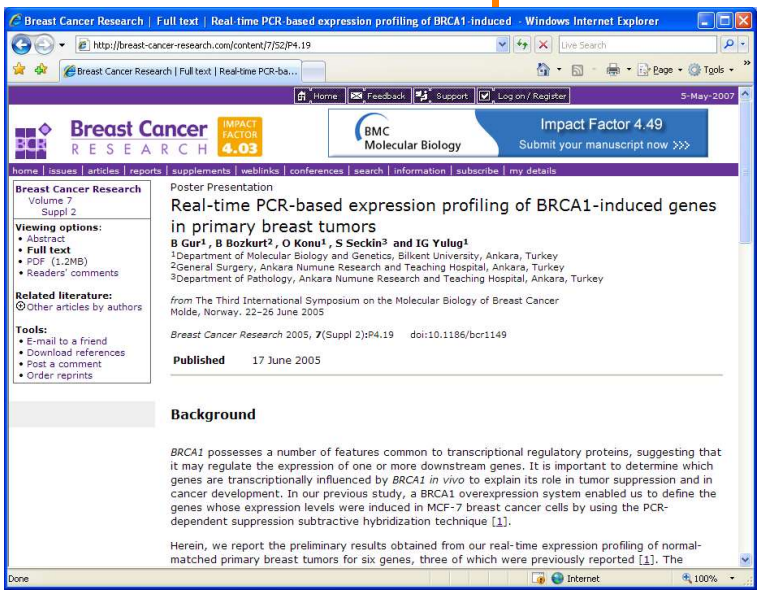
Real-Time PCR in Gene Expression Analysis

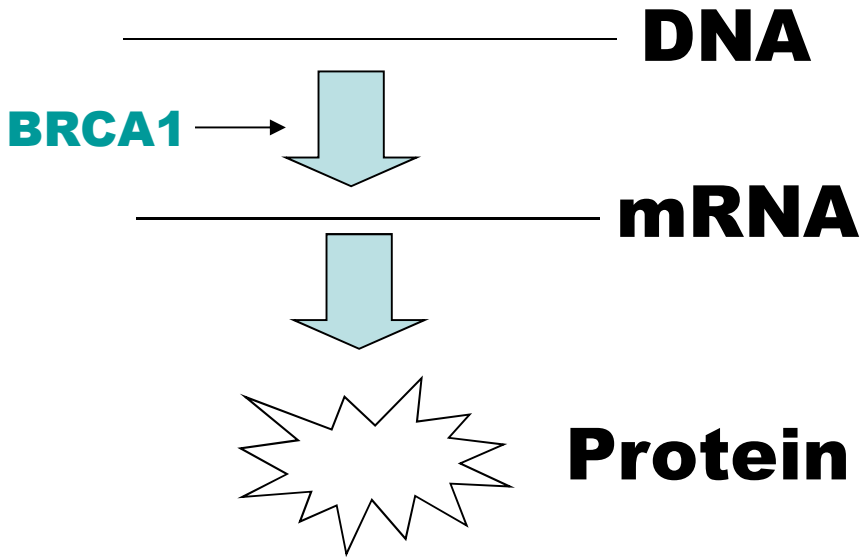
Example: BRCA1 Expression Profiling

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.



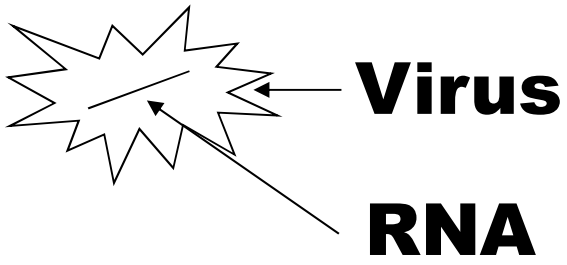


Real-Time PCR in Disease Management

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.

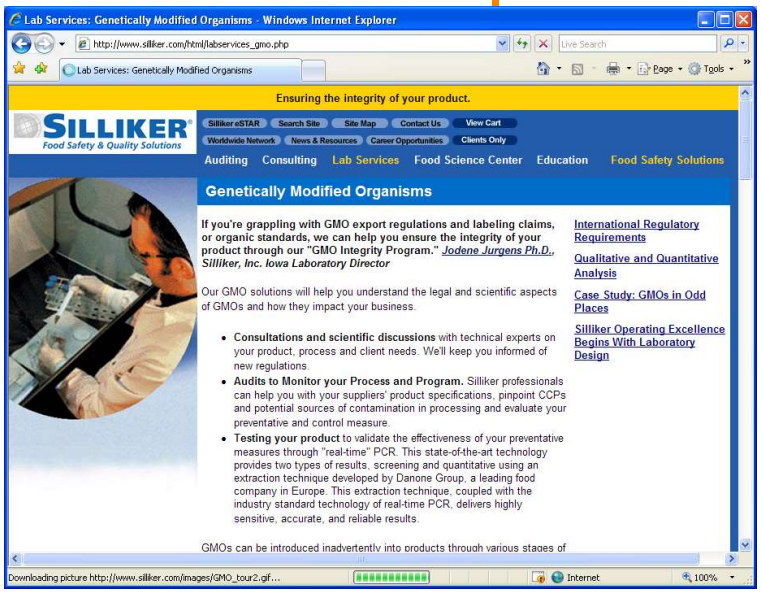


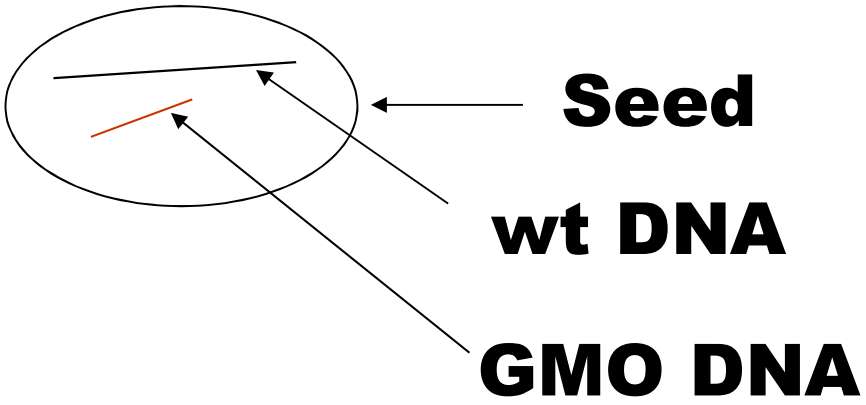
Real-Time PCR in Food Testing

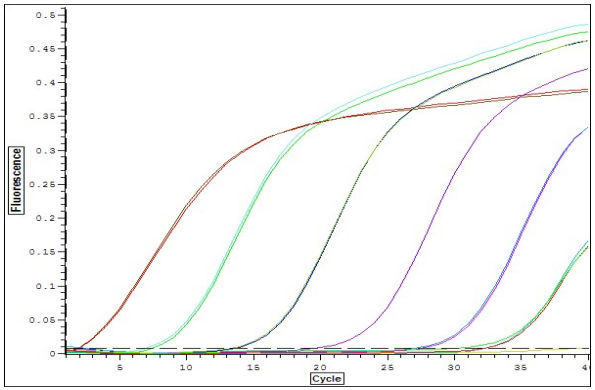
Example: Determining percentage of GMO food content

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.







Part 2:

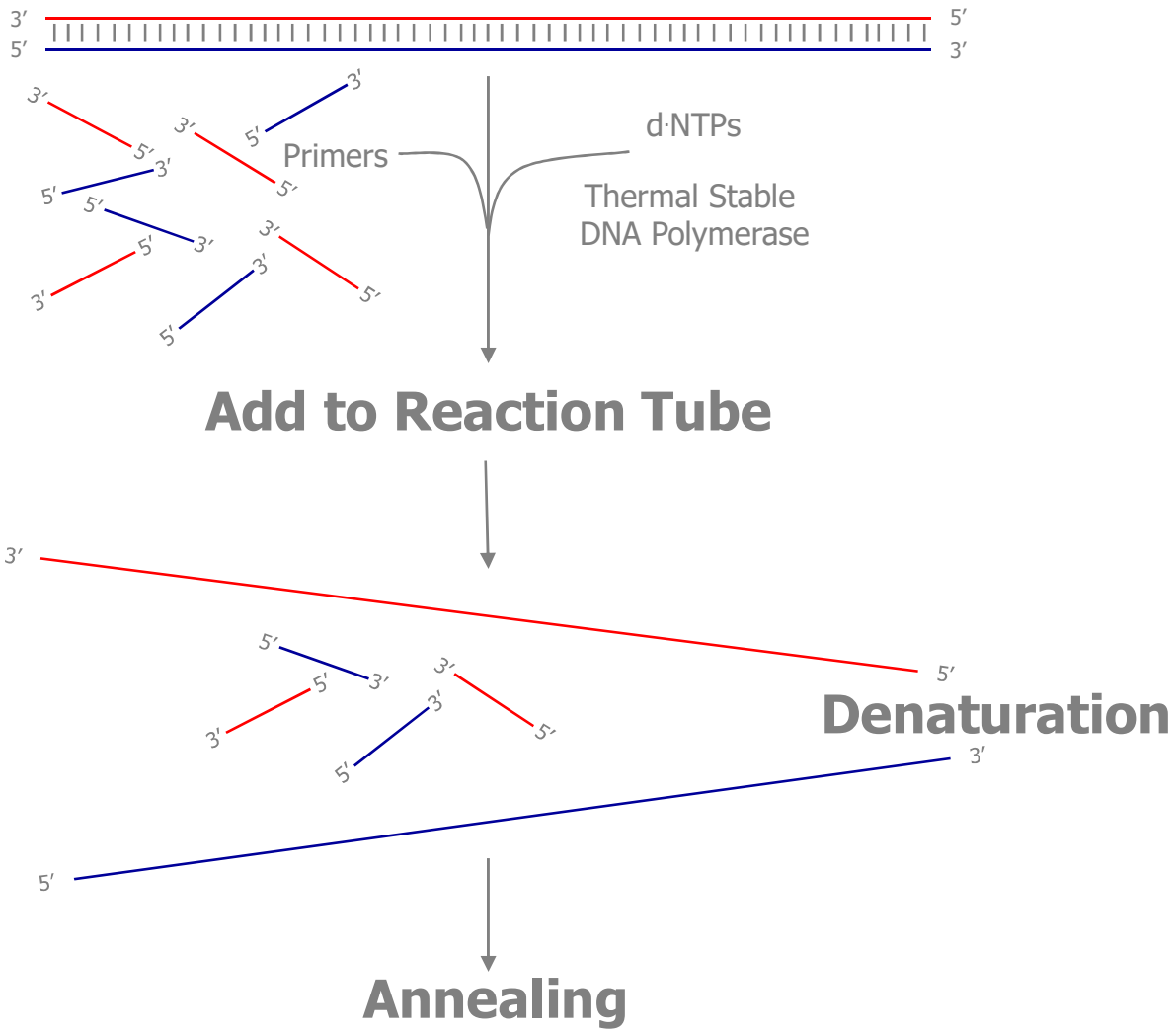
How does Real-Time PCR work?

How does real-time PCR work?

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

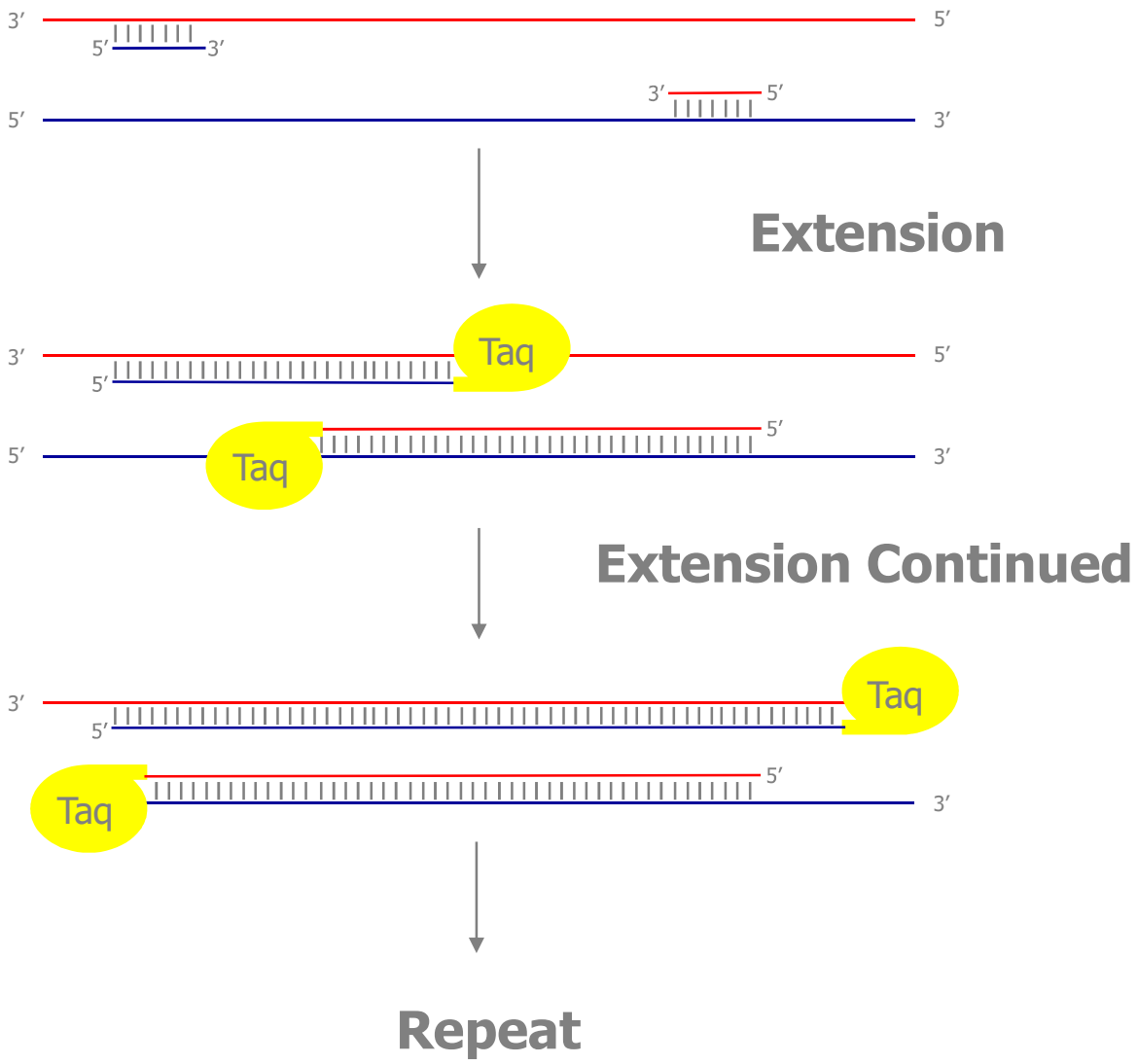
The Polymerase Chain Reaction

How does PCR work??



The Polymerase Chain Reaction

How does PCR work??

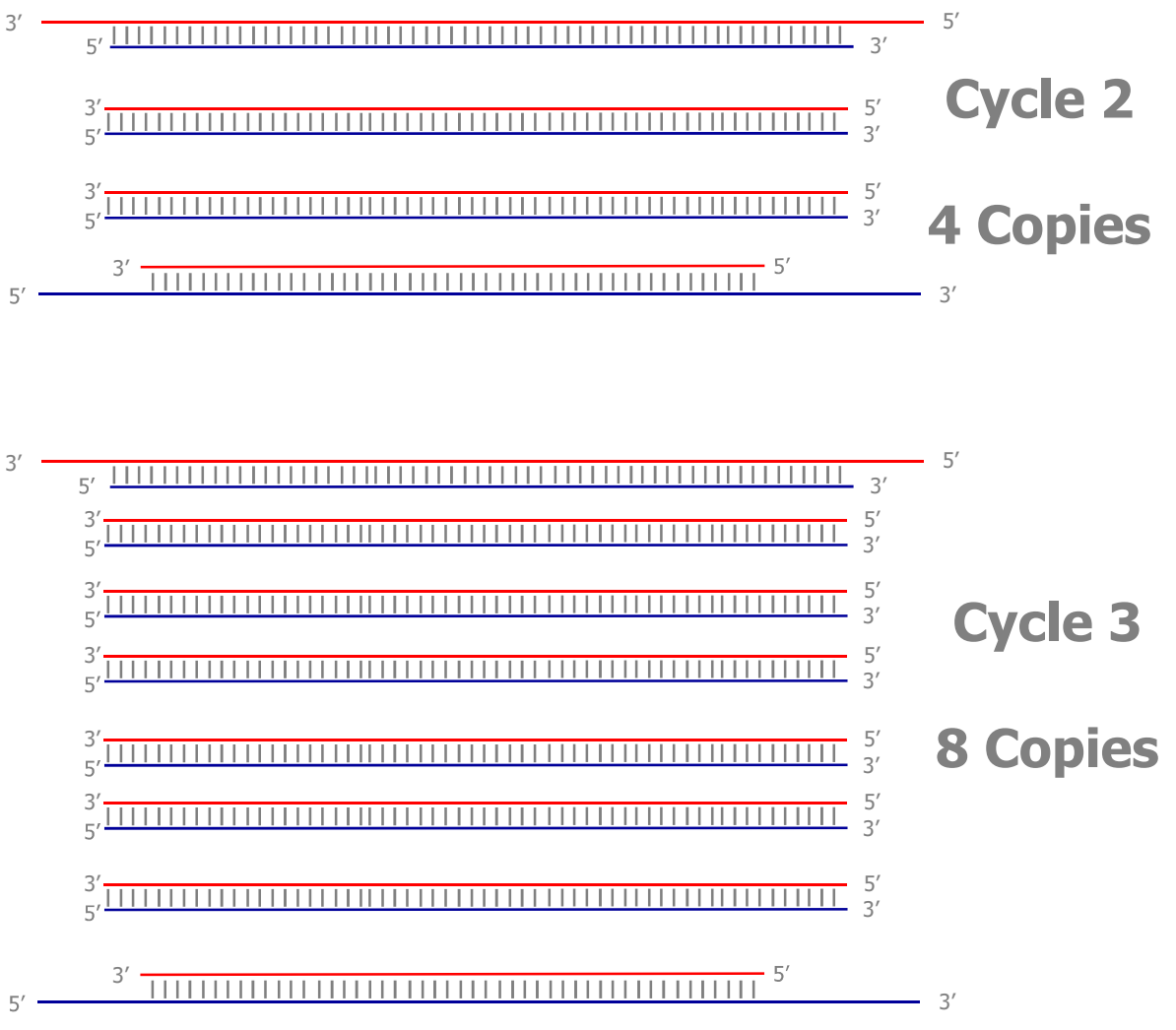


Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

The Polymerase Chain Reaction

How does PCR work??



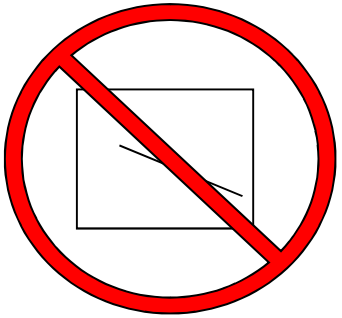
How does Real-Time PCR work?

…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…

Most importantly, we'll start by imagining the PCR itself, and only then will we draw graphs to illustrate what's going on.

NO GRAPHS (yet)



Imagining Real-Time PCR

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, 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.

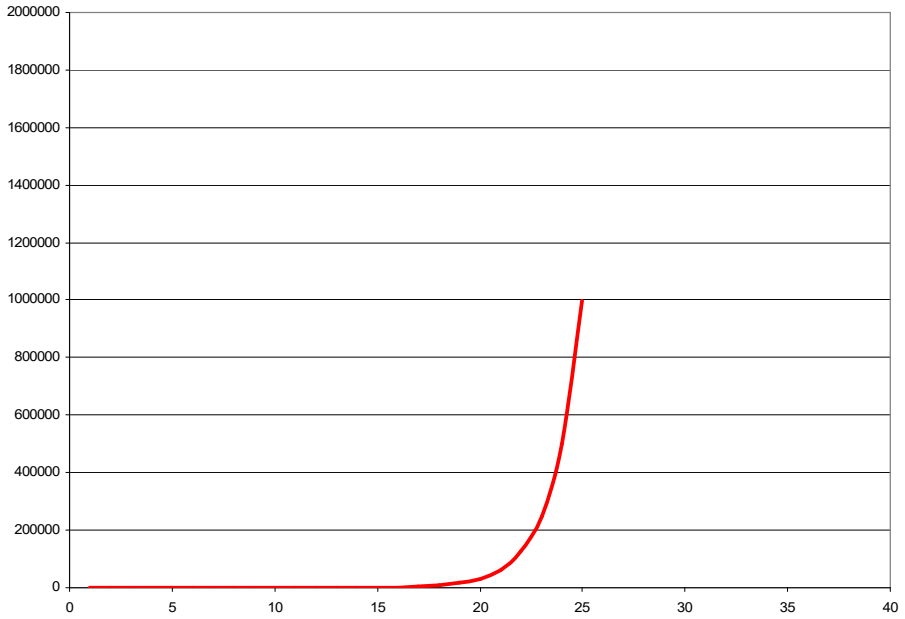
And what about cycle 22?

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:

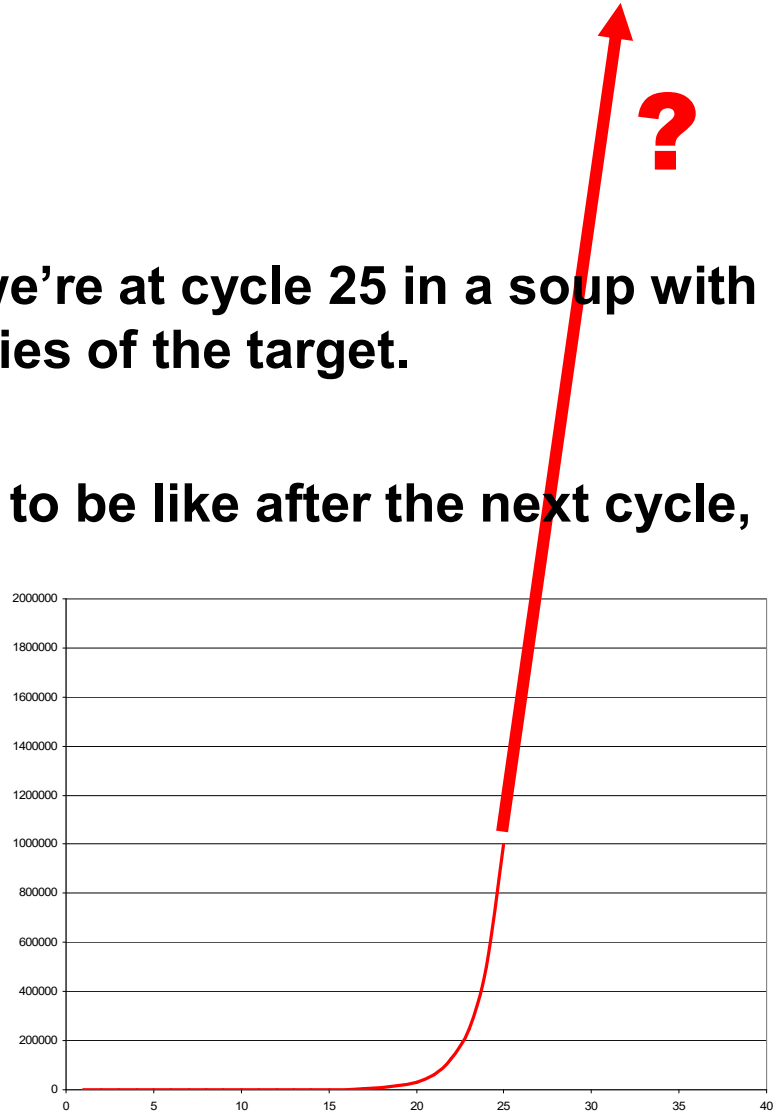


Imagining Real-Time PCR

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?



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 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!!!!

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.

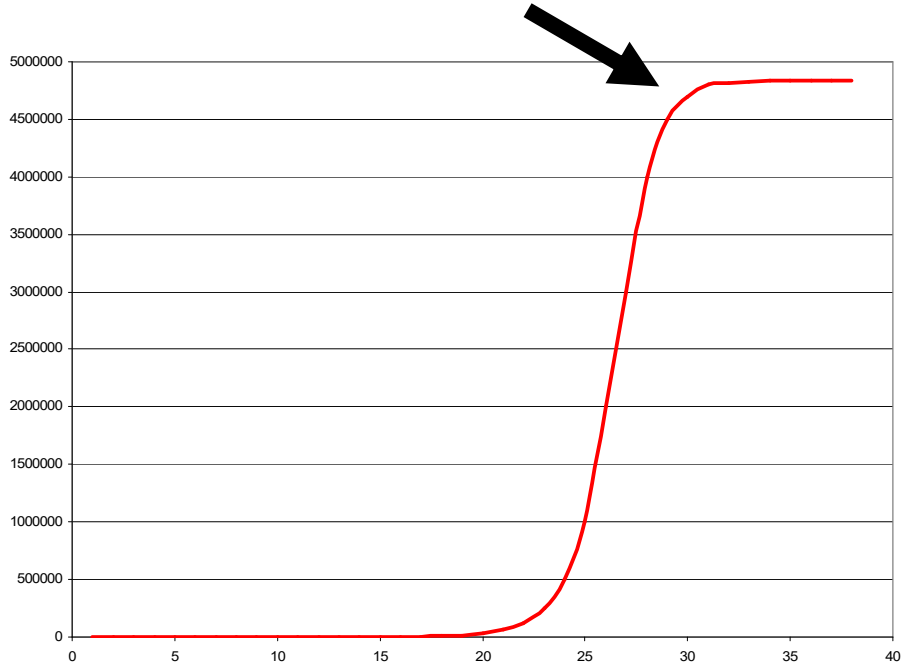
Realistically, at the chain reaction progresses, it gets exponentially harder to find primers, and nucleotides. And the polymerase is wearing out.

So exponential growth does not go on forever!

Imagining Real-Time PCR

So where are we going?

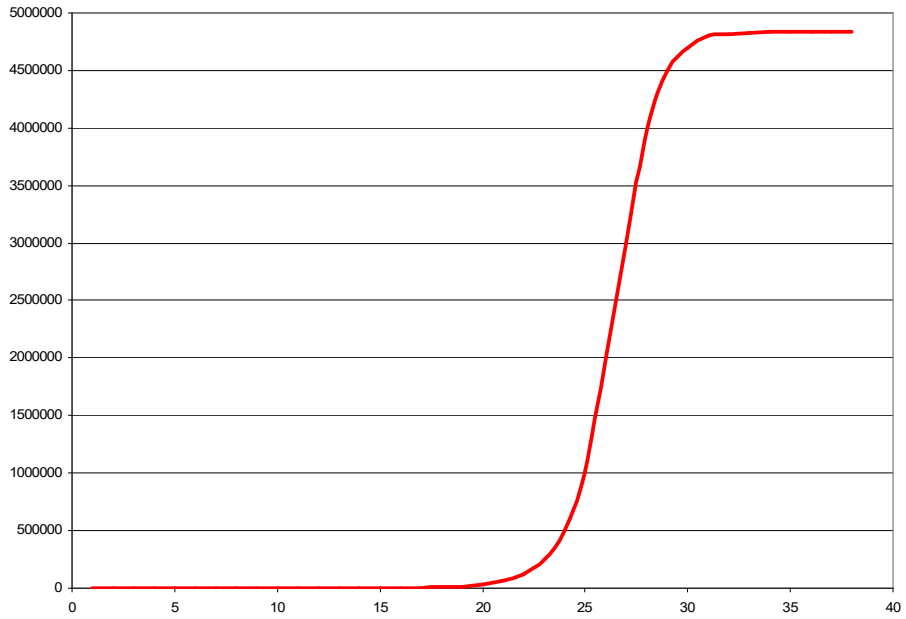
If we plot the amount of DNA in our tube going forward from cycle 25, we see that it actually looks like this:



Imaging Real-Time PCR

Measuring Quantities

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



Imaging Real-Time PCR

Measuring Quantities

Let's imagine that you start with <u>four</u> times as much DNA as I do…picture our two tubes at cycle 25 and work backwards a few cycles.

Cycle 25 →

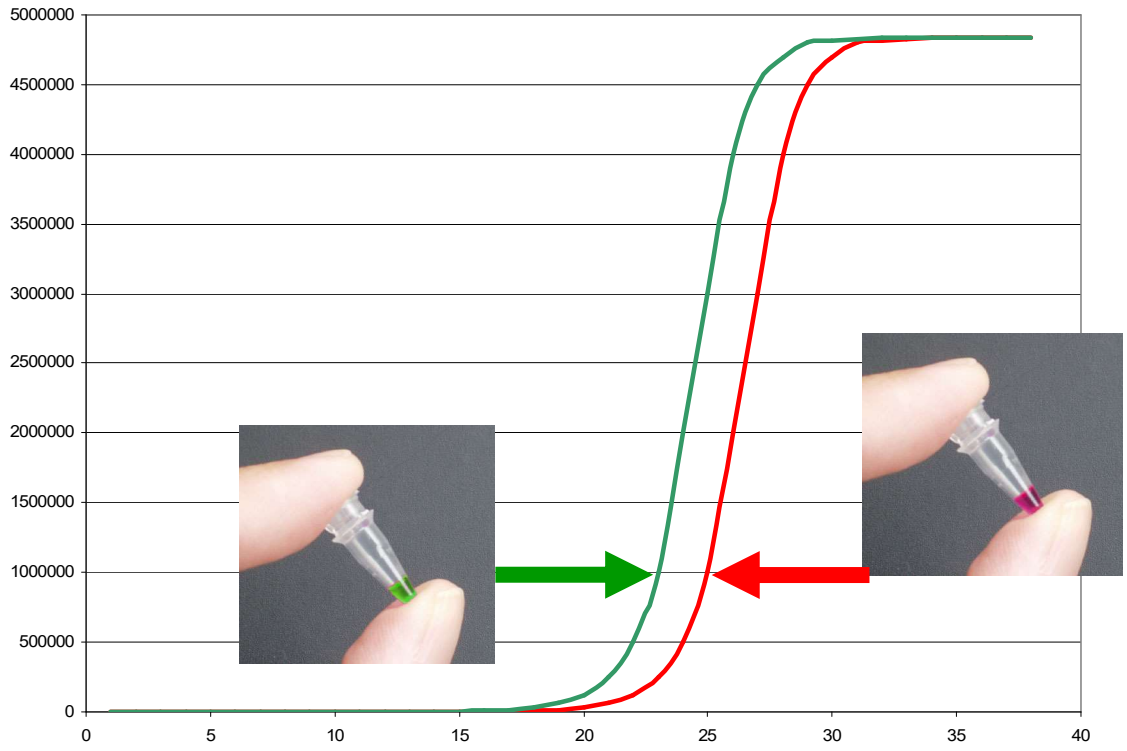
Cycle	Me	You
23	250,000	1,000,000
24	500,000	2,000,000
25	1,000,000	4,000,000

Imaging 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

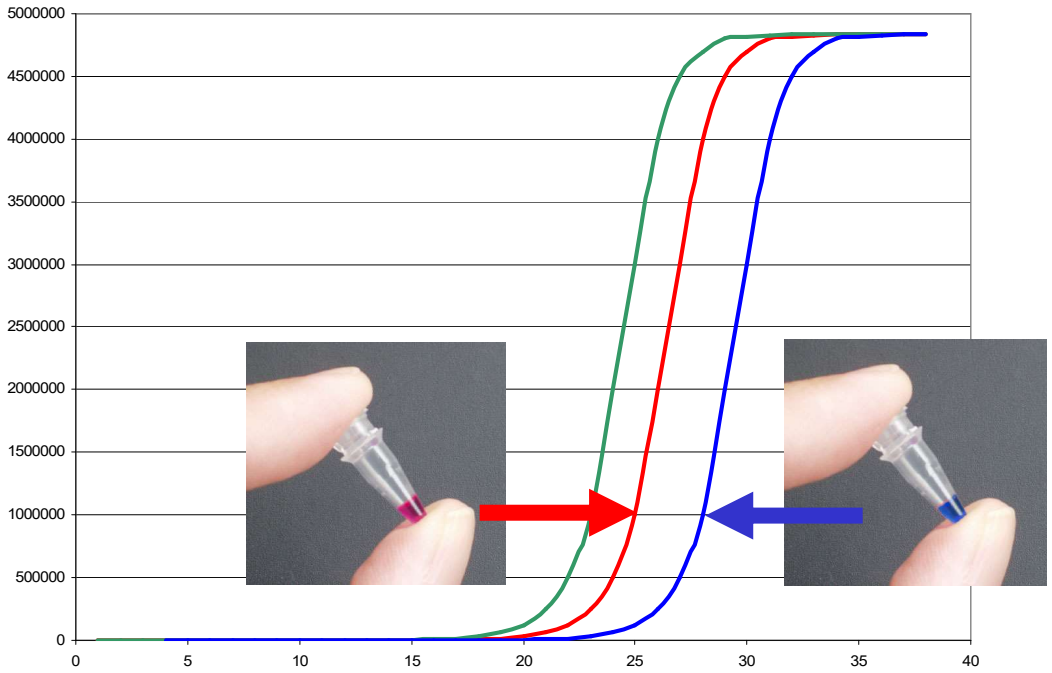
Imagining Real-Time PCR

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!



Imaging Real-Time PCR

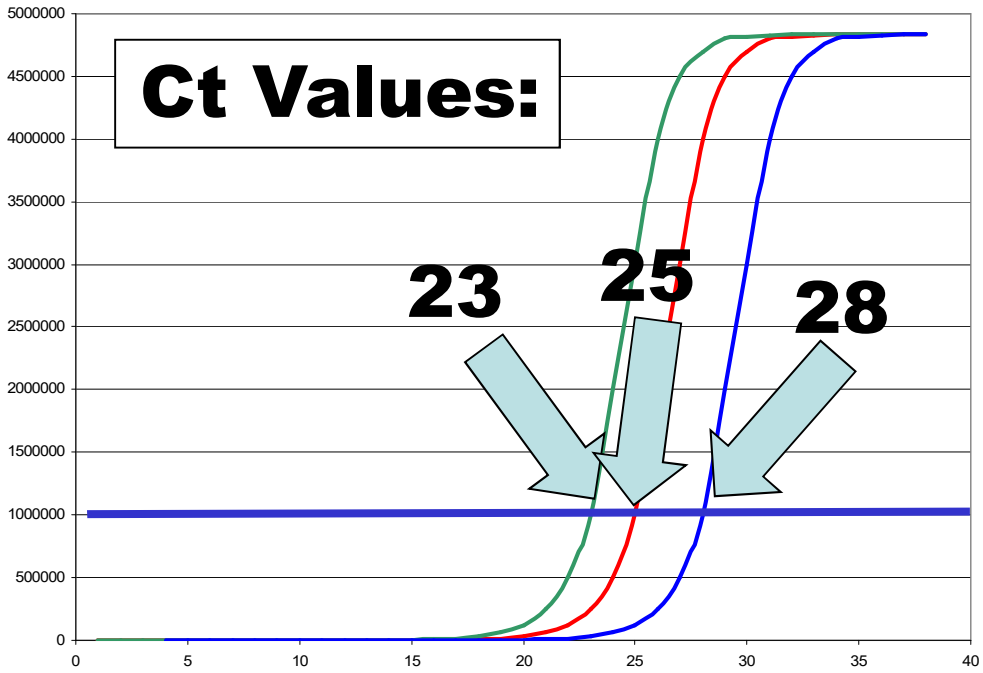
Measuring Quantities

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:

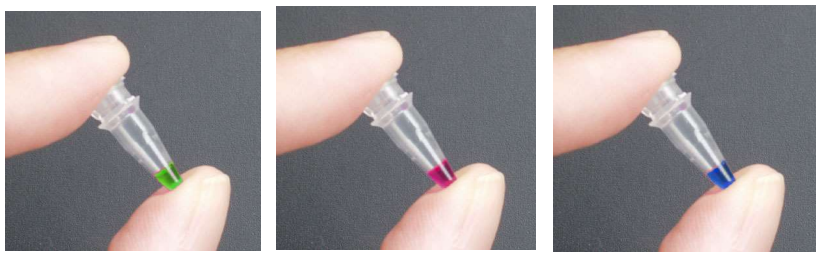
Quantity = 2^{Ct}



Imaging Real-Time PCR

Measuring Quantities

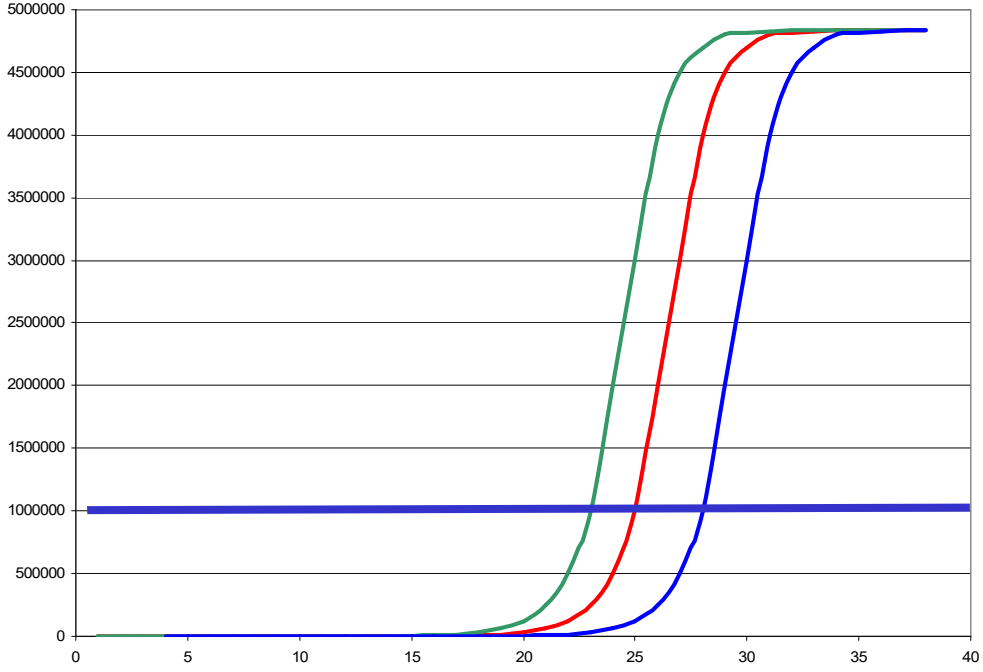
Let's recap…



4 units
Ct=23

1 unit
Ct=25

1/8 unit
Ct=28

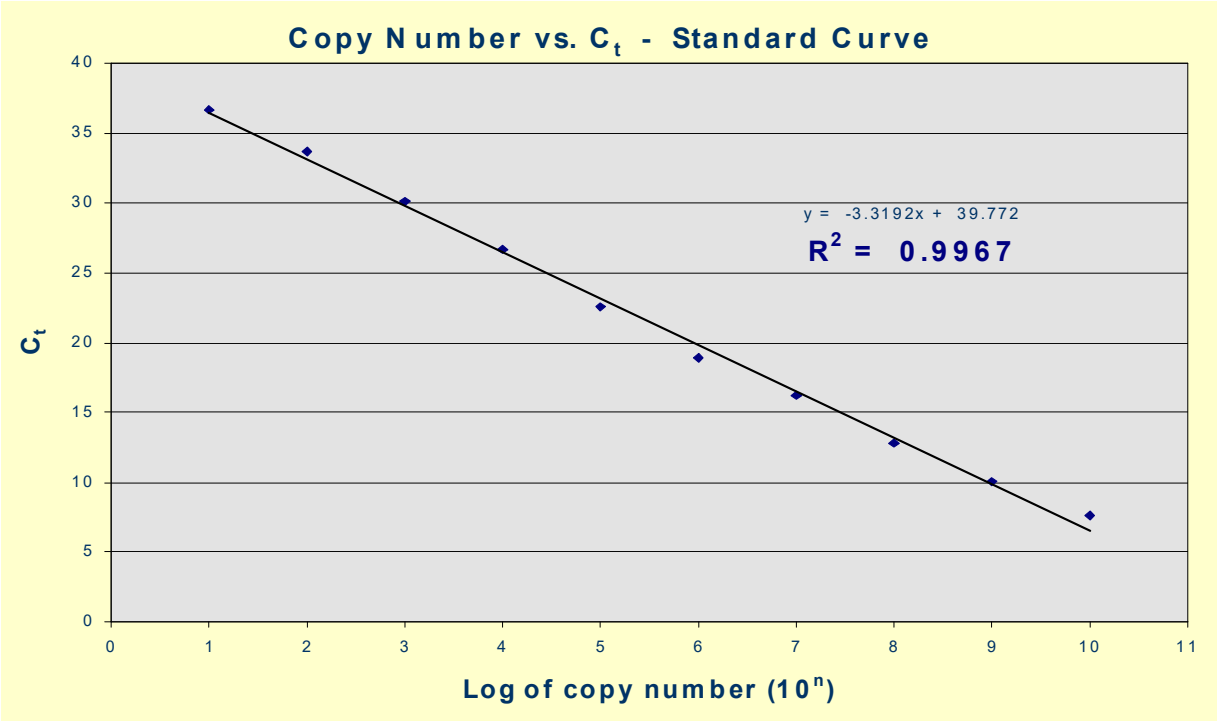


Imaging Real-Time PCR

Measuring Quantities

There's a DIRECT relationship between the starting amount of DNA, and the cycle number that you'll reach an arbitrary number of DNA copies (Ct value).

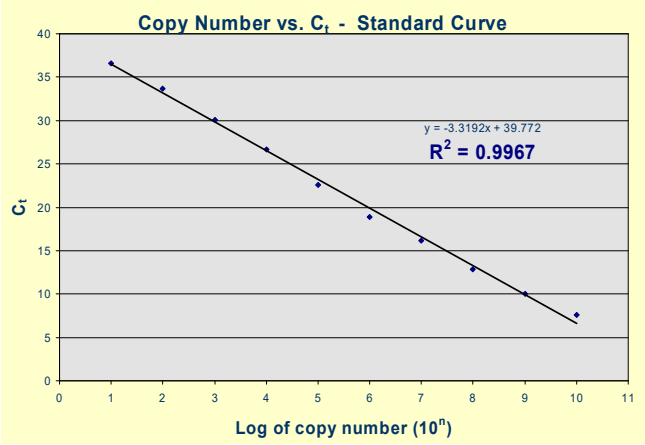
DNA amount ≈ 2 Cycle Number



Imaging Real-Time PCR

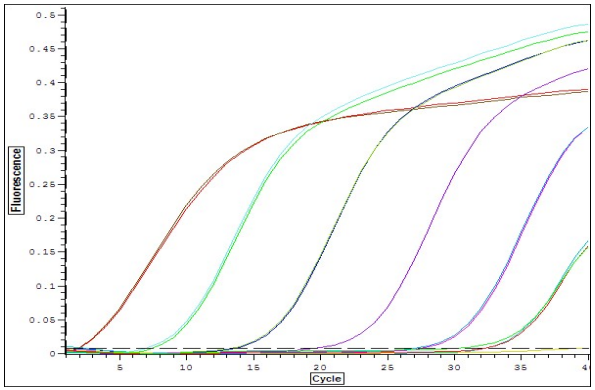
Measuring Quantities

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 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!

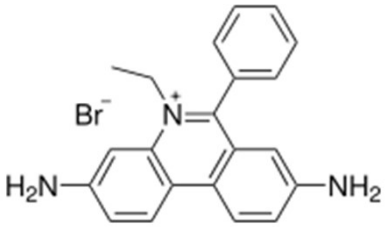
Ethidium bromide and SYBR Green I dye are two such reagents.

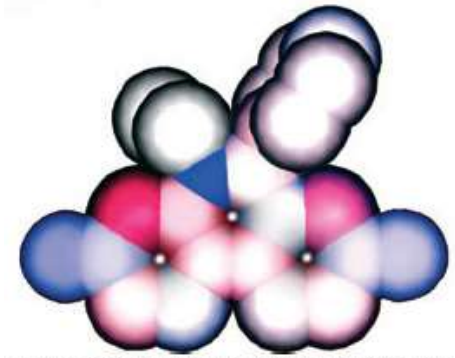
They bind to double-stranded DNA and emit light when illuminated with a specific wavelength.

SYBR Green I dye fluoresces much more brightly than ethidium.

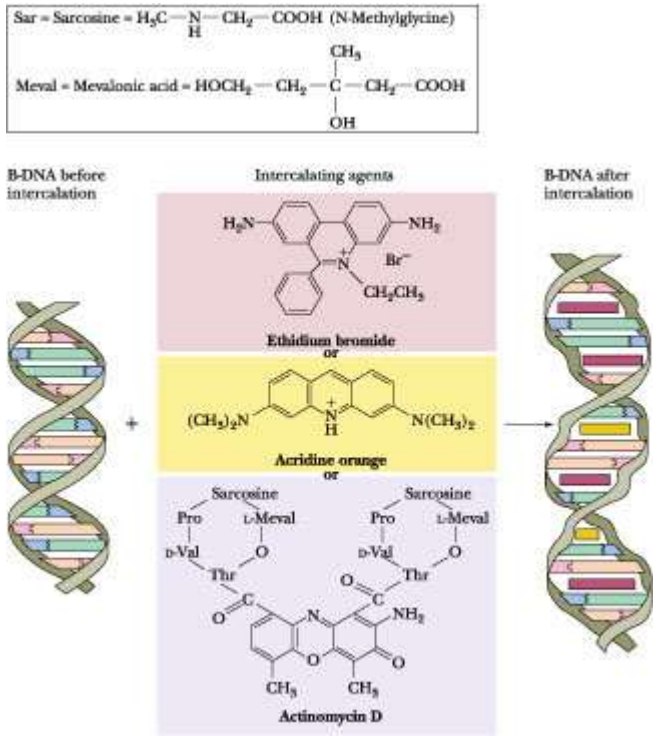
Measuring DNA: Ethidium Bromide

Ethidium Bromide





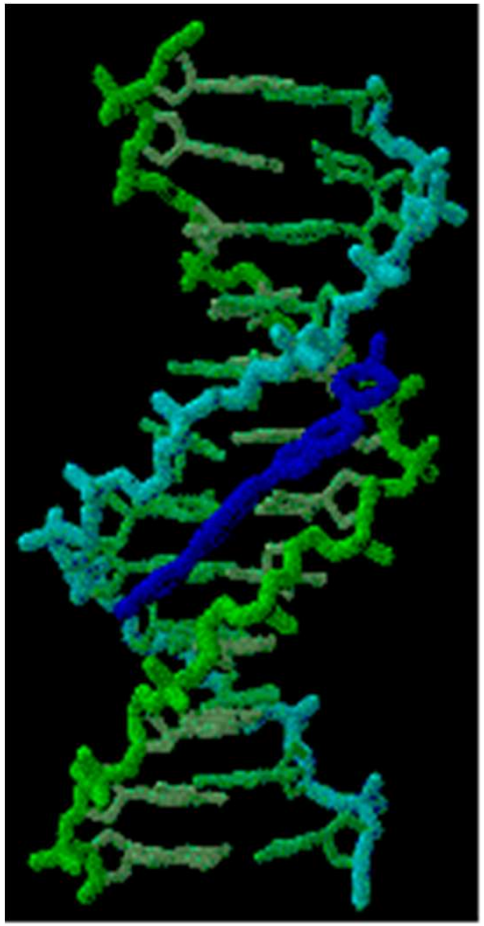
http://www.bioorganic-chemistry.com/ethidium_electronic_text.pdf

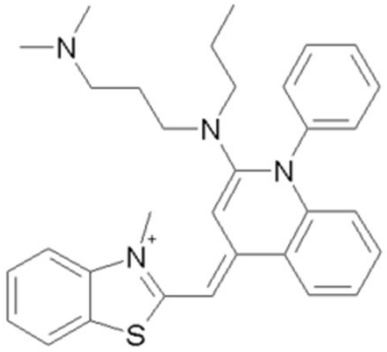


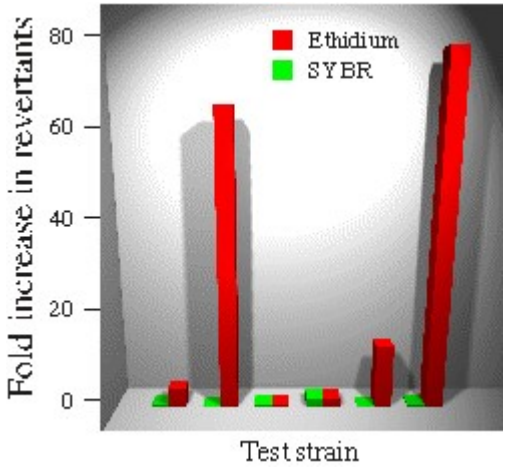
http://www.web.virginia.edu/Heidi/chapter12/chp12.htm

Measuring DNA: SYBR Green I

SYBR Green I



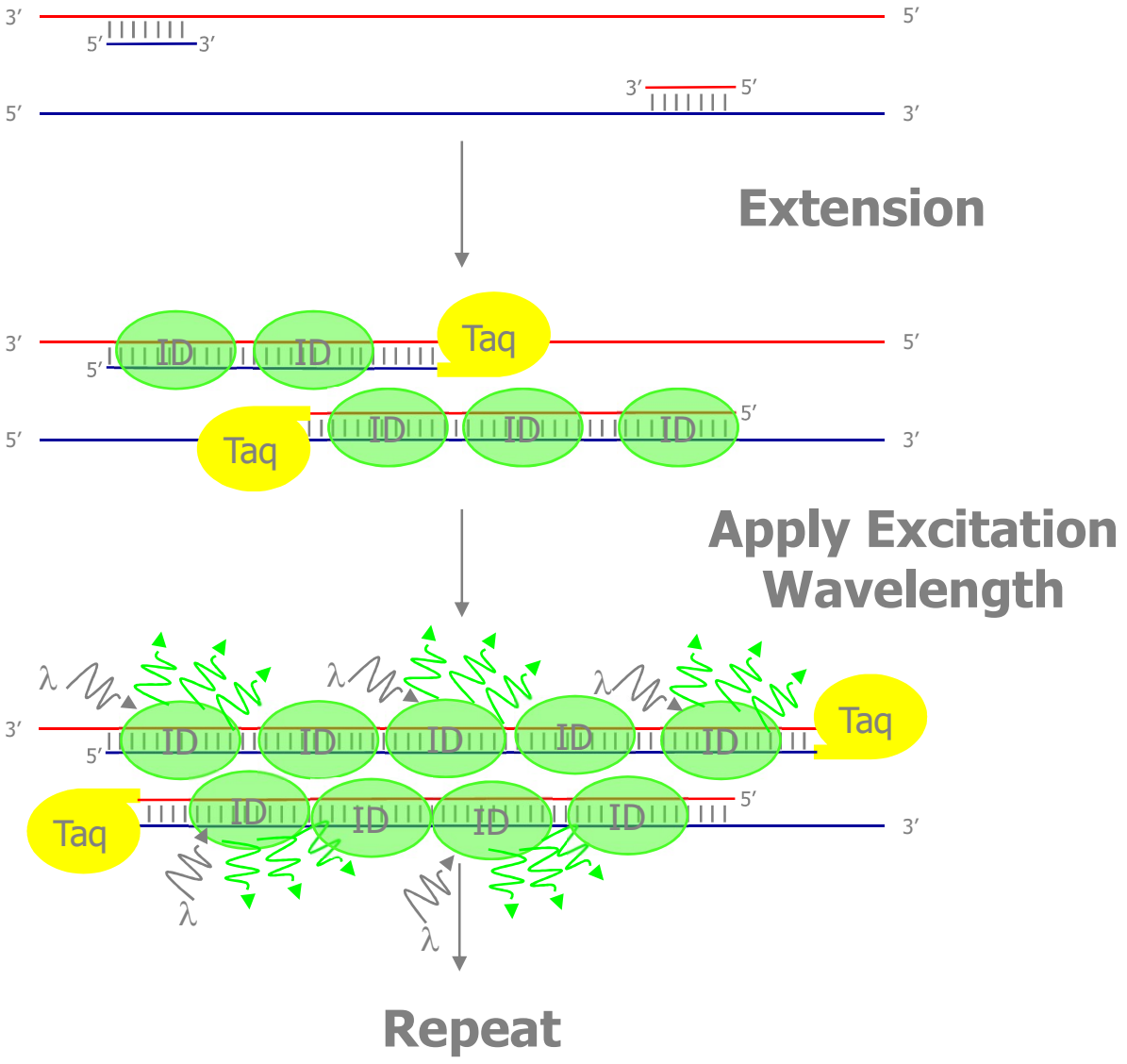




Ames test results from Molecular Probes
Singer et al., Mutat. Res. 1999, 439: 37- 47

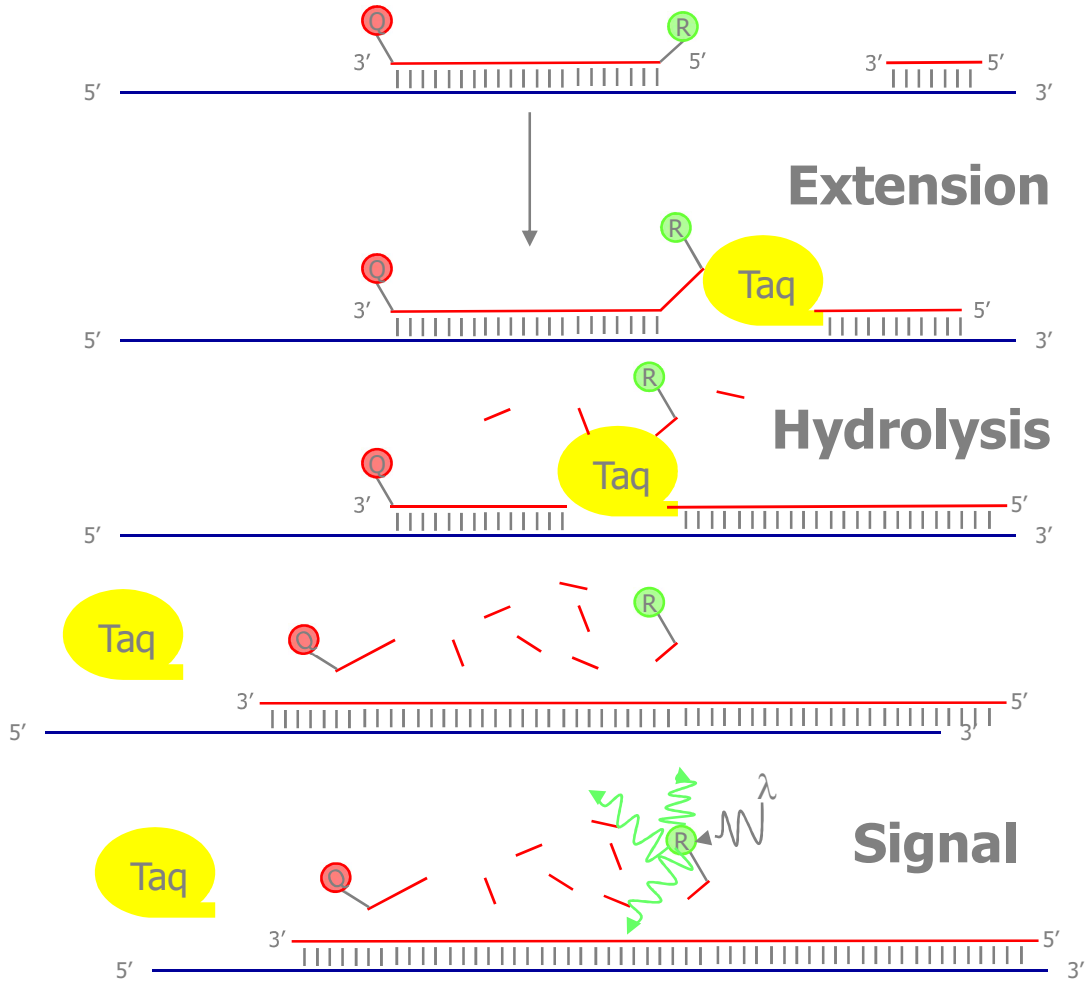
Fluorescent Dyes in PCR

Intercalating Dyes



Fluorescent Dyes in PCR

Probes



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

Real-time PCR instruments consist of THREE main components:

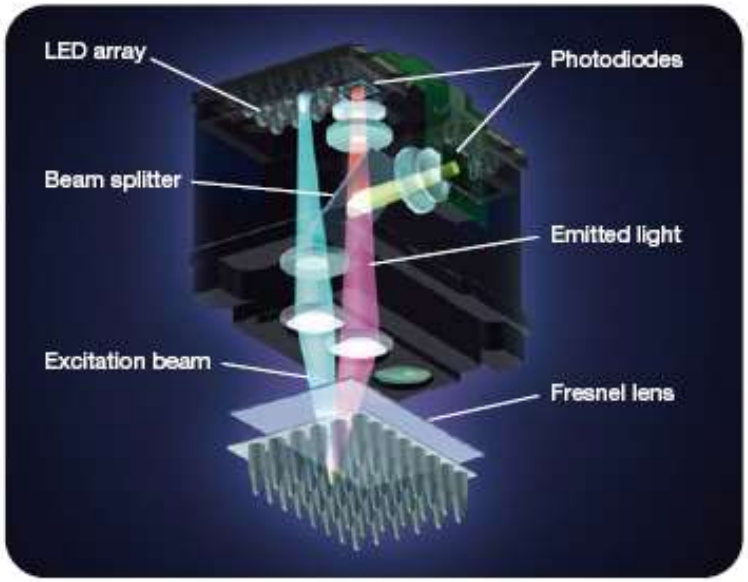
1. Thermal Cycler (PCR machine)
2. Optical Module (to detect fluorescence in the tubes during the run)
3. Computer (to translate the fluorescence data into meaningful results)

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.

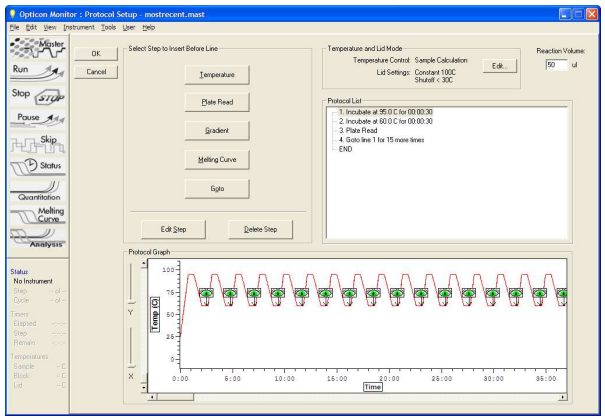


What Type of Software is used with Real-Time PCR?

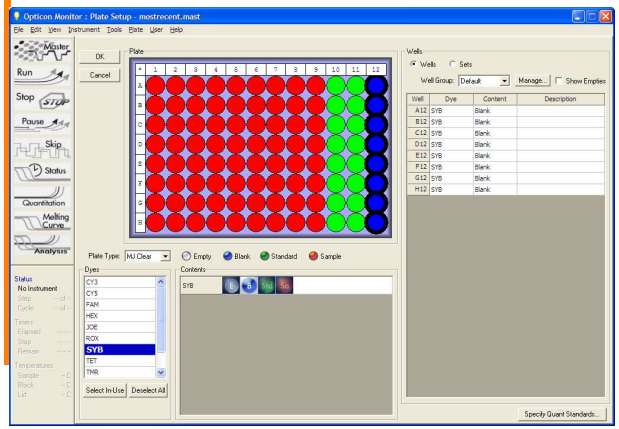
The real-time software converts the fluorescent signals in each well to meaningful data.

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

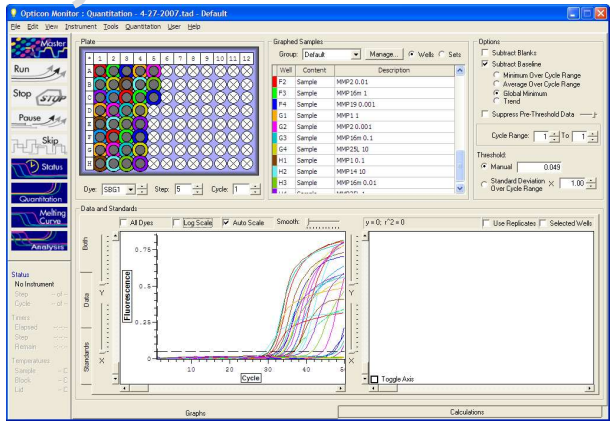
1

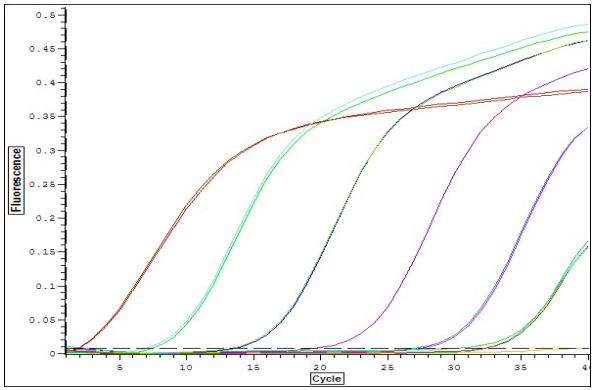


2



3,4





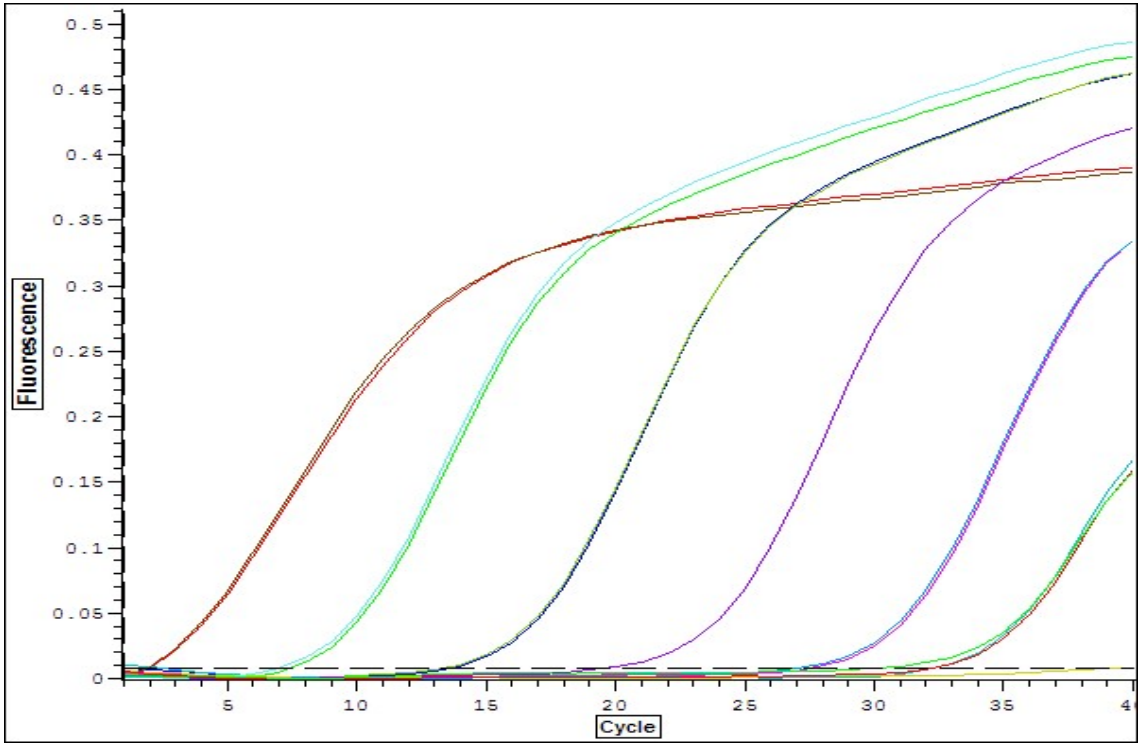
Part 4:

What does real-time data look like?

Real-Time PCR

Actual Data

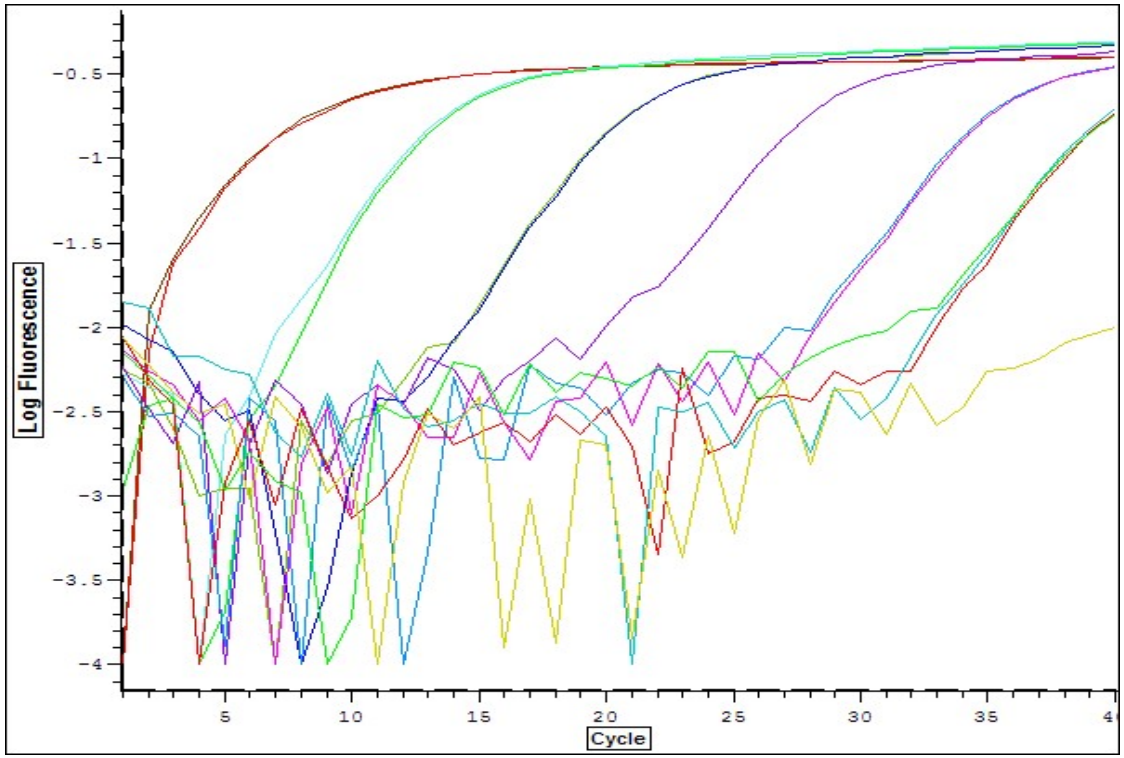
- **This is some actual data from a recent real-time PCR run.**
- **Data like this can easily be generated by preparing a dilution series of DNA.**



Real-Time PCR

Actual Data

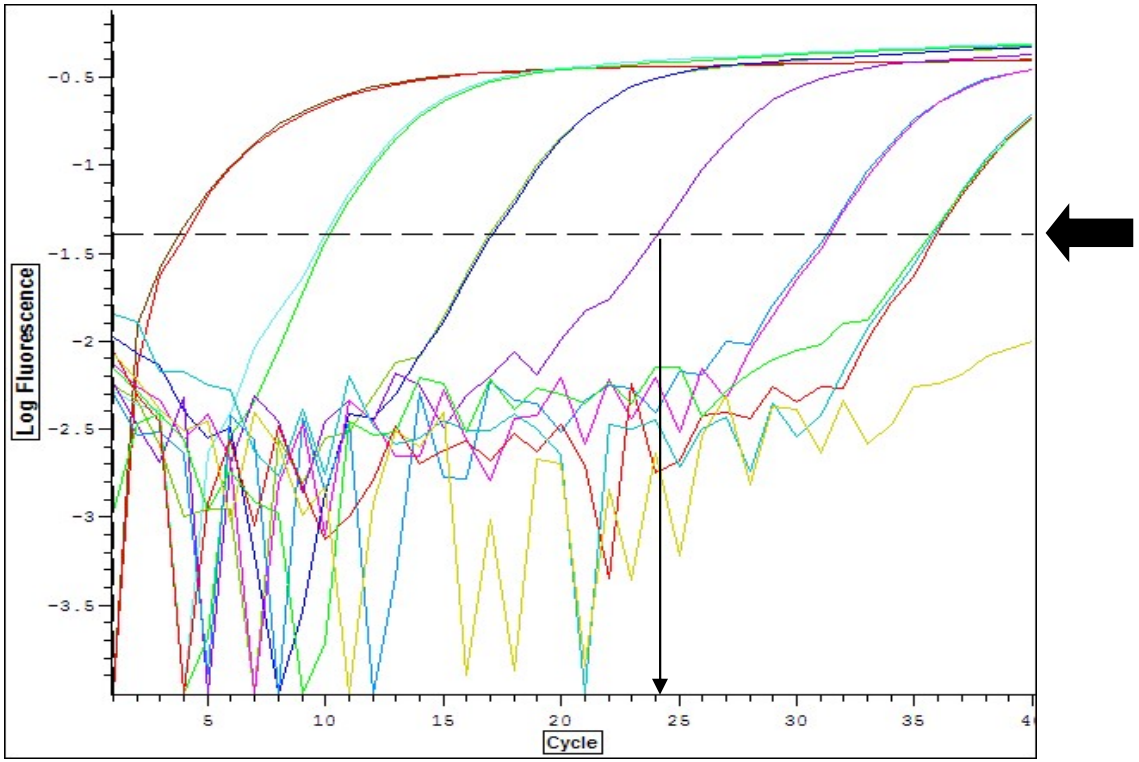
- **The same data set in log view**



Real-Time PCR

Setting Thresholds

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

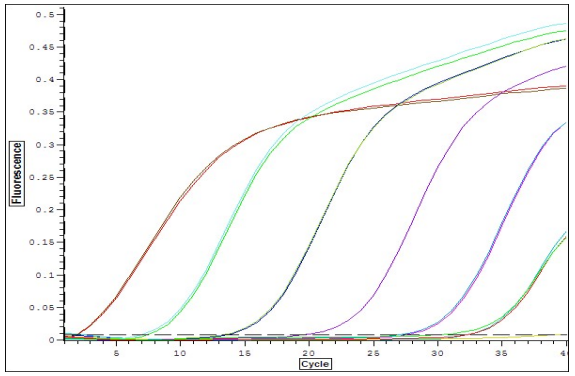


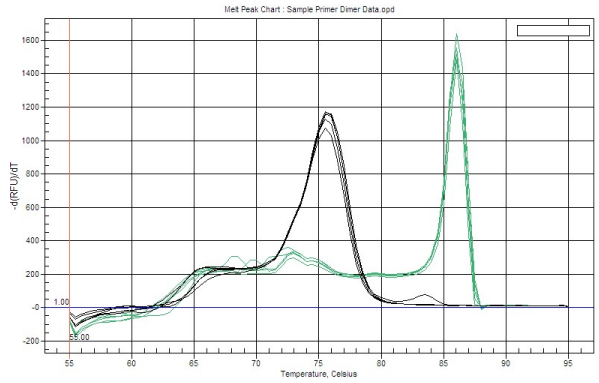
- **Ct values can then be used to calculate quantities of template DNA.**

Real-Time PCR

Actual Data

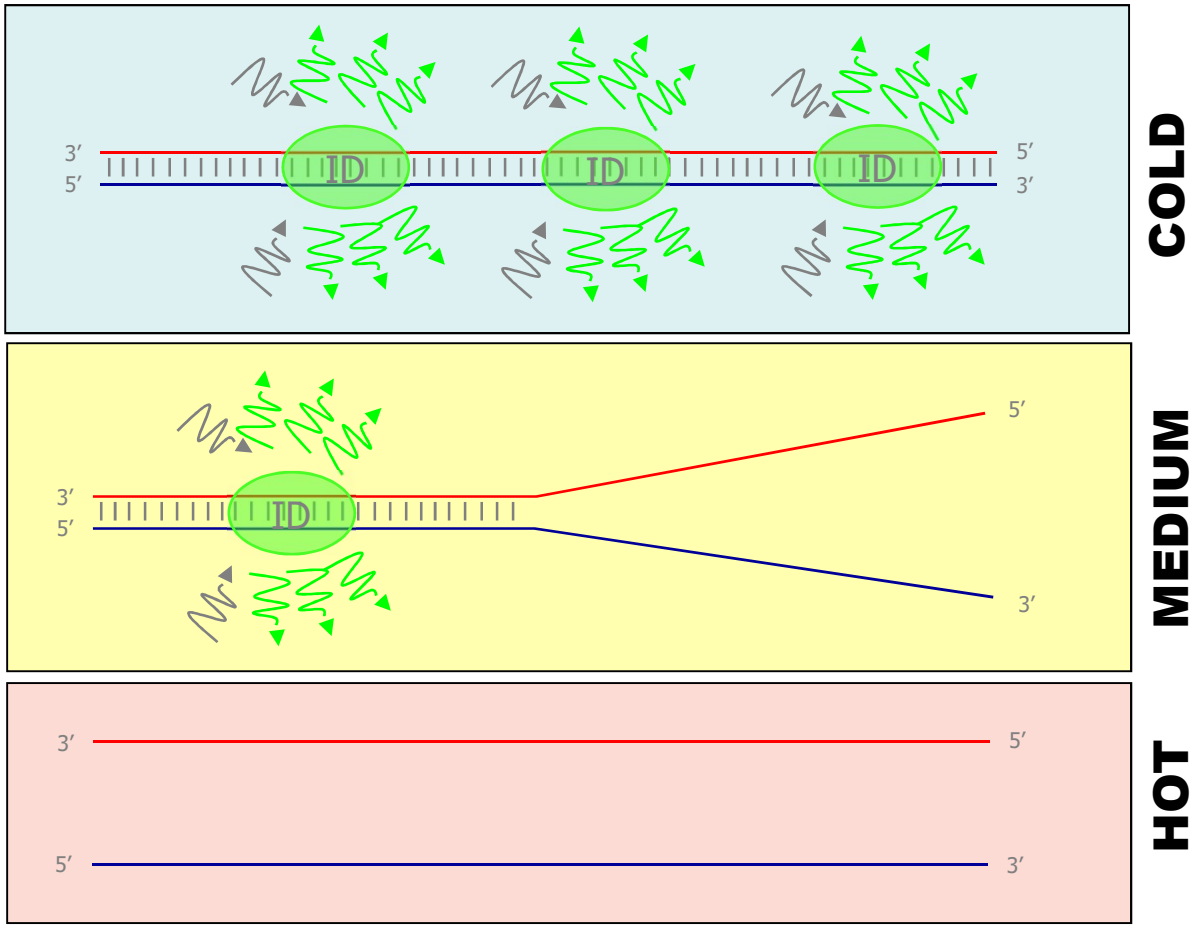
- **The fluorescence data collected during PCR tells us "how much" … but there is another type of analysis we can do that tells us "what"!**





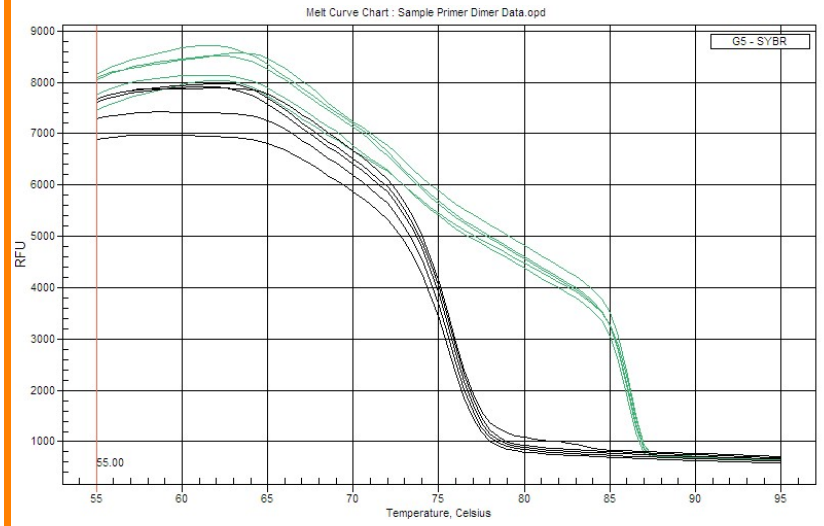
Real-Time PCR – the Concept of MELT CURVES…

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

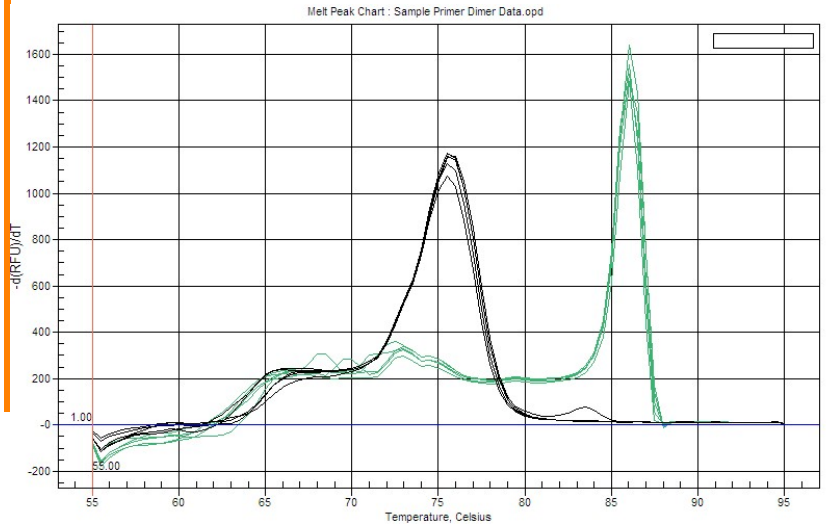


Real-Time PCR – the Concept of MELT CURVES…

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



RFU vs T

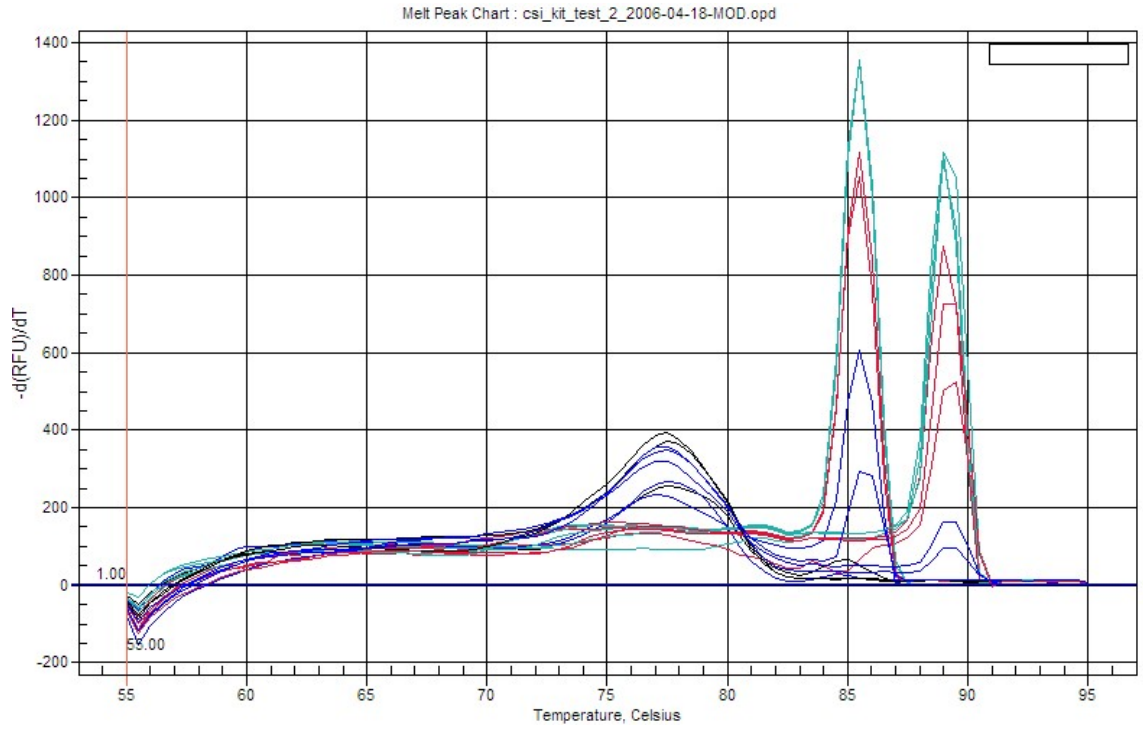


dRFU/dT

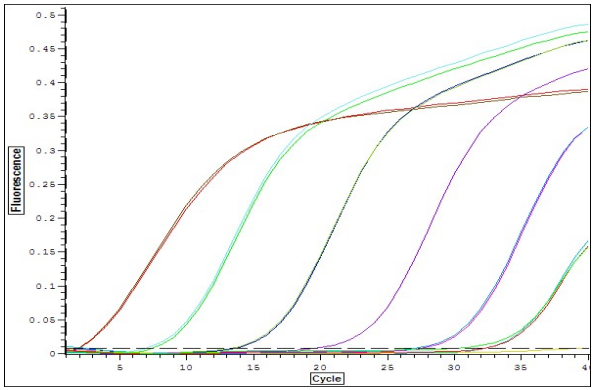
Real-Time PCR

The Concept of MELT CURVES

- **Different amplicons will have different melt peaks.**
- **Primer-Dimers will have a very different melt peak.**



Color key: Green=100X, Red=10000X, Blue=1000000X , Black=NTC.



Part 5:

How can we use the Crime Scene Investigator kit to demonstrate real-time PCR?

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit

An Overview

TYPICAL WORKFLOW

- Introduction to DNA profiling
- Set up PCR reactions
- Electrophorese PCR products
- Analysis and interpretation of results

Target audience

- The Crime Scene Investigator PCR Basics™ Kit is intended to be an introduction to the polymerase chain reaction (PCR)
- Students will have a much better appreciation of the kit if they have some understanding of DNA structure and function

What is DNA profiling?

DNA profiling is the use of molecular genetic methods to determine the exact genotype of a DNA sample in a way the results can basically distinguish one human being from another

The unique genotype of each sample is called a DNA profile.

Since humans are 99.9% identical where do crime scene investigators look for differences in DNA profiles?

Crime Scene Investigators search in areas of the genome that are unique from individual to individual and are "anonymous" (control no known trait or function)
The areas examined are Short Tandem Repeats or STR's

STR region

Example of an STR

The TH01 locus contains repeats of TCAT.

CCC TCAT TCAT TCAT TCAT TCAT TCAT AAA

This example has 6 TCAT repeats.

There are more than 20 known TH01 alleles.

Each individual inherits 1 allele from each parent.

Determining genotypes for individuals using STRs

Ms. Smith's TH01 locus for her two chromosomes is given below.

What is her genotype?

MOM'S CHROMOSOME

CCC TCAT TCAT TCAT TCAT TCAT TCAT AAA

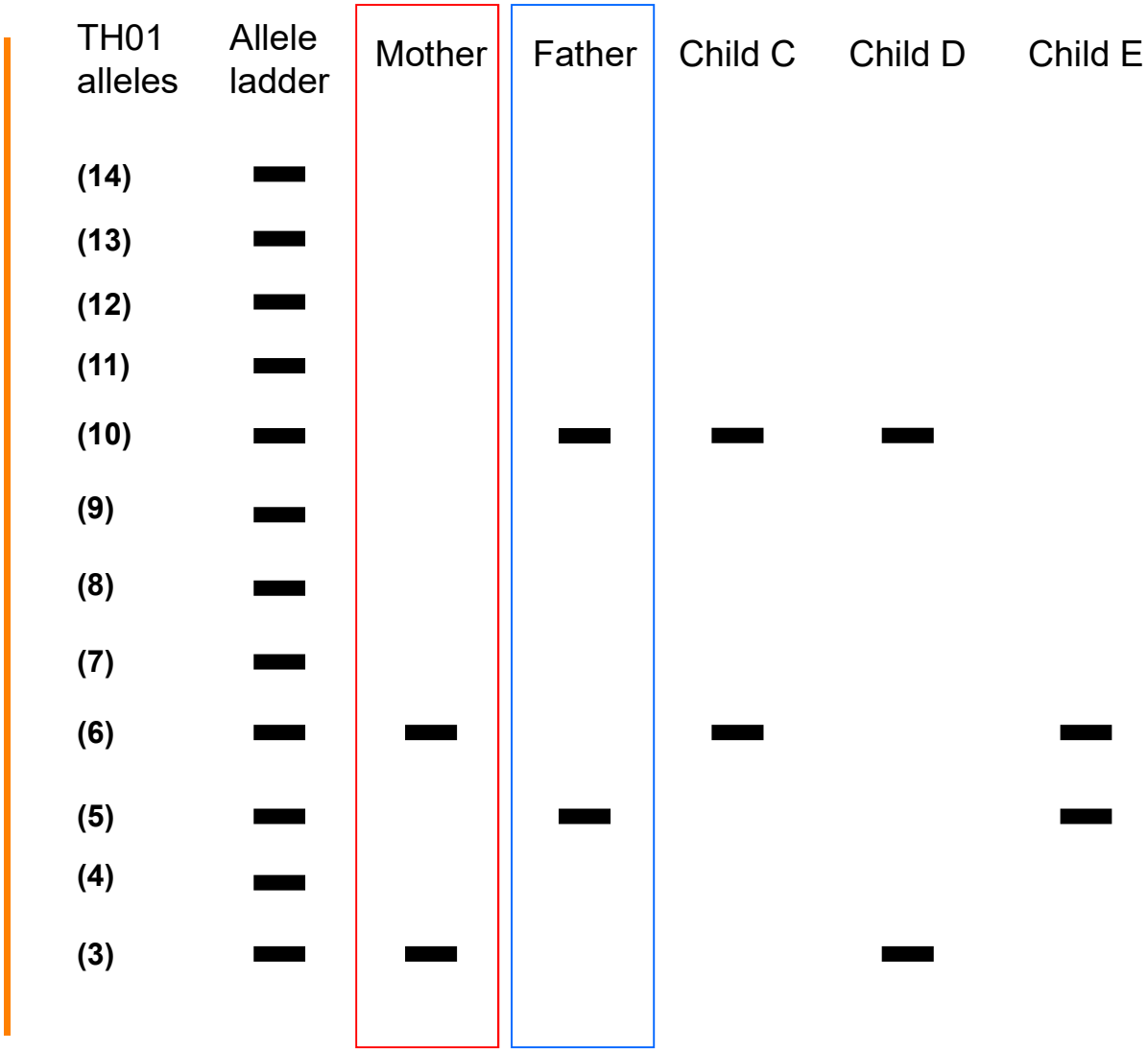
DAD'S CHROMOSOME

CCC TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT TCAT AAA

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

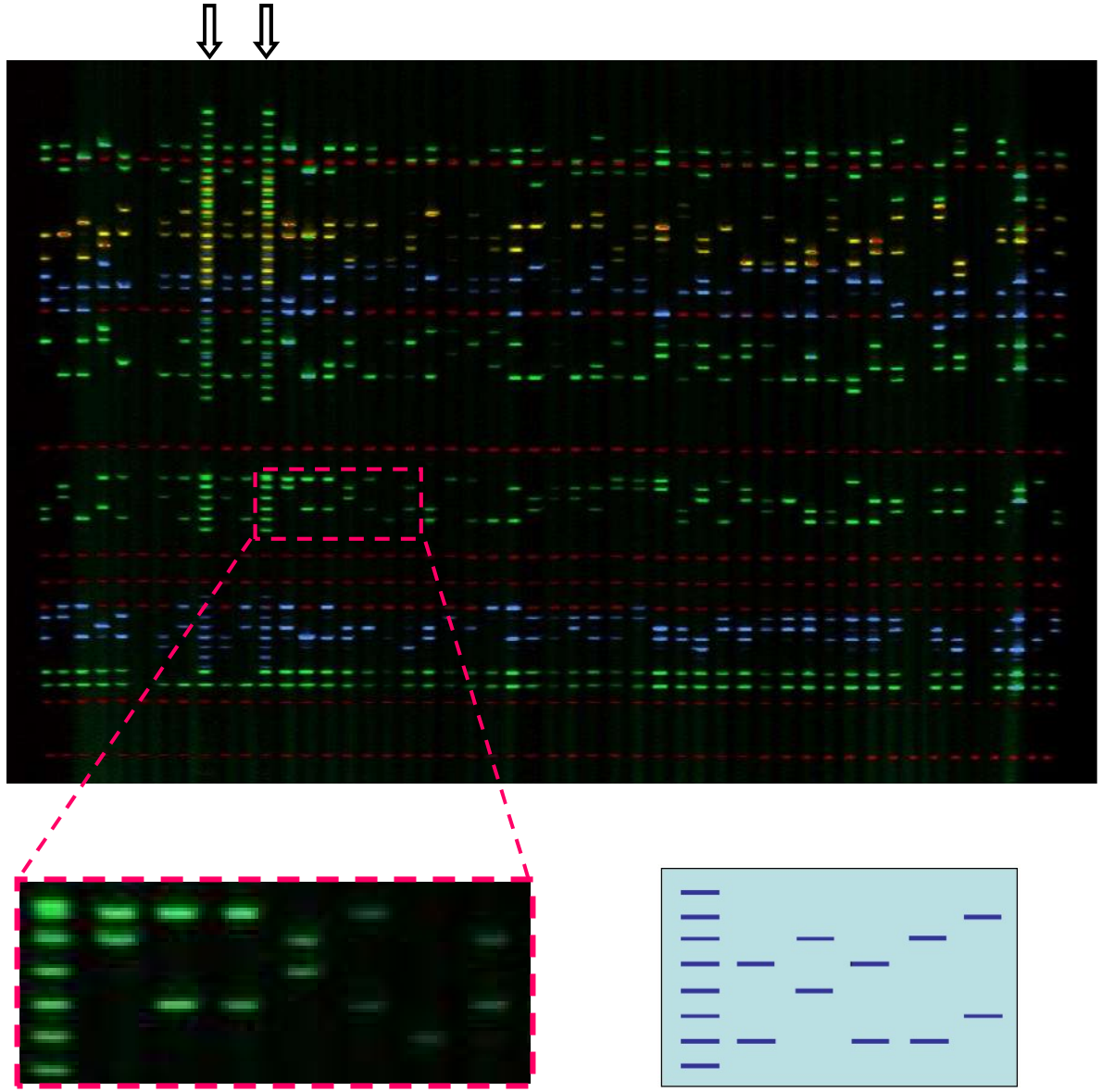
To visualize PCR products Crime Scene investigators use gel electrophoresis



Real STR analysis

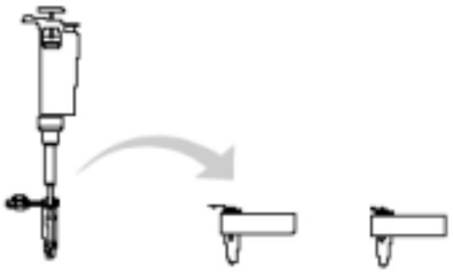
Four different fluorescent tags have been used to identify 7 amplified loci

Allele ladders are indicated by arrows



How the Crime Scene Kit works:

Set up PCR reactions

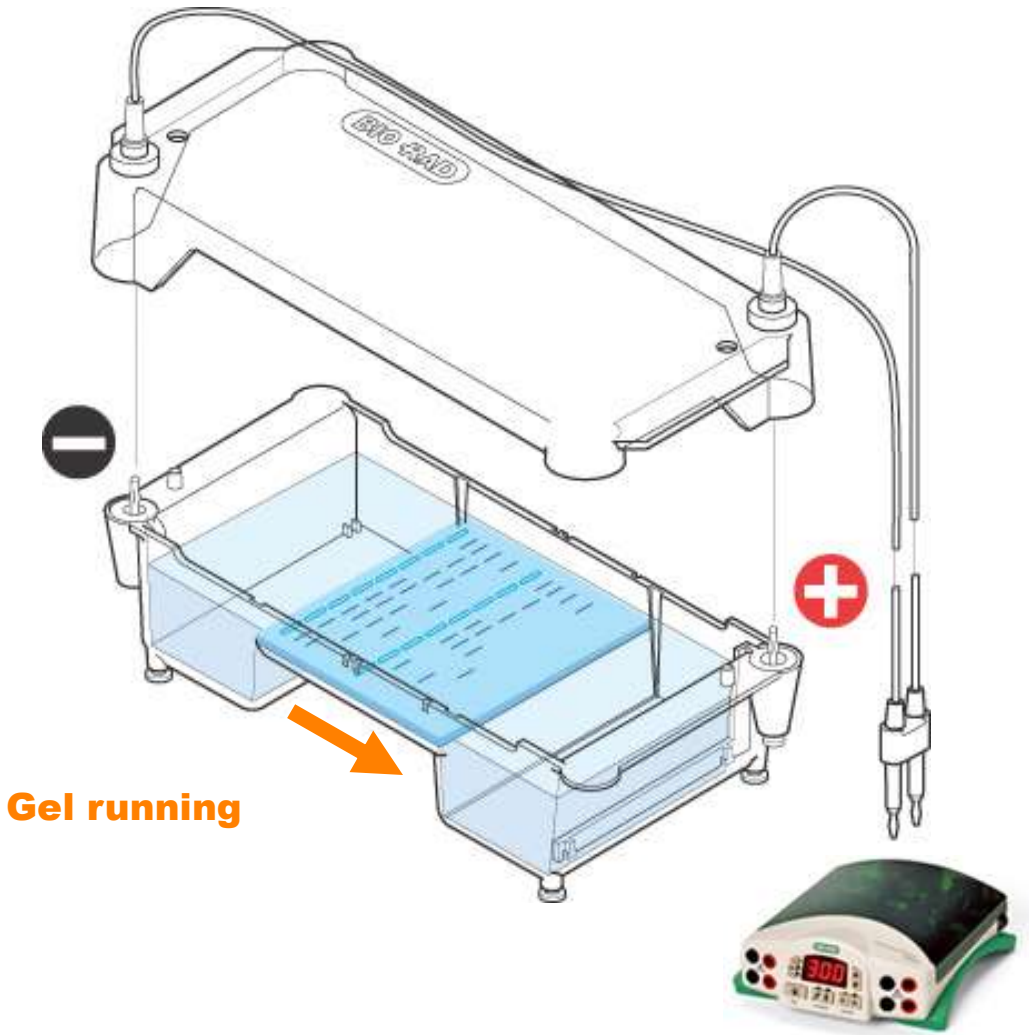


1. Find the PCR tubes at your station. Label them ‘CS’ for Crime Scene DNA, ‘A’ for Suspect A DNA, ‘B’ for Suspect B DNA, ‘C’ for Suspect C DNA, and ‘D’ for Suspect D DNA.
2. Keeping the tubes on ice, add 20 µl of Master Mix + blue primers to each tube.
3. Keeping the tubes on ice, add 20 µl of each DNA to the appropriately labeled tube.
4. USE A FRESH TIP EACH TIME!
5. Mix and put in thermal cycler
6. Cycle ~3 hours

Agarose Electrophoresis Running

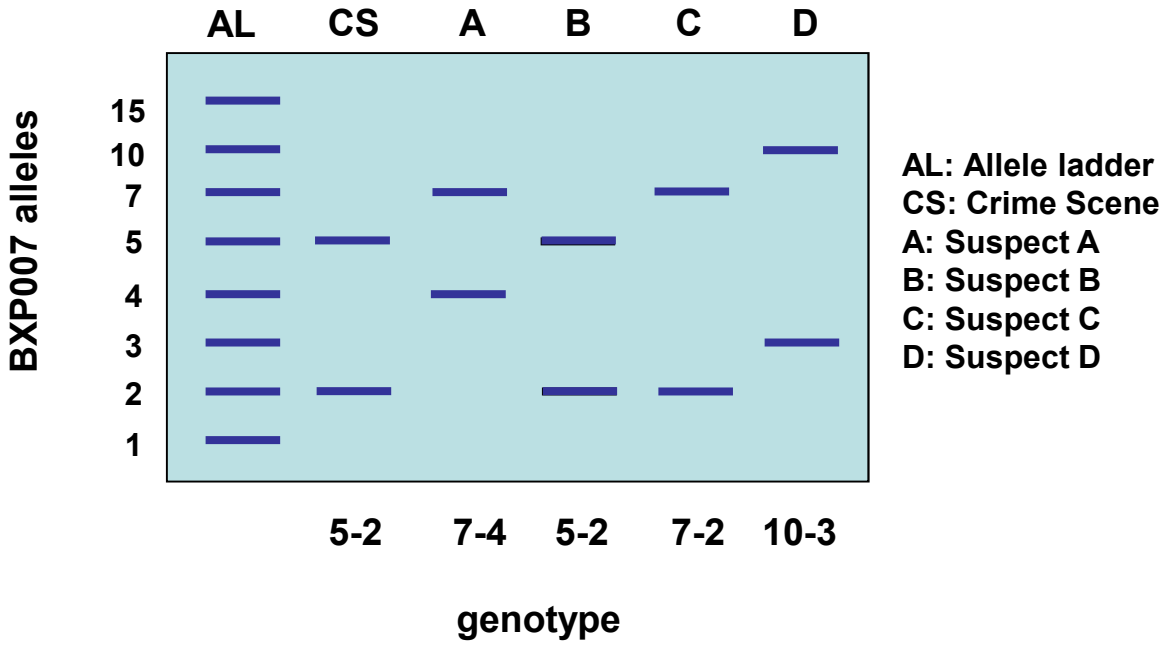
Agarose gel sieves
- DNA fragments according to size
  - Small fragments move farther than large fragments

Use a 3% gel to separate small fragment sizes



Analysis of Results:

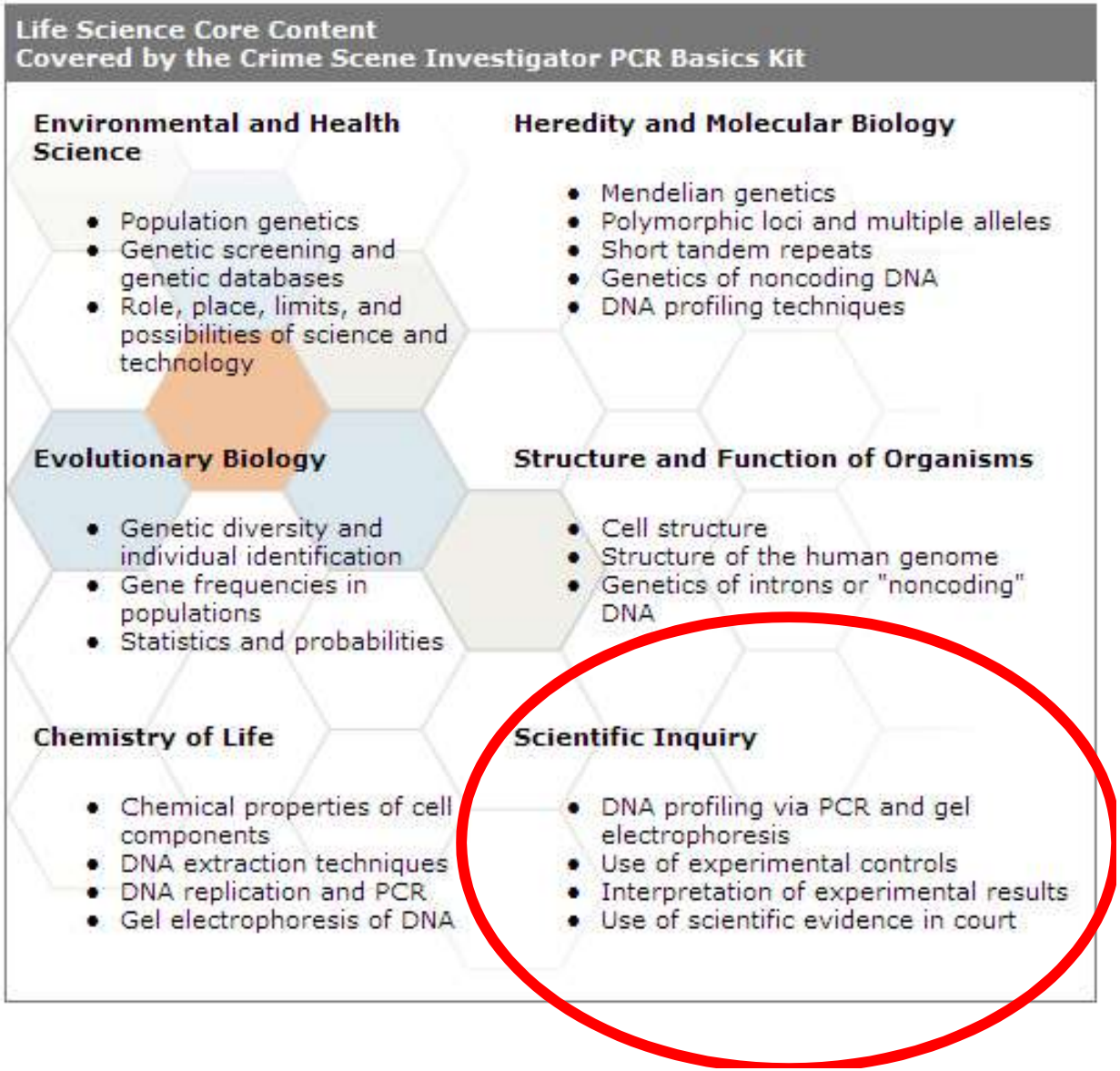
Who can't be excluded?



Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Core Content (Crime Scene Kit)



Crime Scene Investigator Kit

So how can we use the Crime Scene Kit to perform real-time PCR???

Two options…

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 1

- Introduction to DNA profiling
- Set up PCR reactions on a real-time PCR instrument, using real-time reagents
- Electrophorese PCR products
- Analysis and interpretation of results

Simply add this step

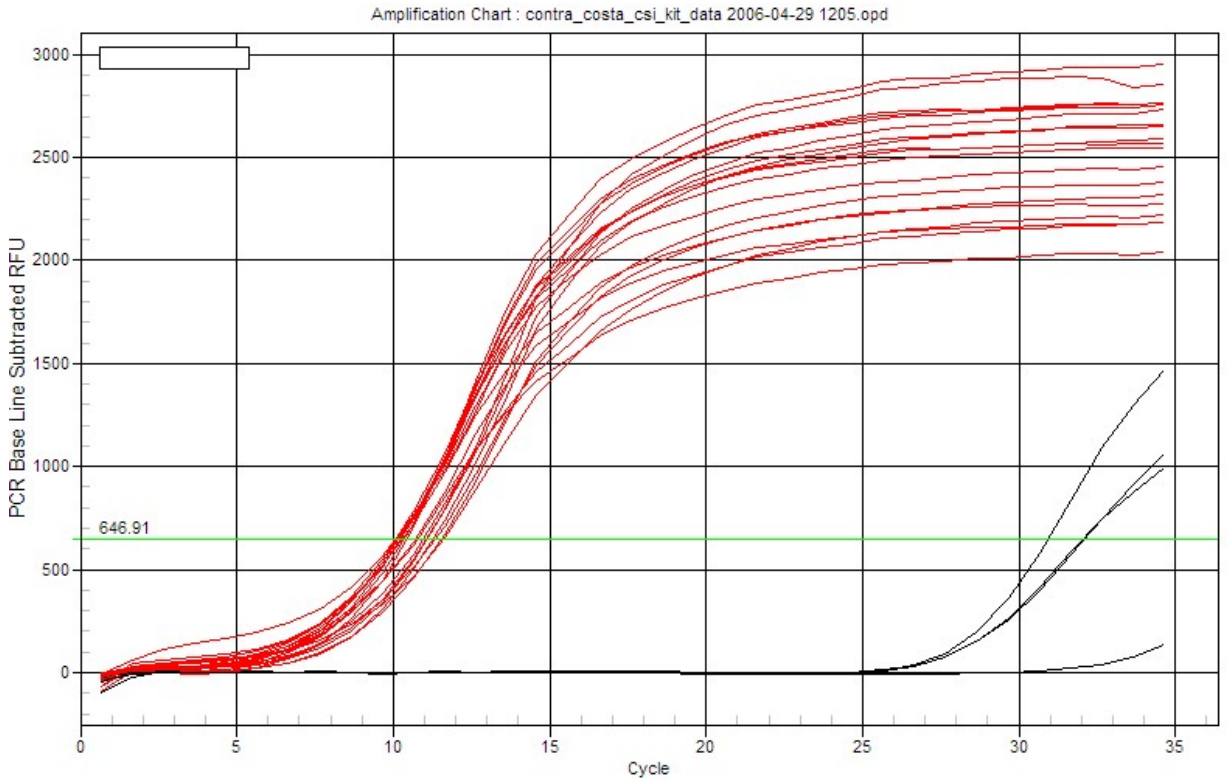
Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 1

- **View the Crime Scene PCR reactions as they occur in real-time!**



Contra Costa College, May 2006

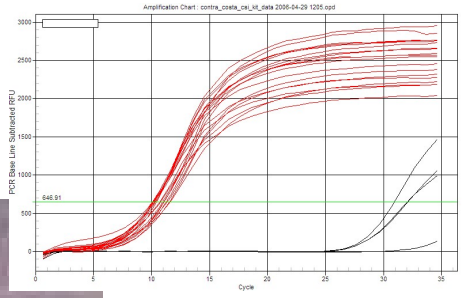
Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

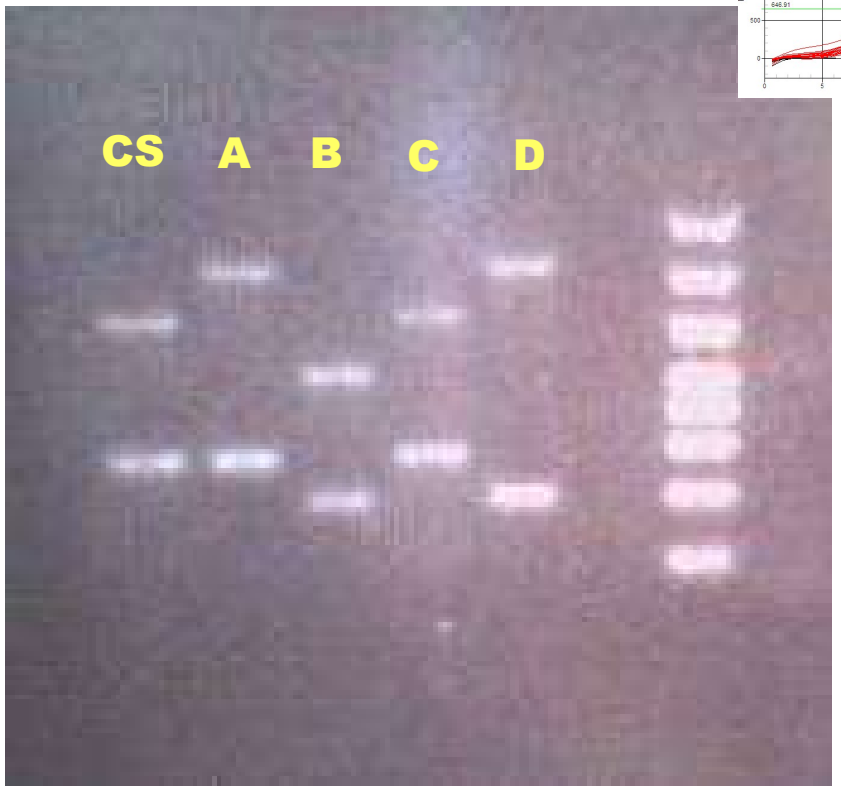
BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 1

- View the Crime Scene PCR reactions as they occur in real-time!





Contra Costa College, May 2006

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 2

- Introduction to DNA profiling
- Set up PCR reactions
- Electrophorese PCR products
- Analysis and interpretation of results

Entirely new protocol.

Use the kit components for a complete Real-Time PCR demonstration…

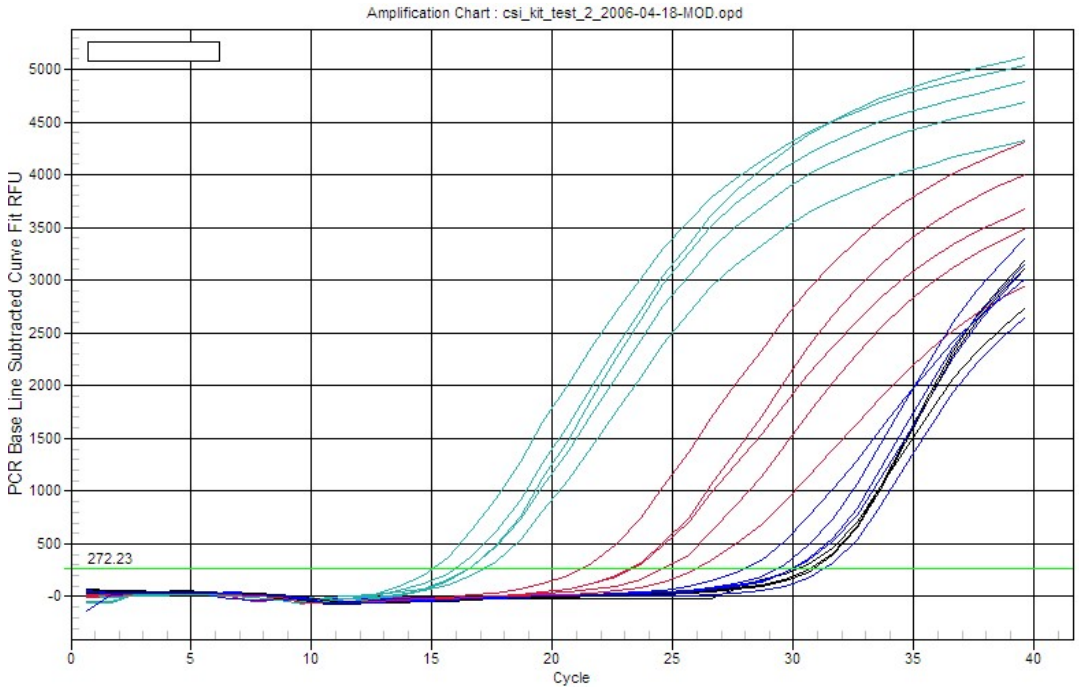
Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 2

- **Use the Crime Scene PCR kit as a source for reliable target DNA and primers.**
- **Use a modified protocol:**
  - **Dilute Crime Scene DNA provided with the kit 100, 10000, 1000000 fold.**
  - **Run reactions with iQ SYBR Green Supermix on a real-time PCR instrument.**

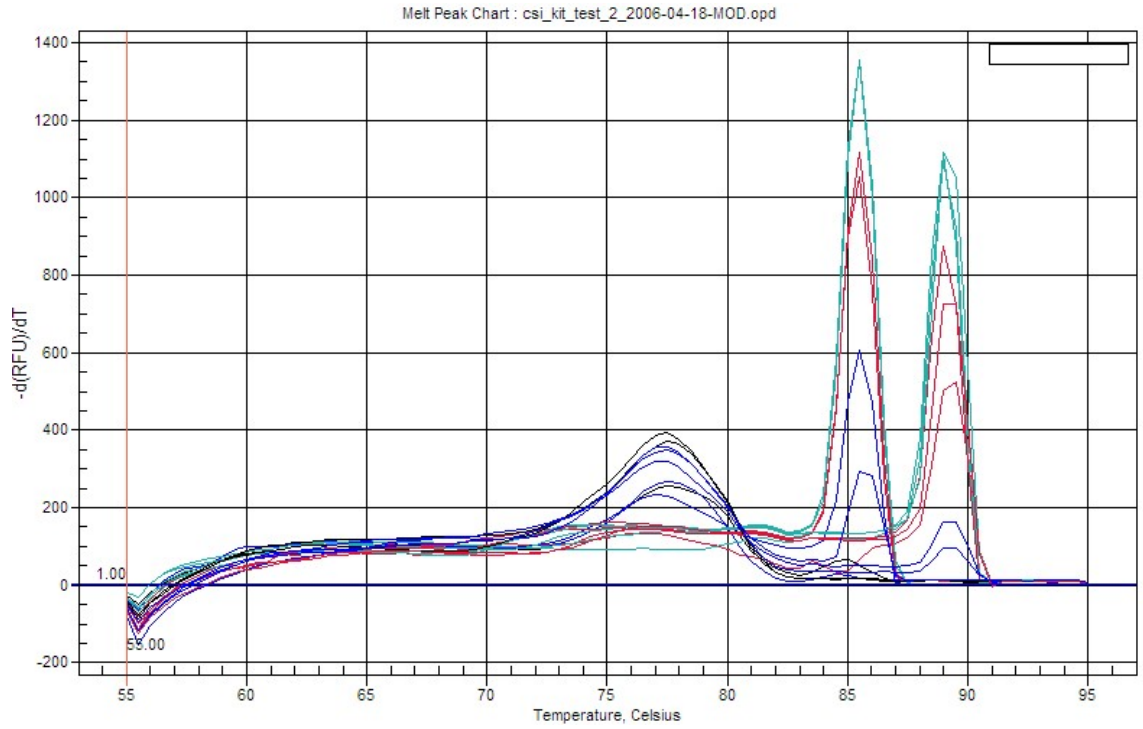


Color key: Green=100X, Red=10000X, Blue=1000000X , Black=NTC.

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 2

- **Use the Crime Scene PCR kit as a source for reliable target DNA and primers.**
- **If different DNA samples are used, interesting melt curves result because of the different amplicons in the kit:**

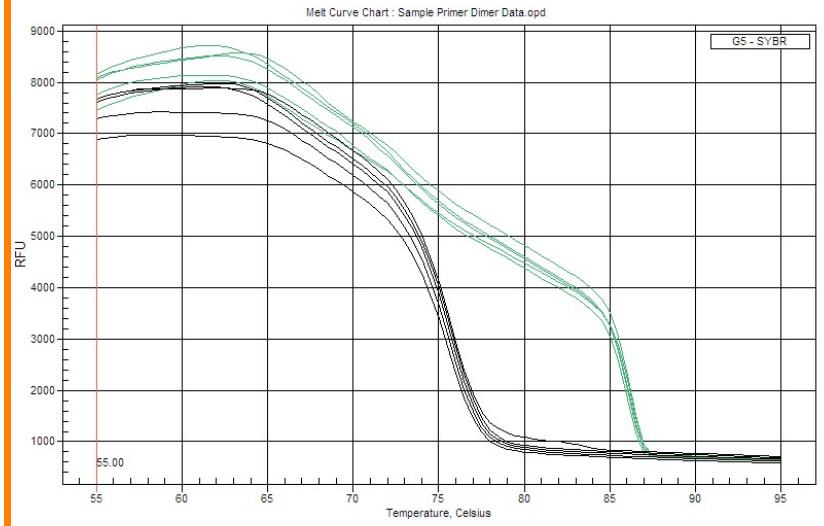


Color key: Green=100X, Red=10000X, Blue=1000000X , Black=NTC.

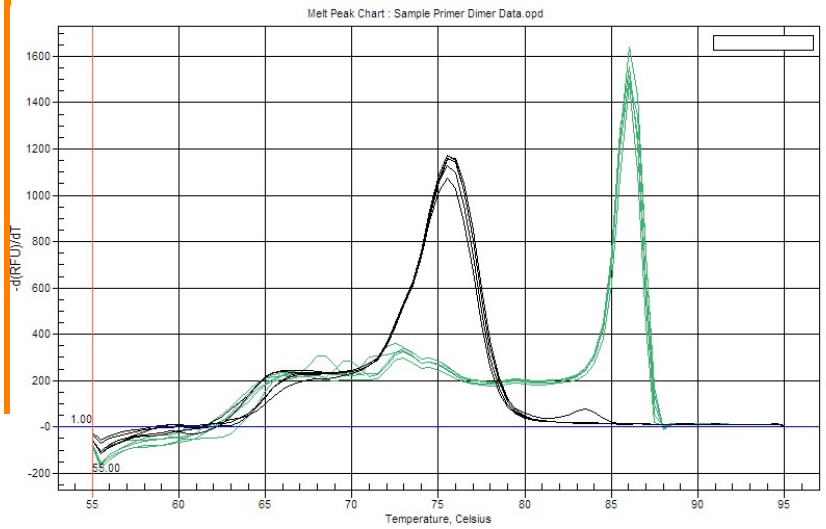
Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 2

- **SIDEBAR: Melt Curve Theory**



RFU vs T



dRFU/dT

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator PCR Basics Kit in REAL-TIME!

Option 2

- **Learning Points**
  - Viewing PCR reactions as they occur in real-time
    - Exciting!
  - Using real-time PCR to quantify DNA
    - Basis of gene expression analysis, disease diagnosis, etc.
  - Measuring pipetting variation
    - Run samples in duplicate for an easy test of reproducibility
  - Importance of experimental controls
    - No template control and positive controls
  - Melt curve analysis
    - Tie concepts of the basic structure of DNA with visible evidence that two strands can anneal and melt.

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Crime Scene Investigator Kit in Real-Time !

- **To run either of the two options, ONLY two additional items are needed!**
- **iQ SYBR Green Supermix**

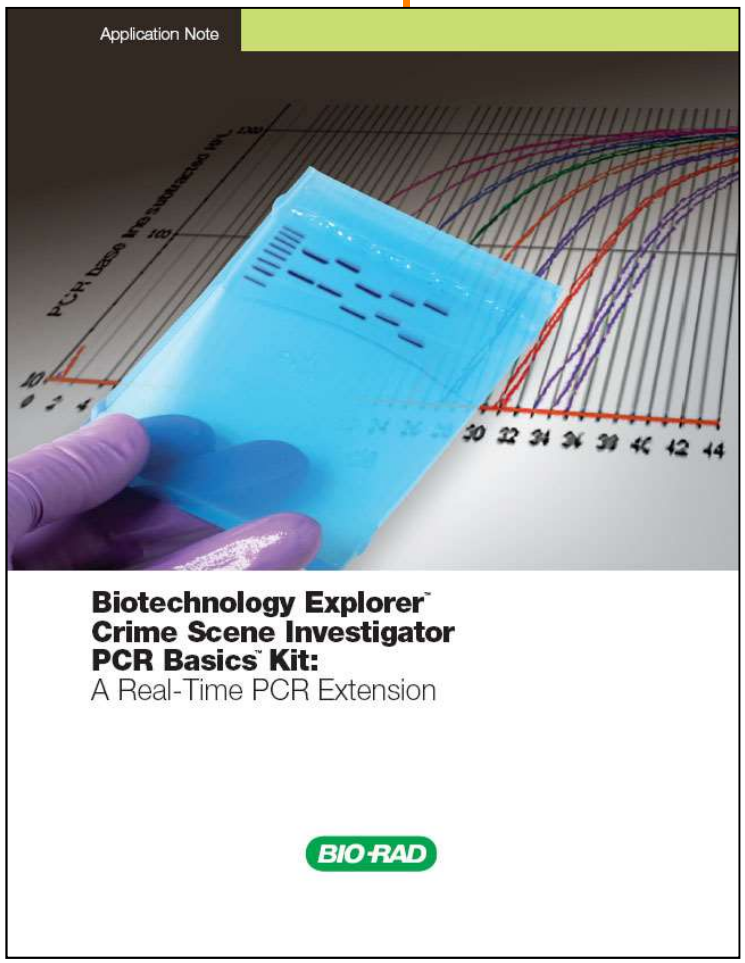
iQ SYBR Green supermix

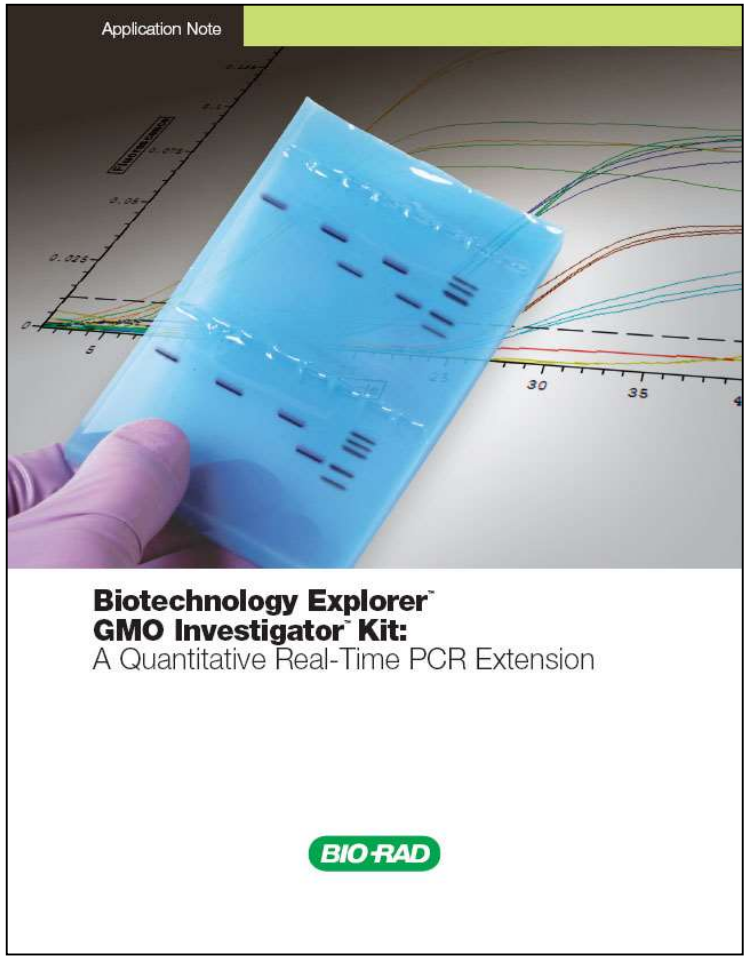
- **A real-time PCR instrument**

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

- **Two Applications Notes are available:**





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: Step-By-Step

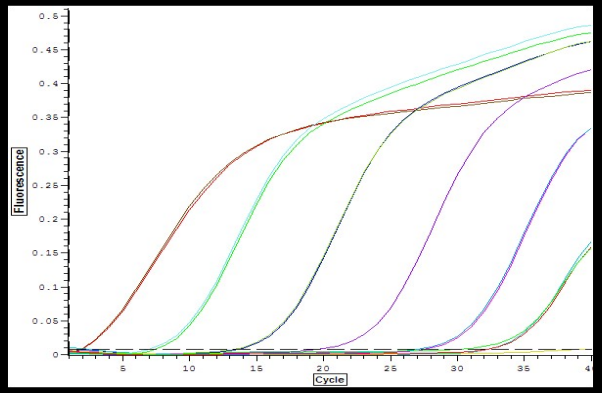
- **Step 1:**
  - Make your DNA dilutions (screw-cap tubes).
  - Dilute your "unknown" DNA 1:100
  - 1 ul of your DNA into 99 ul of water.
- **Step 2:**
  - Prepare your PCR tubes.
  - Add 20 ul of the spiked SYBR Green Supermix (contains 0.2 ul of Crime Scene Primers) to your four PCR tubes.
- **Step 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. 52C for 30 sec (extend / anneal)**
  - **4. Plate read (captures fluorescence data)**
  - **5. Goto Step 2 for 39 more times**

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD



Real-Time PCR

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

Biotechnology Explorer™ CAPTIVATING SCIENCE EDUCATION

BIO-RAD

Webinars

- **Enzyme Kinetics — A Biofuels Case Study**
- **Real-Time PCR — What You Need To Know and Why You Should Teach It!**
- **Proteins — Where DNA Takes on Form and Function**
- **From plants to sequence: a six week college biology lab course**
- **From singleplex to multiplex: making the most out of your realtime experiments**

explorer.bio-rad.com→Support→Webinars