# GENETICS AND MOLECULAR BIOLOGY FOR ENVIRONMENTAL ANALYSIS

# MOLECULAR ECOLOGY
## LESSON 5: DNA AMPLIFICATION

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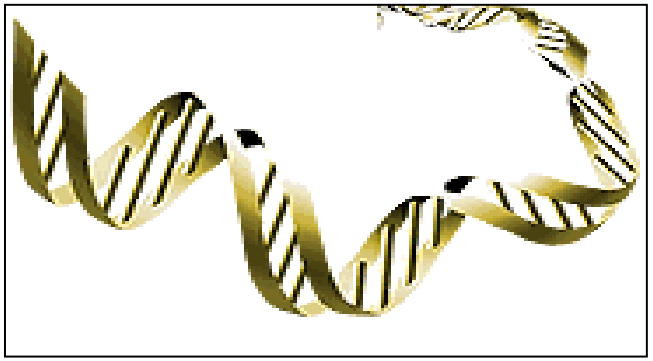
# The Problem

**How do we identify and detect a specific sequence in a genome?**

# The Problem:

## How do we identify and detect a specific sequence in a genome?

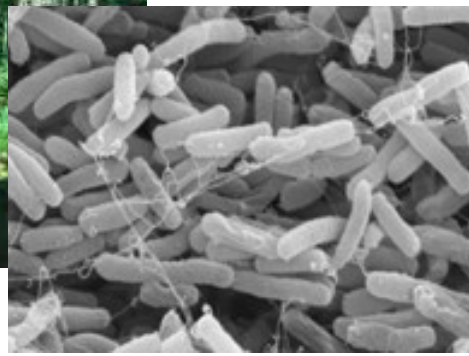


- **TWO BIG ISSUES:**
  - There are a LOT of other sequences in a genome that we're not interested in detecting. (SPECIFICITY)
  - The amount of DNA in samples we're interested in is VERY small. (AMPLIFICATION)

# The Problem:

# Specificity

**How do we identify and detect a specific sequence in a genome?**




- Pine: 68 billion bp
- Corn: 5.0 billion bp
- Soybean: 1.1 billion bp
- Human: 3.4 billion bp
- Housefly: 900 million bp
- Rice: 400 million bp
- E. coli: 4.6 million bp
- HIV: 9.7 thousand bp

# The Problem:

# Specificity

## Just How Big Is 3.4 Billion?

- The human genome is 3.4 B bp
- If the bases were written in standard 10-point type, on a tape measure...
- ...The tape would stretch for 5,366 MILES!
- **Identifying a 500bp sequence in a genome would be like finding a section of this tape measure only 4 feet long!**

# The Problem:

# Amplification

**How many molecules do we need to be able to see them?**

- To be visible on an agarose gel, need around 10 ng DNA for fluorescent stain.
- For a 500-bp product band, weighing 660 g/mol.bp, therefore need 10e-9 / (500*660) = 3.03e-14 moles.
- Avogadro's number = 6.02e23.
- Therefore need 1.8e10 copies!

- In other words, to "see" a single "gene", the DNA in a sample of 100 cells would have to be multiplied 180 million times!!!!!

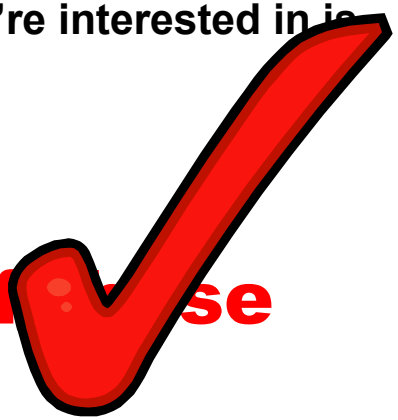
# The Problem:

SPECIFICITY

AMPLIFICATION

## Specificity Amplification

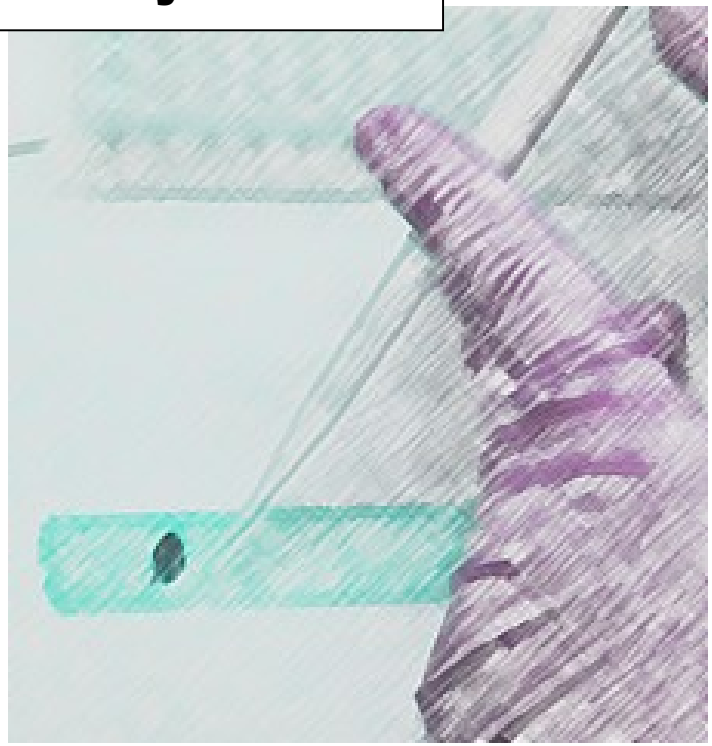
- **How do we identify and detect a specific sequence in a genome?**
- **TWO BIG ISSUES:**
  - **There are a LOT of other sequences in a genome that we're not interested in detecting.**
  - **The amount of DNA in samples we're interested in is VERY small.**



**PCR solves BOTH of these issues!!!**

# So what's PCR used for?

- Forensic DNA detection
- Identifying transgenic plants
- Detection and quantification of viral infection
- Cloning
- Detection of ancient DNA
- Gene expression analysis



# PCR History

# The Invention

In what has been called by some the greatest achievement of modern molecular biology, **Kary B. Mullis** developed the **polymerase chain reaction** (PCR) in 1983. PCR allows the rapid synthesis of designated fragments of **DNA**. Using the technique, over one billion copies can be synthesized in a matter of hours.

PCR is valuable to scientists by assisting **gene mapping**, the study of gene functions, cell identification, and to forensic scientists in criminal identification. Cetus Corporation, Mullis' employer at the time of his discovery, was the first to commercialize the PCR process. In 1991, Cetus sold the PCR patent to Hoffman-La Roche for a price of $300 million. It is currently an indispensable tool for molecular biologists and the development of genetic engineering.

# Mr. PCR: Kary B. Mullis

**(1944 - )**
**The inventor of the DNA synthesis process known as the Polymerase Chain Reaction (PCR). The process is an invaluable tool to today's molecular biologists and biotechnology corporations.**

**Mullis, born in Lenoir, North Carolina, attended the University of Georgia Tech for his undergraduate work in chemistry, and then obtained a Ph. D. in biochemistry from Cal Berkeley.**

**In 1983, working for Cetus Corporation, Mullis developed the Polymerase Chain Reaction, a technique for the rapid synthesis of a DNA sequence. The simple process involved heating a vial containing the DNA fragment to split the two strands of the DNA molecule, adding oligonucleotide primers to bring about reproduction, and finally using polymerase to replicate the DNA strands. Each cycle doubles the amount of DNA, so multiple cycles increase the amount of DNA exponentially, creating huge numbers of copies of the DNA fragment.**
**Mullis left Cetus in 1986. For his development of PCR, he was co-awarded the Nobel Prize in chemistry in 1993.**

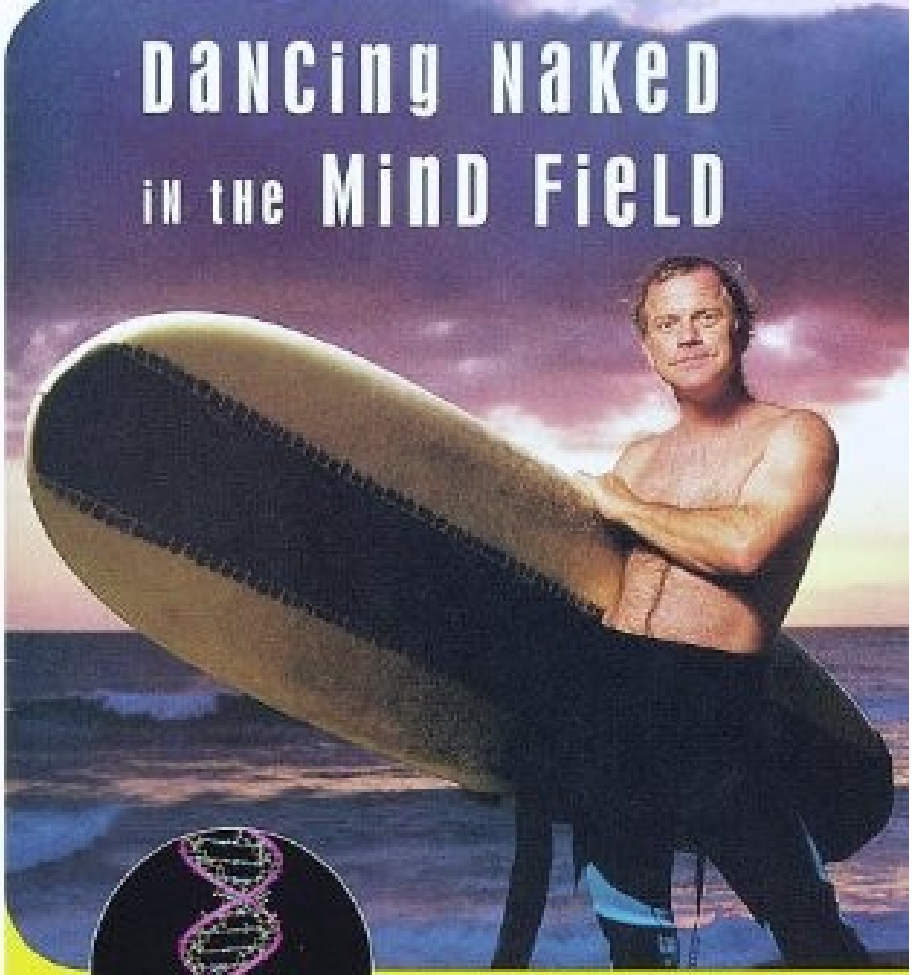
# The Invention of PCR

**The process, which Dr. Mullis conceptualized in 1983, is hailed as one of the monumental scientific techniques of the twentieth century. A method of amplifying DNA, PCR multiplies a single, microscopic strand of the genetic material billions of times within hours. Mullis explains:**

***"It was a chemical procedure that would make the structures of the molecules of our genes as easy to see as billboards in the desert and as easy to manipulate as Tinkertoys....It would find infectious diseases by detecting the genes of pathogens that were difficult or impossible to culture....The field of molecular paleobiology would blossom because of P.C.R. Its practitioners would inquire into the specifics of evolution from the DNA in ancient specimens....And when DNA was finally found on other planets, it would be P.C.R. that would tell us whether we had been there before."***

http://www.osumu.org/mu/events_lectures1b.htm

# Dancing Naked in the Mind Field





WINNER OF THE NOBEL PRIZE IN CHEMISTRY

## Kary Mullis

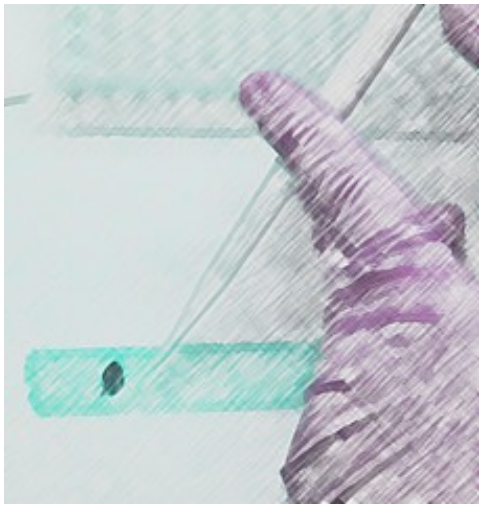
"Kary Mullis, perhaps the weirdest human ever to win the Nobel Prize in Chemistry, [has written] a chatty, rambling, funny, iconoclastic tour through the wonderland that is [his] mind."

—*THE WASHINGTON POST*

# Practical Uses of PCR

# Uses of PCR:

## Forensics



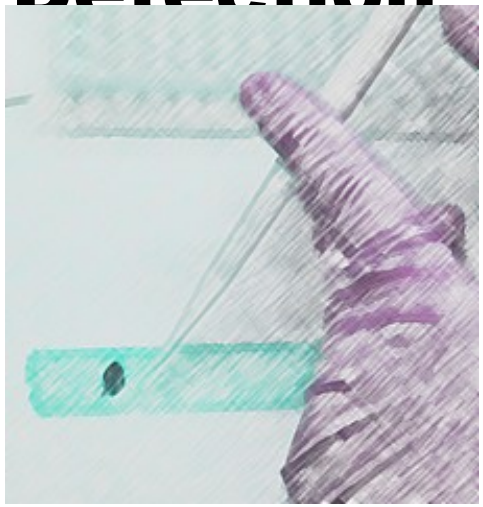
PCR's ability to amplify even the smallest amount of DNA from samples collected at a crime scene gives the method great power when used in criminal forensics.

The DNA from body fluid, hair, or other tissue samples is amplified to create a nearly unique pattern for each individual. This pattern can then be compared to suspects in the case.

The infamous OJ Simpson case was the first one in which the technique of PCR became widely publicized.

# Uses of PCR:

## GMO Food Detection



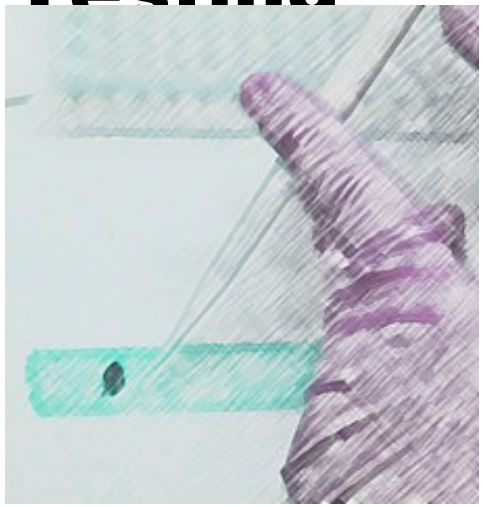
Genetically-modified foods (GMO foods) are widely grown in the USA and other countries.

For various reasons, some countries require exporters to indicate the percentage of GMO content in grain and food shipments.

PCR can be used to accurately measure the exact quantity of genetically-modified food in a shipment, by “looking” at the DNA that makes up the food!

# Uses of PCR:

## Paternity Testing



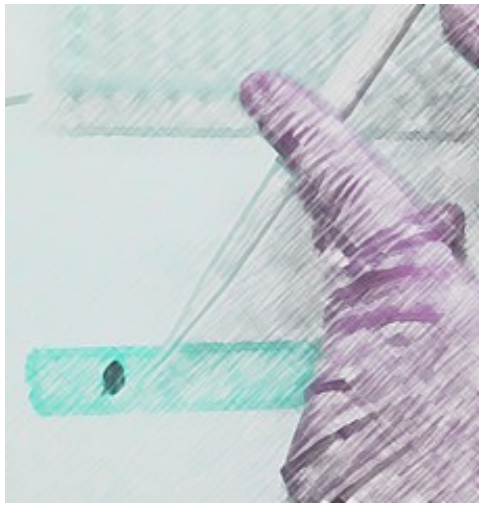
PCR's power at identifying individual genetic makeup has made it invaluable for use in paternity testing.

By amplifying specific DNA fragments from parents or close relatives, it is possible to reconstruct relatedness between individuals.

PCR can not only identify relationships between people today, but can also be used to identify historical family relationships!

# Uses of PCR:

# Archaeology



PCR has been used for many scientific studes in the field of archaeology:

Reconstructing the Dead Sea Scrolls.

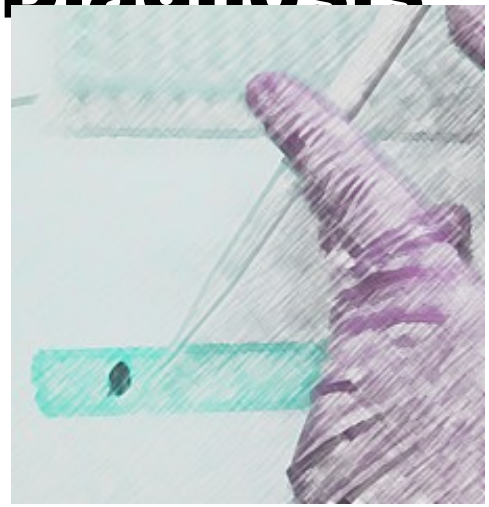
Identification of paint pigments in cave paintings.

Determining relatedness between individuals in ancient ossuaries.

Constructing dinosaurs from blood preserved in amber specimens. (!)

# Uses of PCR:

## Disease Diagnosis



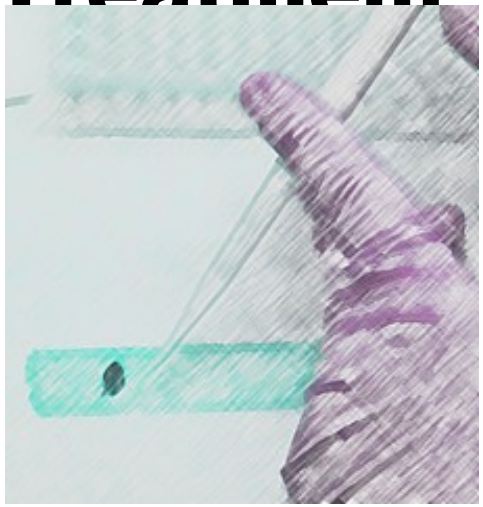
PCR is now invaluable in modern disease diagnosis.

PCR can identify disease-causing organisms much earlier than other methods, since it looks for the DNA of the organism itself, not its proteins or its effect on our immune system.

PCR has even been used to diagnose diseases of the past, by amplifying minute amounts of disease-related DNA in preserved specimens.

# Uses of PCR:

## Disease Treatment

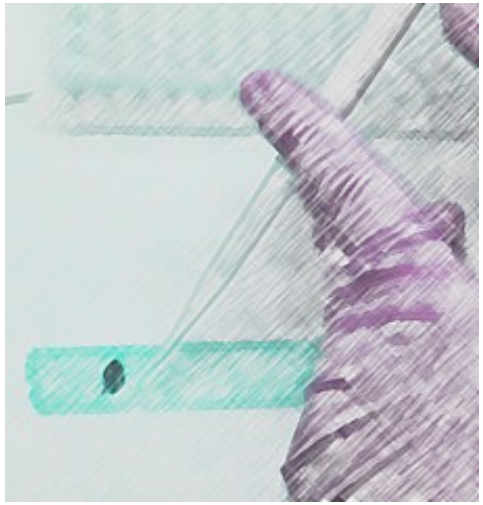


PCR can not only be used in disease diagnosis, but also as an aid in the treatment of diseases.

For example, real-time PCR is used to directly monitor the amount of HIV virus in patients suffering from infection. By monitoring the amount of virus present, the drug therapy can be continually adjusted to maximize virus suppression.

# Uses of PCR:

# Wildlife Conservation



Because PCR can be used to identify not only individuals, but also can differentiate between species, it is often used in wildlife conservation research.

PCR can be used to monitor trade in products made from endangered species.

PCR can be used to monitor ecosystems for the presence of certain species.

PCR can be used even to monitor and identify individual animals!

# How PCR Works

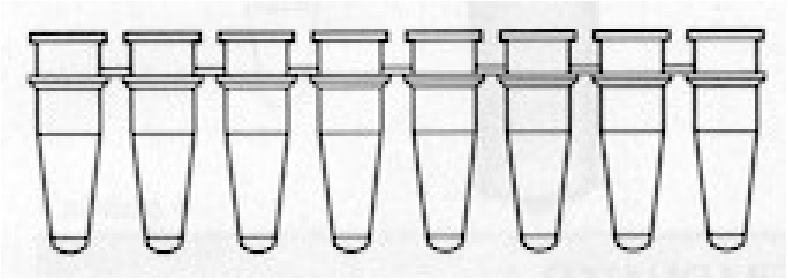
# How PCR works

- **Animated .GIF #2**

# The PCR Reaction Chemistry

# PCR Reaction Components

- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg++ ions
- DNA Polymerase



# PCR Reaction:

# Water

- **Water**
  - **The medium for all other components.**

# PCR Reaction:

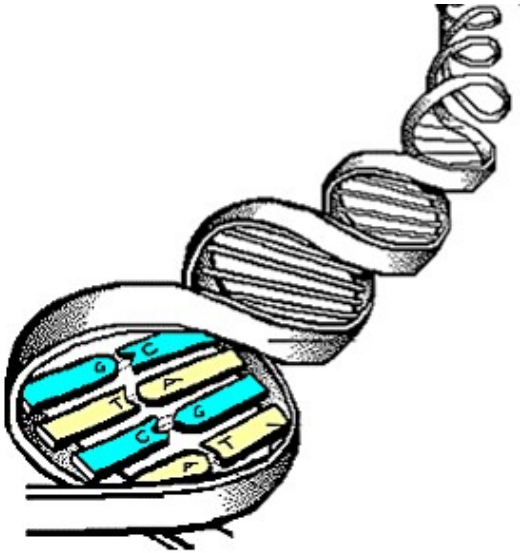
# Buffer

- **Water**
- **Buffer**
  - **Stabilizes the DNA polymerase, DNA, and nucleotides**
  - **500 mM KCl**
  - **100 mM Tris-HCl, pH 8.3**
  - **Triton X-100 or Tween**

# PCR Reaction:

# Template DNA

- **Water**
- **Buffer**
- **DNA template**
  - **Contains region to be amplified**
  - **Any DNA desired**
  - **Should be free of polymerase inhibitors**



# PCR Reaction:

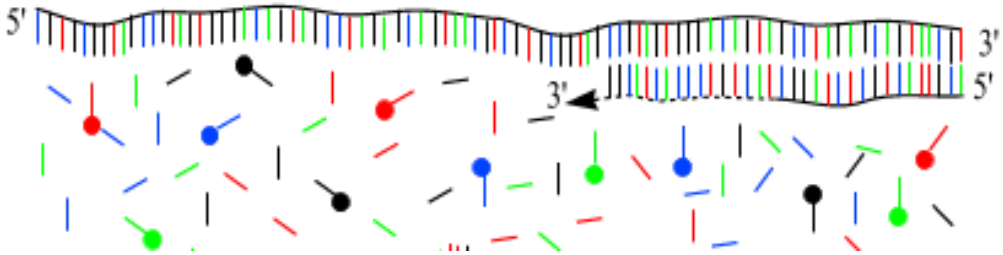
# Primers

- Water
- Buffer
- DNA template
- Primers
  - Specific for ends of amplified region
  - Forward and Reverse
  - Annealing temps should be known
    - Depends on primer length, GC content, etc.
  - Length 15-30 nt
  - Conc 0.1 – 1.0 uM (pMol/ul)

# PCR Reaction:

# Nucleotides

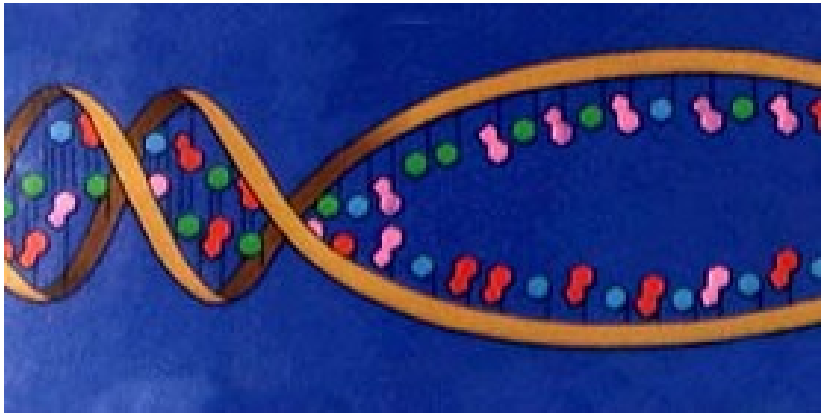
- Water
- Buffer
- DNA template
- Primers
- **Nucleotides**
  - Added to the growing chain
  - Activated NTP's
  - dATP, dGTP, dCTP, dTTP
  - Stored at 10mM, pH 7.0



# PCR Reaction:

# Magnesium

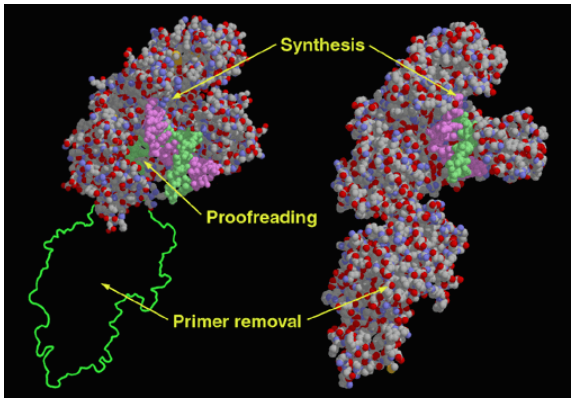
- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- **Mg++ ions**
  - Essential co-factor of DNA polymerase
  - Too little:  Enzyme won't work.
  - Stabilizes the DNA double-helix
  - Too much:  DNA extra stable, non-specific priming, band smearing
  - Used at 0.5 to 3.5 uM in the assay

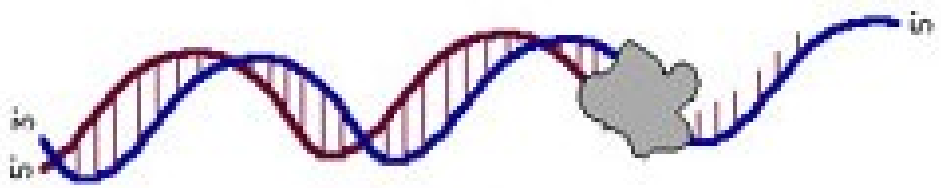


# PCR Reaction:

# Polymerase

- Water
- Buffer
- DNA template
- Primers
- Nucleotides
- Mg++ ions
- **DNA Polymerase**
  - The enzyme that does the extension
  - TAQ or similar
  - Heat-stable
  - Approx 1 U / rxn



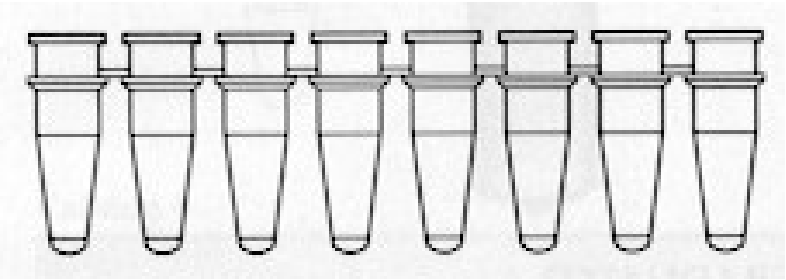



# PCR Reaction Components

# Review

**Summary:**

- **Water**
- **Buffer**
- **DNA template**
- **Primers**
- **Nucleotides**
- **Mg++ ions**
- **DNA Polymerase**



# Setting Up PCR Reactions

# A Typical PCR Reaction

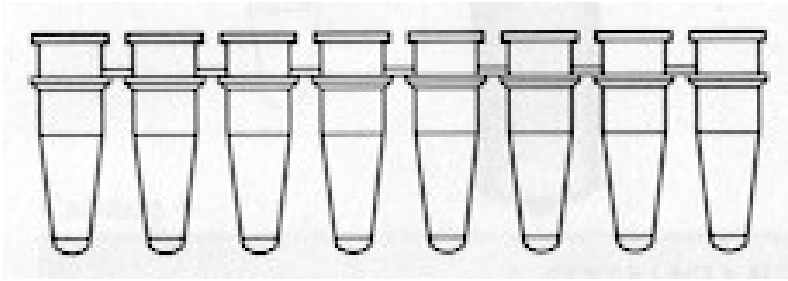
| | |
|---|---|
| Sterile Water | 38.0 ul |
| 10X PCR Buffer | 5.0 ul |
| MgCl2 (50mM) | 2.5 ul |
| dNTP's (10mM each) | 1.0 ul |
| PrimerFWD (25 pmol/ul) | 1.0 ul |
| PrimerREV | 1.0 ul |
| DNA Polymerase | 0.5 ul |
| DNA Template | 1.0 ul |
| | |
| Total Volume | 50.0 ul |

# Mixing Common Reagents Saves Time

| Component | 1X | 20X |
|---|---|---|
| Sterile Water | 38.0 ul | 760 ul |
| 10X PCR Buffer | 5.0 ul | 100 ul |
| MgCl2 (50mM) | 2.5 ul | 50 ul |
| dNTP's (10mM each) | 1.0 ul | 20 ul |
| PrimerFWD (25 pmol/ul) | 1.0 ul | 20 ul |
| PrimerREV | 1.0 ul | 20 ul |
| DNA Polymerase | 0.5 ul | 10 ul |
| DNA Template | 1.0 ul | -- |
| Total Volume | 50.0 ul | 980 ul |

Add DNA
as last step

Aliquot
49 ul



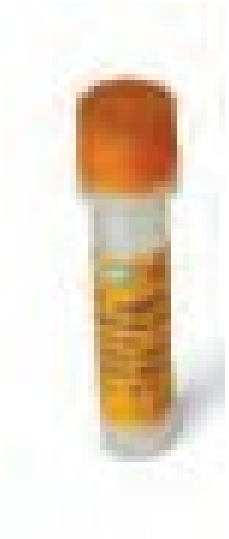
# An Even Simpler Approach:

# Mastermix

Sterile Water

10X PCR Buffer

MgCl2
dNTP's
DNA Polymerase
Primer FWD
Primer REV
DNA Template

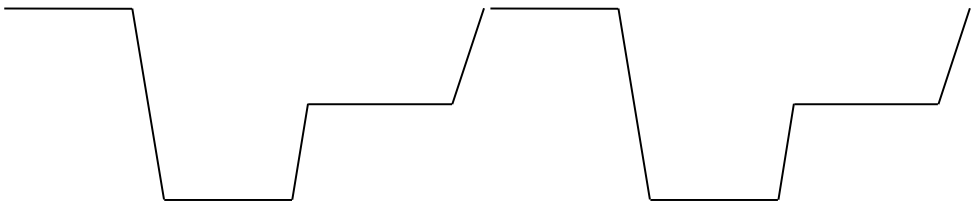


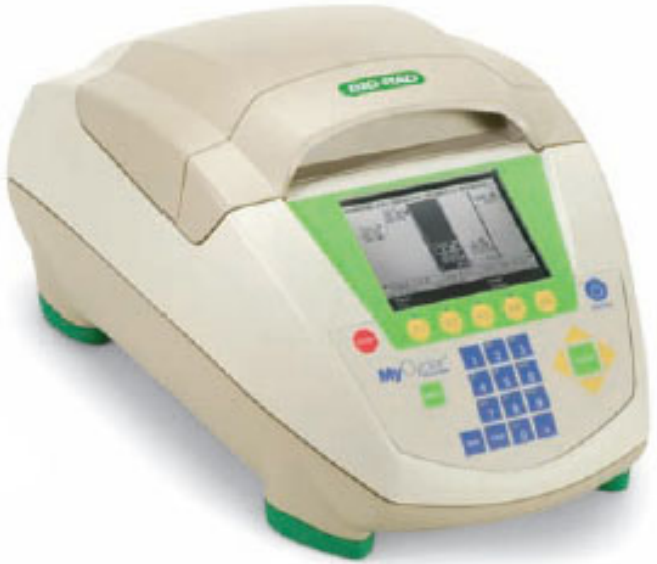
| MASTERMIX | 19.6 ul |
|---|---|
| Sterile Water | |
| 10X PCR Buffer | |
| MgCl2 | |
| dNTP's | |
| DNA Polymerase | |
| Primers Fwd+Rev | 0.4 ul |
| DNA Template | 20.0 ul |
| Total Volume | 40.0 |

# Programming the Thermal Cycler

# Typical Thermal Cycler Conditions

| | | | |
|---|---|---|---|
| 1. | Initial Denaturation | 95 C | 3 min |
| 2. | DNA Denaturation | 95 C | 1 min |
| 3. | Primer Annealing | 65 C | 1 min |
| 4. | Primer Extension | 72 C | 1 min |
| 5. | Go to step #2, repeat 39 more times | | |
| 6. | End | | |

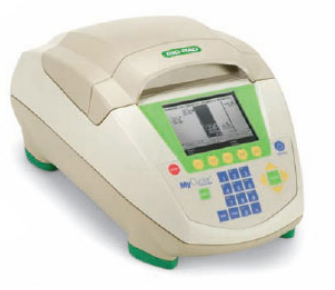




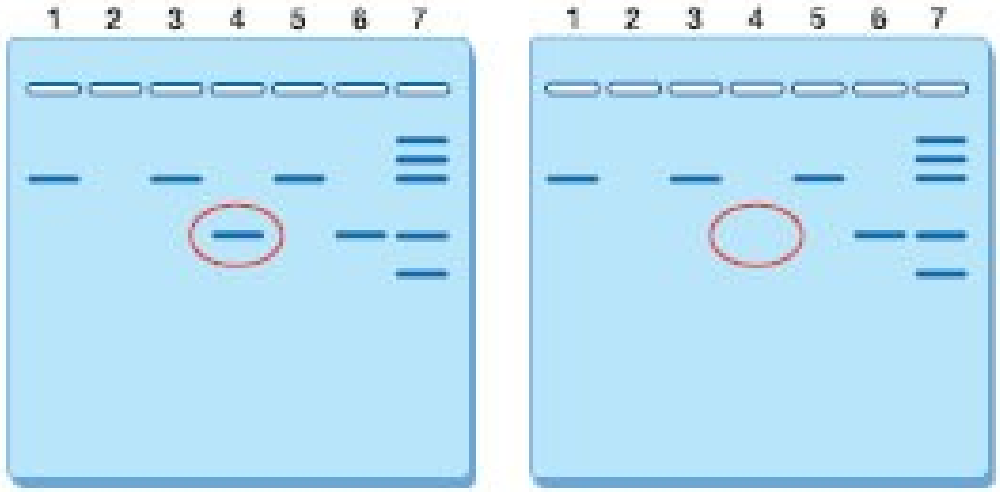
# Analyzing the Amplified DNA

# PCR

# Visualizing Results

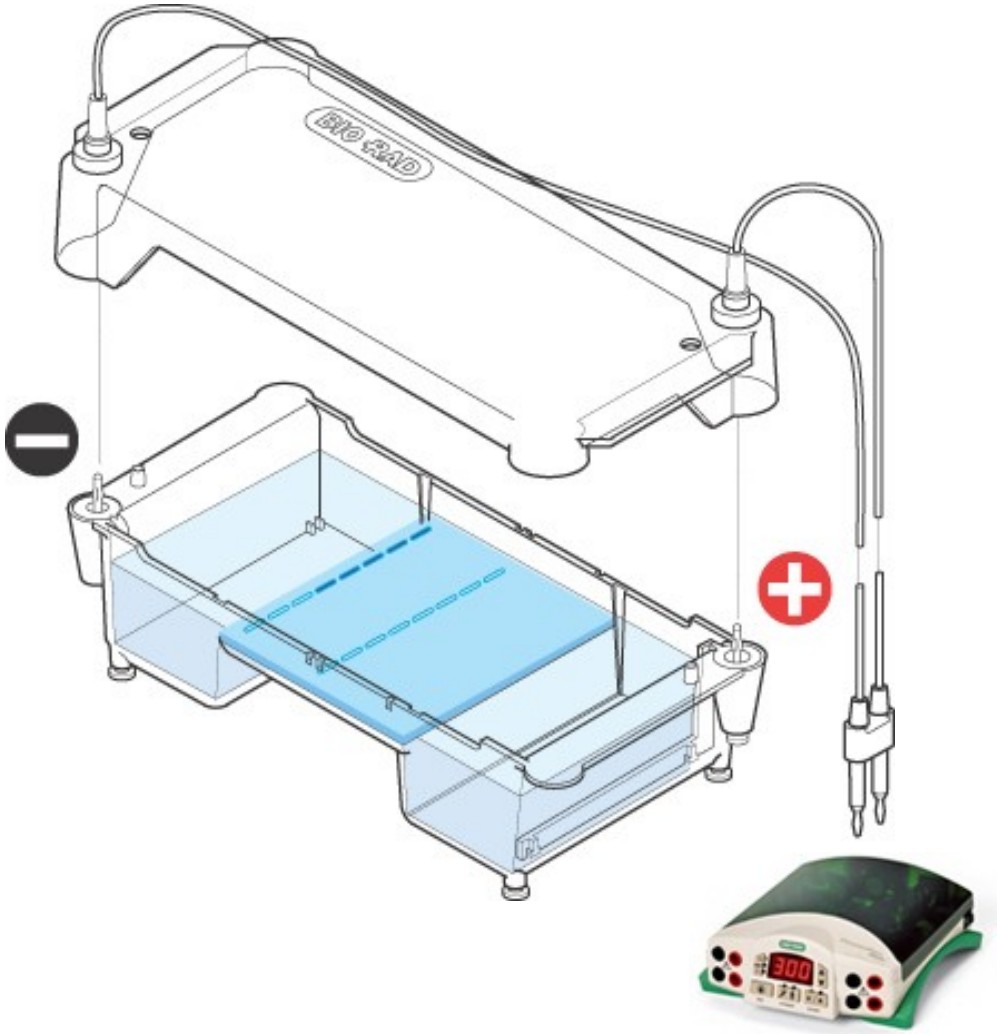


- After thermal cycling, tubes are taken out of the PCR machine.
- Contents of tubes are loaded onto an agarose gel.
- DNA is separated by size using an electric field.
- DNA is then stained.
- PCR products are visible as different “bands”.



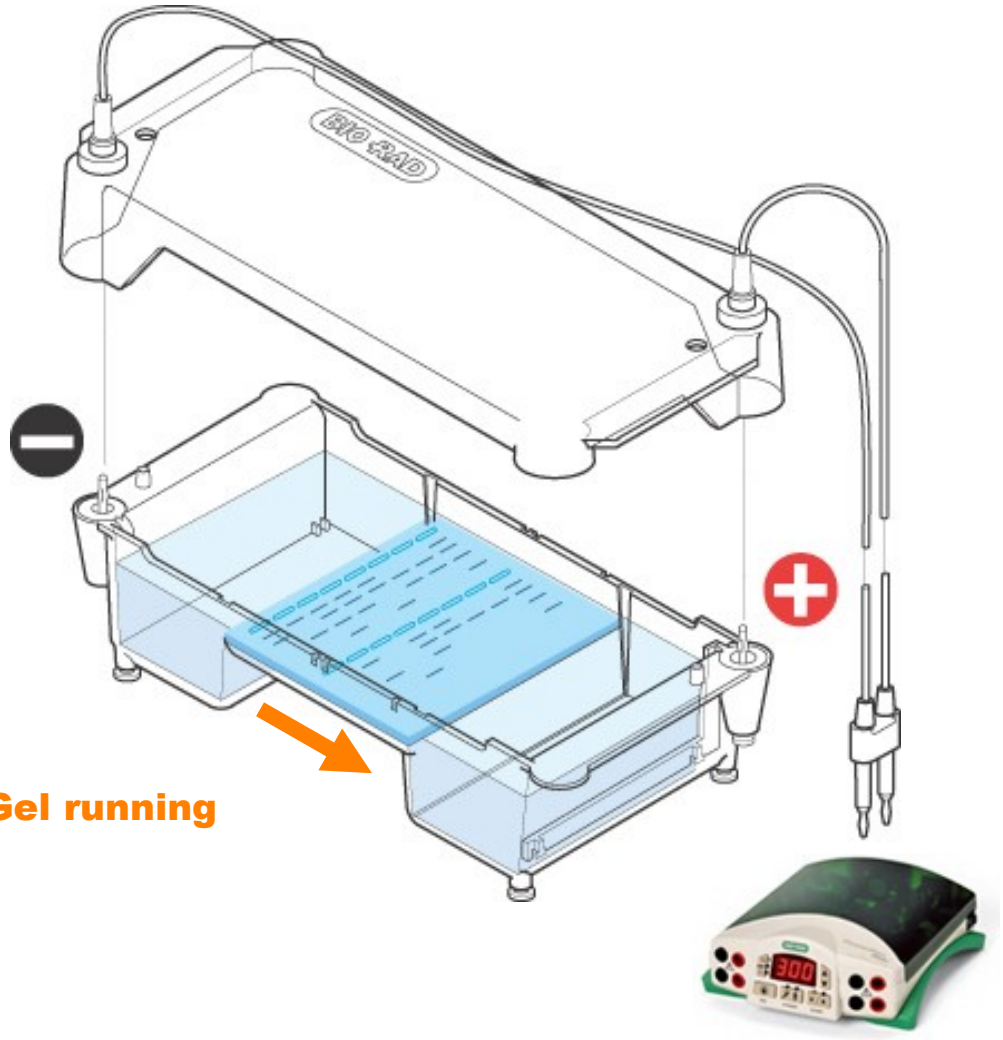
# PCR

# Visualizing Results



# PCR

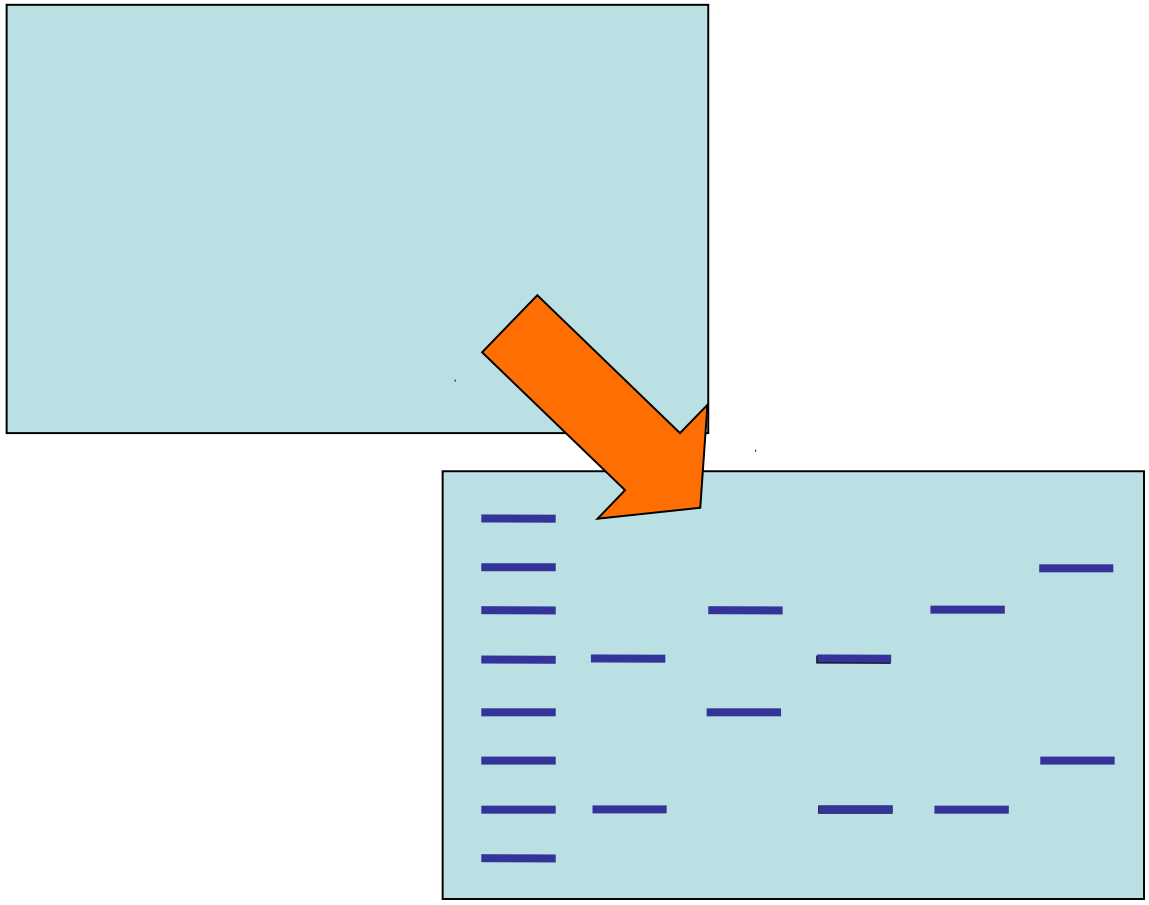
# Visualizing Results



# PCR

# Visualizing Results

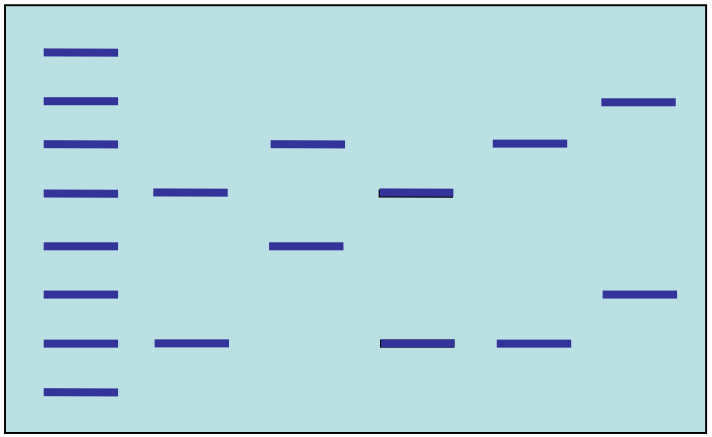
**After the gel has run, it is stained to reveal the DNA bands:**



# PCR

# Visualizing Results

**The final result of the traditional PCR procedure is a gel with a series of bands:**



**Bands can be compared against each other, and to known size-standards, to determine the presence or absence of a specific amplification product.**

# PRIMER DESIGN

- 1) Requires knowledge of the target sequence (i.e. we can’t use PCR to amplify an unknown sequence)
- 2) Use can designate subsection of gene to amplify.
- 3) Primers must have similar “melting temperatures” (e.g. 60ºC)

# CALCULATING MELTING TEMPERATURE (TM)

- Based on thermodynamic binding properties of double-stranded DNA.
- G-C pairing has higher energy than A-T binding!
- There are many methods for calculating Tm, and generally speaking it is more important to use the same method (consistency) rather than making exact predictions (accuracy).
- Common method: every G or C is assigned a

# CALCULATING MELTING TEMPERATURE (TM)

**Tm = 4(G + C) + 2(A + T) ºC**

5’-ACGTGTGTCAGCTGTAGTCG-3’

=4 x C
=7 x G
} = 11

=3 x A
=6 x T
} = 9

Tm = 4(11) + 2(9)

Tm = 62ºC

This method of calculating Tm is limited to short sequences, and really only useful in PCR primer design, where the annealing temperature is expected to be near 60ºC.

# EXAMPLE OF PCR PRIMER DESIGN

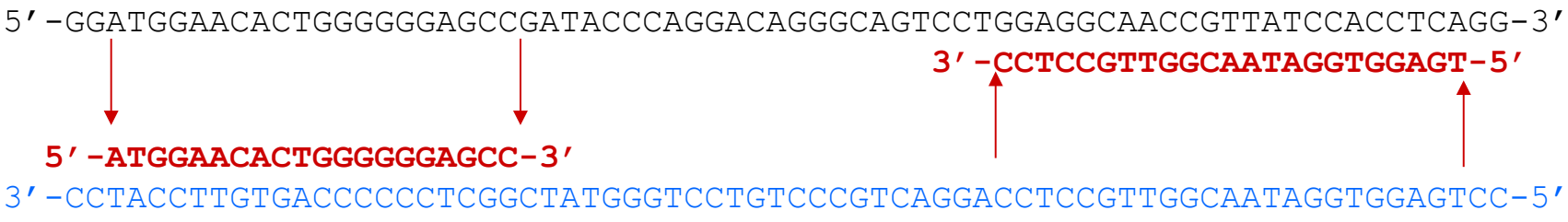
1) Target Sequence:

GGATGGAACACTGGGGGGAGCCGATACCCAGGACAGGGCAGTCCTGGAGGCAACCGTTATCCACCTCAGG

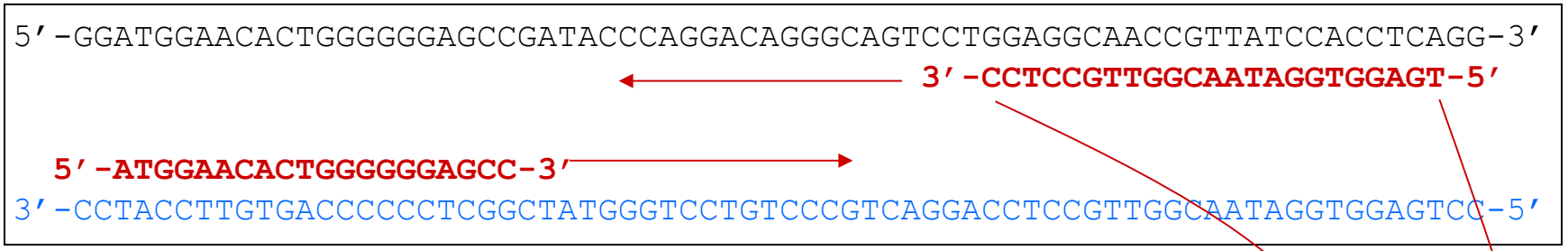
2) Note that the complementary strand is present (but not shown in sequence file):

GGATGGAACACTGGGGGGAGCCGATACCCAGGACAGGGCAGTCCTGGAGGCAACCGTTATCCACCTCAGG
CCTACCTTGTGACCCCCCTCGGCTATGGGTCCTGTCCCGTCAGGACCTCCGTTGGCAATAGGTGGAGTCC

3) Primers are extracted from sequence:



# EXAMPLE OF PCR PRIMER DESIGN




Amplified Region, which includes Primer Sequences

Primers (WRITTEN 5’ to 3’ !!!)

“Forward” Primer: **ATGGAACACTGGGGGGAGCC**
“Reverse” Primer: **TGAGGTGGATAACGGTTGCCTCC**

# PCR PRIMER DESIGN

- Computationally, PCR Primer design involves the coordination of:

1) Selecting 2 different primers that are:
   a) Any distance apart (there are length limits in PCR!)
   b) -OR- A specified distance apart (e.g. “PCR product will be 300 base-pairs long”)
   c) -OR- Span a specific subsequence or “motif” (e.g. “PCR product includes exons #1 and #2”)

# PCR PRIMER DESIGN

- 2) Selecting 2 different primers that are nearly/exactly the same melting temperature (Tm). This is usually 60ºC.

- 3) Many times PCR Primers are designed with a “GC Clamp”. This simply means that the 3’ end of the primers is a G or a C, which binds more tightly (than AT) and the Polymerase initiates synthesis more efficiently.

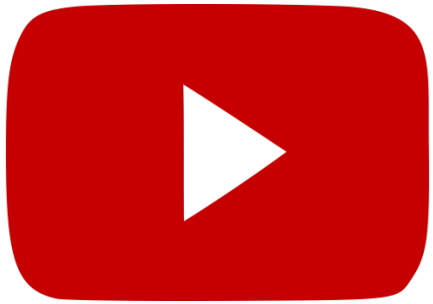
- 4) Finally, better results from PCR reactions occur if the two primers are not complementary to each other, or contain

# SEVERAL SOFTWARE FOR PRIMER DESIGN

- http://bioinfo.ut.ee/primer3/
- http://www.bioinformatics.nl/cgi-bin/primer3plus/primer

# HOW TO ASSEMBLE AND CHECK A PCR

- VIDEO



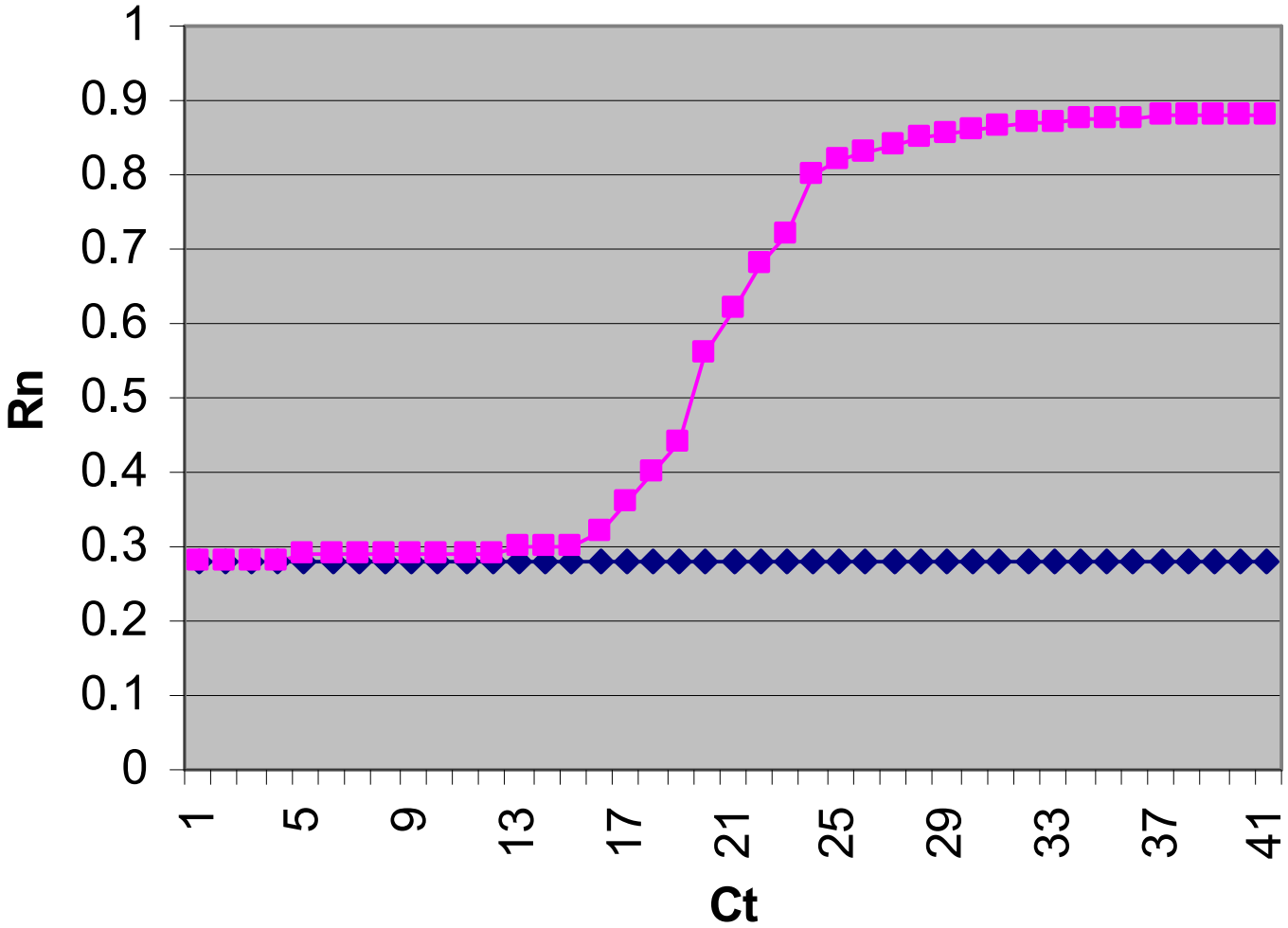
# SEQUENCE DETECTION APPLICATIONS

- End point PCR: simple +/- results
  - PCR product detection (pathogens, transgenes)
  - Genotyping (allelic discrimination, single nucleotide polymorphisms-SNPs)
- Real time PCR: complex results
  - Absolute quantitation
  - Relative quantitation
  - PCR interrogation (optimization)

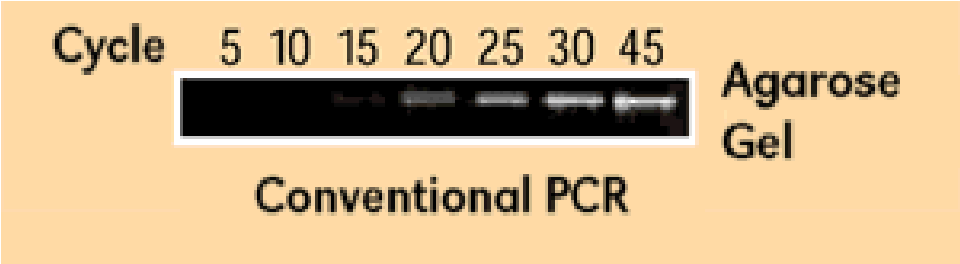
# QPCR DETECTION SYSTEMS

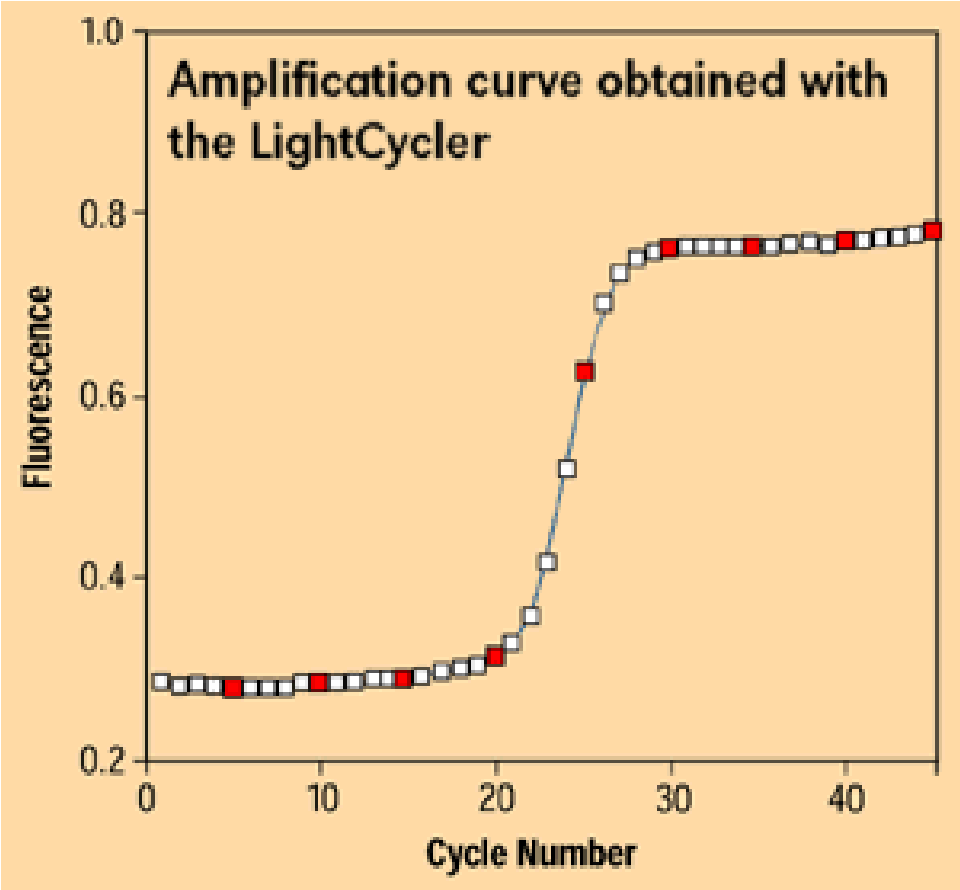
- DNA-specific dyes bind and fluoresce double-stranded DNA nonspecifically.
- Hybridization probes only bind and fluoresce the intended PCR product.
- Primer-incorporated probes label the PCR product.

# MODEL OF SINGLE AMPLIFICATION PLOT



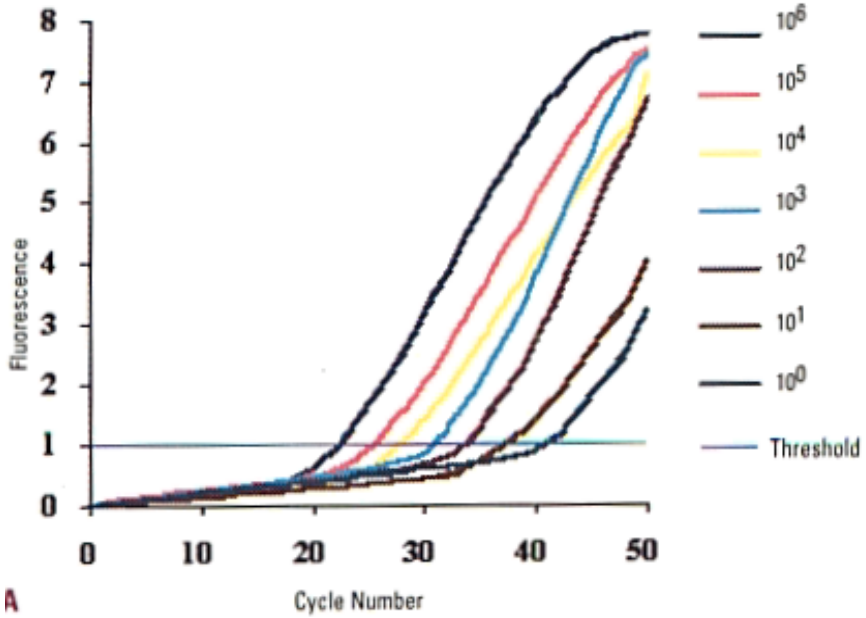
# GEL ANALYSIS VS FLUORESCENCE

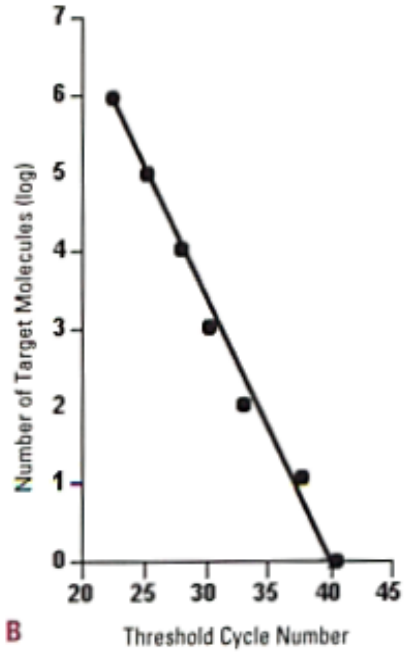


# QUANTITATIVE PCR (QPCR)

- A threshold level of fluorescence is determined based on signal and background.
- Input is **inversely proportional** to “threshold” cycle (cycle at which fluorescence crosses the threshold fluorescence level).

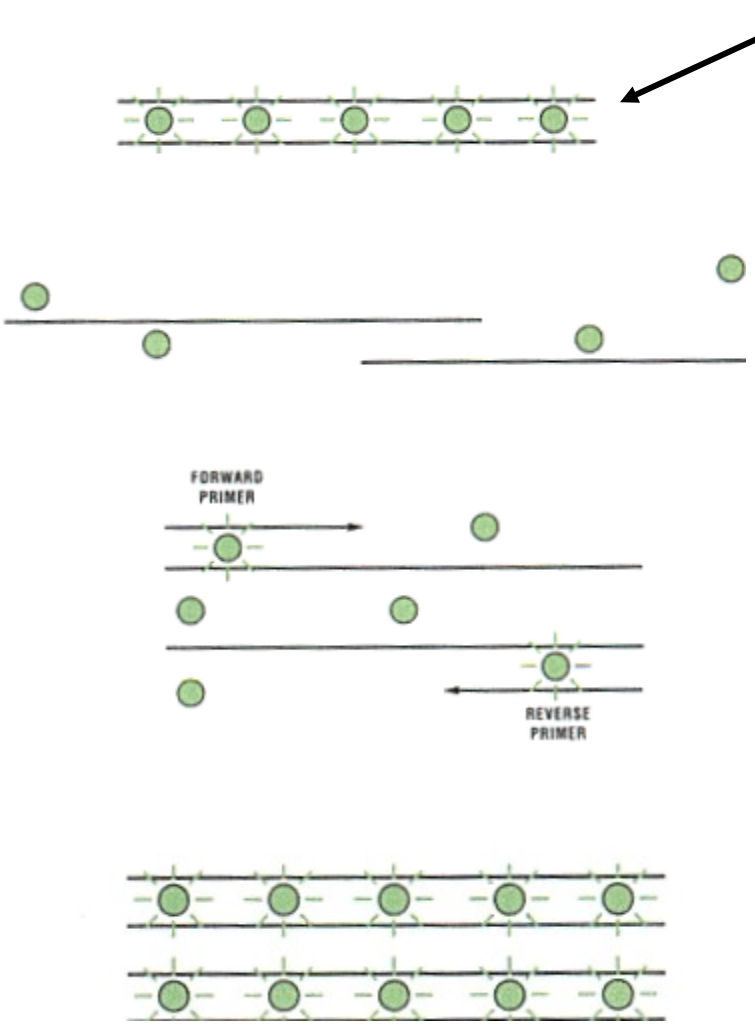


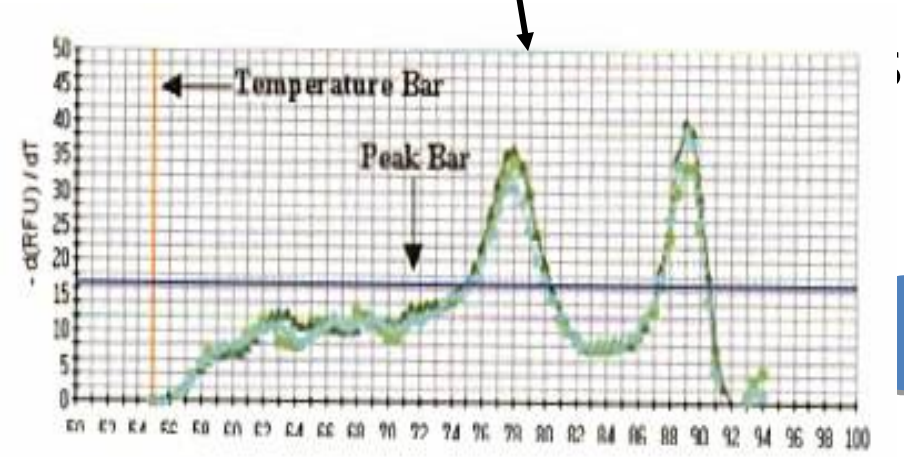
# QPCR DETECTION SYSTEMS

- DNA-specific dyes
  - Ethidium bromide
  - SyBr® green
  - Eva® green
- Hybridization probes
  - Cleavage-based (TaqMan®)
  - Displaceable (Molecular Beacons®, FRET®)
- Primer-incorporated probes

# QPCR: SYBR® GREEN



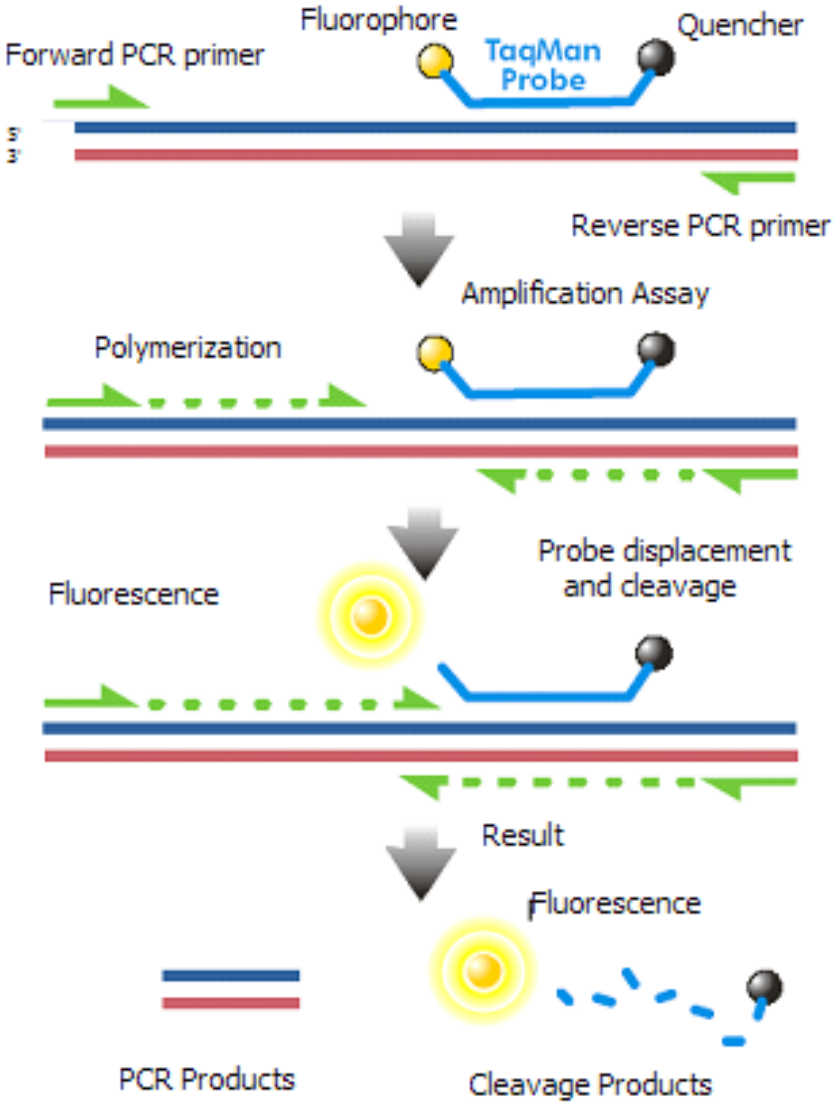

- Binds minor groove of double-
stranded DNA.
- Product can be further tested
in a post-amplification melt



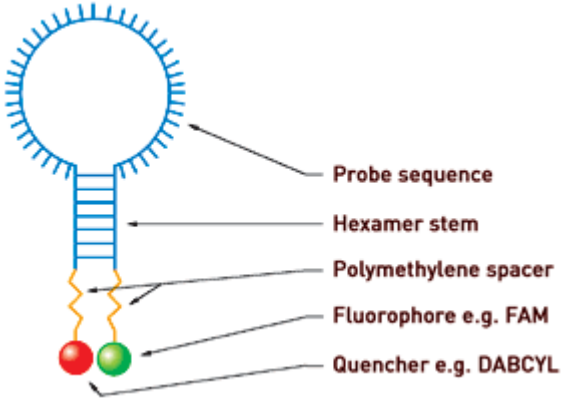
# REAL-TIME PCR LABELED PROBES

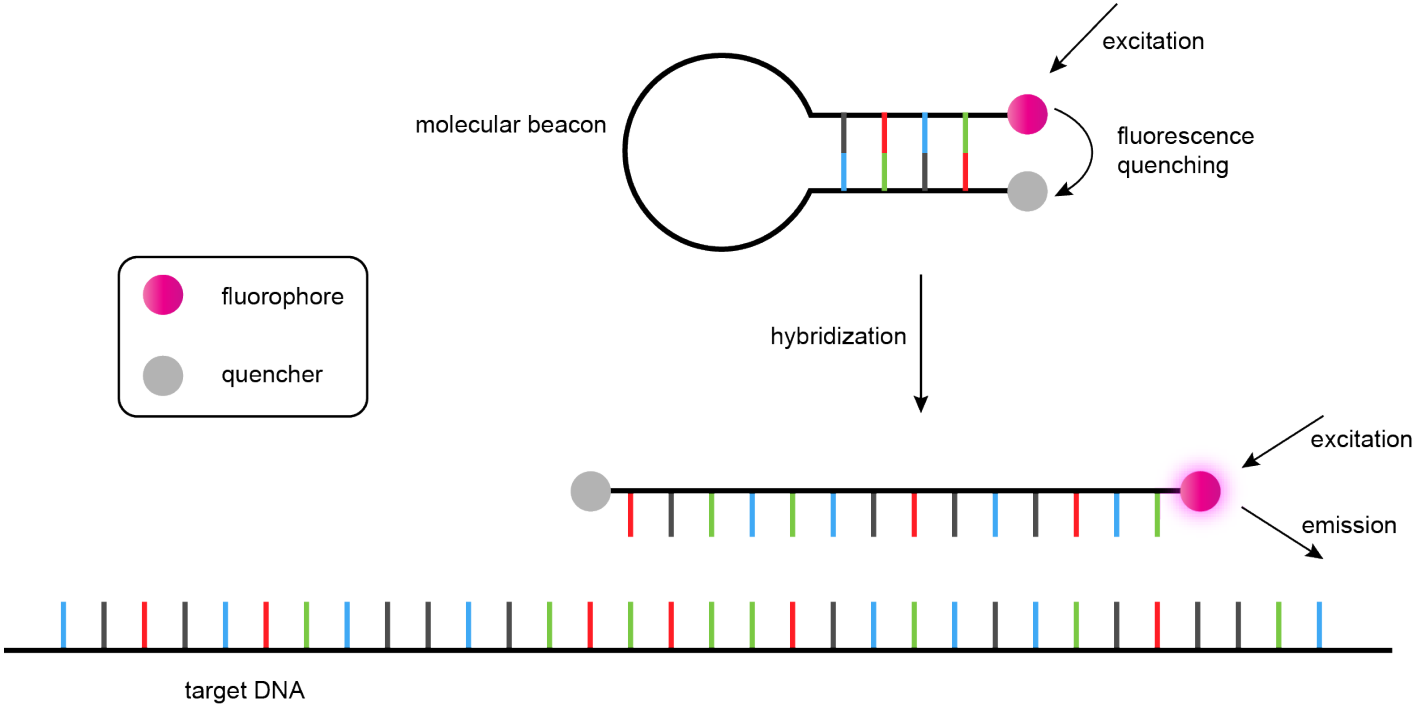
- Cleavage-based probes
  - TaqMan Assay
  - Fluorescent reporter at 5’ end and a quencher at 3’ end
- Molecular beacons
  - Hairpin loop structure
  - Fluorescent reporter at 5’ end and a quencher at 3’ end
- FRET probes
  - Fluorescence resonance energy transfer probes

# CLEAVAGE-BASED ASSAY: TAQMAN 5’-3’ EXONUCLEASE

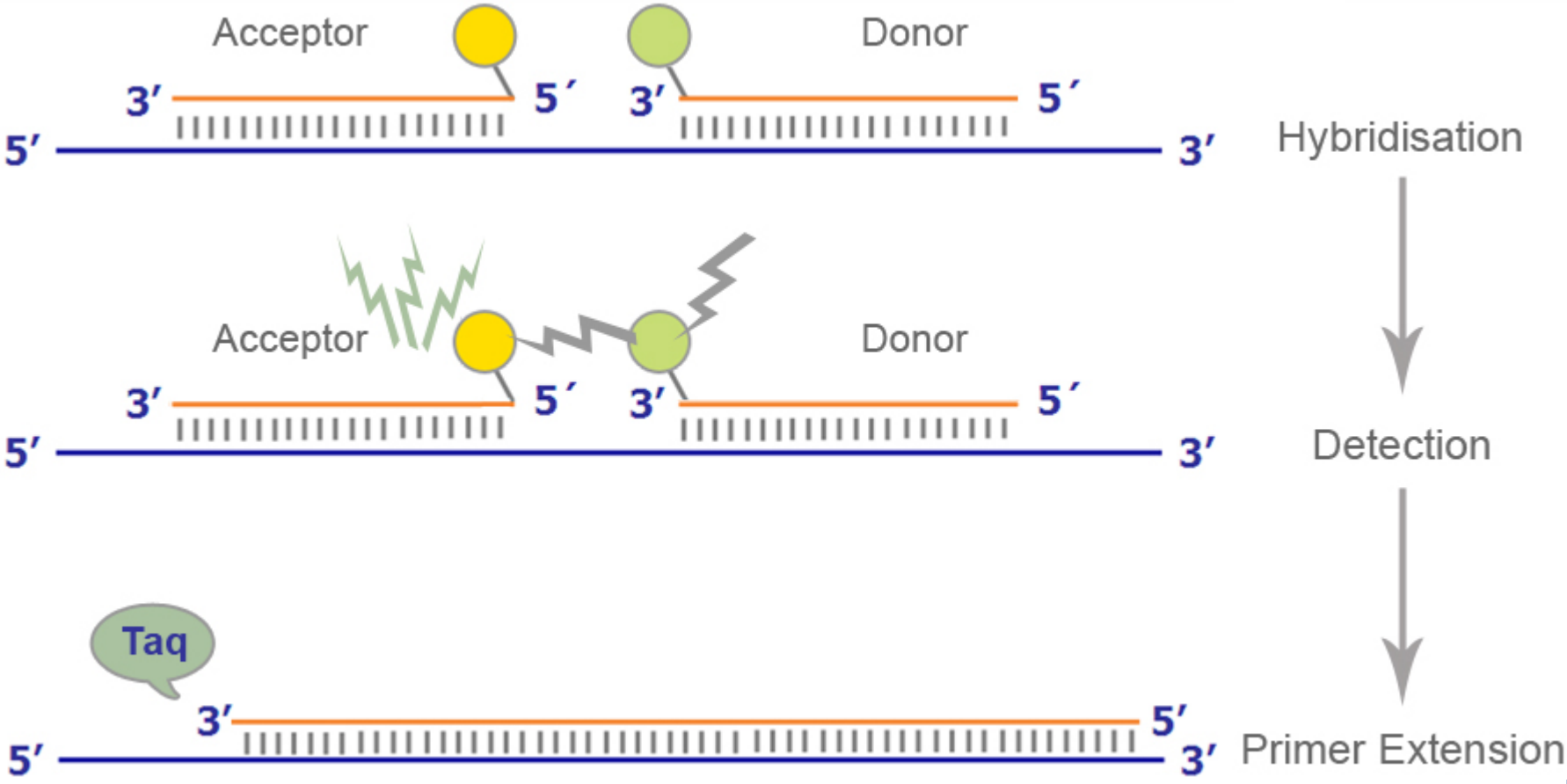


# MOLECULAR BEACON ASSAY



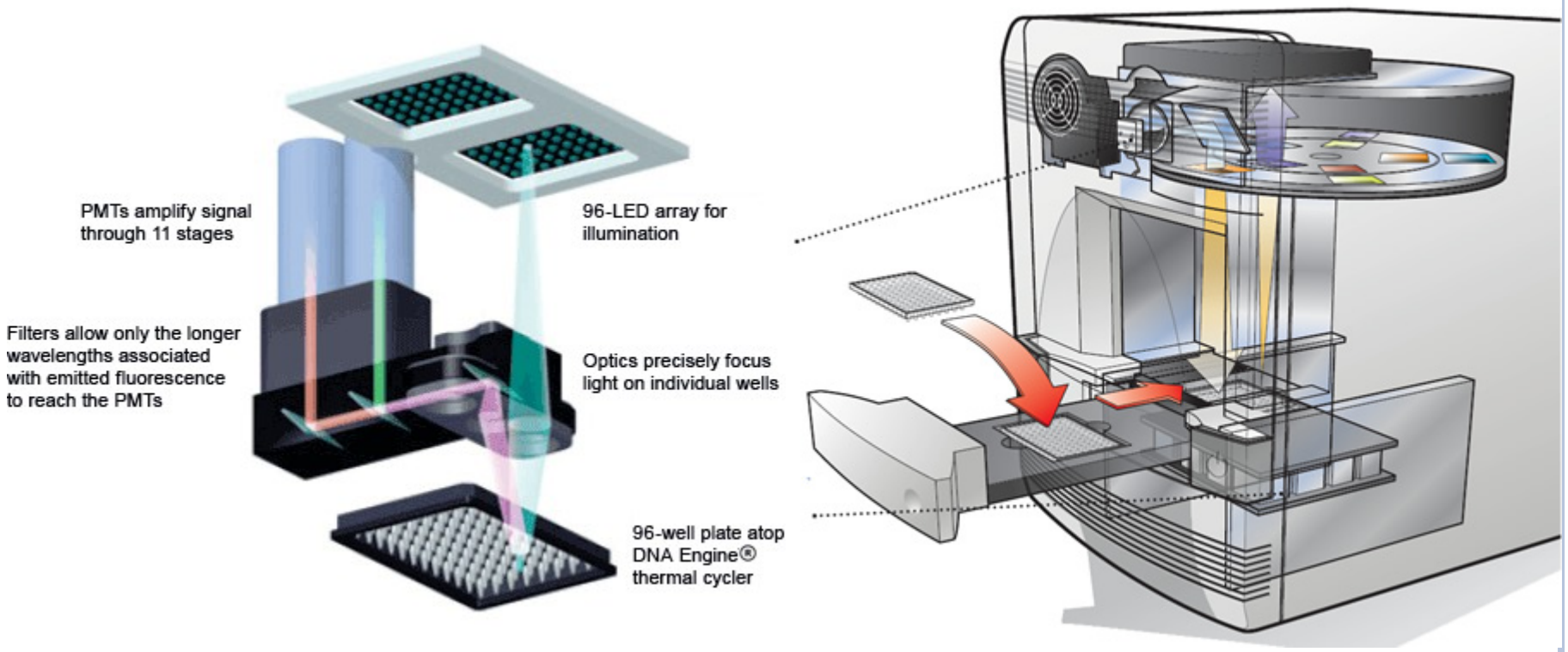



# FRET PROBE


Acceptor
Donor
3′
5′
5′
3′
Hybridisation
Acceptor
Donor
Detection
Taq
Primer Extension

# QPCR DETECTION SYSTEMS

- Thermal cyclers with fluorescent detection and specialized software.
- PCR reaction takes place in optically clear plates, tubes, or capillaries



# SUMMARY

- PCR is a method to specifically amplify target sequences in a complex mixture.
- The primers determine what sequences are amplified (specificity).
- Contamination control is important in laboratories performing PCR.
- Quantitative PCR offers the advantage of quantifying target.
- In addition to PCR, signal and probe amplification methods are available for use in the clinical laboratory.