nibrt
National Institute for Bioprocessing Research and Training

L8 Bioanalytical Techniques

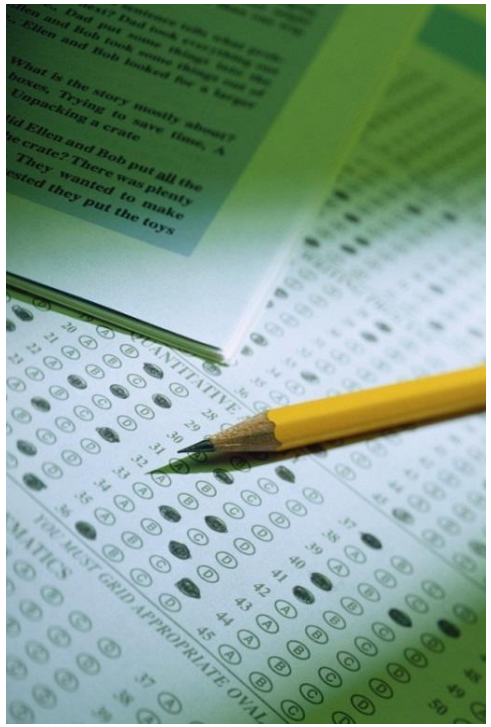
Lecture 7 -PCR

Reminder: MCQ 2

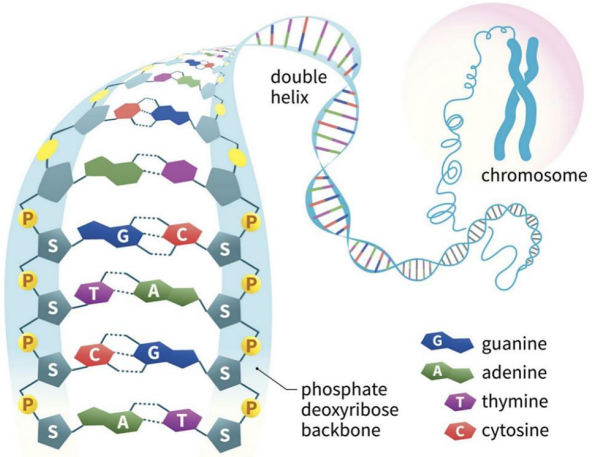
- 15 questions in 45min
- Based on lectures 5-7
- Opens 12.00pm

  Wednesday 19th March
- Closes 12.00pm

  Friday 21st March
- Worth 10% of overall grade
- Negative marking will apply (-10% of a mark for incorrect answer!)



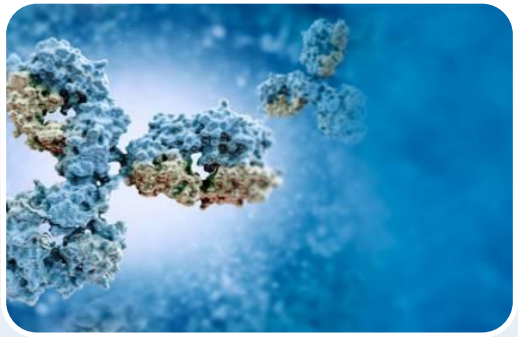
Learning Objectives



1. Discuss the basic principles of PCR

2. Discuss the differences between traditional PCR and qPCR

3. Discuss applications of PCR in Biopharma



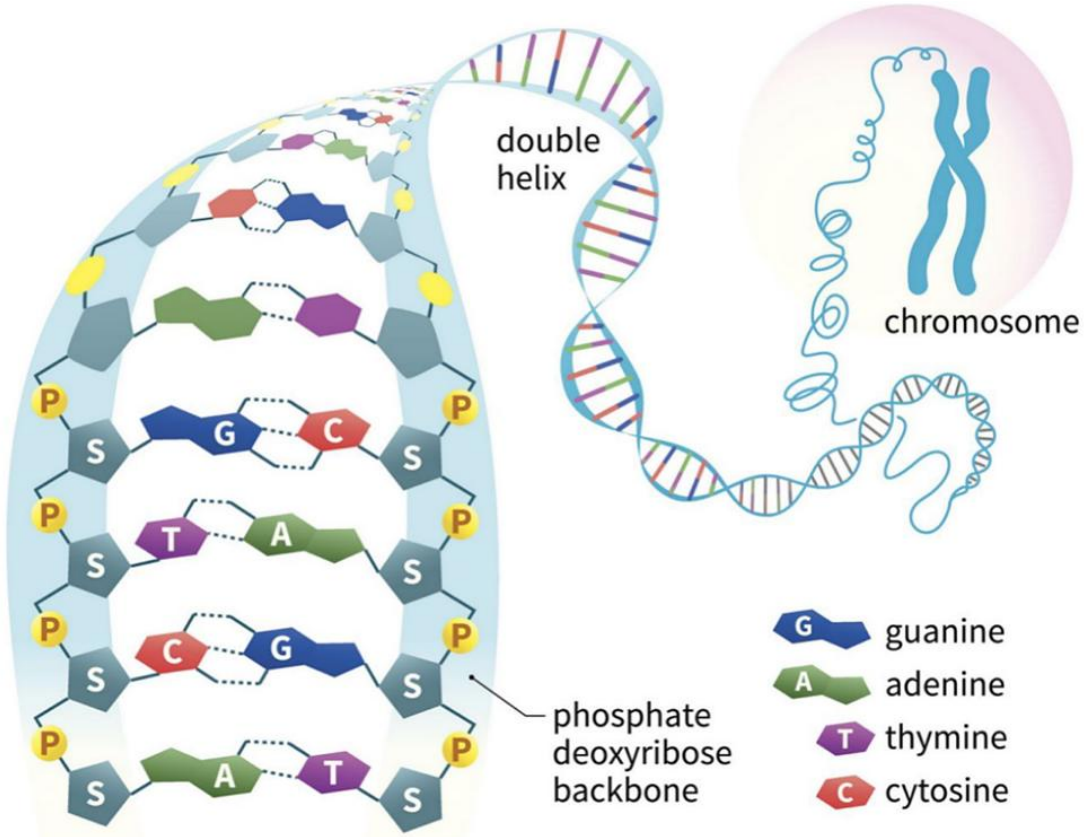
Topics

- Basic Principles of PCR
- Traditional PCR
- Real Time (Quantitative) PCR, qPCR
- Applications

Polymerase Chain Reaction

- The Polymerase chain reaction (PCR) is a rapid, inexpensive and simple means of producing millions of copies of a stretch of **deoxyribonucleic acid (DNA)** from minute quantities of starting material.

- PCR is simply **DNA replication** (molecular photocopying) in a test tube.



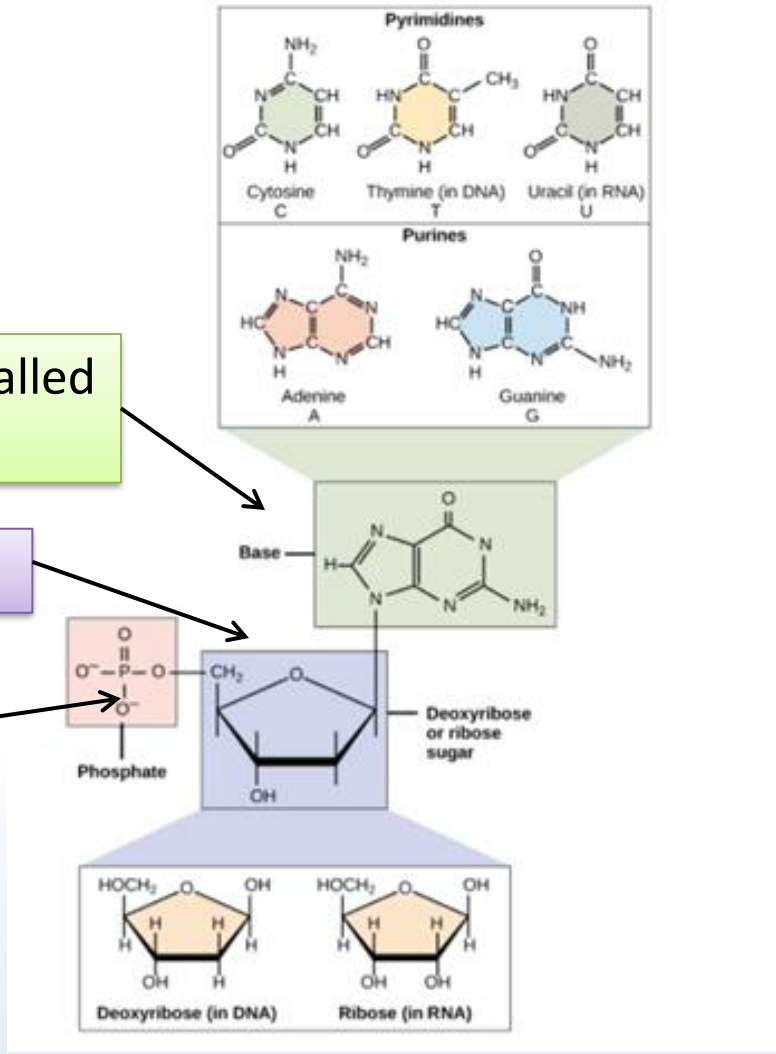
Building blocks of DNA and RNA

- Each nucleotide is made up of three parts:

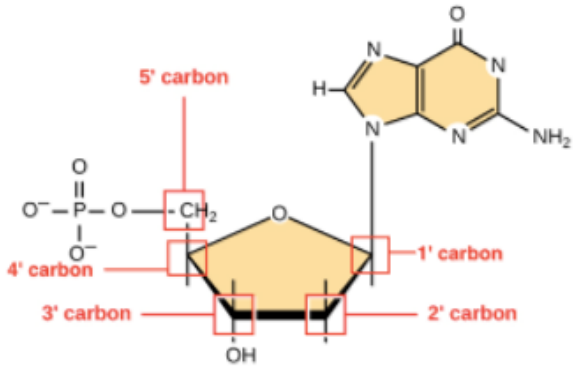
nitrogen-containing ring structure called a nitrogenous base

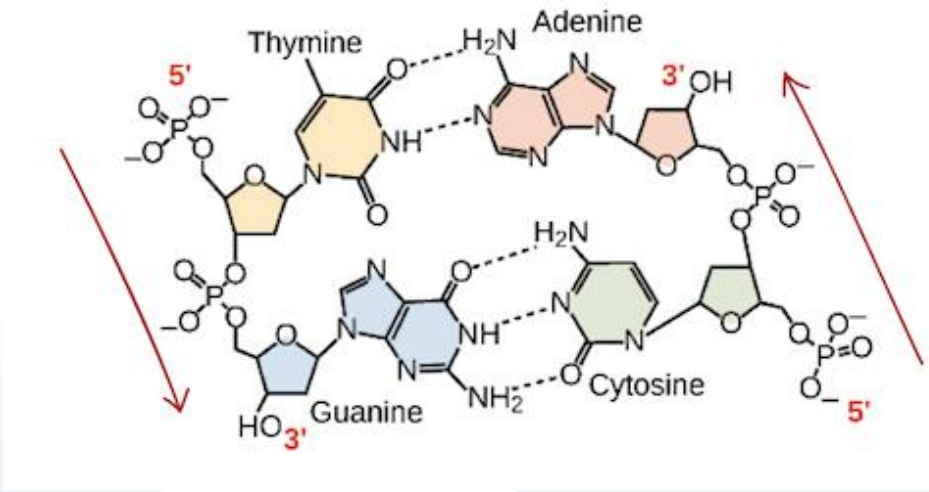
five-carbon sugar

at least one phosphate group



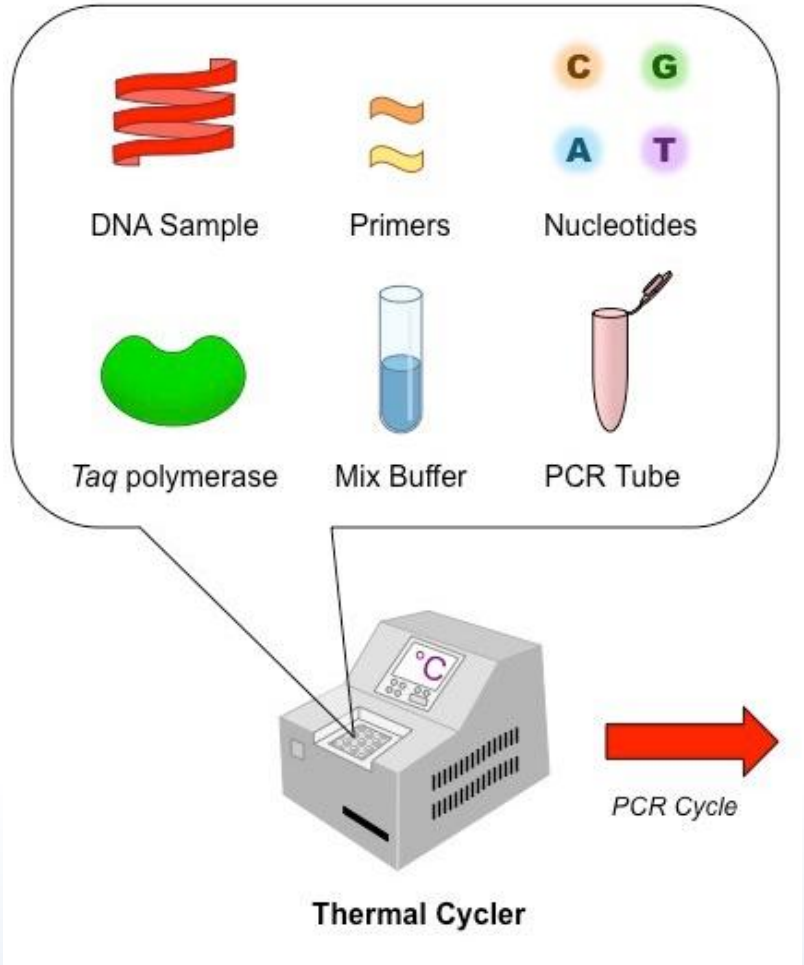
Building of DNA and RNA





- DNA sequences are usually written in the **5' to 3' direction**
- As new nucleotides are added to a strand of DNA or RNA, the strand grows at its 3’ end, with the **5’ phosphate** of an incoming nucleotide **attaching to the hydroxyl group at the 3’ end** of the chain.
- The two strands of the helix run in **opposite directions,** meaning that the 5’ end of one strand is paired up with the 3’ end of its matching strand
- DNA consists on complementary strands:
  - **Adenine : Thymine**
  - **Cytosine : Guanine**

PCR Components

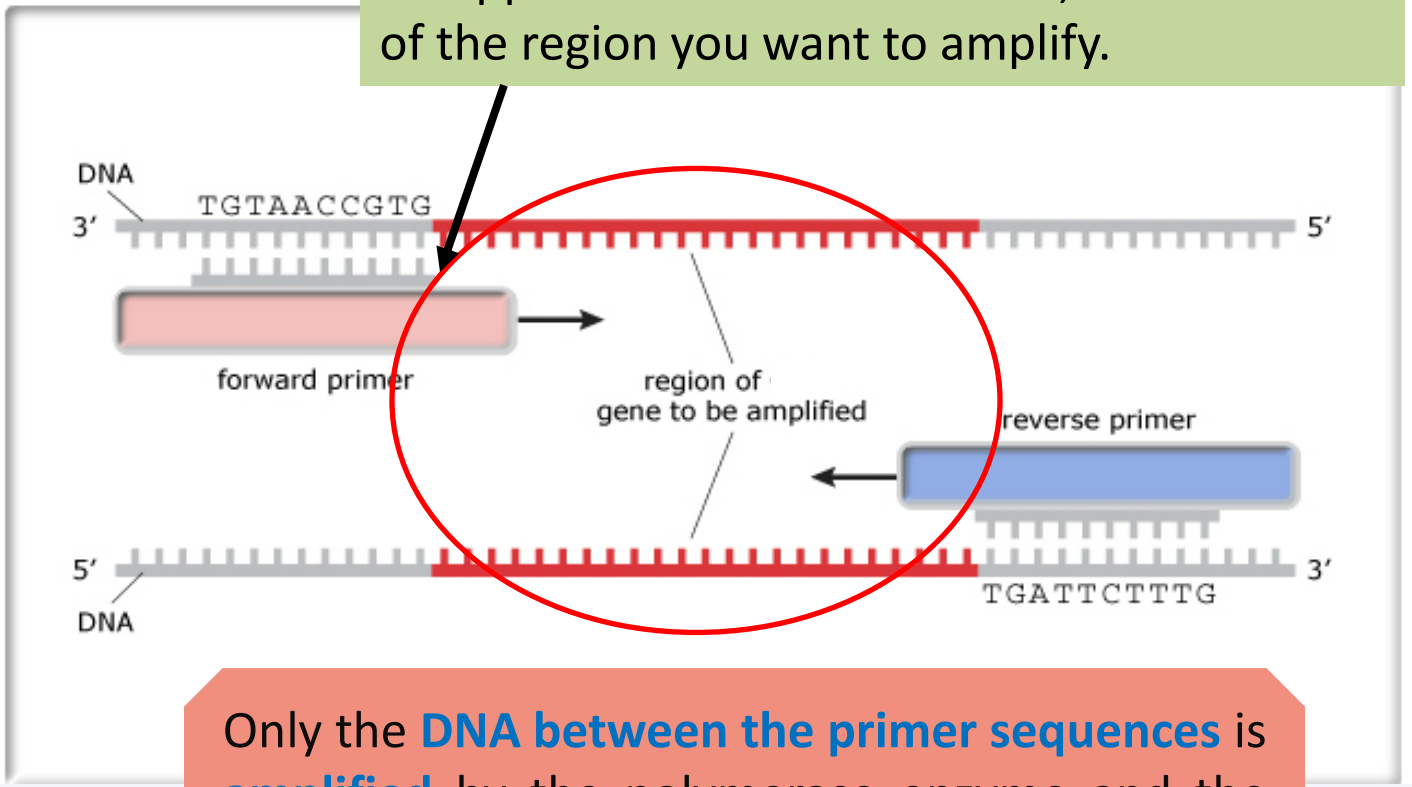


http://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html

Primers

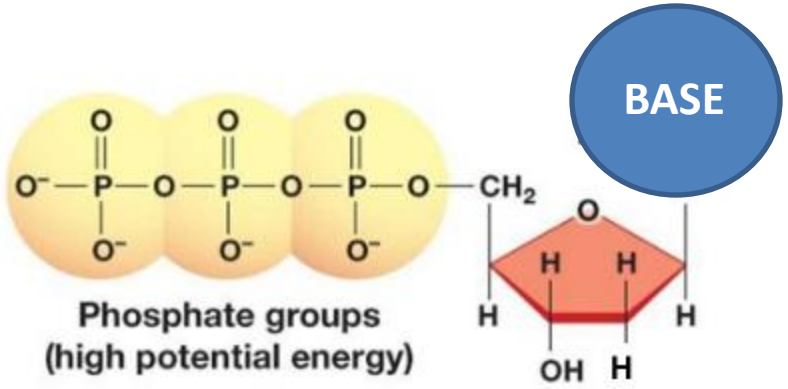
- PCR primers are **short, single stranded pieces of DNA**, usually 20-30 base pair in length
- Primers are uniquely designed to **select the fragment to be amplified** from the test DNA sample
- They are made to be **complementary** to sequences on the test DNA sample
- Primers are necessary because DNA polymerase can only attach onto an existing piece of DNA to start copying.

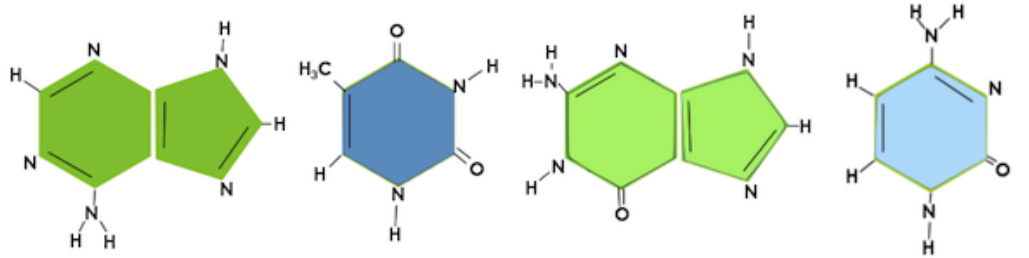
Primers **anneal** to complementary sequences on opposite strands of the DNA, on either side of the region you want to amplify.



Only the **DNA between the primer sequences** is **amplified** by the polymerase enzyme and the rest of the PCR components.

Nucleotides



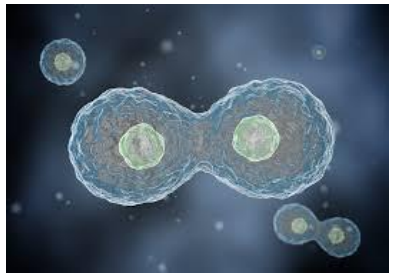


Sensitive to repeat freeze-thaw cycles

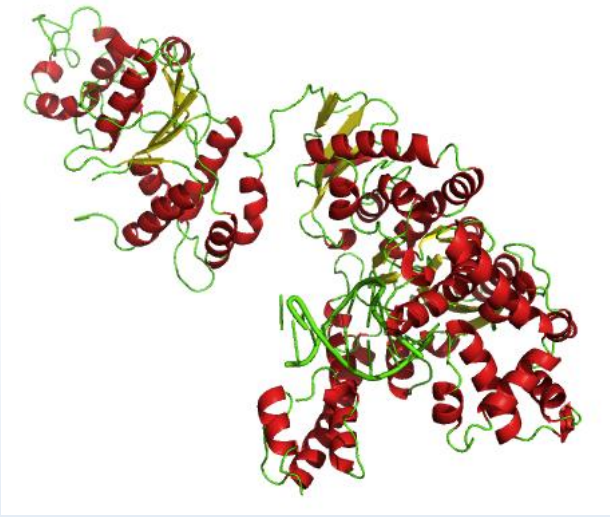
- Deoxynucleoside triphosphates (dNTPs) - four nucleotides containing triphosphate groups
- **DNA building-blocks** from which the DNA polymerase synthesizes a new DNA strand

http://www.unc.edu/depts/our/hhmi/hhmi-ft_learning_modules/plantmodule/step2.html

Polymerase



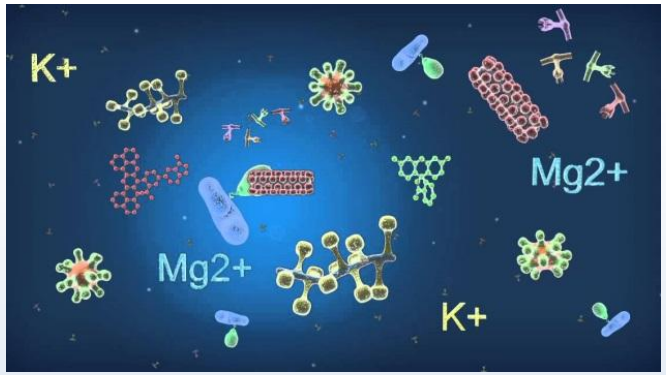
- In our body, a naturally occurring complex of proteins called **DNA Polymerase,** copies a cell's DNA before it divides in two.
- Polymerase detects and attaches to the **bound primer, extending the sequence** using the dNTPs to compliment the sample DNA sequence.
- DNA polymerase used for PCR reactions comes from a strain of bacteria called *Thermus aquaticus* and it is called **Taq polymerase**
- Taq polymerase is stable at 95°C
- Other polymerases available but differ in:
  processivity, fidelity and persistence



Taq polymerase

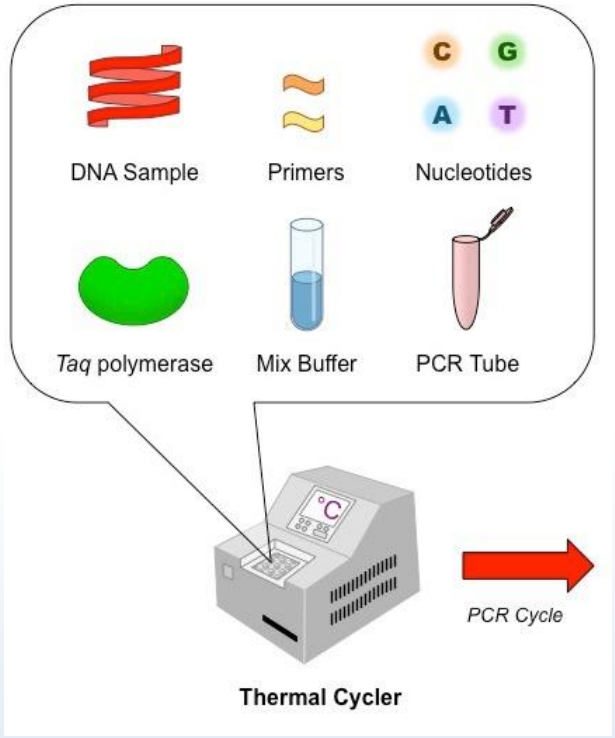
PCR buffer and tubes

- PCR Reagent Buffer - provides an **optimal pH and salt environment** for the reaction
- $MgCl_2$ in the buffer supplies the **Mg^{2+}** divalent cations required as a **cofactor and a catalyst for polymerase activity**
- PCR tubes need to be thin walled to permit **favourable thermal conductivity** to allow for rapid thermal equilibration in a thermal cycler

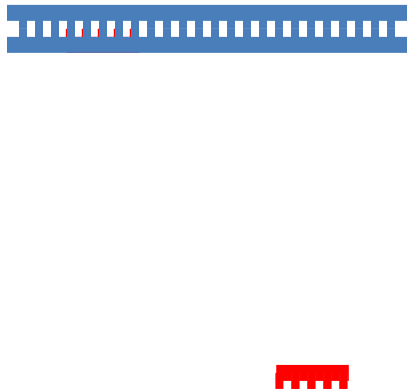


Thermal cycler

- Thermal cycler - a piece of equipment that enables the reactions to be **heated at different temperatures**.
- The thermal cycling parameters are critical to a successful PCR experiment



Thermal Cycling



Taq polymerase extends the primers, synthesizing new strands of DNA.

Template Denaturation (96°C):

Causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

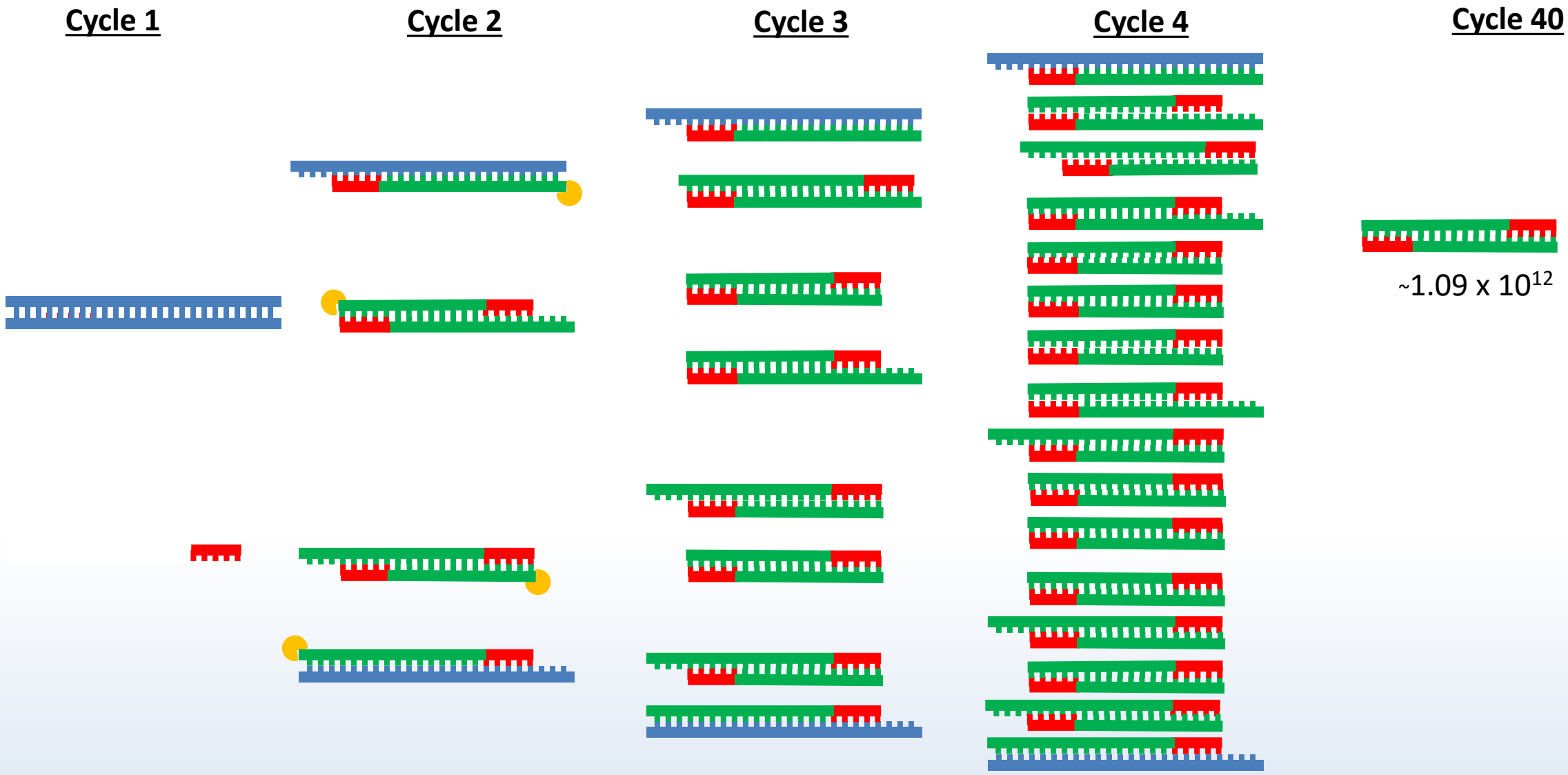
Primer Annealing (55 - 65°C)

Primers bind to their complementary sequences on the single-stranded template DNA.

Primer Extension (72°C)

30-40x

PCR Amplification

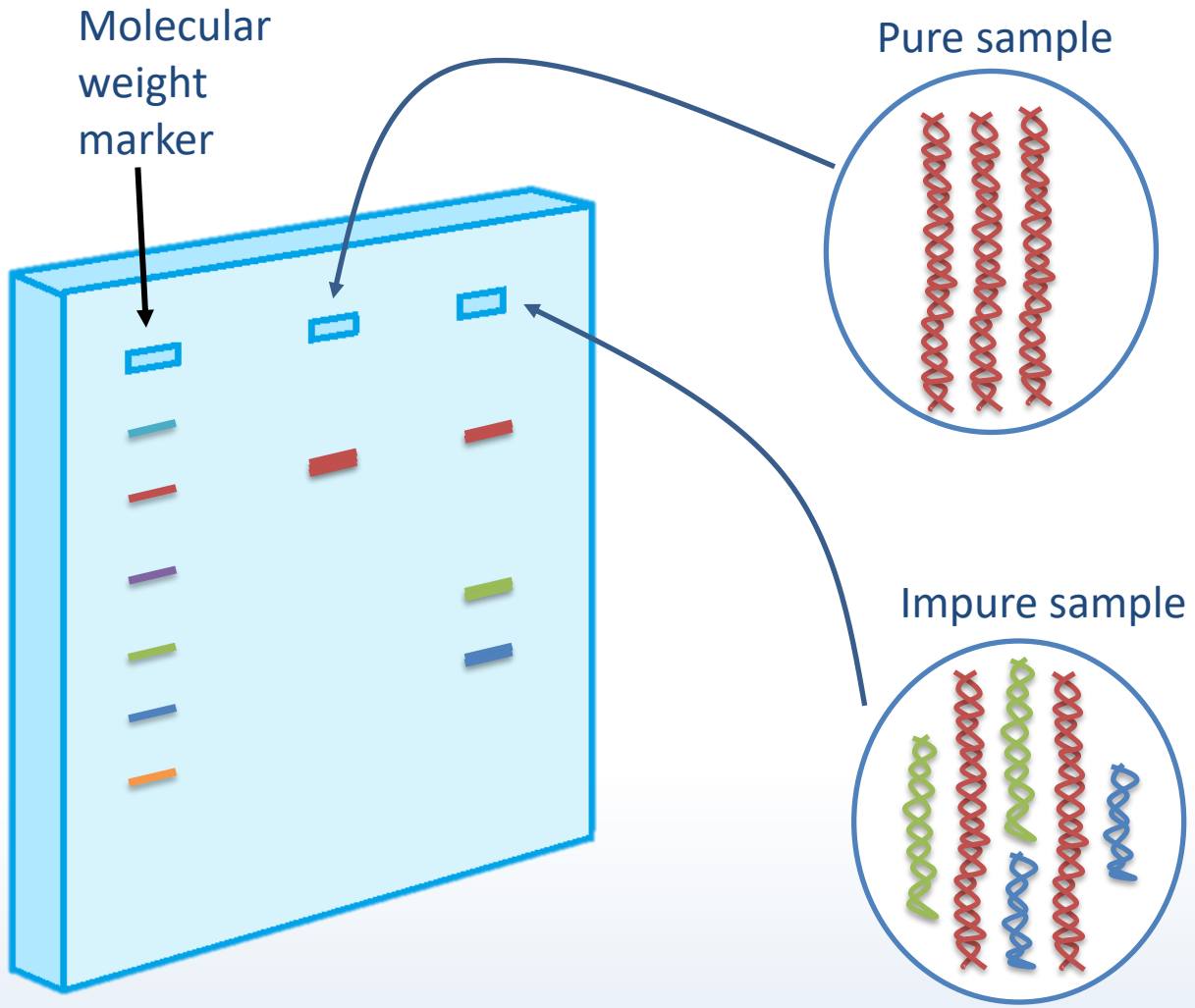


Topics

- Basic Principles of PCR
- Traditional PCR
- Real Time (Quantitative) PCR, qPCR
- Applications

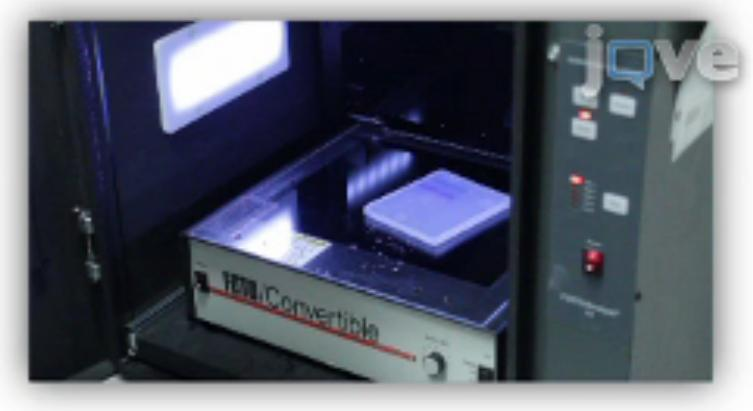
Visualisation of the PCR results

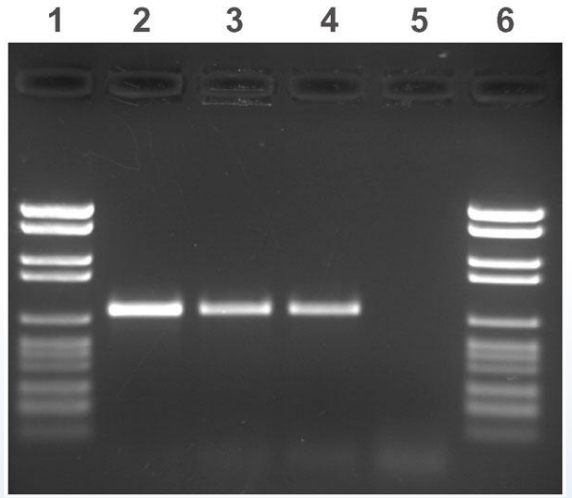
- The results of a PCR reaction are usually visualised using **agarose gel electrophoresis**
- Nucleic acids are inherently **negatively charged**
  - Due to sugar-phosphate backbone
  - Will migrate towards **anode**
  - Longer strands will have greater difficulty moving through pores of gel
  - DNA separates based on **size**
- Agarose is a **polysaccharide** (branched sugar) from *red algae and* is dissolved in **electrophoresis buffer**



Agarose Gel Electrophoresis

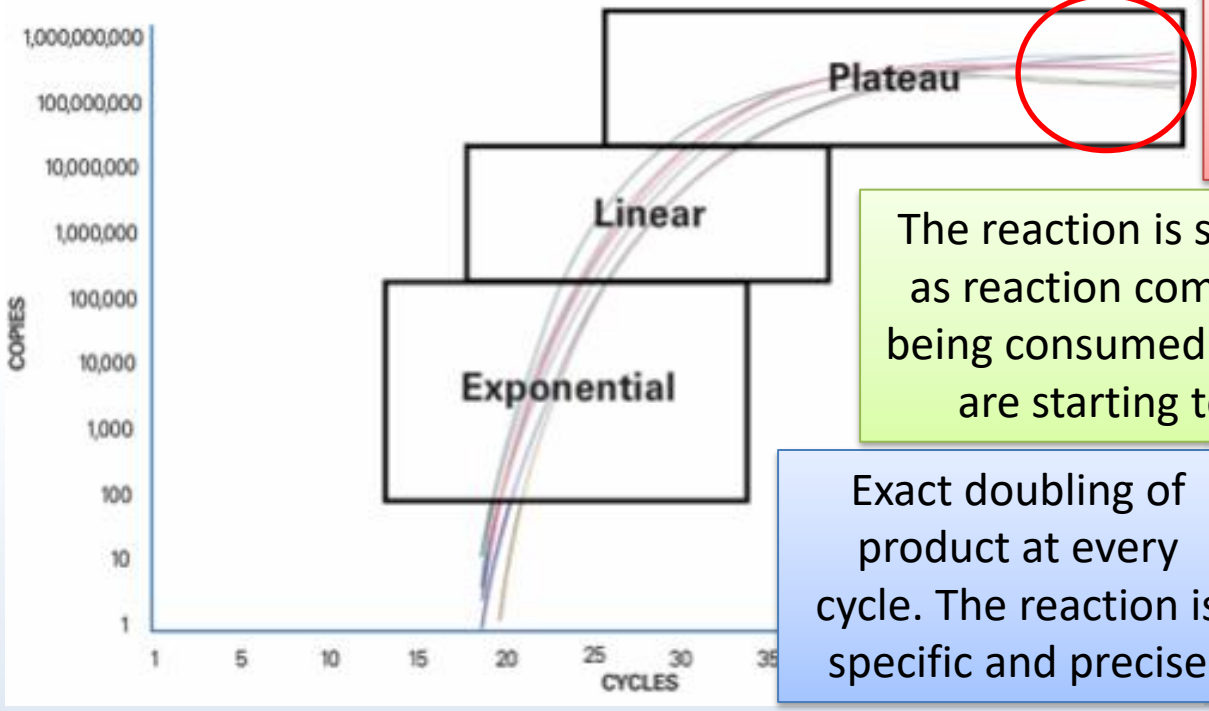
- **Nucleic acid stain** is also added to the gel mixture before it sets
  - Typically Ethidium Bromide – potential carcinogen!
  - Alternatives have been developed, such as SYBR Gold and SYBR Safe dye
- Stains bind to the DNA/RNA and cause them to fluoresce under UV light (or in the case of SYBR Safe a blue-light transilluminator can be used)





Disadvantages of Traditional PCR

- Poor Precision, Low sensitivity and resolution (Agarose Gel resolution ~10 fold)
- Using a dye for quantification is not precise
- Results are not expressed as numbers
- Size-based discrimination only
- Agarose gel results are obtained from **the end point** of the reaction



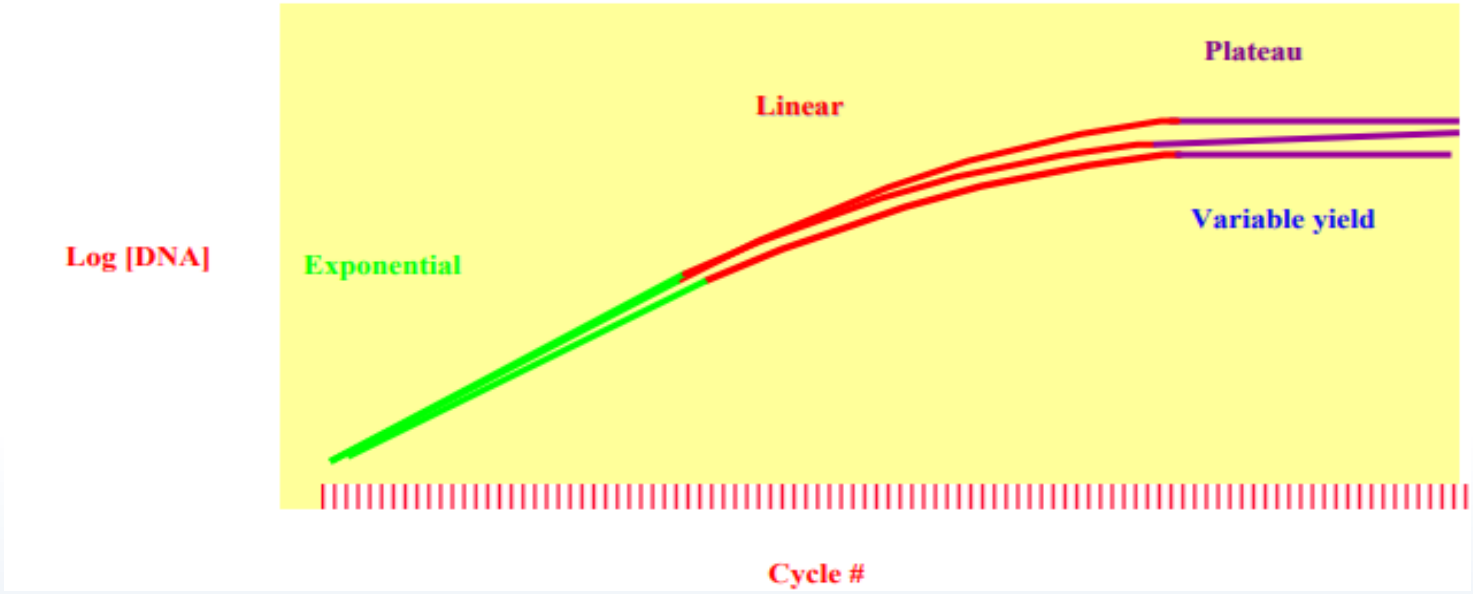
The reaction has stopped, no more products are being made

The reaction is slowing down as reaction components are being consumed and products are starting to degrade

Exact doubling of product at every cycle. The reaction is specific and precise

Disadvantages of Traditional PCR

- High variability beyond the Exponential phase
- Three replicates with the same starting quantity , but different quantities at the plateau phase



Can we obtain data from the exponential phase?

Topics

- Basic Principles of PCR
- Traditional PCR
- Real Time (Quantitative) PCR, qPCR
- Applications

Quantitative PCR (qPCR)

- Theoretically, there is a **quantitative relationship** between amount of **starting target sample** and amount of **PCR product** at any given cycle number

- qPCR allows us to monitor the increase in PCR product formation as it occurs (in the **exponential phase)**

- Enables **quantitative determination** of the starting amounts of DNA in a sample.

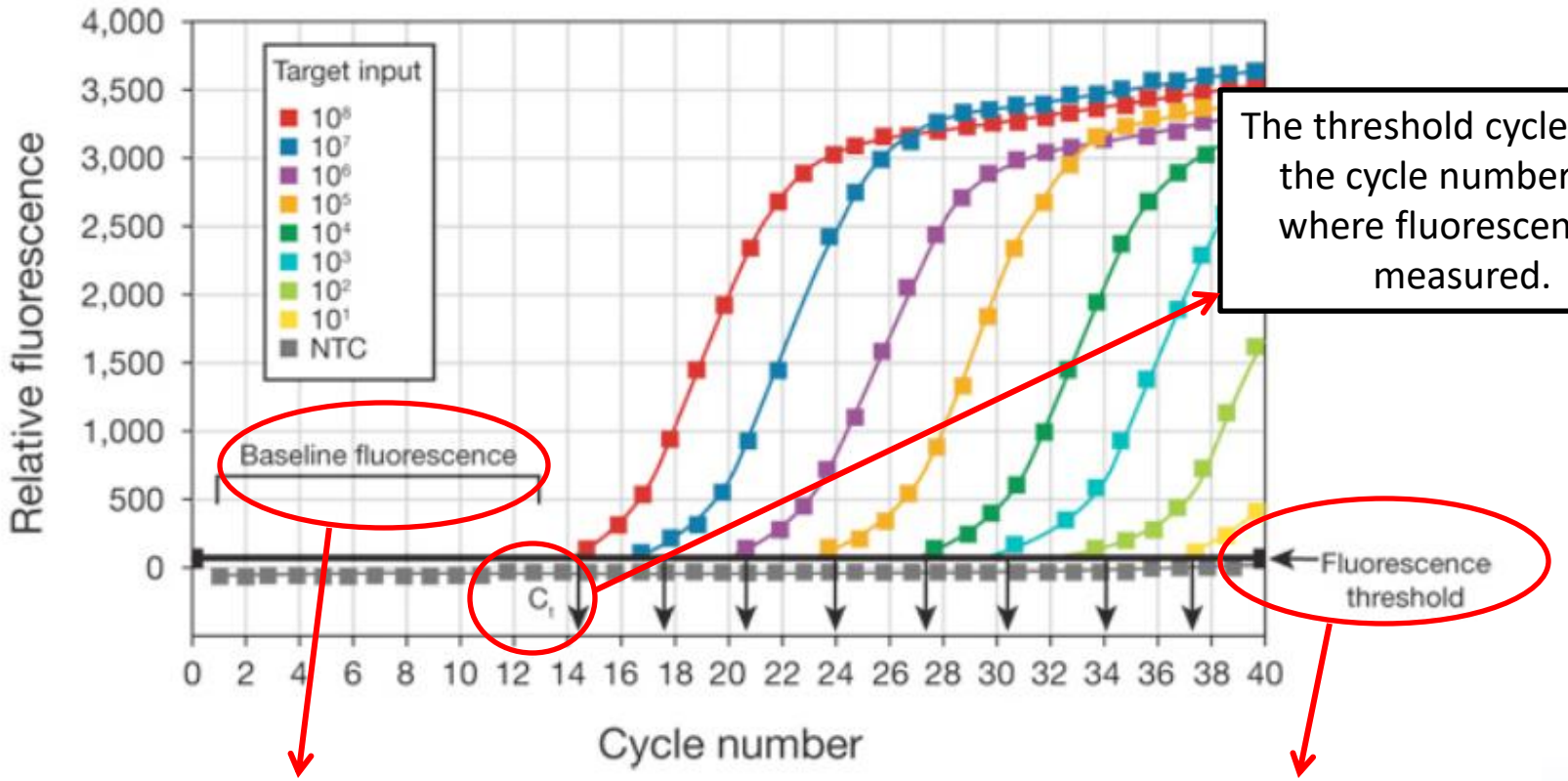
Real-time PCR

- In real-time PCR, **the amount of DNA is measured after each cycle via fluorescent dyes** that yield increasing fluorescent signal in direct proportion to the number of PCR product molecules generated.

- The change in fluorescence over the course of the reaction is measured by an instrument that combines **thermal cycling with fluorescent dye scanning capability**

- By plotting **fluorescence against the cycle number**, the real-time PCR instrument generates **an amplification plot** that represents the accumulation of product over the duration of the entire PCR reaction

Amplification Curve



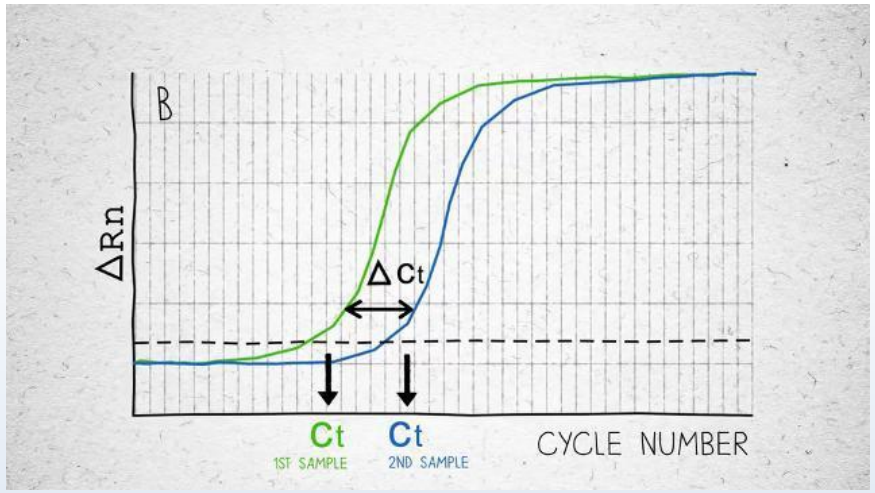
The threshold cycle (Ct) is the cycle number and where fluorescence is measured.

The baseline of the real-time PCR reaction has minimal change in fluorescent signal. This is the background or the “noise” of the reaction.

The threshold of the real-time PCR reaction is the level of signal signifying the beginning of the exponential phase.

Quantitative PCR

- In other words, Ct it is the point at which the reaction returns a positive result
- The Ct is used to calculate the initial DNA copy number, because the **Ct value is inversely related to the starting amount of target** (e.g. a sample with twice the starting amount will yield a Ct one cycle earlier than a sample that contained half as many copies of the target prior to amplification)

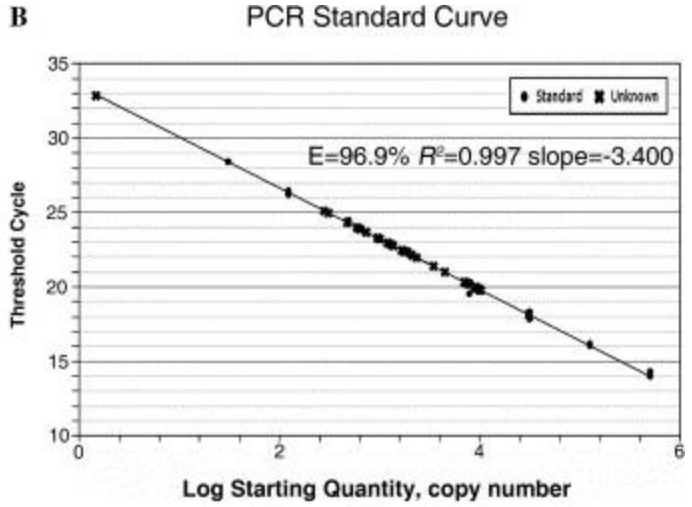


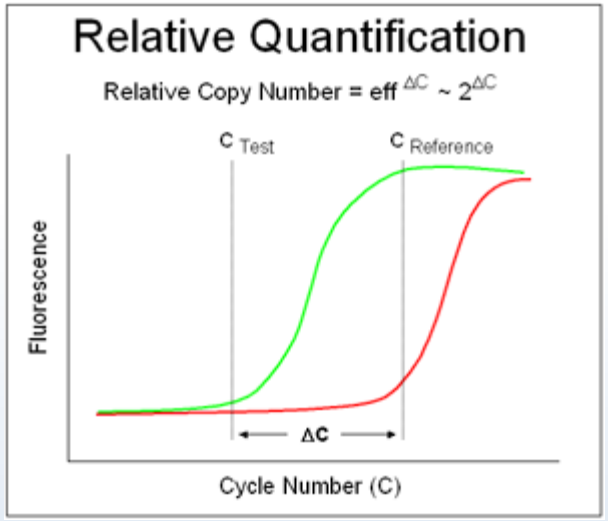
Quantification Analysis

- The Ct is used to calculate the initial DNA copy number, because the **Ct value is inversely related to the starting amount of target**

- **Absolute quantification -** Samples of known quantity are serially diluted and then amplified to generate a standard curve. Unknown samples are then quantified by comparison with this curve.

- **Relative quantification -** The expression of a gene of interest in one sample is compared to expression of the same gene in another sample. The results are expressed as fold change (increase or decrease) in expression of one in relation to the other.

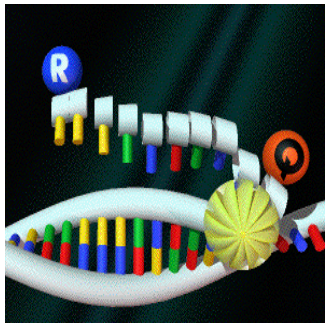




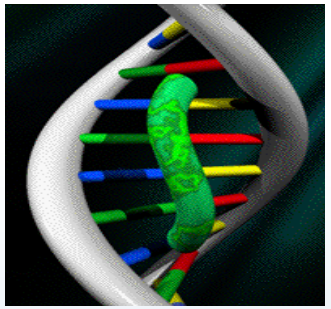
Quantification methods

- There are two key methods used to detect the number of DNA segments amplified:

1. Dye-labeled, sequence specific oligonucleotide probes e.g. **TaqMan assays**

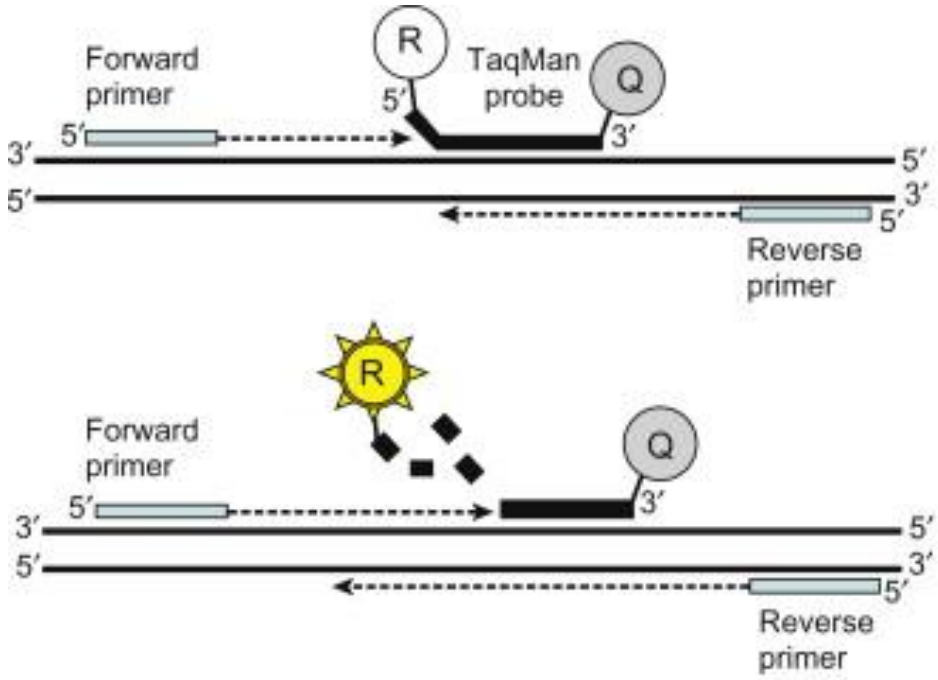


1. DNA-binding dyes **(SYBR Green)**



TaqMan® PCR technology

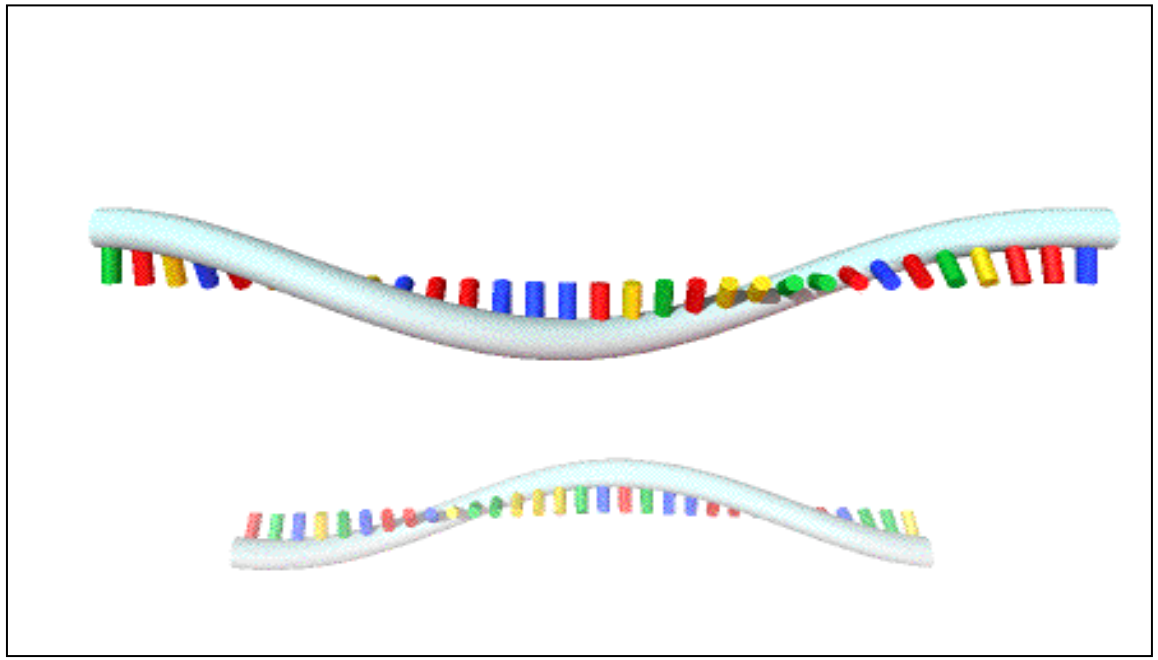
- Sequence-specific probe that **anneals between the forward and reverse primers**.
- Labelled with a **reporter (R)** and a **quencher (Q) dye.**
  - R is usually a green dye (e.g. fluorescein)
  - Q is typically a red dye (e.g. TAMRA)
- When the probe is intact **Q interferes with R - little to no fluorescence**.
- As the PCR primer extends, the probe is cleaved from the DNA strand
- **Cleavage separates R and Q – increase in fluorescence.**



https://doi.org/10.1016/B978-0-12-374513-2.00003-8

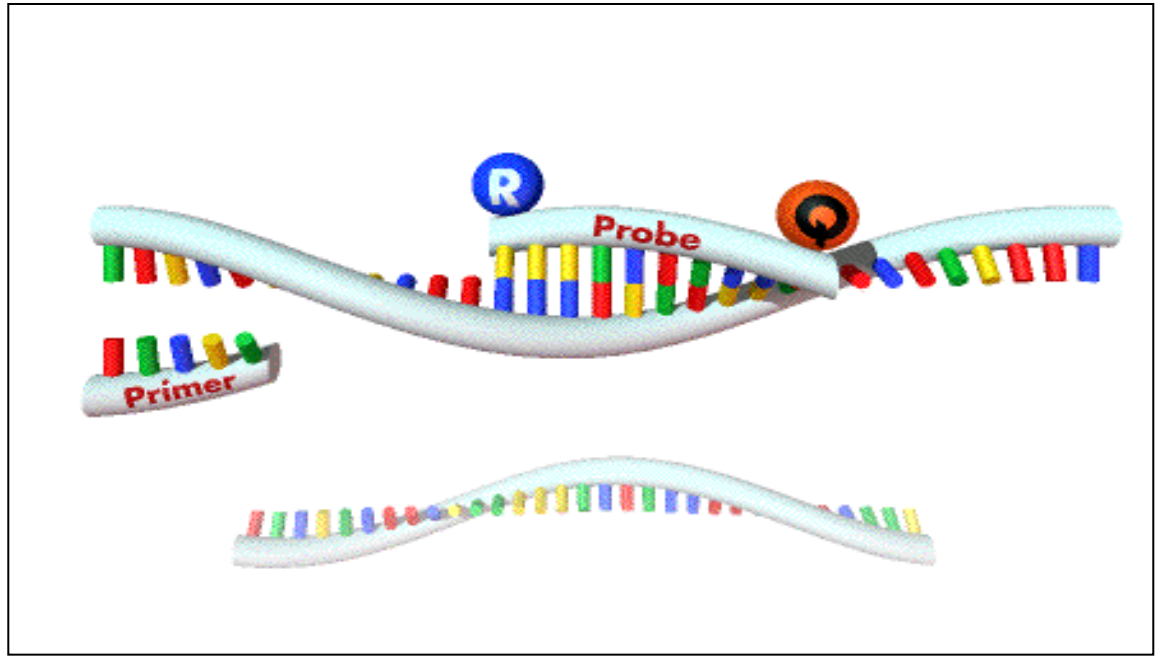
5' Nuclease Assay with TaqMan® Probes

1. Denaturation



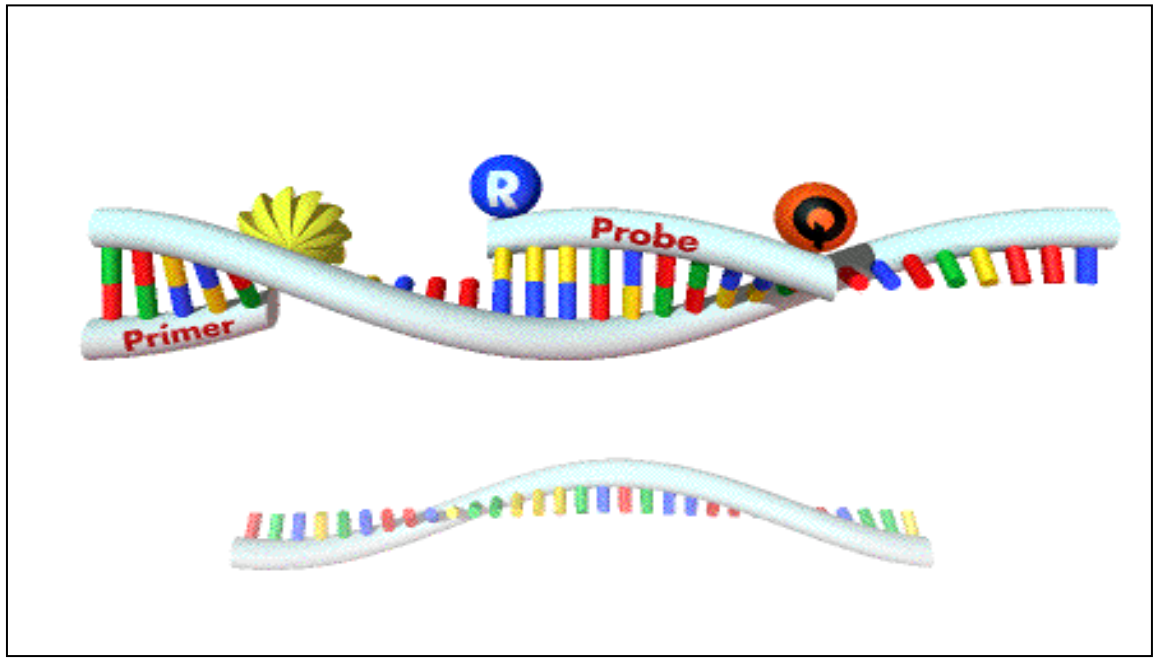
5' Nuclease Assay with TaqMan® Probes

1. Denaturation
2. Annealing of Primers and Probe
    - Probe is designed to have a **slightly higher annealing temperature** compared to the PCR primers so the probe will be **hybridized when extension of the primers begin**.



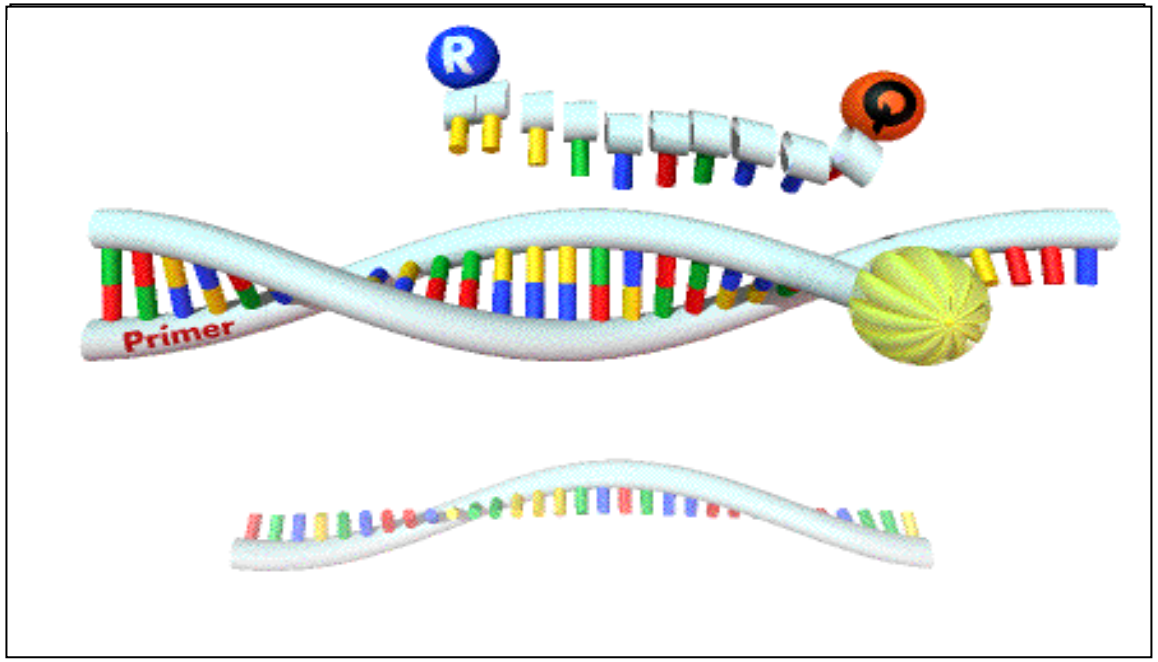
5' Nuclease Assay with TaqMan® Probes

1. Denaturation
2. Annealing of Primers and Probe
    - Probe is designed to have a **slightly higher annealing temperature** compared to the PCR primers so the probe will be **hybridized when extension of the primers begin**.
3. DNA Polymerase and Probe Cleavage



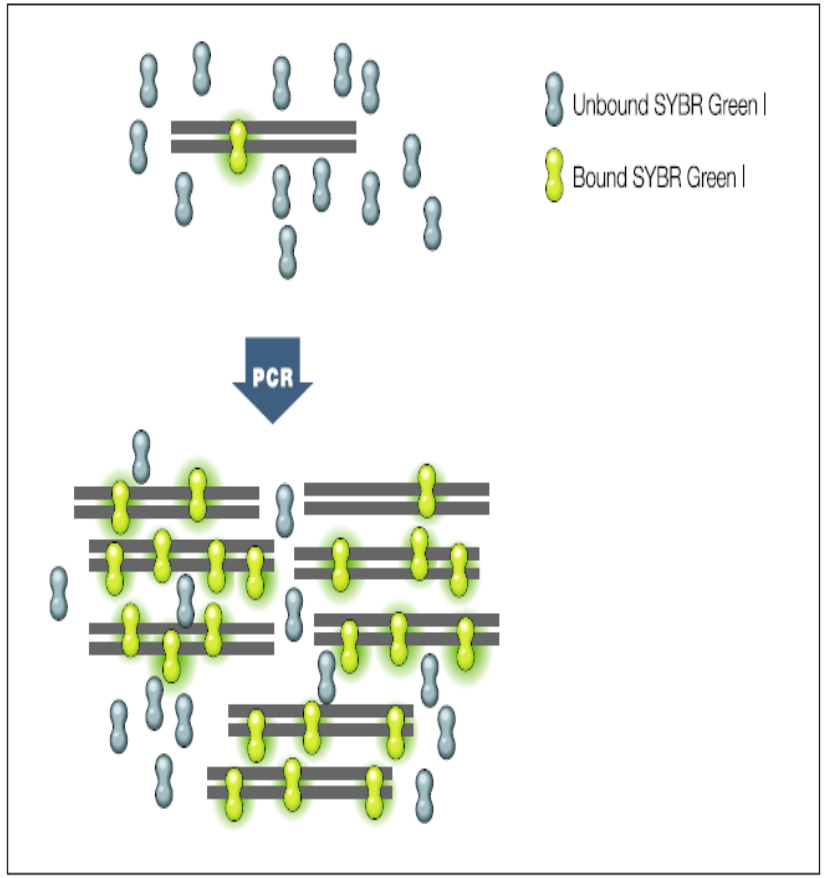
5' Nuclease Assay with TaqMan® Probes

1. Denaturation
2. Annealing of Primers and Probe
   - Probe is designed to have a **slightly higher annealing temperature** compared to the PCR primers so the probe will be **hybridized when extension of the primers begin**.
3. DNA Polymerase and Probe Cleavage
4. AmpliTaq Gold® DNA Polymerase and Probe Cleavage



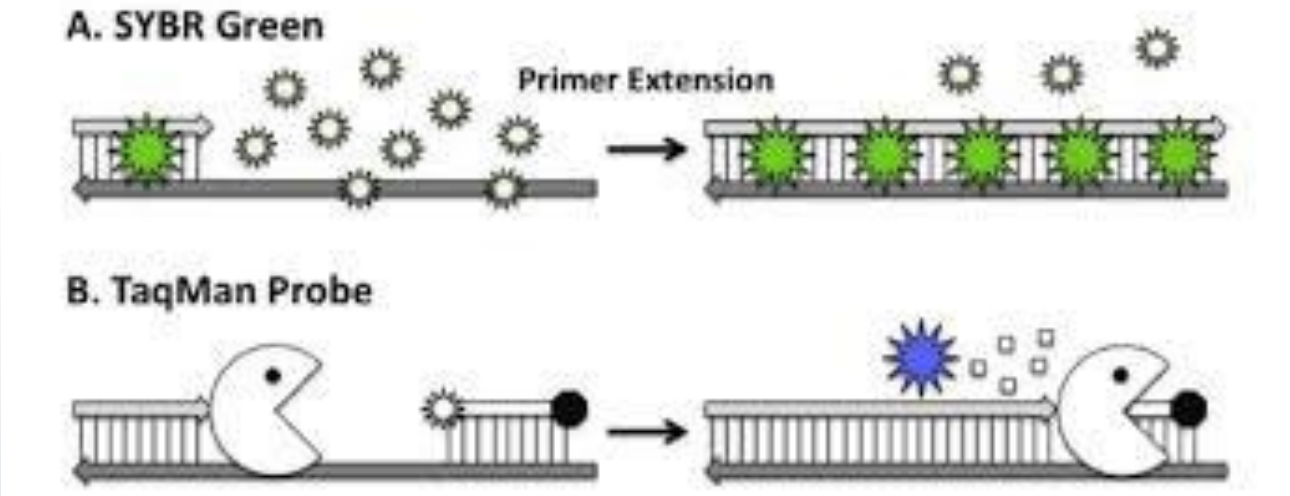
SYBR Green I

- SYBR Green I is a DNA dye that binds non-discriminately to double-stranded DNA (dsDNA).
- SYBR Green I exhibits **minimal fluorescence when it is free in solution**, but its **fluorescence increases** dramatically (up to 1000-fold) upon **binding to dsDNA** (new DNA)
- As more double stranded amplicons are produced, SYBR Green dye signal will increase respectively.



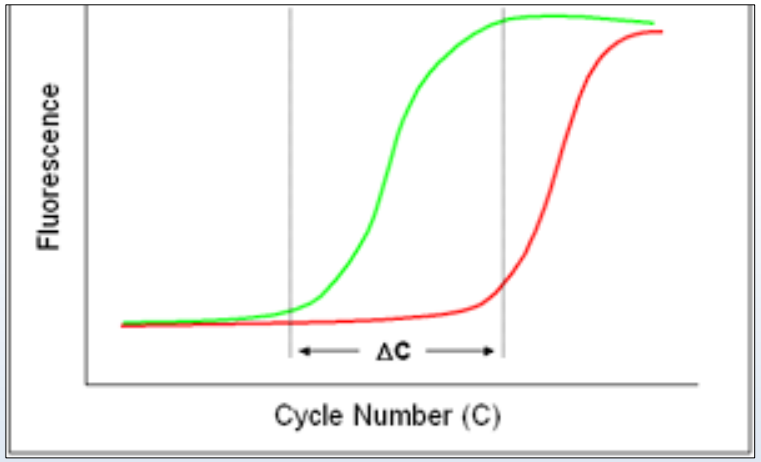
Advantages of SYBR green

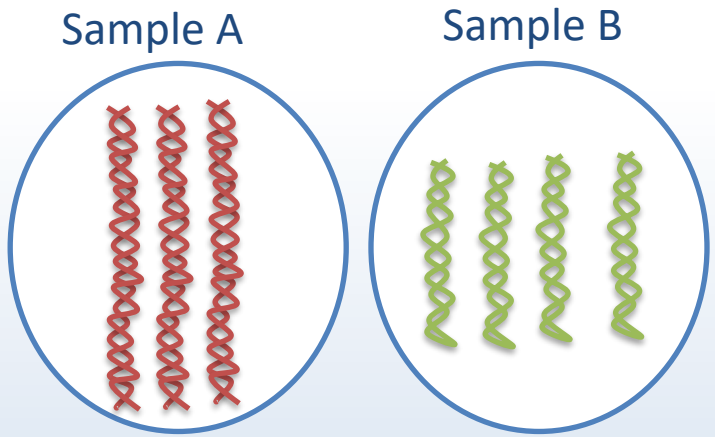
- Simple assay design (only two primers are needed; probe design is not necessary).
- Ability to test multiple genes quickly without designing multiple reported probes.
- Lower initial cost (probes cost more).



Disadvantage of SYBR green

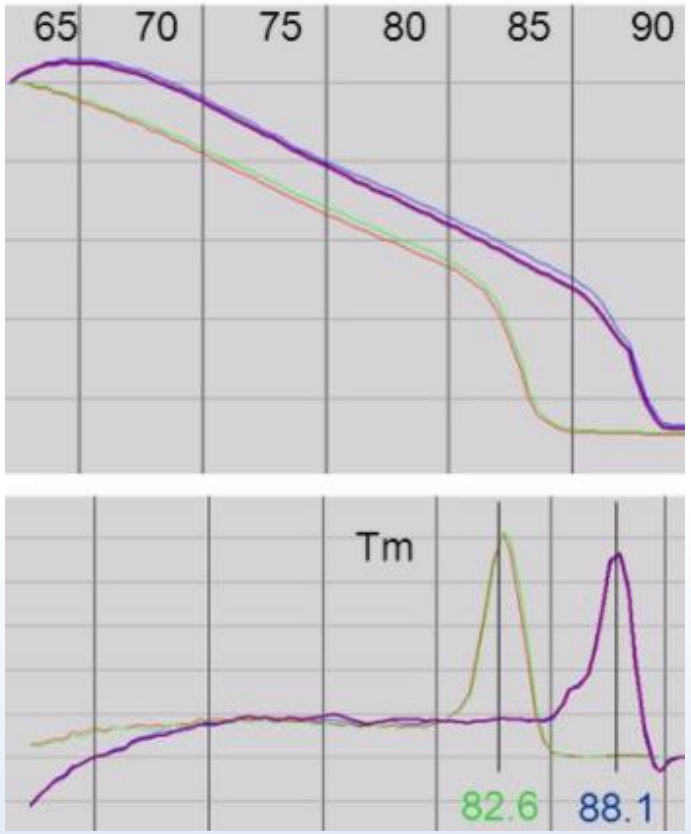
- SYBR Green technology is **not product specific**.
- Binds to any double-stranded DNA, cannot distinguish between the amplification of target DNA and **primer-dimers**.
- **False positives** – amplification of non-target sequences
- For this reason, when using SYBR Green I it is prudent to verify that target DNA is being amplified; this is commonly done by post amplification **melt-curve analysis**





Melt Curve Analysis

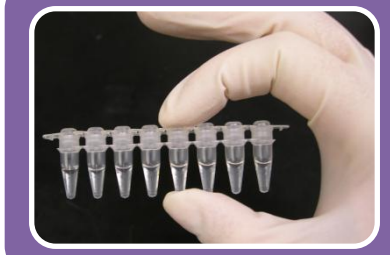
- Increasing temperature denatures DNA and SYBR Green **fluorescence decreases**
- **Melting temperature (Tm)** – **50% drop in fluorescence**, is product specific.
- It can be plotted as:
  - fluorescence vs temperature (melt curve)
  - $\frac{\Delta\ fluorescence}{\Delta\ temperature}$ vs Temperature (melting peak)
- Melting Curve analysis confirms the **PCR product identity**
  - **Homogenous product** should have only **one melting peak**
  - Identifies **non specific products** such as primer-dimers
    - ➢ Usually lower melting temperature and broad peak



Topics

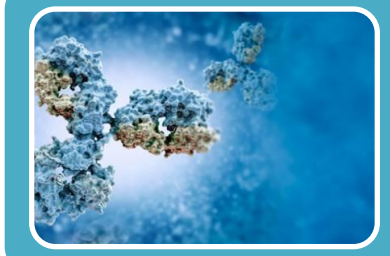
- Basic Principles of PCR
- Traditional PCR
- Real Time (Quantitative) PCR, qPCR
- Applications

Applications of PCR



Medicine– genetic analysis, diagnostics, pathogen detection

Forensic Science/Archaeology e.g. can produce a DNA profile from a trace sample of blood i.e. human identification.



Bio/Pharmaceutical Science e.g. can rapidly detect and quantify virus/retrovirus, residual host cell DNA , mycoplasma etc.

Applications of Real time PCR in the Pharmaceutical Industry

- Quantitative PCR assays are available for a variety of applications, such as the following:
  - Detection or quantification of **Host Cell DNA** in cell banks, bulk harvest material, finished product etc.
  - Detection or quantification of **DNA or RNA viruses and other contaminants** in cell banks, bulk harvest material, finished product etc.

Host Cell DNA detection

- The regulatory authorities state that manufacturers of biologicals should quantify the amount of residual DNA in final products manufactured from continuous mammalian cell lines.

- The World Health Organization (WHO) and the European Union (EU) -10 ng of residual DNA per dose.
- The Food and Drug Administration (FDA) - 100 pg per dose

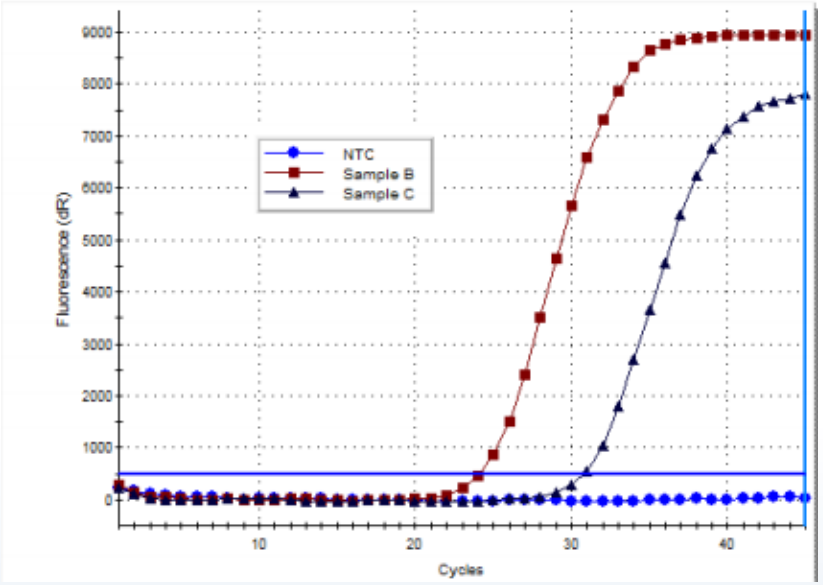
- TaqMan® Residual DNA Chinese Hamster Ovary kit.
- TaqMan® Residual DNA Escherichia coli kit.

Detection of Retroviruses and Endogenous Retrovirus-Like Particles

- Some cell lines, such as rodent cells, harbour endogenous retroviruses.
- The retroviral particles must be quantified in unprocessed bulk fluids and in cell lines.
- Quantitative PCR assays are available to enumerate viral copy numbers and have real advantages (such as considerably shorter turnaround times and higher sensitivity) over traditional methods.

Detection of Contamination

- PCR also a common technique for microbial surveillance



Sample B (red) and C (black) show contamination with Mycoplasma DNA, compared to no template control (NTC, blue)

https://www.applichem.com/fileadmin/produktinfo/a9019_en.pdf

Topics

- Basic Principles of PCR
- Traditional PCR
- Real Time (Quantitative) PCR, qPCR
- Applications

Thank You