Real-Time PCR

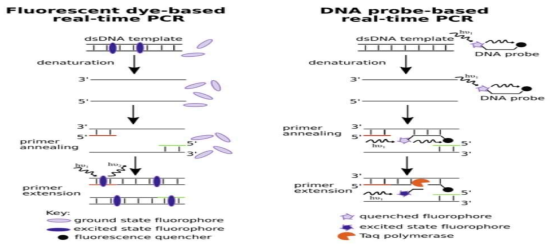
Topic You Need to Know

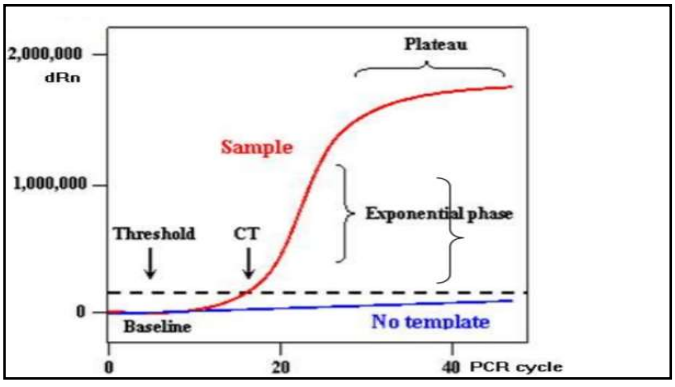
1. What is real-time PCR?
2. Mechanism of Real-time PCR
3. Types of Real-time PCR probes used
4. Advantages
5. Disadvantages

What is Real-time PCR ?

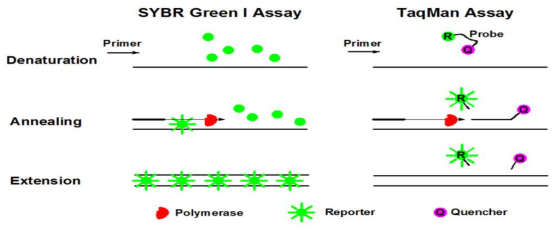
- A Real-time PCR is a laboratory technique of molecular biology based on PCR. It monitors the amplification of target DNA molecule during the PCR in real time & not at its end, as in conventional PCR.
- qPCR

Mechanism of Real-time PCR

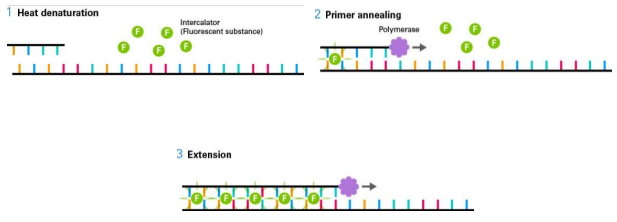




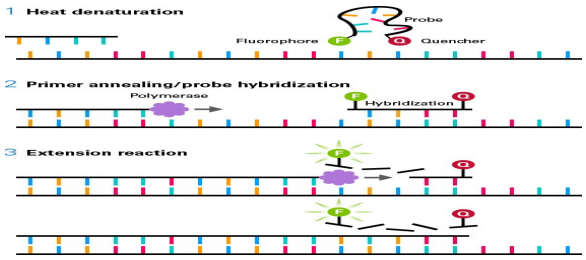
Types of Real-time PCR probes



SYBR Green Dye



Taqman Probe



Taqman vs. SYBR Green

TaqMan Probe

Advantages:
- Increased specificity
- Use when the most accurate quantitation of PCR product accumulation is desired.
- Option of detecting multiple genes in the same well (multiplexing).

Disadvantages:
- Relative high cost of labeled probe.

SYBR Green

Advantages:
- Relative low cost of primers.
- No fluorescent-labeled probes required.

Disadvantages:
- Less specific – only primers determine specificity.
- Specific and non-specific double-stranded PCR products generate the same fluorescence signal upon binding SYBR Green I dye.
- Not possible to multiplex multiple gene targets.

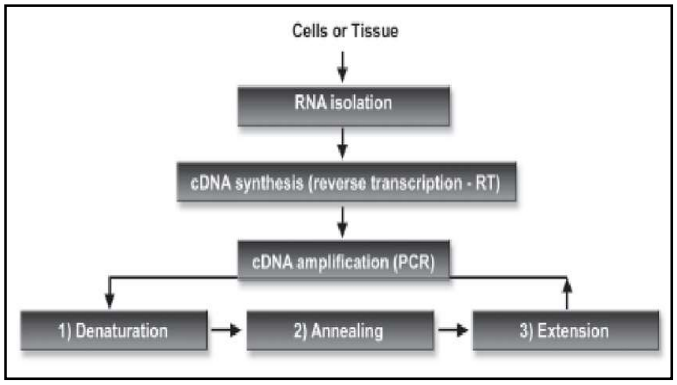
4. Advantages of Realtime-PCR

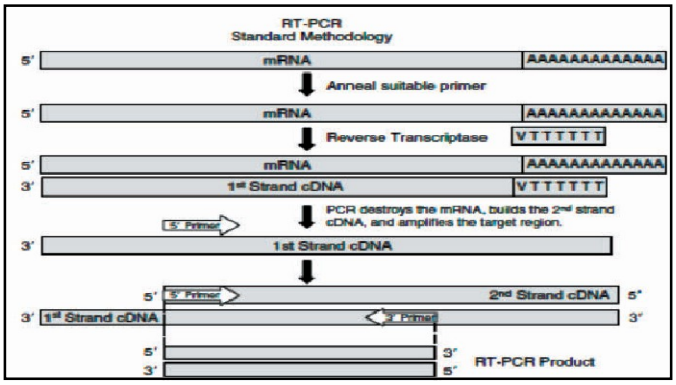
1. Wide dynamic range of quantification (7–8 log decades).
2. High technical sensitivity (< 5 copies).
3. High precision (< 2% CV of CT values).
4. No post-PCR steps like running of gel or sequencing etc.
5. Non-specific amplification can be detected by melt curve analysis of PCR products.
6. Minimized risk of cross contamination.
7. High throughput.
8. Multiplex approach possible.
9. Saves lot of time.

5. Disadvantages of Realtime-PCR

- At very low or high levels of DNA precision may suffer.
- Can give the incorrect quantity if DNA sample is degraded
- qPCR assumes the sample is quantified at same efficiency as the calibrant sample
- Results are calculated based on the calibrants. If the calibrants are made up wrong then the quantities of the samples will be wrong.

Reverse Transcription PCR





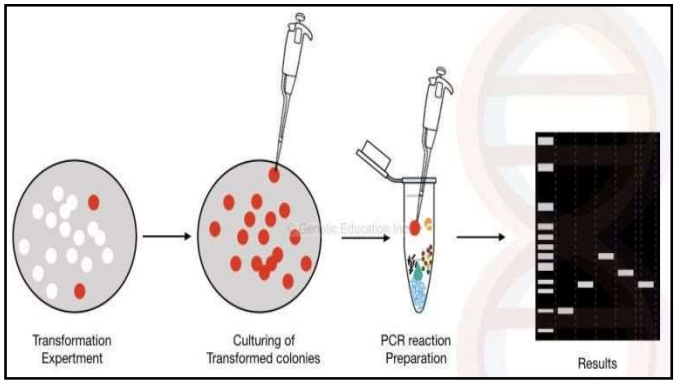
Changes Need to Consider

1. Primers used:
   i. Oligo dT Primer
   ii. Random Primer
   iii. Gene Specific Primer/ Sequence Specific Primer
2. Reverse Transcriptase Enzyme used:
   i. MMLV RT Enzyme
   ii. SS III RT Enzyme

Colony PCR

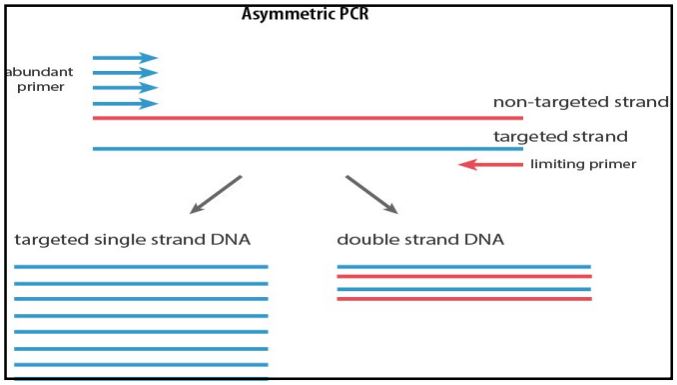
- It is used for screening recombinants from transformation products.
- **Procedure:**

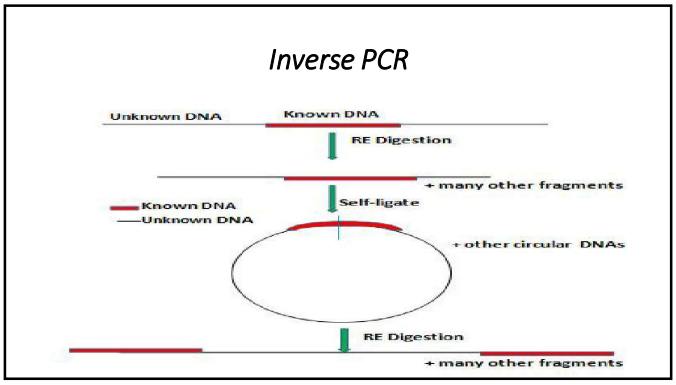
1. Transformation
2. Selection of colony
3. Put the colony in sterile water
4. Heat the mixture
5. Chilling on ice
6. Addition of PCR mix
7. PCR performed

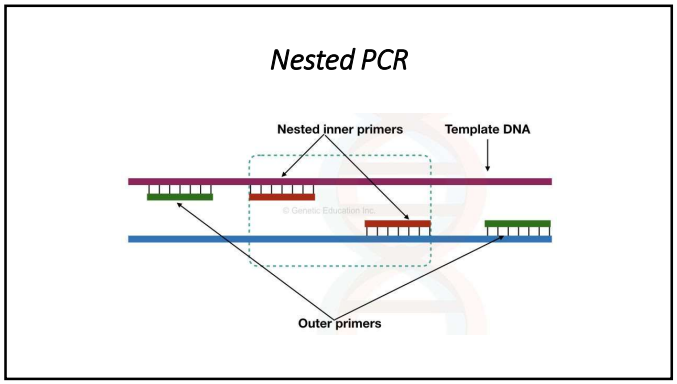


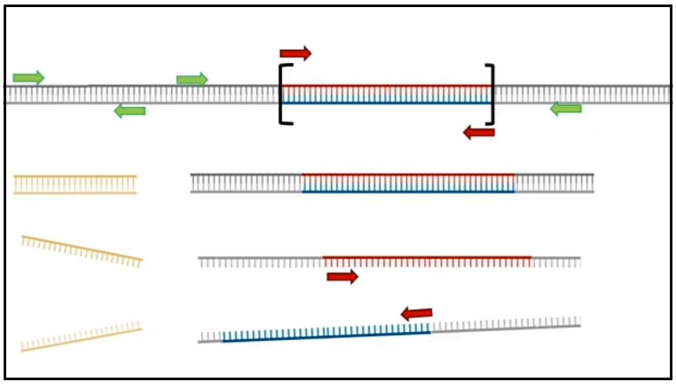
Asymmetric PCR

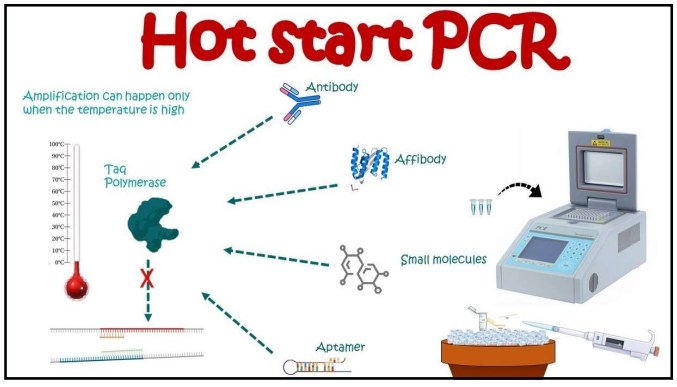
- Asymmertic PCR is used to preferentially amplify one strand of the target DNA more than other.
- Asymmetric PCR is used to form single stranded DNA from double stranded DNA, which is then used for DNA sequencing etc.
- The whole PCR process is similar to regular PCR except that the amount of primer for the targeted strand is much more than that of the non-targeted strand.
- As the PCR reaction progresses the lower concentration limiting primer get used up. After the depletion of limited primer, linear synthesis of the targeted single stranded DNA are formed from the excess primer.

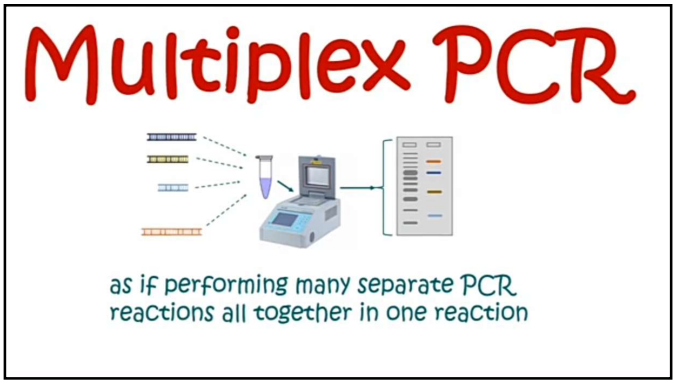

Asymmetric PCR
abundant primer
non-targeted strand
targeted strand
limiting primer
targeted single strand DNA
double strand DNA


Inverse PCR
Unknown DNA
Known DNA
RE Digestion
+ many other fragments
Known DNA
Unknown DNA
Self-ligate
+ other circular DNAs
RE Digestion
+ many other fragments

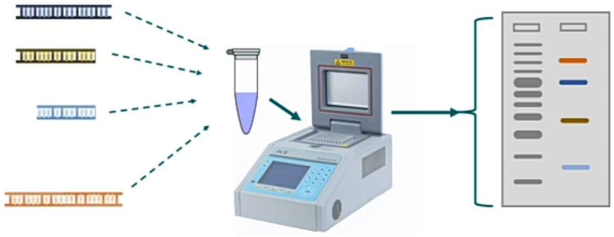

Nested PCR
Nested inner primers
Template DNA
Outer primers



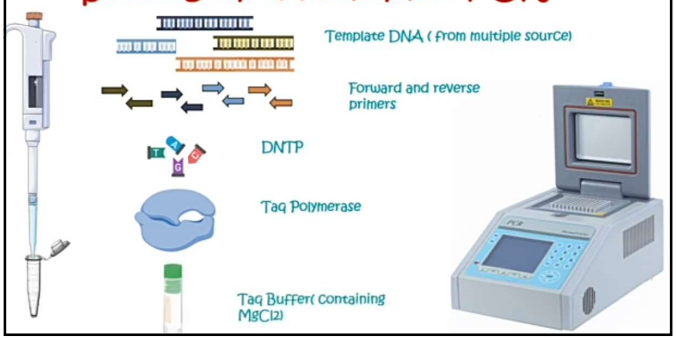

Hot start PCR
Amplification can happen only when the temperature is high
Antibody
Taq Polymerase
Affibody
Small molecules
Aptamer


Multiplex PCR
as if performing many separate PCR reactions all together in one reaction

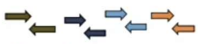
Multiplex PCR is the simultaneous detection of multiple targets in a single reaction well, with a different pair of primers for each target.



Setting up a multiplex PCR



Criteria for a successful multiplex PCR



Forward and reverse primer sets

The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR.

The primer design should ensure all primers pairs are highly specific

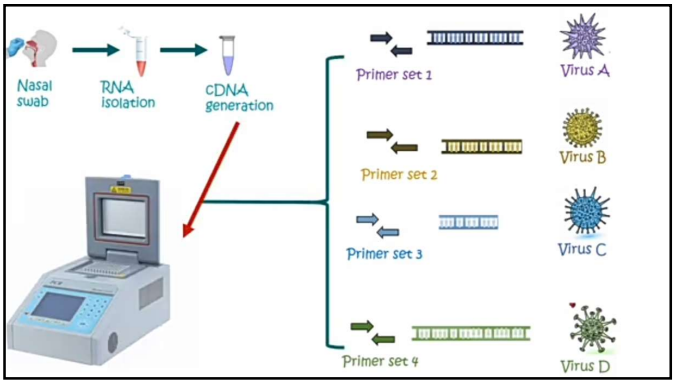
Amplicon size should not differ too much

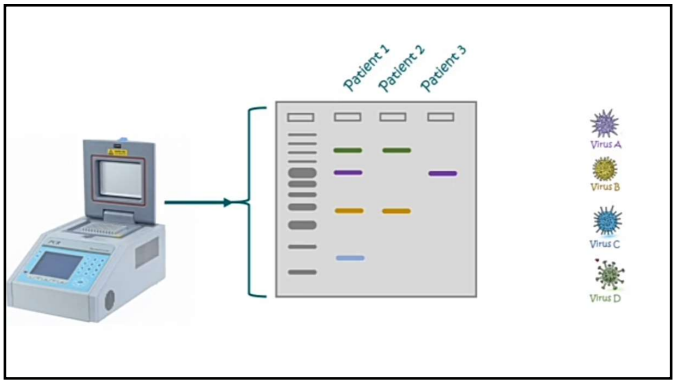
one target might get amplified while other might not.

Setting the polymerization time optimally is important

Some of the applications of multiplex PCR include

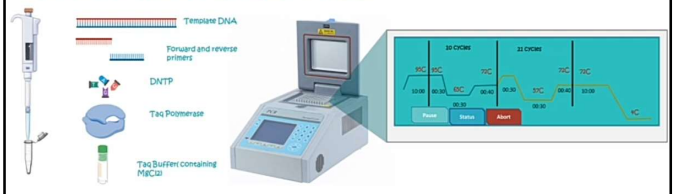
- Pathogen Identification
- High Throughput SNP Genotyping
- Mutation Analysis
- Gene Deletion Analysis
- Linkage Analysis



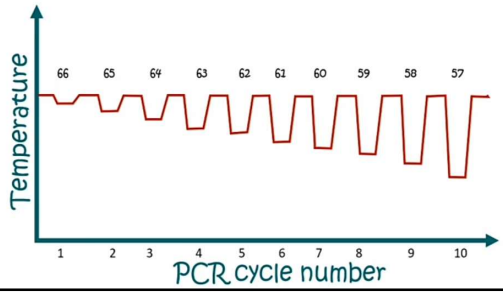


Touch Down-PCR

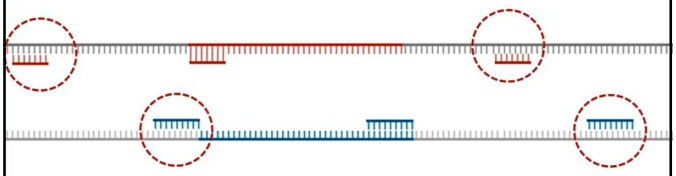
TD-PCR is a modification of PCR in which the initial annealing temperature is higher than the optimal Tm of the primers and is gradually reduced over subsequent cycles until the Tm temperature or "touchdown temperature" is reached.



This is much like the touchdown of an airplane.



Starting with temperatures higher than the calculated Tm in the initial cycles, TD-PCR favours only accumulation of amplicons whose primer-template complementarity is the highest.



TD-PCR results in:

Specificity
Better yield