Real-Time _ PCR Basics

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PCR From classical to real time

- PCR has completely revolutionized the detection of RNA and DNA.

- Real-time PCR is a bespoke form of the Polymerase Chain Reaction that maximizes the potential of the technique.

PCR From classical to real time

- Traditional PCR has advanced from detection at the end-point of the reaction to detection while the reaction is occurring.

- To understand Real Time PCR it's easier to begin with the principles of a basic PCR

Principles of PCR

- PCR is a technique for amplifying DNA.
- 2 reasons why you may want to amplify DNA.
- 1- simply create multiple copies of a rare piece of DNA. For example a forensic scientist may want to amplify a tiny piece of DNA from a crime scene.
- 2- More commonly /compare 2 different samples of DNA to see which is the more abundant.

Principles of PCR

- Because DNA is microscopic you cannot see which sample contains the most DNA.

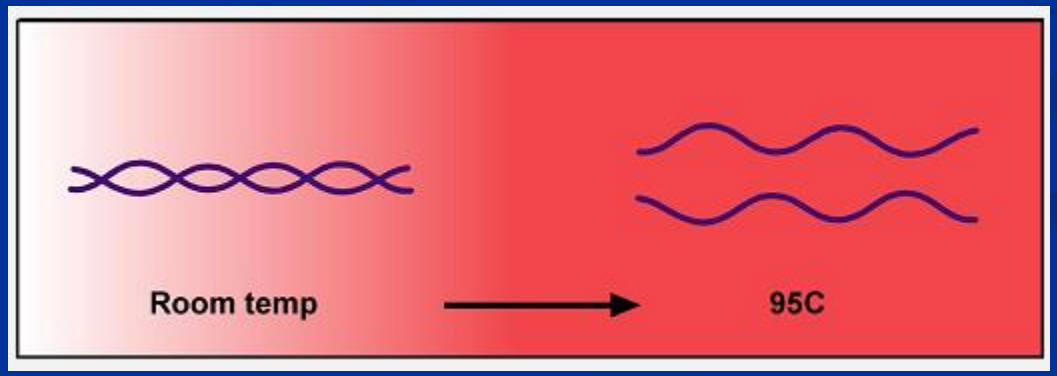
- However, if you amplify both samples at the same rate, you can calculate which sample was the biggest to begin with by establishing which is the biggest after amplification .

Principles of PCR

- A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point.
- you can choose which gene you wish the polymerase to amplify in a mixed DNA sample by adding small pieces of DNA complimentary to your gene of interest. These small pieces of DNA are known as primers

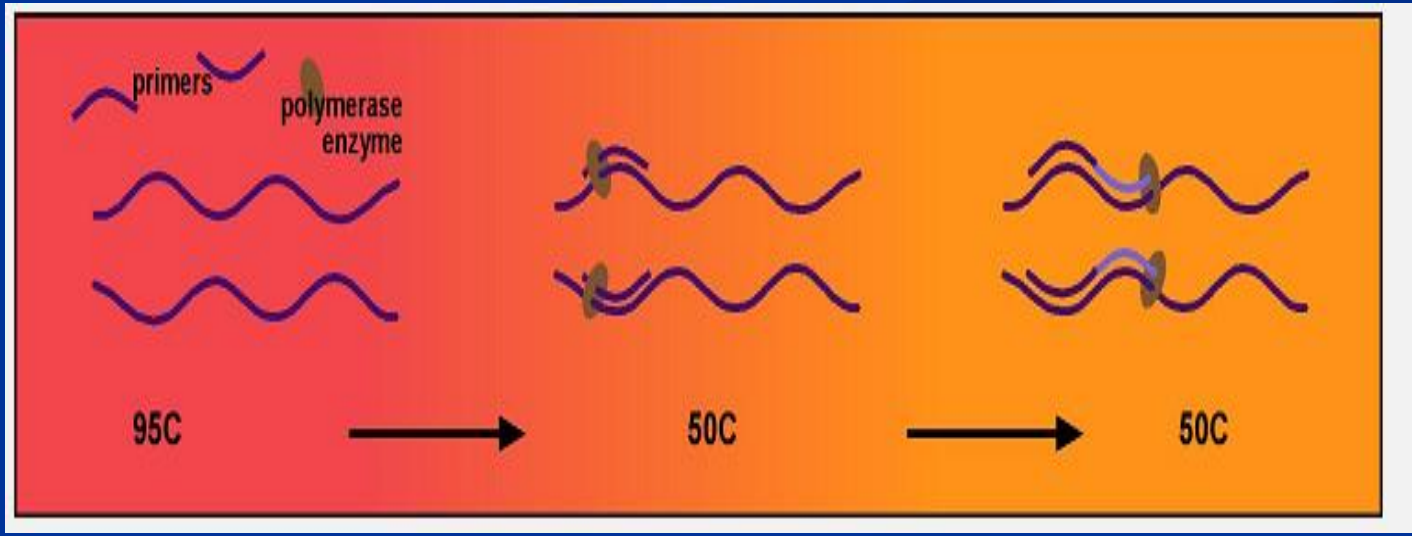
Principles of PCR

- .During a PCR, changes in temperature are used to control the activity of the polymerase and the binding of primers.
- To begin the reaction the temperature is raised to 95°C. At this temperature all double stranded DNA is “melted” in to single strands:



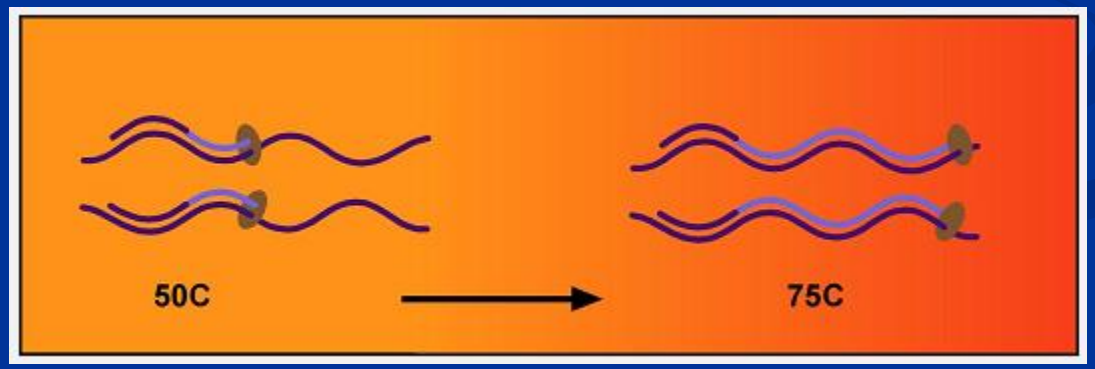
Principles of PCR

- The temperature is then lowered to ~50°C. This allows the primers to bind to your gene of interest. Thus the polymerase has somewhere to bind and can begin copying the DNA strand:



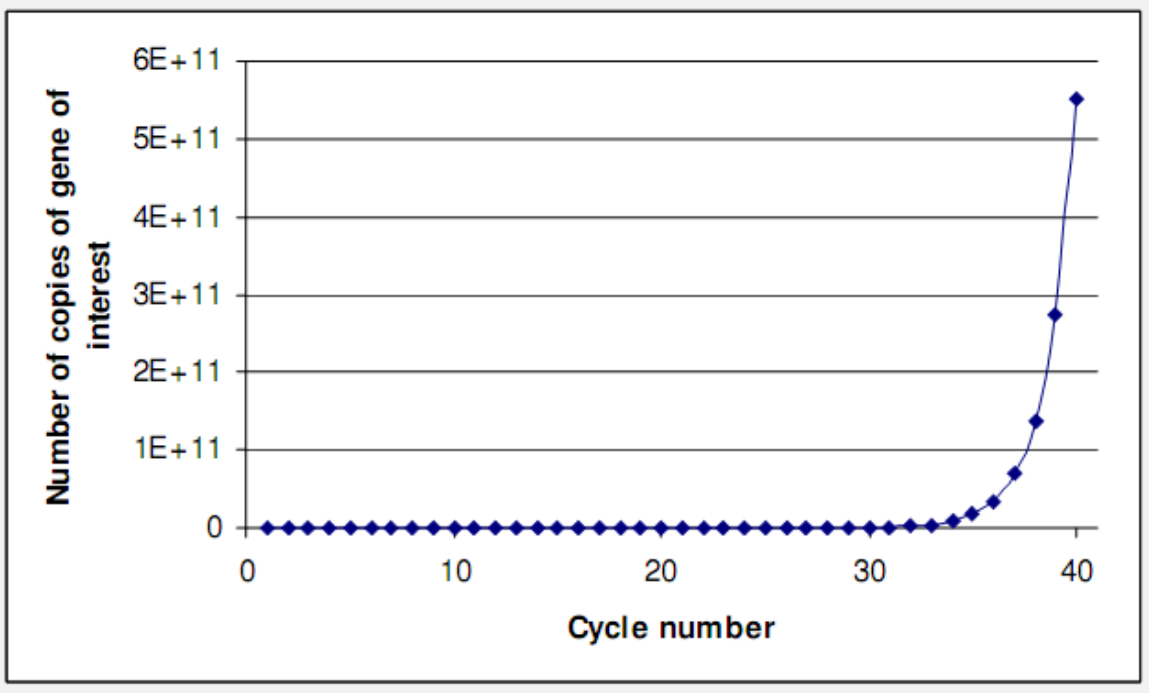
Principles of PCR

- The optimal temperature for the polymerase to operate is 72°C so at this point the temperature is raised to 72°C to allow the enzyme to work faster. There are now twice as many copies of your gene of interest as when you started:



Principles of PCR

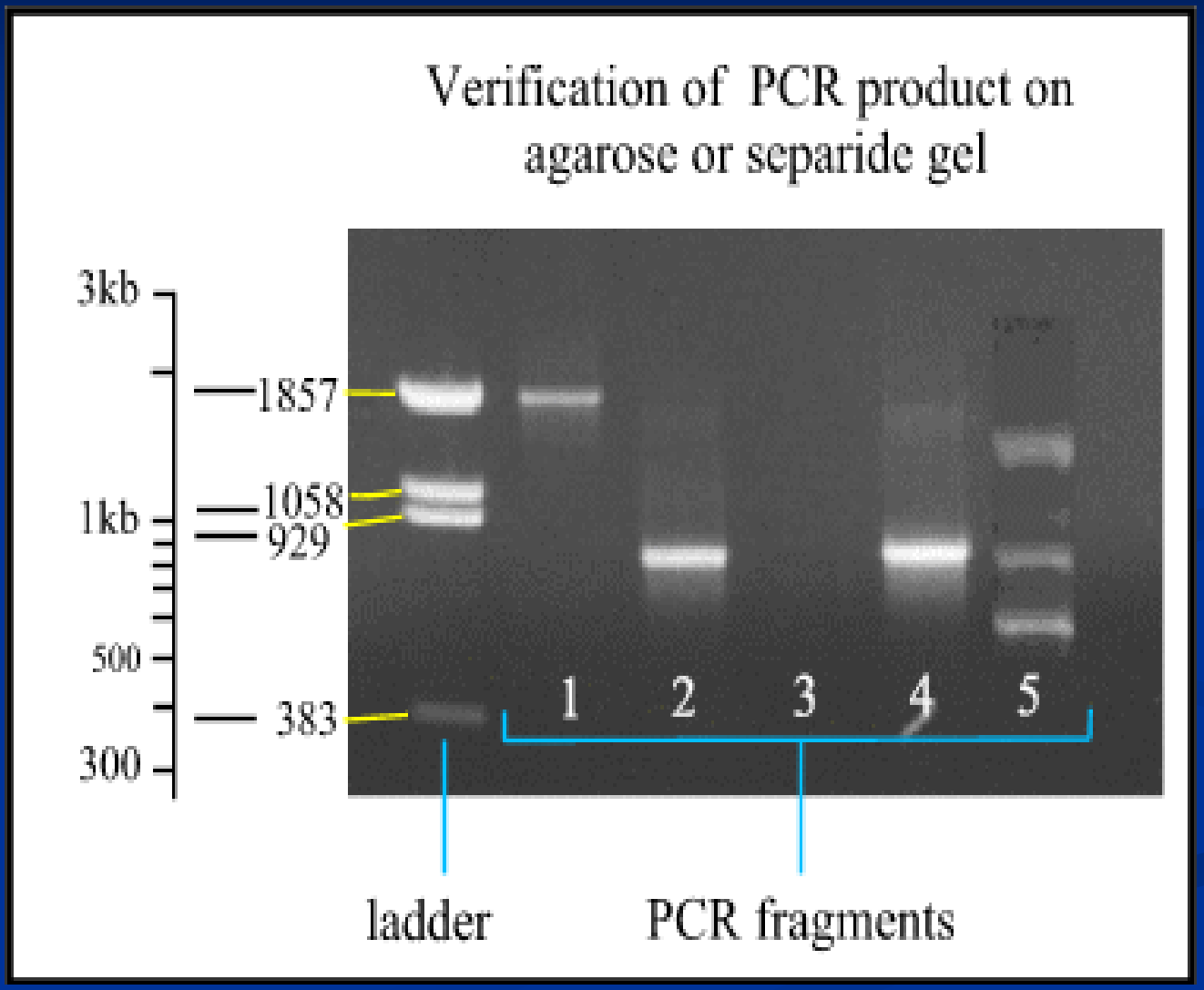
- The cycle of changing temperatures (95°C, 50°C and 72°C) is then repeated and two copies become four. Another cycle and four become eight, and so on... for 40-50 cycles



Principles of PCR

- After amplifying your gene in to many millions of copies it is possible to run the amplified DNA out on a polyacrilamide or Agarose gel and stain it with a dye which makes is visible. The bigger the visible band, the more copies of your gene you have created.
- Hence if you are comparing 2 samples e.g. one from a healthy patient and one from a cancer patient, you can see in which sample your gene of interest was expressed most highly.

Principles of PCR



Real-Time PCR

- Real-Time PCR is identical to a simple PCR except that :
- the progress of the reaction in monitored by a camera or detector in “real-time”.
- There are a number of techniques that are used to allow the progress of a PCR to be monitored.

Methods of monitoring DNA amplification in "real-time":

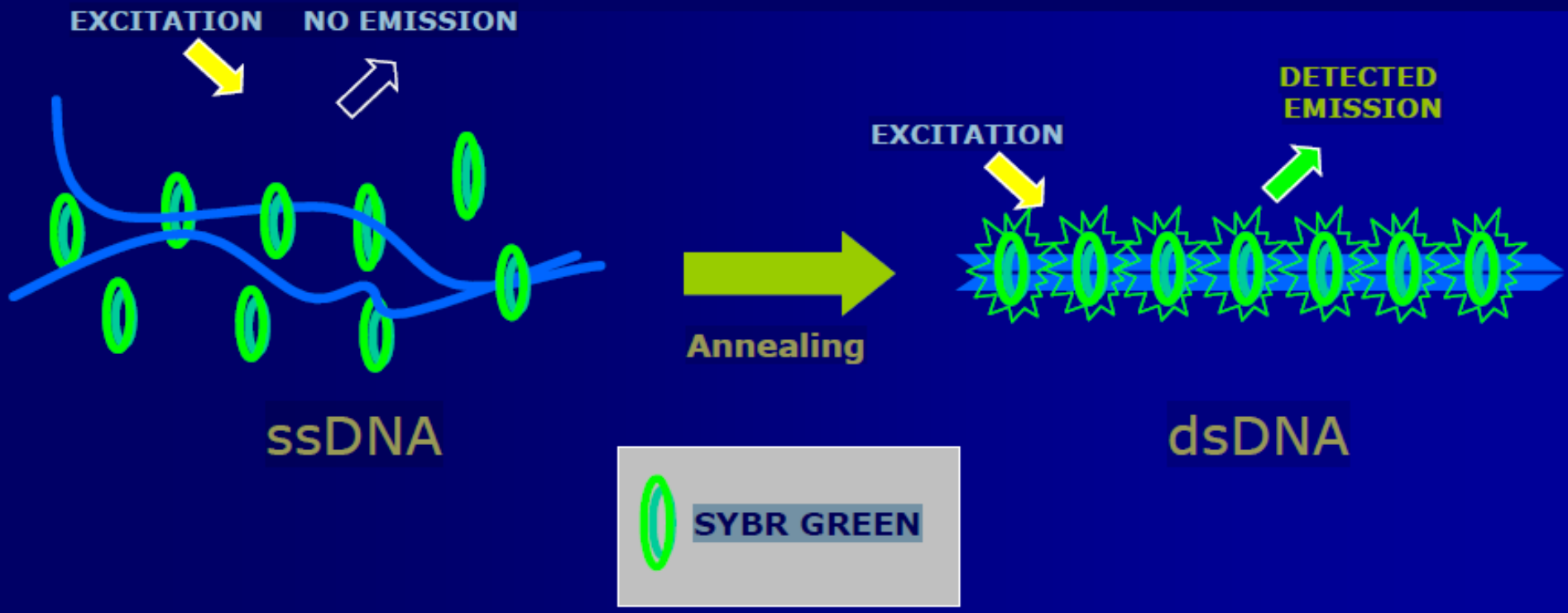
- Each technique uses some kind of fluorescent marker which binds to the DNA. Hence as the number of gene copies increases during the reaction so the fluorescence increases.
- This is advantageous because the efficiency and rate of the reaction can be seen.
- There is also no need to run the PCR product out on a gel after the reaction.

Fluorescent dyes

- Intercalating fluorescent dyes (e.g. SYBR green) are the simplest and cheapest way to monitor a PCR in real-time. These dyes fluoresce only when bound to double-stranded DNA. So as the number of copies of DNA increases during the reaction so the fluorescence increases.
- The major disadvantage of using a dye such as this is the lack of specificity. This dye will report the amplification of any DNA not just your gene of interest.

Fluorescent dyes

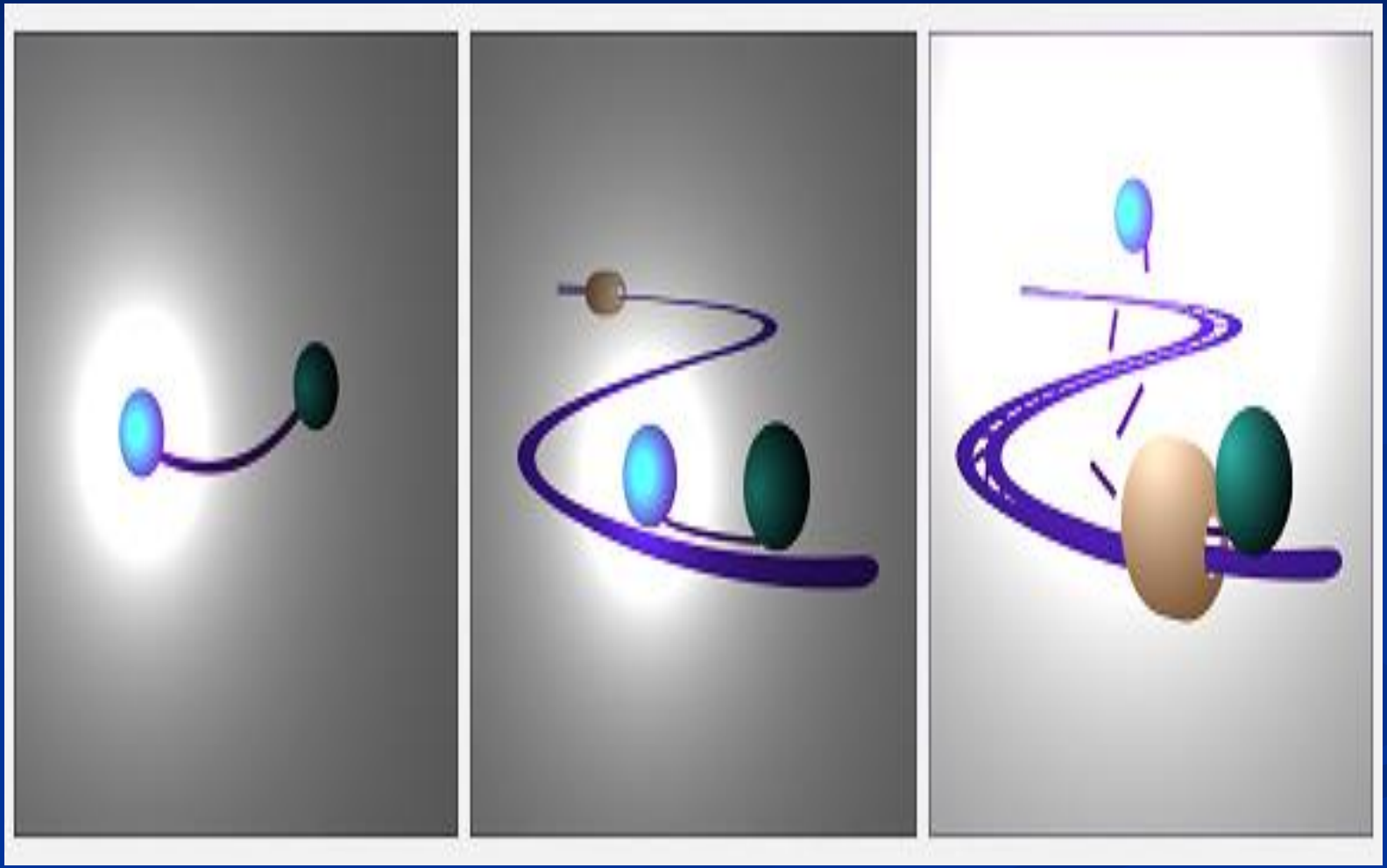
SYBR Green I



Fluorescent probes

- Fluorescent probes are pieces of DNA complimentary to your gene of interest that are labeled with a fluorescent dye.
- The simplest and most commonly used type of probe is the Taqman-type probe. These probes are labeled with a fluorescent reporter molecule at one end and a quencher molecule (capable of quenching the fluorescence of the reporter) at the other.

Fluorescent probes



Fluorescent probes

- Hence under normal circumstances the fluorescent emission from the probe is low.
- during the PCR the probe binds to the gene of interest and becomes cleaved by the polymerase. Hence the reporter and quencher are physically separated and the fluorescence increases.

molecular beacon

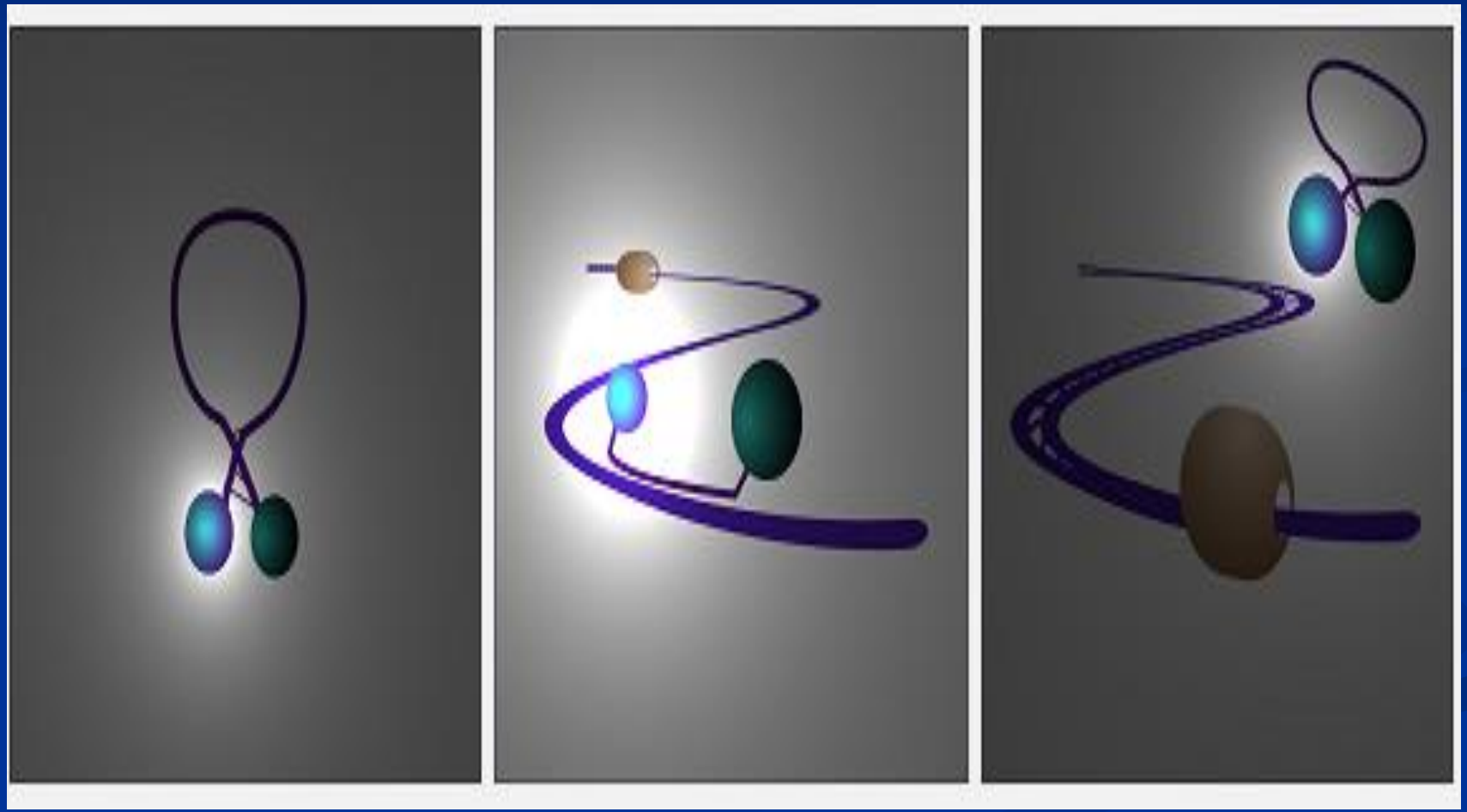
- small pieces of DNA complimentary to gene of interest labeled with a fluorescent reporter and a quencher molecule on opposite ends.

- designed to fold on to themselves to bring the reporter and quencher in to closer proximity and minimize fluorescent emission.

molecular beacon

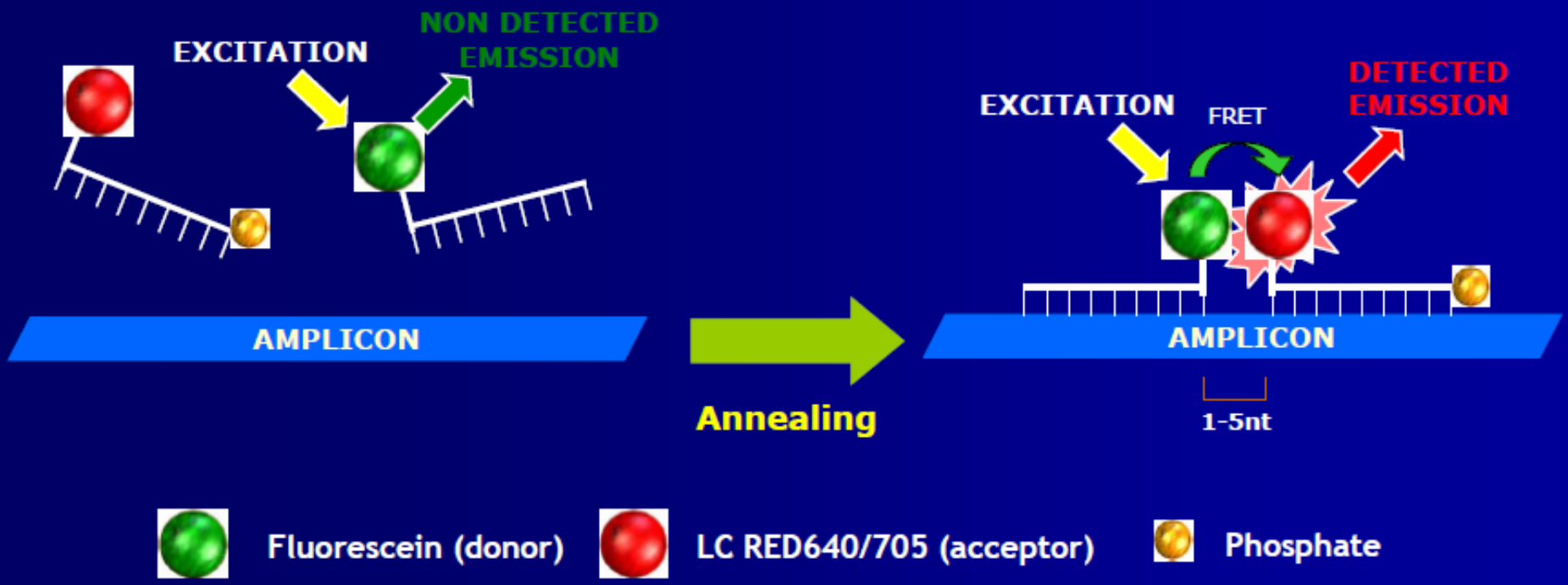
- when the probe binds to the gene of interest the probe takes up a linear confirmation and the reporter and quencher are separated.
- This results in the desired increase in fluorescence.
- Molecular beacon probes are not cleaved by the polymerase but are simply “knocked off” again.

molecular beacon

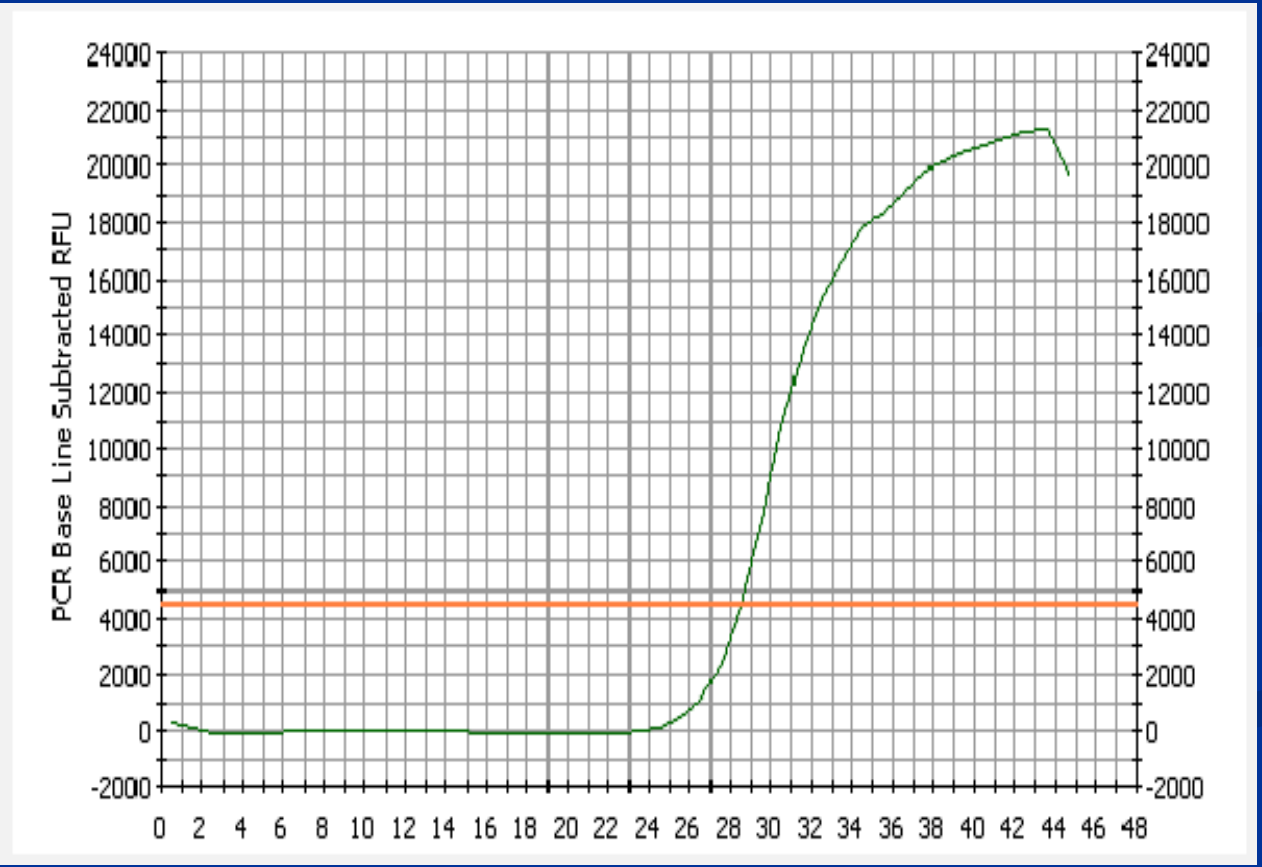


Hybridization probes

Principle: adjacent hybridisation and FRET



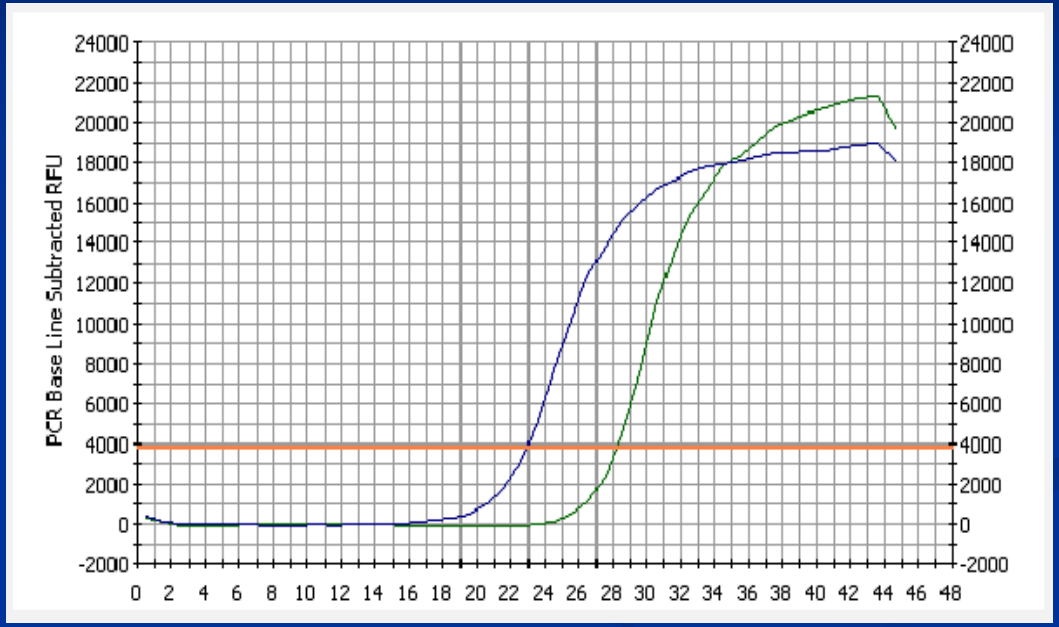
- The output from a real-time PCR reaction is in the form of a graph showing the number of PCR cycles (1 cycle = 90°C, 50°C, 72°C) against the increasing fluorescence. This is known as an amplification plot:



Ct (cross threshold) value

- The horizontal line on the graph represents a "**threshold**" set by the user.
- The point at which the amplification plot crosses this threshold is known as the **Ct (cross threshold)** value.
- the Ct value for a sample the greater the starting amount of DNA in the sample.

- if two amplification plots are compared it is simple to deduce which sample contained the greatest amount of the DNA of interest by the **Ct value:**



- Blue sample Ct value=23. Green sample Ct value=28. Therefore the blue sample contained 32 (2^5) times more of the gene of interest than the green sample

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