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Real Time PCR: "An Advanced tool in Disease Diagnosis"

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What is PCR?

- Specifically targets and amplifies a single sequence from within a complex mixture of DNA
- In vitro DNA amplification technique invented by Karry Mullis in 1985
- In the last decade PCR has revolutionized the detection of infectious pathogens because of its high sensitivity and specificity
- Automated for routine use in laboratories worldwide
- PCR is DNA replication in a test tube
- Real-Time PCR
- Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection

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- PCR is DNA replication in a test tube
- It is a technique used to monitor the progress of a PCR reaction in real-time.
- At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified.
- It is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds.
- It is also known as a quantitative polymerase chain reaction (qPCR), which is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR).
- qPCR is a powerful technique that allows exponential amplification of DNA sequences.

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- A PCR reaction needs a pair of primers that are complementary to the sequence of interest. Primers are extended by the DNA polymerase.
- The copies produced after the extension, so-called amplicons, are re-amplified with the same primers leading thus to exponential amplification of the DNA molecules.
- After amplification, however, gel electrophoresis is used to analyze the amplified PCR products and this makes conventional PCR time consuming; since the reaction must finish before proceeding with the post-PCR analysis. Real-Time PCR overcomes this problem.
- The term "real-time" denotes that it can monitor the progress of the amplification when the process is going on in contrast to the conventional PCR method where analysis is possible only after the process is completed.

Principle of Real Time PCR:

This same principle of amplification of PCR is employed in real-time PCR. But instead of looking at bands on a gel at the end of the reaction, the process is monitored in "real-time". The reaction is placed into a real-time PCR machine that watches the reaction occur with a camera or detector.Although many different techniques are used to monitor the progress of a PCR reaction, all have one thing in common. They all link the amplification of DNA to the generation of fluorescence which can simply be detected with a camera during each PCR cycle. Hence, as the number of gene copies increases during the reaction, so does the fluorescence, indicating the progress of the reaction.

Real time Definitions:

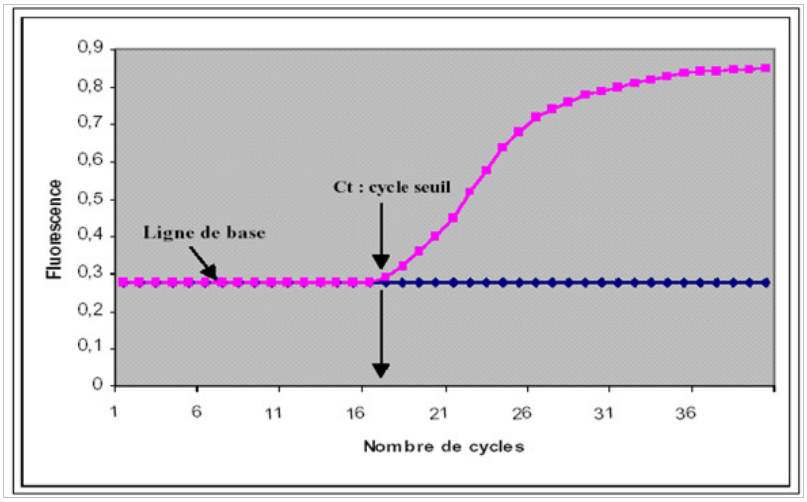
Baseline: The initial cycles prior to amplification in which there is little change in fluorescence signal

Threshold: The level of detection or the point at which a reaction reaches a fluorescent intensity above baseline

CT (Cycle Threshold): The cycle at which the sample's amplification curve crosses the threshold

Δ Rn (Reporter normalized): Change in reporter signal (as compared to background noise) normalized to a passive fluorescent reference dye

Threshold cycle = Ct: The concept of the threshold cycle is at the heart of accurate and reproducible quantification using fluorescence-based PCR



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It corresponds to the cycle from which one observes a statistically significant increase in standardized fluorescence

Steps of Real Time PCR (Protocol)

A. Amplification

1. Denaturation

High temperature incubation is used to "melt" double- stranded DNA into single strands and loosen secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if template GC content is high.

2. Annealing

During annealing, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used that is based on the calculated melting temperature (Tm) of the primers (5°C below the Tm of the primer).

3. Extension

At 70-72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

B. Detection

* The detection is based on fluorescence technology.
* The specimen is first kept in proper well and subjected to thermal cycle like in the normal PCR.
* The machine, however, in the Real Time PCR is subjected to tungsten or halogen source that lead to fluoresce the marker added to the sample and the signal is amplified with the amplification of copy number of sample DNA.
* The emitted signal is detected by an detector and sent to computer after conversion into digital signal that is displayed on screen.
* The signal can be detected when it comes up the threshold level (lowest detection level of the detector).

Various Methods of Real Time PCR:

- Minor groove binding dye
- Hydrolysis probe (Taqman probe)
- Hybridization probe
- Molecular Beacons
- Scorpion

Fluorescence Markers used in Real Time PCR:

There are many different markers used in Real Time PCR but the most common of them include:

Krishi Netra
ISSN : 3048-5207

1. **Taqman probe.**

2. **SYBR Green.**

Taqman Probe

- It is a hydrolysis probe which bear a reporter dye, often fluorescein (FAM) at its 5' end and a quencher tetramethylrhodamine (TAMRA), attached to the 3' end of the oligonucleotide.
- Unders normal conditions, the probe remains coiled on itself bringing the fluorescence dye near the quencher, which inhibits or quenches of fluorescent signal of the dye.
- The oligonucleotide of the Taqpolymerase has a homologous region with the target gene and thus when the target sequence is present in the mixture, it binds with the sample DNA.
- As the taq polymerase start to synthesize new DNA strand in the extension stage, it causes degradation of the probe by 5' end nuclease activity and the fluorescein is separated from the quencher as a result of which fluorescence signal is generated.
- As this procedure continues, in each cycle the number of signal molecule increases, causing the increase in fluorescence which is positively related with the amplification of the target.

Hybridisation probes technique:

In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridisation probes can anneal, resulting in higher fluorescence signals.

Scorpion probe

- Scorpion are PCR primer with a stemloop tail containing a fluorophore and a quencher.
- This stem loop is separated from the PCR primer sequence by a PCR stopper, which is chemical modification that prevents the PCR from copying the stem loop sequence of the scorpion primer.
- At the annealing phase, the probe sequence in the scorpion tail curls back to hybridize to the target sequence in the PCR product.as the tail of the scorpion and PCR product are now the part of the same strand of DNA.

SYBR Green

- This is a dye that emits prominent fluorescent signal when it binds at the minor groove of DNA, nonspecifically.
- Other fluorescent dyes like Ethidium Bromide or Acridine Orange can also be used but SYBR Green is better used for its higher signal intensity.
- SYBR Green is more preferred than the Taqman Probe as it can provide information about each cycle of amplification as well as about the melting temperature which is not obtained from the Taqman probe.

Krishi Netra
ISSN : 3048-5207

- However, its disadvantage is the lack of specificity as compared to Taqman Probe.

Advantages of SYBR Green I Dye:

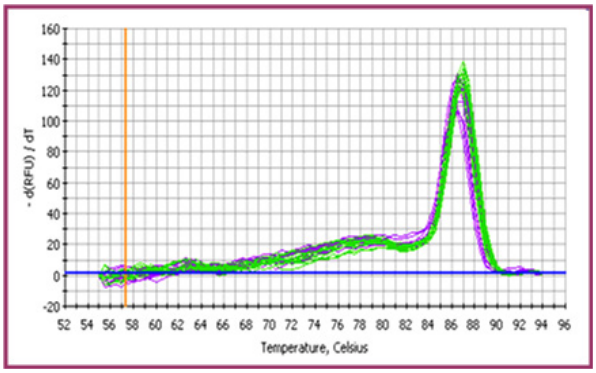
- It can be used to monitor the amplification of any double-stranded DNA sequence.
- No probe is required, which reduces assay setup and running costs.

Disadvantage of SYBR Green I Dye:

- It may generate false positive signals; i.e., because the SYBR Green I dye binds to any double-stranded DNA.
- It can also bind to nonspecific double-stranded DNA sequences.

Use of SYBR Green in Melting curve analysis

- The principle is very simple
- DNA fragment is having a specific melting temperature depending upon their nucleotides.
- As temperature increases, the PCR product will melt.
- At a specific temperature ds DNA becomes single stranded where SYBR green can not bind and there will be absence of fluoresce.



- If mutation is there, then there will be change in melting temperature.

ADVANTAGES REAL-TIME PCR

* Monitor amplification in real-time
* High sample throughput (~200 samples/day)
* Low contamination risk (sealed reactions)
* Very sensitive (3pg or 1 genome eq of DNA)
* Broad dynamic range (10 - 1010 copies)
* Reproducible (CV < 2.0 %)
* No post-PCR processing
* Ultra-rapid cycling (30min Vs 3 hours)

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* Accurate quantification

* Confirmation of PCR product (Melting Curve Analysis)

* Software driven operation

DISADVANTAGES OF REAL-TIME PCR

* Current technology has limited capacity for multiplexing

* Simultaneous detection of 3-5 targets is the limit

* Development of protocols needs high level of technical expertise

* Skill and/or support (requires R&D capacity)

* High capital equipment costs (Rs. 15 – 20 lakhs)

It has many advantages over the normal PCR:

- It gives a look in to the reaction that is help to decide which reactions have worked well and which have failed.
- The efficiency of the reaction can be precisely calculated.
- There is no need to run the PCR product out on a gel after the reaction as the melt curve analysis serve the purpose.
- The real-time PCR data can be used to perform truly quantitative analysis of gene expression. In comparison, old fashioned PCR was only ever semi-quantitative at best.
- Faster than normal PCR.
- Less complexity at the quantification of sample etc….

Thus, unlike the ordinary preparative PCR, Real Time PCR allows the success of multiple PCR reaction to be determined automatically after only a few cycles, without separate analysis of each reaction, and avoids the problem of “false negatives”.