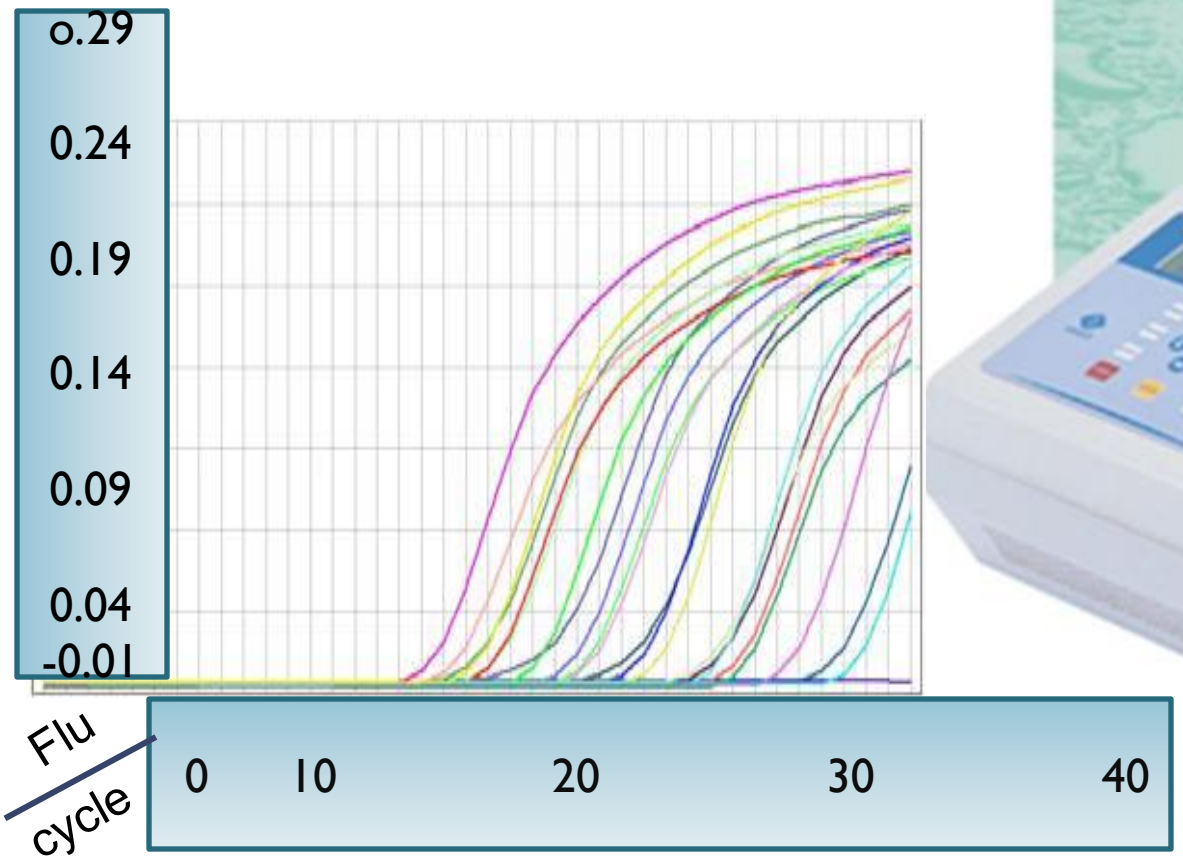
REAL – TIME POLYMERASE CHAIN REACTION



There is three basic steps which are in common with all type of PCR

Thermal denaturation :

In this step DNAs are denatured mostly by temperature about 94˚c & single stranded DNAs are made.

(in some cases It's done by helicase)

Primer annealing :

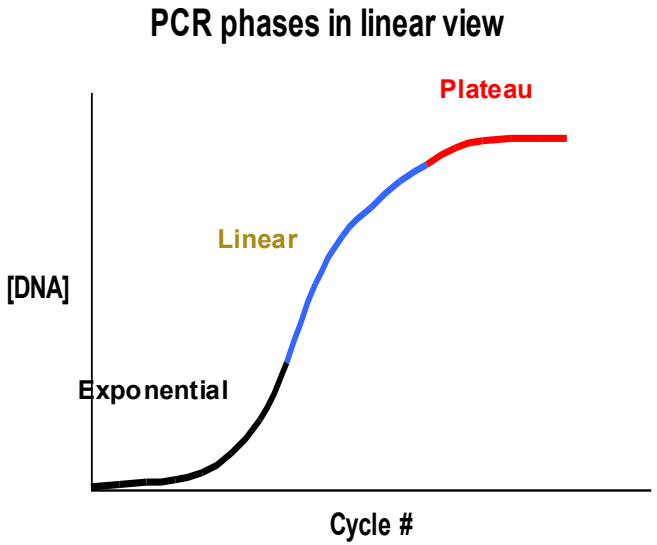
In this step Primers are attached to ssDNA by their complementary regions.

Extension or polymerization :

This is done by a temperature resistance polymerase named **Taq polymerase** which is extracted from **Thermus aquaticus.**

PCR phases

- **Exponential**
  - If 100% efficiency – exact doubling of products. Specific and precise
- **Linear**
  - High variability. Reaction components are being consumed and PCR products are starting to degrade.
- **Plateau**
  - End-point analysis. The reaction has stopped and if left for long – degradation of PCR products.



Advantages & disadvantages

* The most accurate & feasible technique to determine the amount & concentration of products.
* Rapid cycling (30 minutes to 2 hours).
* Specific & sensitive.
* Not much more expensive.

* * * * *

* Pollution.
* Poor precision.
* Hard to get quantitative data.

Real Time PCR

Quantitative Real-Time PCR is an important technique for quantifying messenger RNA levels (gene expression) and DNA gene levels (copy number) in biological samples.

Additional benefits of Real-Time quantitative PCR include sensitivity and a wide dynamic range. As few as 10 copies of an RNA/DNA target can be detected.

Considered to be the most sensitive method for the detection and quantification of gene expression

Real Time -PCR

What is Real Time-PCR ?

- **Real-Time PCR is a specialized technique that allows a PCR reaction to be visualized "in real time" as the reaction progresses.**

- **This enables researchers to quantify the amount of DNA in the sample at the start of the reaction!**

- **It differs from standard PCR in a way that it can detect the amplified product as the reaction progresses with time but in standard PCR the amplified product is detected at the end of the reaction by agarose gel electrophoresis.**

Why Real Time? What's wrong with Agarose Gels ?

- **End point analysis**
  - End point result is time consuming
  - End point is variable from sample to sample, while gels may not be able to resolve these variability's in yields.
- **Low resolution**
- **Low sensitivity**
- **Size-based discrimination only**

Real-time PCR enables the amount of starting material to be quantified

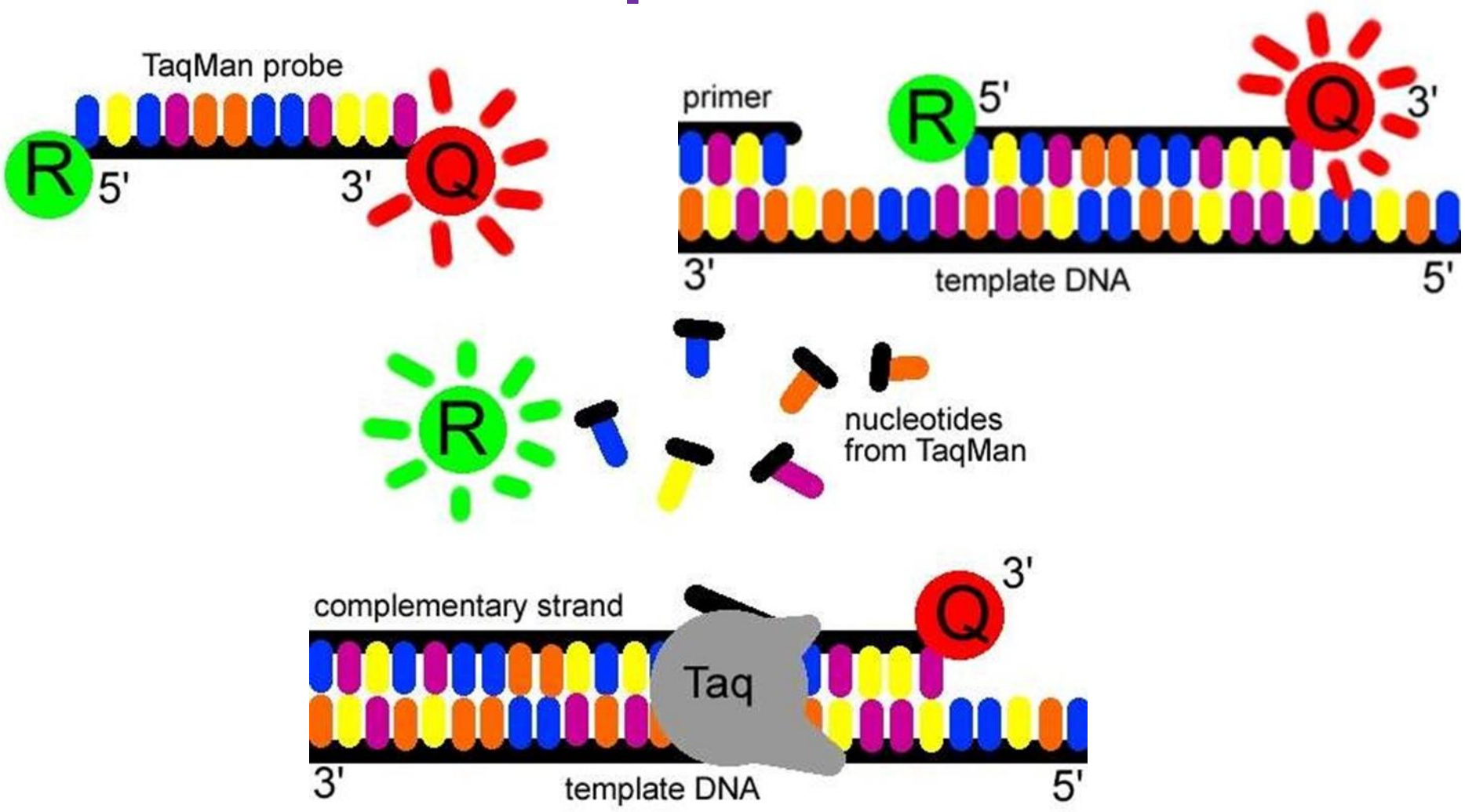
Real Time Principle

- **It is based on the detection and quantitation of a fluorescent reporter**

- **In stead of measuring the endpoint we focus on the first significant increase in the amount of PCR product.**

- **If there are only a few DNA molecules at the beginning of the PCR then relatively little product will be made, but if there are many starting molecules then the product yield will be higher.**

For Real Time PCR we need a specific probe with a fluorescent reporter



Real-Time PCR

- Real-time PCR is a variant of PCR technology that allows the the detectionof PCR products as they accumulate in "real-time" during the PCR amplification process.
- All real-time PCR systems rely upon the detection and quantitation of a fluorescent reporter

  The signal of which increases in direct proportion to the amount of PCR product in a reaction

A commonly misused acronym …

RT-PCR:

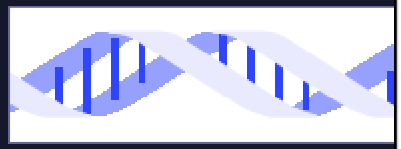
Reverse Transcription Polymerase Chain Reaction
- Or –
~~Real-Time Polymerase Chain Reaction?~~

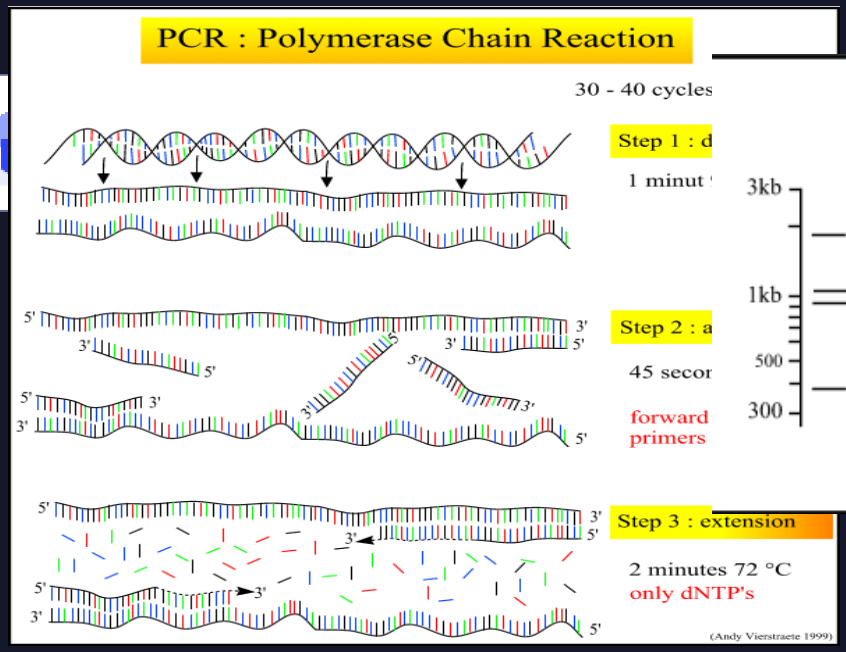
RT-PCR = Reverse Transcription Polymerase Chain Reaction (may be applied to both conventional and Real-time PCR systems).

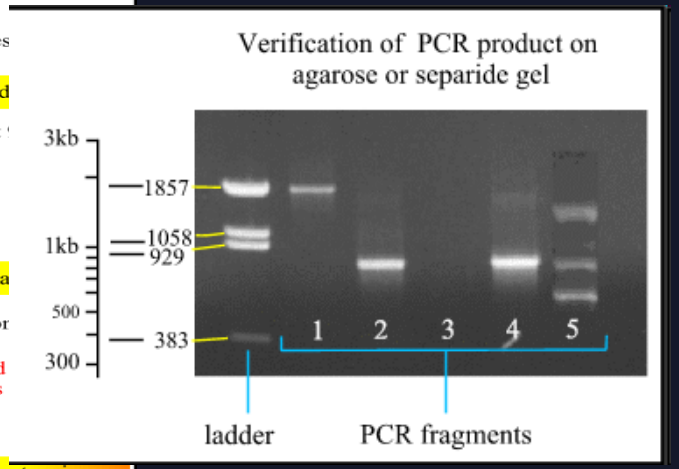
qPCR = Real-Time PCR when used as a quantitative tool.

An Overview of General PCR

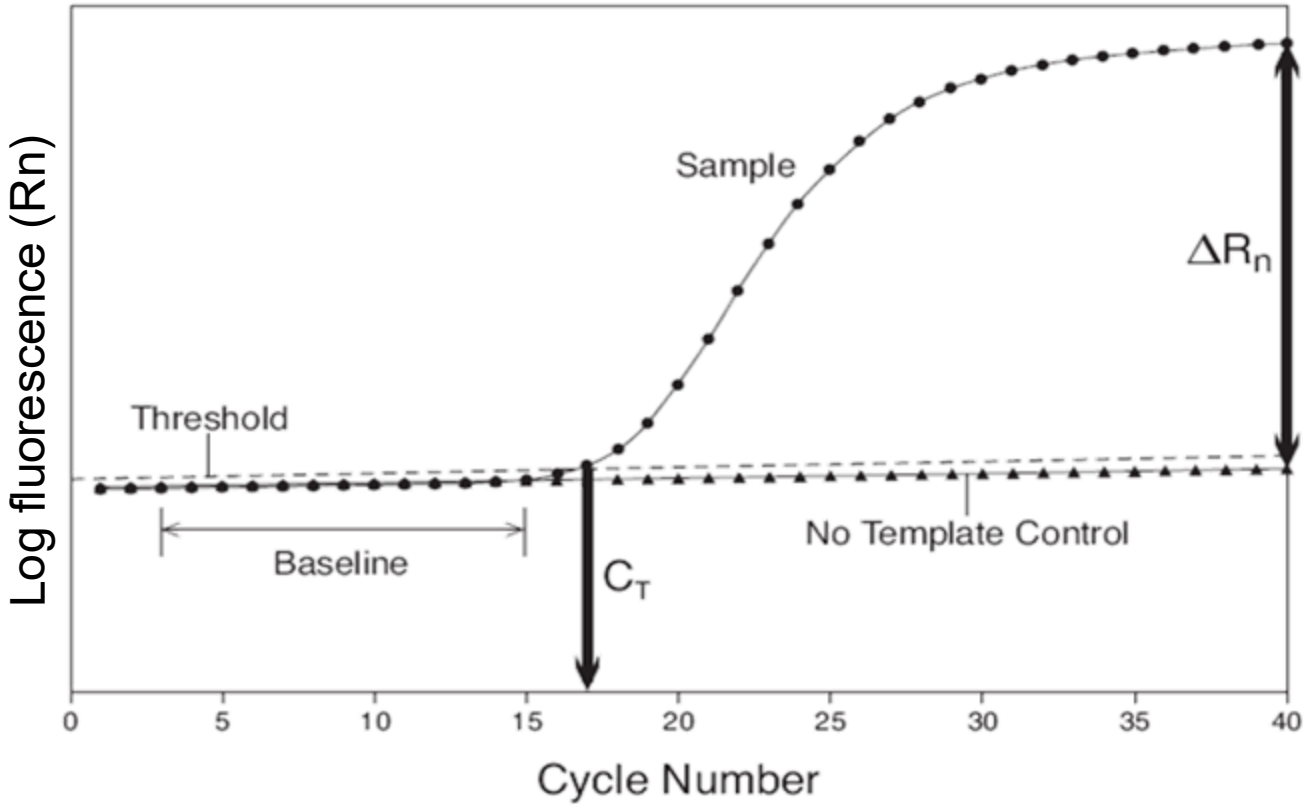
DNA Extraction → **PCR** → **Visualization of PCR Products by Electrophoresis**







The Basic of Real time PCR



Baseline – The baseline phase contains all the amplification that is below the level of detection of the real time instrument.

Threshold – where the threshold and the amplification plot intersect defines C_T. Can be set manually/automatically

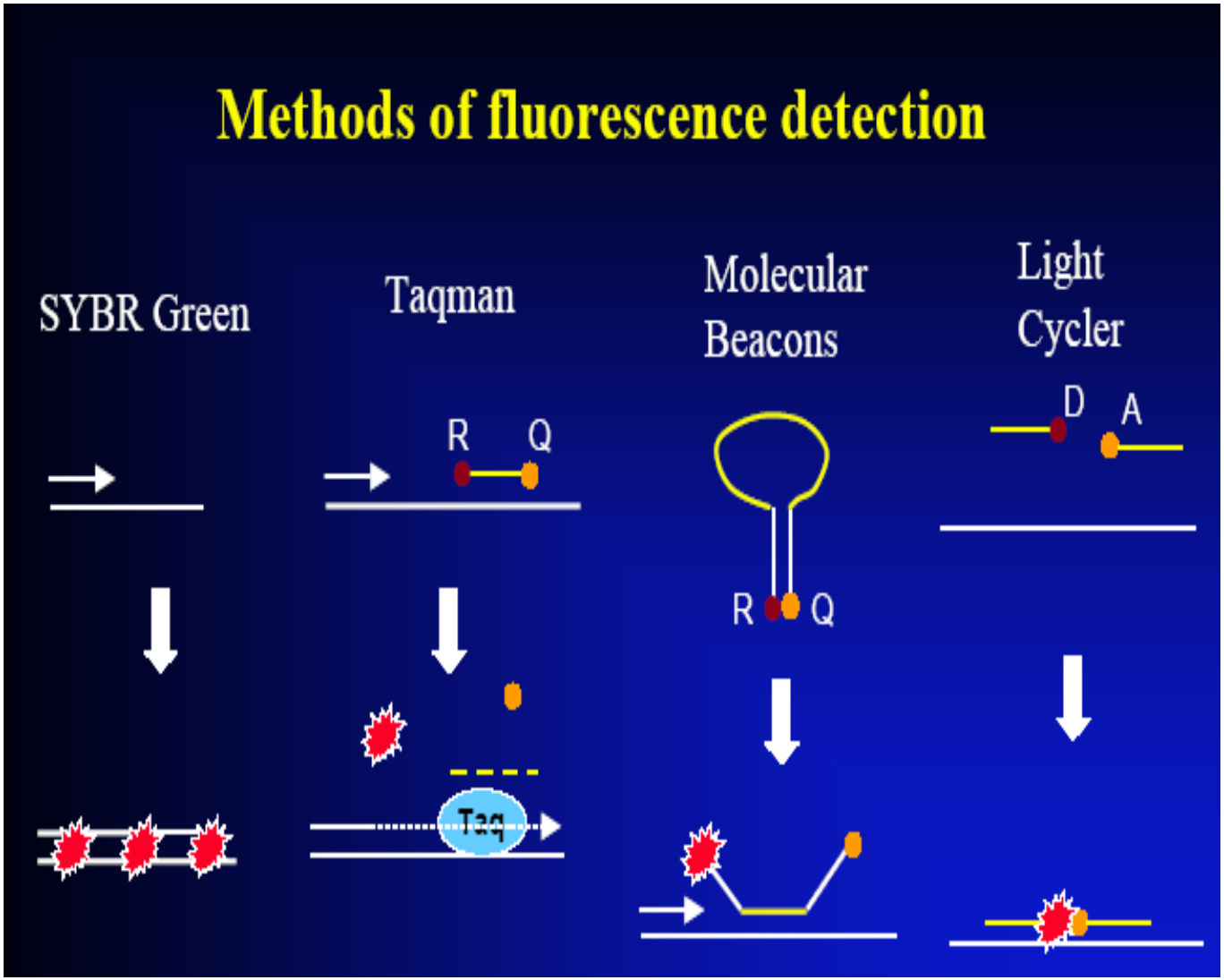
C_T – (cycle threshold) the cycle number where the fluorescence passes the threshold

ΔR_n – (R_n-baseline)

NTC – no template control

ΔRn is plotted against cycle numbers to produce the amplification curves and gives the C_T value.

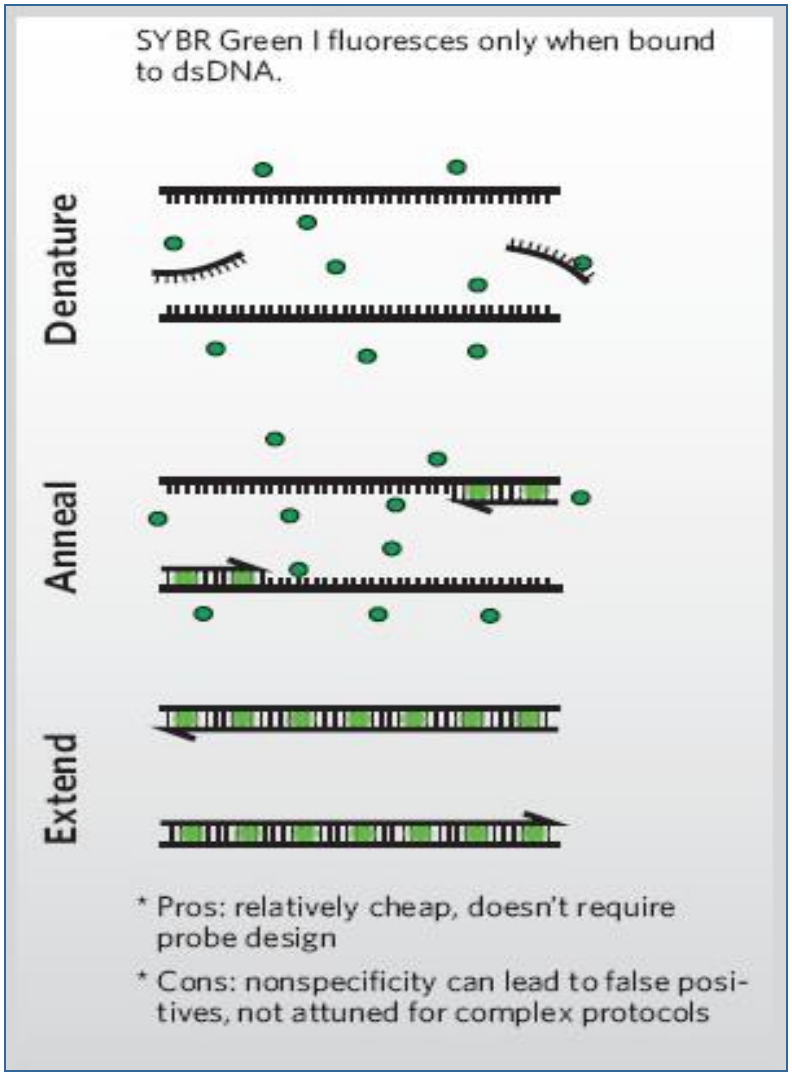
Detection in real time PCR

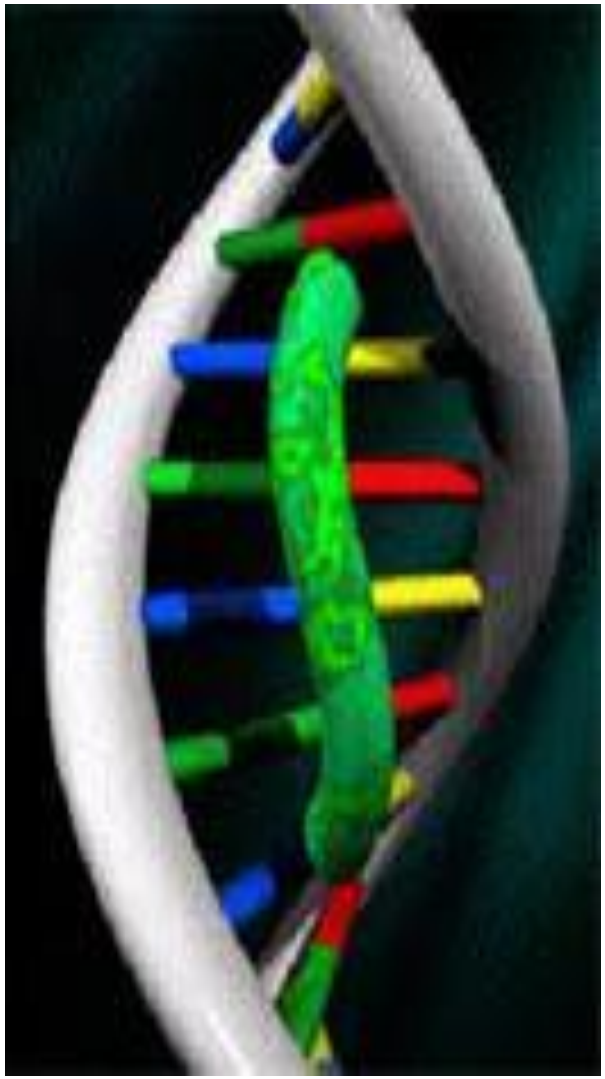


CYBR Green Chemistry

- CYBR Green is the most widely used double-strand DNA specific dye
- It binds to the minor groove of the DNA double helix
- In solution, the unbound dye exhibits very little fluorescence
- When CYBR Green dye binds to double stranded DNA, the fluorescent is substantially enhanced
- As more double stranded amplicons are produced SYBR green dye signal will increase

SYBR® green


SYBR Green I fluoresces only when bound to dsDNA.
Denature
Anneal
Extend
* Pros: relatively cheap, doesn't require probe design
* Cons: nonspecificity can lead to false positives, not attuned for complex protocols



Advantage and disadvantage of CYBR Green Method

Advantage

- Inexpensive
- No probe is required
- Easy to use

Disadvantage

- SYBR Green will bind to any double stranded DNA (e.g. primer dimers, non-specific reaction products)
- Overestimation of target concentration
- Non-specific background in very late cycles

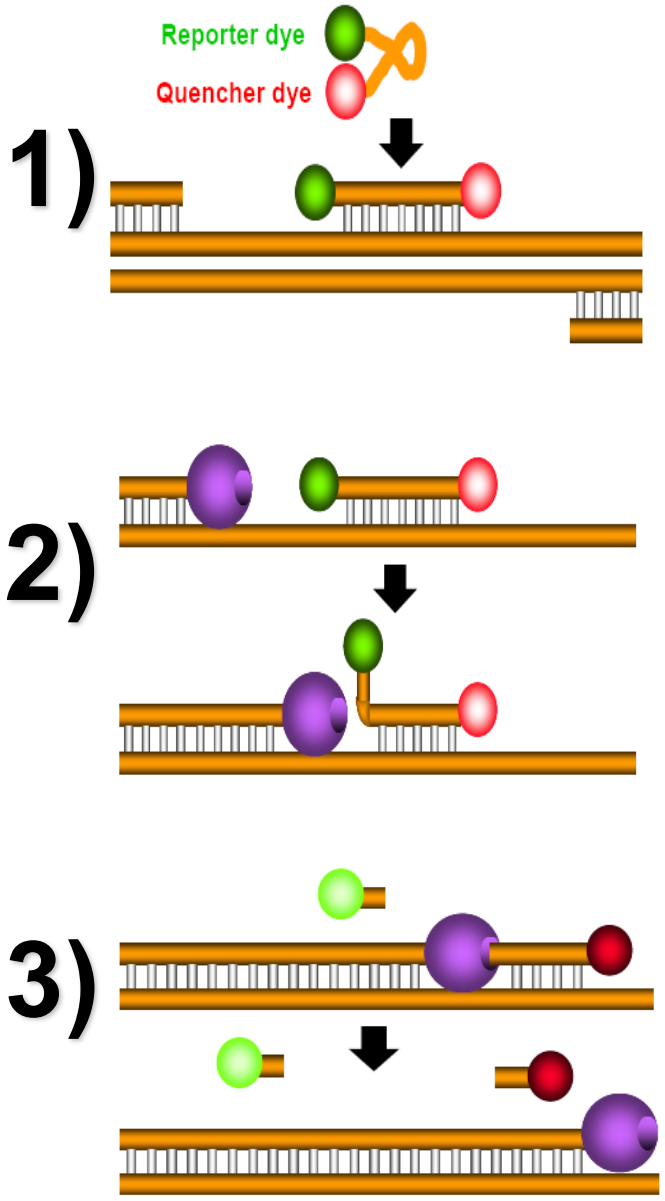
Taqman

1) Denaturation and hybridization of probe.

2) Extension of primer and strand displacement of probe.

3) Cleavage of probe and fluorescence from the reporter dye.

Fluorescence from reporter dye is directly proportional to the number of amplicons generated



TaqMan Chemistry

TaqMan chemistry requires two more additional components with traditional PCR

What's in general PCR ?

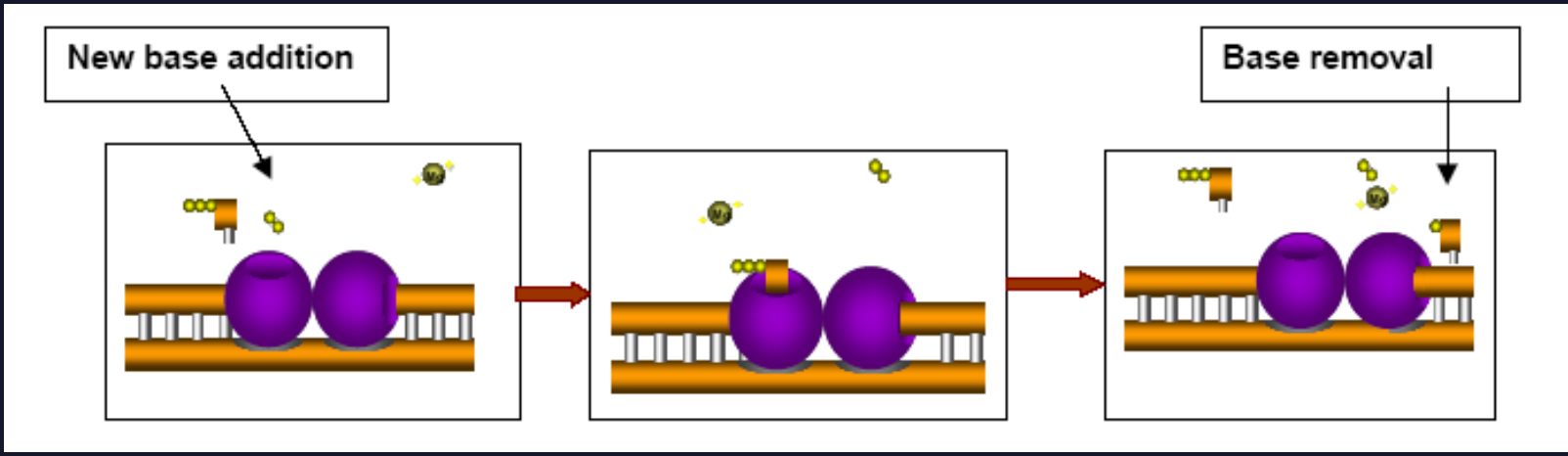
- Template DNA
- Reaction buffer
- Nucleotides (dNTPs)
- Primers (Forward & Reverse)
- *Taq* DNA polymerase

What's new in TaqMan ?

- Template DNA
- Reaction buffer
- Nucleotides (dNTPs)
- Primers (Forward & Reverse)
- *AmpliTaq* Gold DNA Polymerase
- TaqMan Probe

AmpliTaq Gold DNA Polymerase

- In addition to its polymerase activity, it has also 5´ exo-nuclease activity
- The 5´ exo-nuclease activity acts upon the surface of the template to remove obstacles downstream of the growing amplicon that may interfere with it's generation



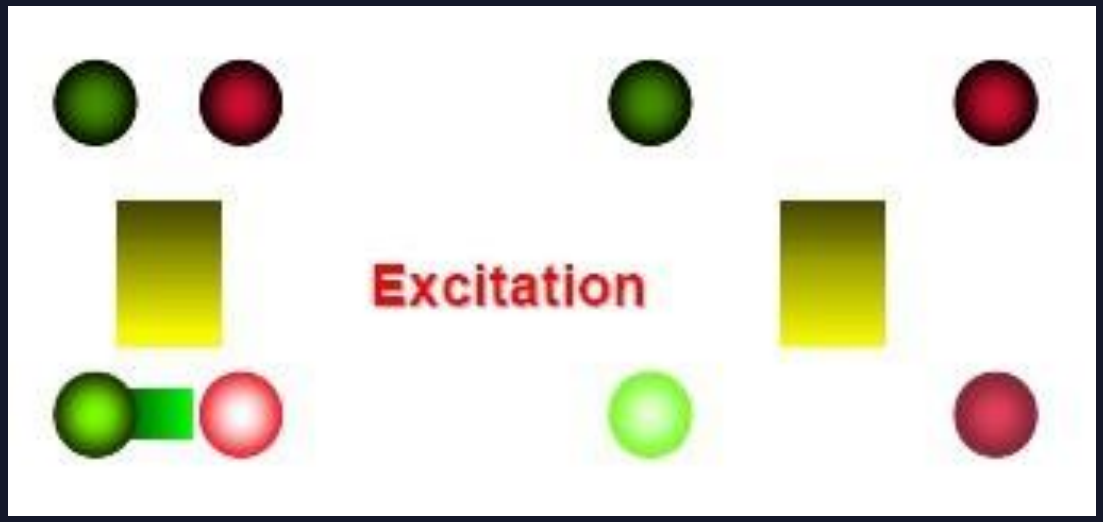
TaqMan Probe

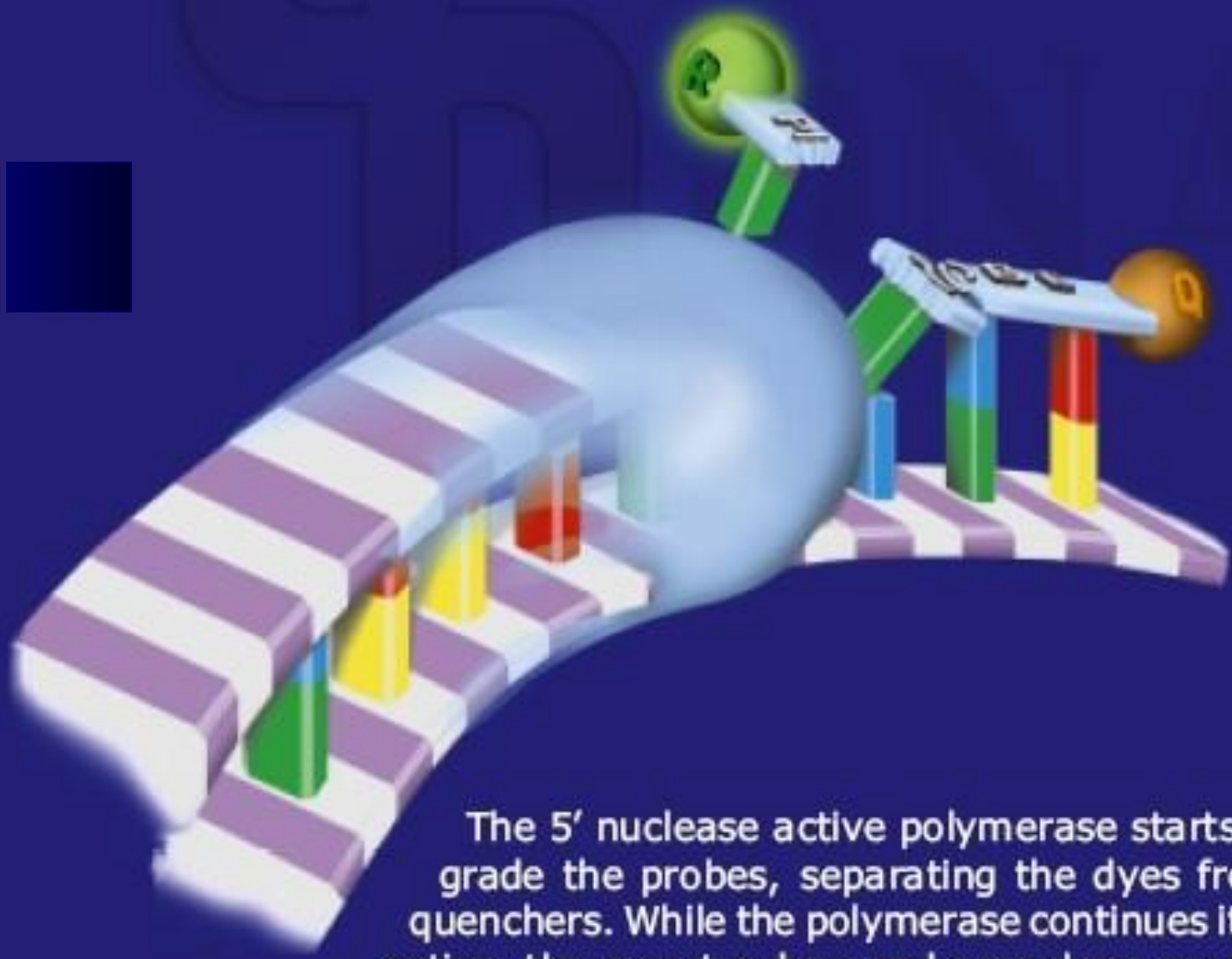
- TaqMan probe is a short DNA sequence with a high energy dye called reporter dye at the 5´ end and a low energy dye called quencher at the 3´ end

- When this probe is intact and excited by a light source, the reported dye emission is suppressed by the quencher dye as a result of close proximity of the dyes. This is known as FRET

Fluorescence Resonance Energy Transfer

When a high energy dye is in close proximity of a low energy dye, there will be a transfer of energy from high to low





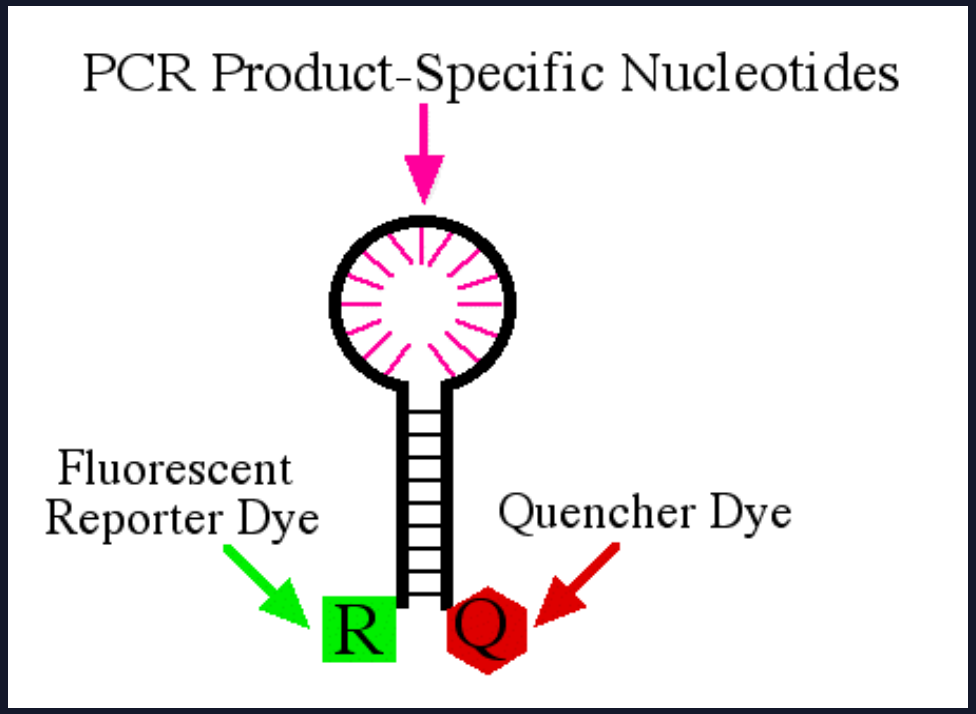
The 5′ nuclease active polymerase starts to degrade the probes, separating the dyes from the quenchers. While the polymerase continues its elongation, the reporter dyes and quenchers are drifting apart, leaving the quenchers unable to absorb fluorescence emitted from the dyes. The result is a detectable fluorescence signal from the sample.

Molecular Beacon

- Molecular beacons are short segments of single stranded DNA that forms a hairpin in its free form
- The loop portion of the molecular beacon is composed of bases that are complementary to one strand of the PCR product the investigator wants to detect and quantify
- Attached to opposite end of the beacon are a fluorescent reported dye and a quencher dye

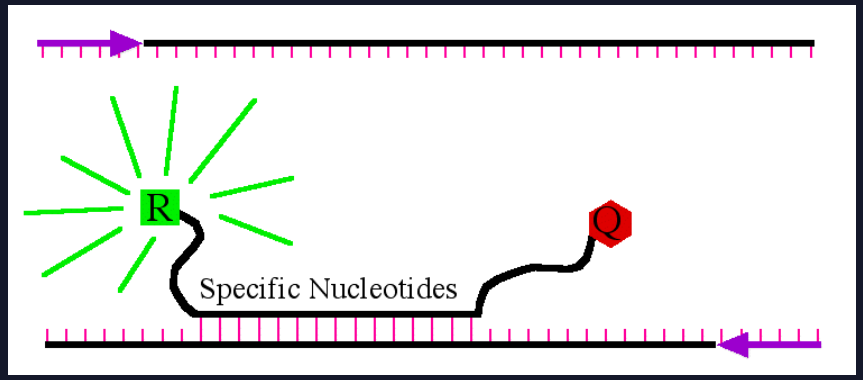
Molecular Beacon

When the molecular beacon is in the hairpin conformation, any fluorescence emitted by the reporter is absorbed by the quencher dye and no fluorescence is detected.



Molecular Beacon

- As the PCR continues, the newly synthesized PCR products are denatured by high temperatures
- At the same time the molecular beacon also is denatured so the hairpin structure is disrupted.
- As the temperatures cool for the next round of primer annealing, the molecular beacon is capable of forming base pairs with the appropriate strand of the PCR product



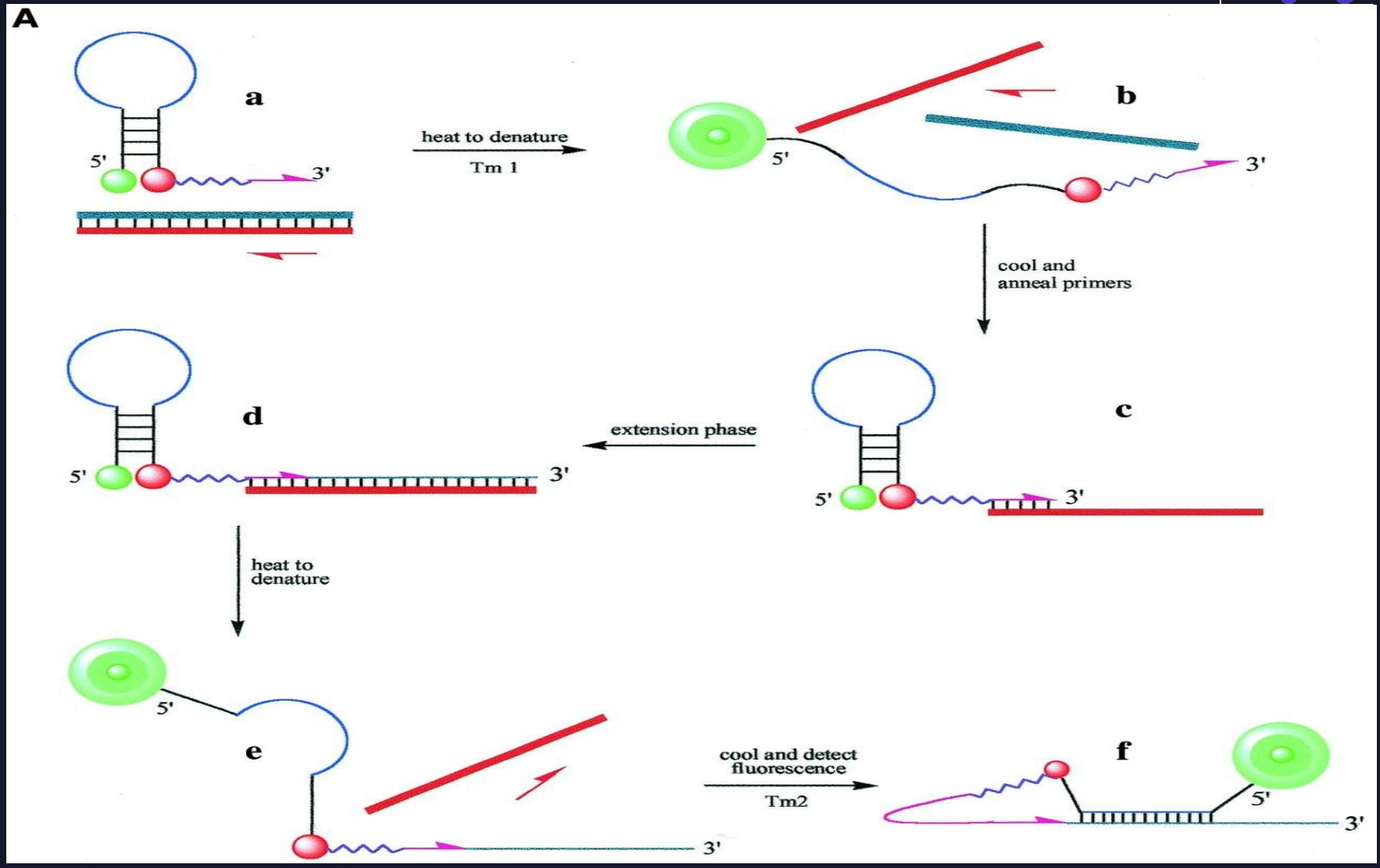
Molecular Beacon

- Molecular beacons that bind to the PCR product remove the ability for the quencher to block fluorescence from the reporter dye
- Molecular beacons that do not bind to the PCR product reform the hairpin structure and thus unable to fluoresce
- Therefore, as PCR product accumulates, there is a linear increase in fluorescence.

Scorpion probe

- Scorpion probe is a bifunctional molecule in which a primer is covalently linked to the probe
- That is why they are sometimes known as "**Scorpion primer & probe"**
- The probe has a self complementary stem sequence with a fluorophore at one end and a quencher at the other end
- In the initial PCR cycle the primer hybridizes to the target and extension occurs due to the action of polymerase
- After denaturation and cooling, the specific probe sequence is able to bind to its complement within the extended amplicon thus opening up the hairpin loop
- The fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation

Scorpion primer & probe



Applications of Real Time PCR

- Quantitation of gene expression
- Drug therapy efficacy / drug monitoring
- Viral quantitation
- Pathogen detection


Thank you!