Advanced laboratory technique
Lab/6
Real Time PCR (RT-PCR)

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Real time PCR

- The Polymerase Chain Reaction (PCR): is a process for the amplification of specific fragments of DNA.
- Real-time polymerase chain reaction: is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR.

Principle of Real-Time PCR:

Real-time PCR (qPCR) works by combining the traditional PCR with a method that allows the quantification of DNA as it is amplified.

1) **Amplification:**

➢DNA Denaturation :The DNA sample is denatured to separate the double-stranded DNA into single strands.

➢ Annealing: Primers specific to the target DNA sequence are then added.

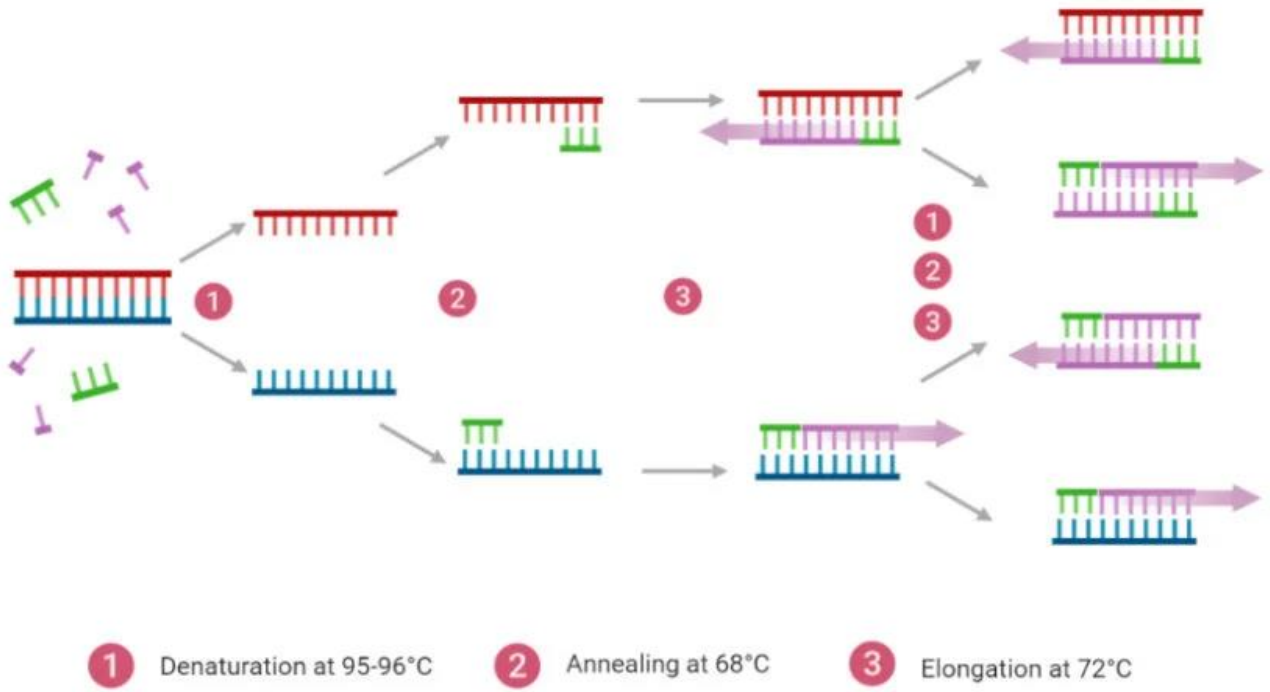
➢Extension: A DNA polymerase enzyme extends the primers to create new DNA strands.

2) **Detection:** During each cycle of amplification, a fluorescent reporter molecule is used to label the DNA product .The fluorescence increases proportionally with the amount of DNA produced.

3) **Quantification:** The fluorescence is measured after each cycle, and the data is analyzed to determine the initial quantity of the target DNA.

Real Time PCR / quantitative PCR (qPCR)

Polymerase chain reaction - PCR



Common detection methods in Real time PCR

There are two detection methods :

(1) By using non-specific fluorescent dyes.

(2) By using sequence-specific fluorescent probes.

Non-specific fluorescent dyes

Non-specific fluorescent dyes, like SYBR Green, bind to any double-stranded DNA (dsDNA) that is produced during PCR. As PCR amplifies the target DNA, the amount of dsDNA increases, and consequently, the fluorescence signal intensifies.

How It Works:

- **Binding:** SYBR Green dye intercalates into the minor groove of dsDNA.
- **Fluorescence:** When bound to dsDNA, the dye emits a fluorescent signal that increases proportionally with the quantity of dsDNA present.
- **Detection:** The real-time PCR machine measures the fluorescence after each cycle, allowing quantification of the DNA.

Sequence-specific fluorescent probes

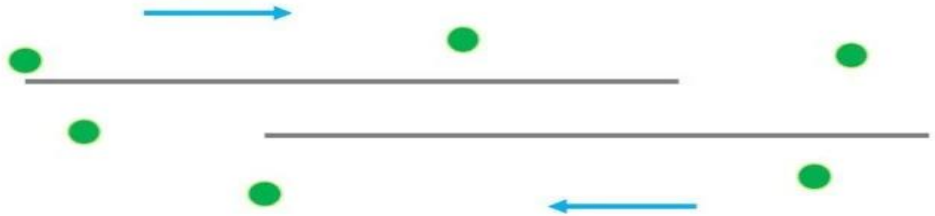
Sequence-specific DNA probes, such as TaqMan probes, offer a highly targeted approach to detecting and quantifying DNA during real-time PCR.

How It Works:

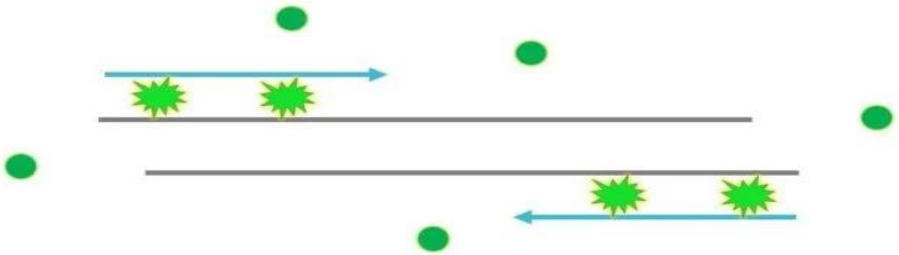
- **Design:** The probe is an oligonucleotide that is designed to be complementary to a specific sequence within the target DNA.
- **Labeling:** Each probe is labeled with a fluorescent reporter dye at one end and a quencher dye at the other.
- **Hybridization:** During the PCR, the probe hybridizes to its complementary target sequence.
- **Cleavage and Fluorescence:** As the DNA polymerase extends the new DNA strand, it cleaves the probe, separating the reporter from the quencher. This separation allows the reporter dye to emit fluorescence, which is measured in real-time.

SYBR

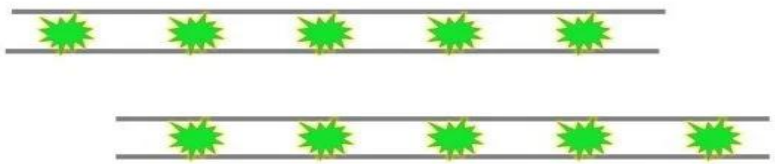
Denature



Polymerization

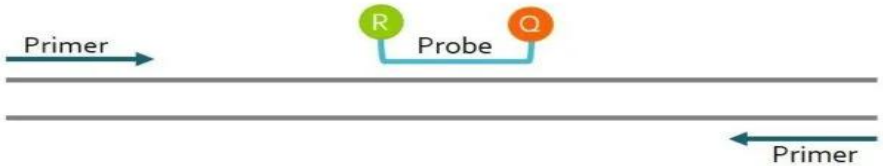


Signal detection (Polymerization completed)

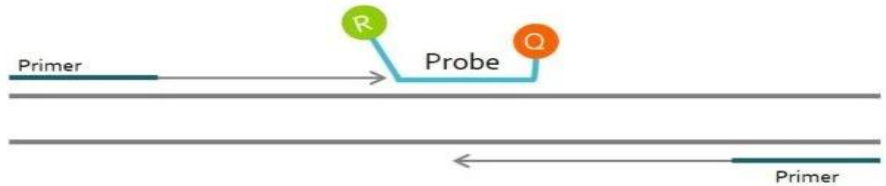


TaqMan

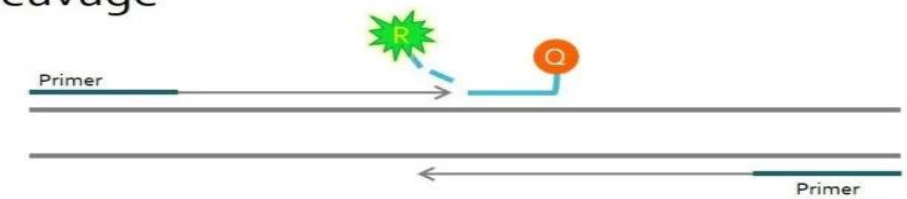
Annealing



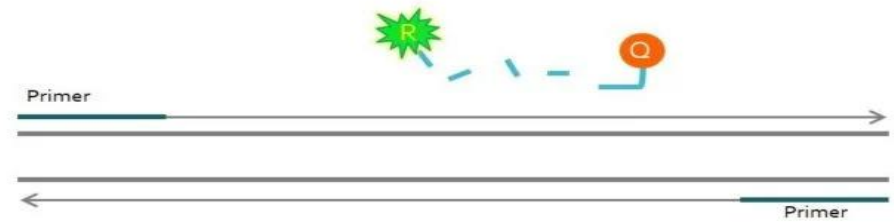
Polymerization & strand displacement



Cleavage



Signal detection (Polymerization completed)



Non-specific fluorescent dyes

Advantages

- **Cost-effective:** Less expensive compared to sequence-specific probes.
- **Versatile:** Can be used for a variety of applications without needing specific probes.

Disadvantages

- **Non-specificity:** Binds to all dsDNA, including non-specific products ,which can lead to inaccurate results.
- **Optimization Required:** Requires careful optimization and validation to ensure specificity and accuracy.

Sequence-specific fluorescent probes

Advantages

- **Specificity:** Reduces the chance of false positives by only binding to a specific DNA sequence.-
- **Quantitative Accuracy:** Provides more accurate quantification compared to non-specific dyes.

Disadvantages

- **Cost:** More expensive due to the need for specific probe design and synthesis.
- **Complexity:** Requires precise design and optimization for each target sequence.

Applications of Real-Time PCR

➢ Real-time PCR is widely used in various fields, including:

✓Gene expression analysis: Measuring the expression levels of specific genes.

✓Pathogen detection: Identifying and quantifying pathogens in clinical and environmental samples.

✓Genetic research: Studying genetic variations and mutations.


THANK YOU