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Real Time PCR and its Applications

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Introduction

In microbiology laboratories, quantitative real-time PCR techniques have been widely developed for the routine detection of infectious illnesses, especially bacterial disorders. The quick identification of bacteria presents directly in specimens using this molecular technology enables early, sensitive, and accurate laboratory confirmation of associated illnesses. A quick evaluation of the existence of genes or gene mutations that cause antibiotic resistance is also possible using this approach. Even though phenotypic resistances are not always predicted by this genetic method, in some circumstances it may aid in improving patient treatment management. The treatment of these infected individuals may undergo significant changes as a result of a method that combines the identification of pathogens, their mechanisms of antibiotic resistance, their virulence factors, and the bacterial load in clinical samples.

Principle

Real-time PCR operates on the same fundamental tenet as traditional PCR, with the added benefit of allowing for real-time monitoring of the reaction result using a fluorogenic probe. Real-time PCR detection devices come in a variety of forms, including molecular beacons, hydrolysis probes, hybridization probes, and dyes that bind double stranded DNA. This test makes use of hydrolysis probes.

In a heat cycler equipped with the ability to illuminate each sample with a beam of light of at least one specific wavelength and measure the fluorescence given off by the excited fluorophore, real-time PCR is carried out. Utilizing the physicochemical qualities of the DNA polymerase, the thermal cycler can also quickly heat and cool samples. A sequence of temperature adjustments that are performed 25–50 times make up the PCR process in general. These cycles typically have three phases:

1. **Denaturation:** this occurs at 95 °C, causing the separation of the nucleic acids double chain.
2. **Annealing:** annealing occurs at around 50- 60 °C, binding the primers with the DNA template.
3. **Extension:** this occurs between 68-72 °C, facilitating polymerization of DNA strands by DNA polymerase.

In order to lessen the signal brought on by the presence of primer dimers when a non-specific dye is employed, the fluorescence is also monitored during a brief temperature phase in four step PCR that lasts only a few seconds in each cycle. This temperature is, for example, 80 °C. The enzyme employed to manufacture the DNA, the quantity of divalent ions and deoxyribonucleotides (dNTPs) in the

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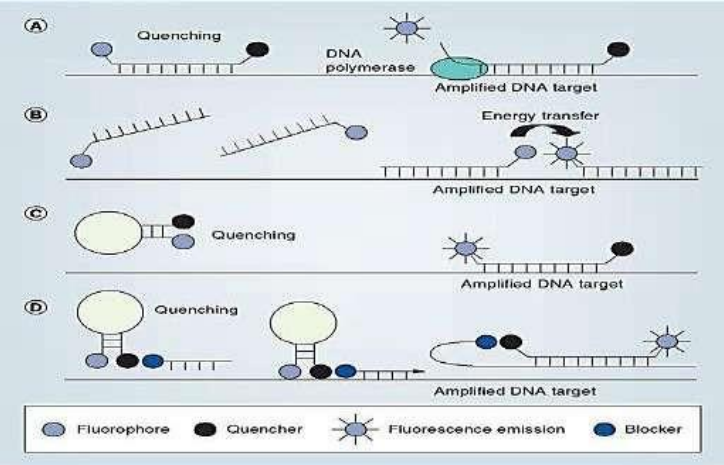
process, and the bonding temperature of the primers are only a few of the many variables that affect the temperatures and timings for each cycle.



The PCR reaction is supplemented with a DNA probe that binds the PCR product in the hydrolysis probe system (as seen in the top image). The DNA probe has a quenching dye, such as black hole quencher (BHQ), on one end and a fluorogenic reporter dye, such as FAM, on the other.

As long as the probe is unhybridized from the target and is nearby, the quencher dye will absorb the emission of the reporter dye. The 5' nuclease activity of Taq-polymerase will hydrolyze the probe when it is hybridised to the target, separating the quencher from the reporter dye. This separation causes the reporter dye's fluorescence emission to rise, which is then measured and recorded by a spectrophotometer. The amount of target template in the samples directly relates to the amount of fluorescence that is captured. Every cycle, the fluorescence signal is observed, showing increases in the PCR product as they happen.

Applications of Real Time PCR

Diagnostic uses: Rapid detection of nucleic acids that are diagnostic of conditions including infectious illnesses, cancer, and genetic anomalies is made possible by the use of diagnostic quantitative PCR. Quantitative PCR assays were introduced to the clinical microbiology laboratory, and they are now used as a tool to detect newly developing illnesses, such as novel flu strains, in diagnostic tests. This has considerably improved the identification of infectious diseases.

Microbiological uses: Microbiologists who study food safety, food deterioration, and fermentation as well as those who examine the microbiological risks associated with the quality of drinking and recreational waters as well as those who seek to safeguard the public's health employ quantitative PCR.

Detection of phytopathogens: To avoid financial losses and protect public health, the agriculture sector works hard to create pathogen-free plant propagules or seedlings. Systems have been created that enable the identification of minute quantities of the host plant's DNA combined with the DNA of the oomycete Phytophthora ramorum, which kills oaks and other species. Based on the amplification of ITS sequences, which are spacers found in the coding region of ribosomal RNA genes and unique

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to each taxon, it is possible to distinguish between the DNA of the pathogen and the plant. This method has also been developed for use in the field to identify the same disease.

Clinical quantification and genotyping: Humans can have viruses owing to direct infection or co-infection, which makes it challenging to diagnose using conventional procedures and can lead to an inaccurate prognosis and course of therapy. Utilizing qPCR enables the quantification and genotyping (strain classification using melting curves) of viruses like the hepatitis B virus. In many circumstances, the level of infection—measured as the number of viral genome copies per unit of the patient's tissue—is important; for instance, the likelihood that the type 1 herpes simplex virus may reactivate is correlated with the number of infected neurons in the ganglia. When a virus integrates into the human genome at any stage of its life cycle, as is the case with the human papillomavirus (HPV), some of its variations are linked to the development of cervical cancer. This quantification can be done with reverse transcription or without it.

Real-time RT-PCR Advantages

- Quick, with outcomes in as little as 3 hours
- Sensitive, multiple sample in vitro surveillance assay.
- Accurate and scalable—processing of several samples is possible.
- Price: a $8/ sample.
- Limits handling of potentially contagious materials.

Can test a variety of sample types.

Real-time RT-PCR disadvantages

- Expensive initial equipment investment;
- Requires proper storage and handling of probes;
- Detects live or inactivated viruses;
- False positives and false negatives.

References

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