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UNIT II (15 Periods)

Molecular methods in clinical microbiology: Applications of PCR, RFLP, Nuclear hybridization methods, Single nucleotide polymorphism
and plasmid finger printing in clinical microbiology
Laboratory tests in chemotherapy: Susceptibility tests: Micro-dilution and macro-dilution broth procedures. Susceptibility
tests:Diffusion test procedures. Susceptibility tests: Tests for bactericidal activity. Automated procedures for antimicrobial susceptibility tests.

References

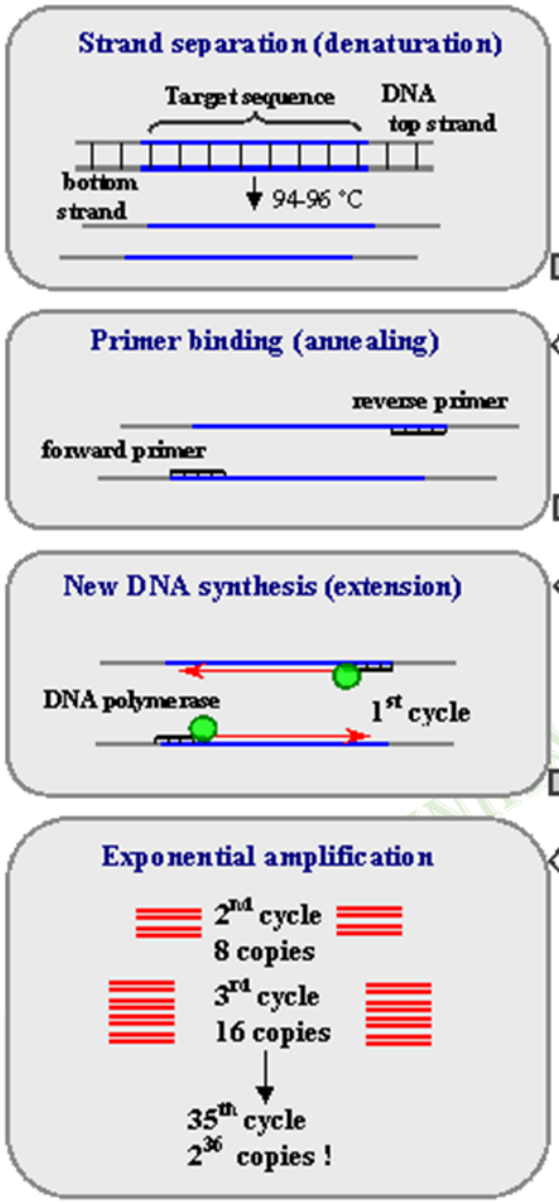
References Open Access

Polymerase Chain Reaction (PCR)

- Polymerase chain reaction (PCR) is a common molecular biology technique developed by Kary B. Mullis, for that he has received the Nobel Prize for Chemistry in 1993.
- PCR is efficient, rapid technique and can amplify DNA or RNA sequences from various sources.
- It is also known as "molecular photocopying"
- PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand.
- DNA polymerase needs a primer to which it can add the first nucleotide.
- At the end of the PCR reaction, the specific sequence will be amplified in billions of copies (amplicons).
- Once the DNA has been sufficiently amplified, the resulting product can be sequenced, analyzed by gel electrophoresis, or cloned into a plasmid for experimental purposes

References

Polymerase Chain Reaction (PCR)



PRINCIPLE: The three basic steps to each amplification cycle include denaturation, annealing and extension.

PROCEDURE

1.DENATURATION the DNA is denatured by heating to 90-95 ˚C, which separtates double-stranded DNA (dsDNA) to single-stranded DNA. The temperature at which 50% of the dsDNA is denatured is known as the melting temperature (T_m) and is determined by the G+C content, the length of the sample, and the concentration of ions (primarily Mg^{2+}).

2.ANNEALING During the annealing step, the sample is cooled to 40-60 ˚C, allowing the primers to attach to the target DNA.

3.EXTENSION. The final PCR step occurs at 70-75 ˚C and is known as extension. During this stage, DNA polymerase extends the DNA from the primers, creating new dsDNA with one old strand and one new strand.

References

Polymerase Chain Reaction (PCR)

- *Final elongation*: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
- *Final hold*: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and maybe employed for short-term storage of the PCR products.
- To check whether the PCR successfully generated the anticipated DNA target region (amplimer or amplicon), agarose gel electrophoresis may be employed for size separation of the PCR products.
- The size of the PCR products is determined by comparison with a DNA ladder, a molecular weight marker which contains DNA fragments of known sizes, which runs on the gel alongside the PCR products.

References

Polymerase Chain Reaction (PCR)

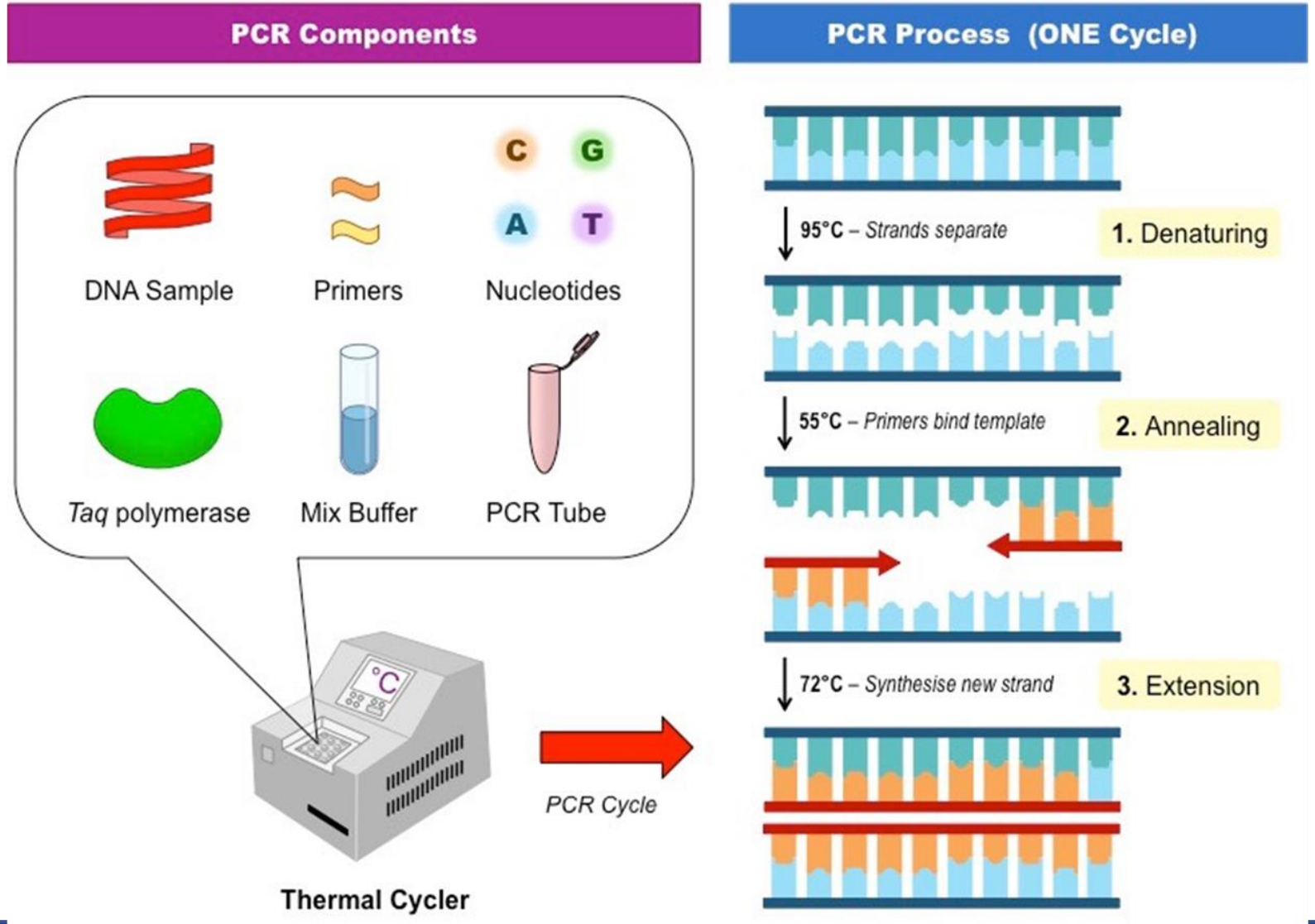
Components of PCR A simple PCR reaction consists of

- **DNA template** the sample DNA that contains the target sequence. At the beginning of the reaction, high temperature is applied to the original double-stranded DNA molecule to separate the strands from each other.
- **DNA polymerase** enzyme that synthesizes new strands of DNA complementary to the target sequence. The first and most commonly used enzyme is *Taq* DNA polymerase (from *Thermis aquaticus*), whereas *Pfu* DNA polymerase (from *Pyrococcus furiosus*) is used widely because of its higher fidelity when copying DNA.
- **Primers** short pieces of single-stranded DNA that are complementary to the target sequence. The polymerase begins synthesizing new DNA from the end of the primer.
- **Nucleotides (dNTPs or deoxynucleotide triphosphates)** single units of the bases A, T, G, and C, which are essentially "building blocks" for new DNA strands.
- **Buffer solution** providing a suitable chemical environment for optimum activity and stability of the DNA polymerase

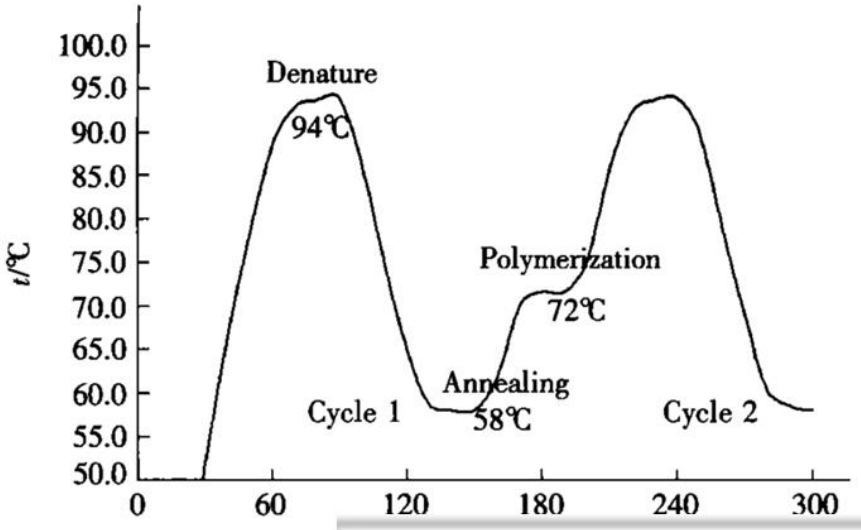
References

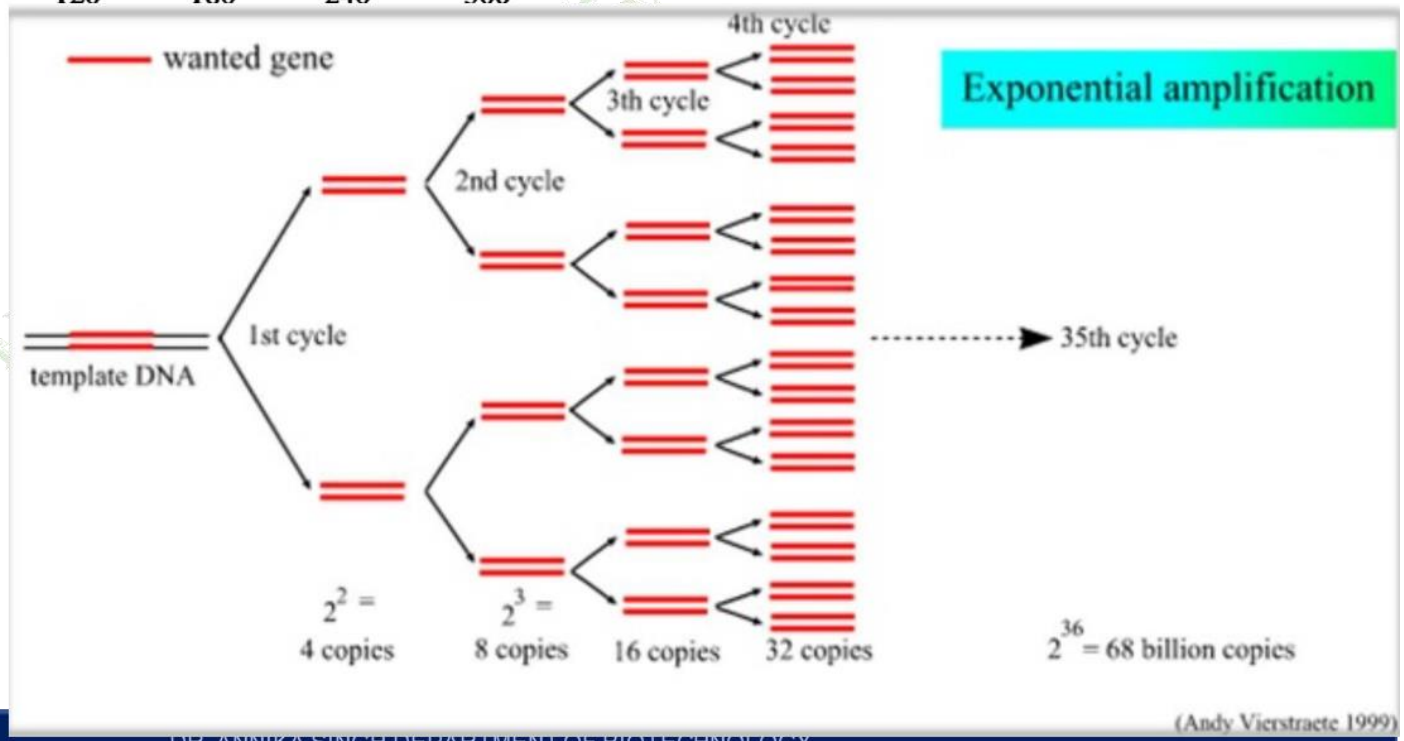
- **Bivalent cations,** typically magnesium (Mg) or manganese (Mn) ions

Polymerase Chain Reaction (PCR)



Polymerase Chain Reaction (PCR)





Polymerase Chain Reaction (PCR)

Hot-start PCR is a technique performed manually by heating the reaction components to the DNA melting temperature (e.g. 95 °C) before adding the polymerase. In this way, non-specific amplification at lower temperatures is prevented. Alternatively, specialized reagents inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody, or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. 'Hot-start/cold-finish PCR' is achieved with new hybrid polymerases that are inactive at ambient temperature and are only activated at elevated temperatures.

Touchdown PCR , the annealing temperature is gradually decreased in later cycles. The annealing temperature in the early cycles is usually 3–5 °C above the standard T_m of the primers used, while in the later cycles it is a similar amount below the T_m. The initial higher annealing temperature leads to greater specificity for primer binding, while the lower temperatures permit more efficient amplification at the end of the reaction.

Assembly PCR (also known as *Polymerase Cycling Assembly* or *PCA*) is the synthesis of long DNA structures by performing PCR on a pool of long oligonucleotides with short overlapping segments, to assemble two or more pieces of DNA into one piece. It involves an initial PCR with primers that have an overlap and a second PCR using the products as the template that generates the final full-length product. This technique may substitute for ligation- based assembly.

References

Polymerase Chain Reaction (PCR)

Multiplex PCR is the simultaneous detection of multiple targets in a single reaction well, with a different pair of primers for each target. This technique requires two or more probes that can be distinguished from each other and detected simultaneously. There is a range of different probe technologies available, all using fluorophores.

Multiplex PCR is used in life science research, clinical diagnostics, and forensic laboratories.

Advantages of using multiplex PCR include:

- More information with less sample
- Higher throughput
- Cost effective — fewer dNTPs, enzymes, and other consumables
- Time saving

References

- **Reverse transcription polymerase chain reaction** (**RT-PCR**) is a laboratory technique combining reverse transcription of RNA into DNA (complementary DNA or cDNA) and amplification of specific DNA targets using polymerase chain reaction (PCR).
- It is primarily used to measure the amount of a specific RNA.
- This is achieved by monitoring the amplification reaction using fluorescence, a technique called real-time PCR or quantitative PCR (qPCR).
- Combined RT-PCR and qPCR are routinely used for analysis of gene expression and quantification of viral RNA in research and clinical settings.

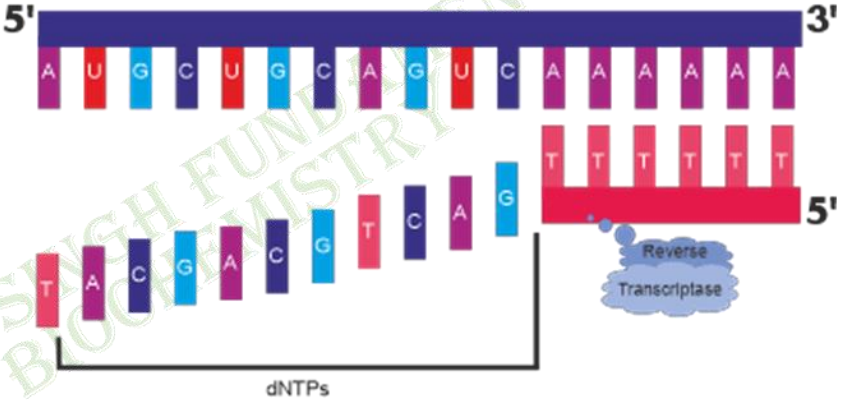
References

Reverse Trancriptase Polymerase Chain Reaction (RT-PCR)

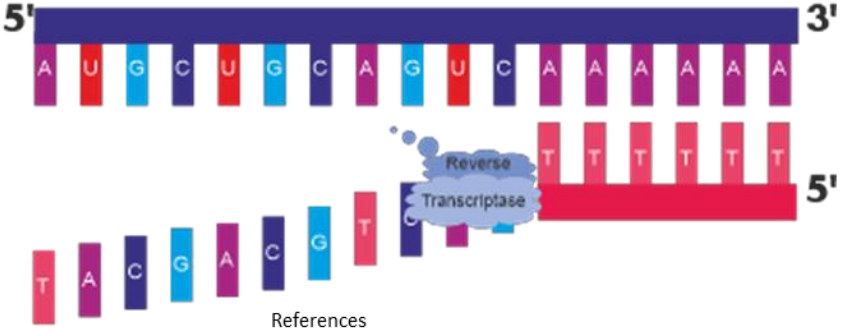
1 a. RNA
RNA consist of Start codon AUG and ends with poly A tail

b. **Oligo dT Primer**
Oligo dr Primer is binding to RNA poly A tail

C. **Reverse Transcriptase**
And
dNTPs

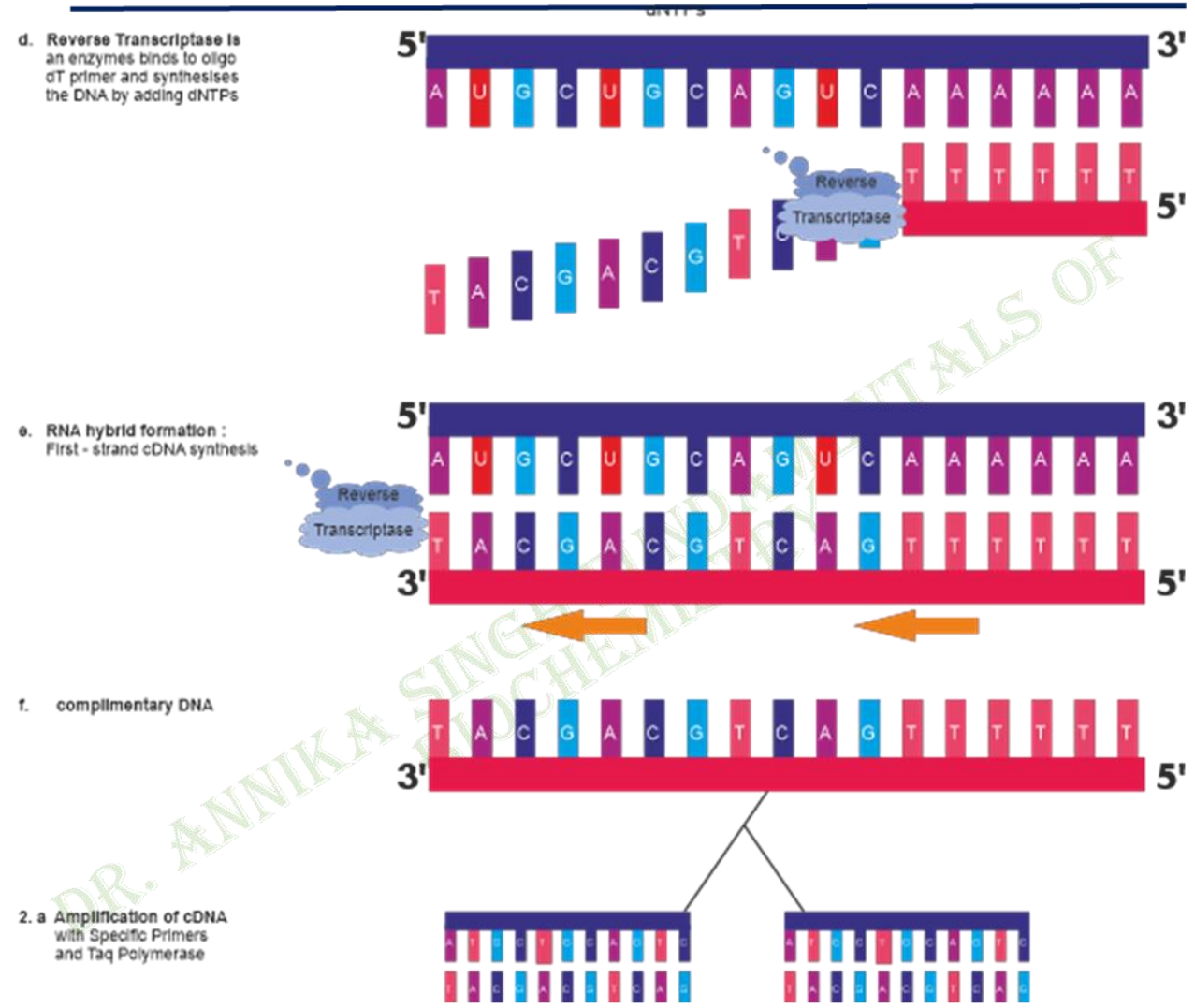


d. **Reverse Transcriptase is** an enzymes binds to oligo dT primer and synthesises the DNA by adding dNTPs



References

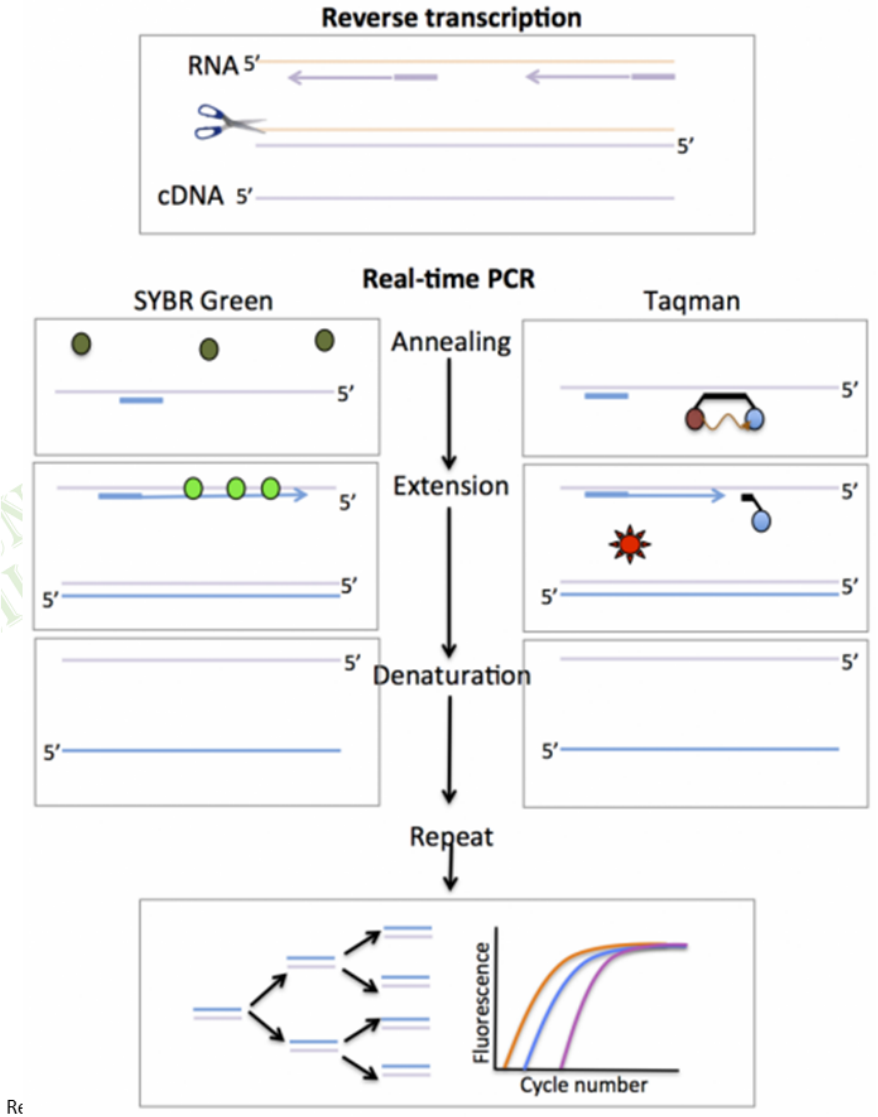
Real Time Polymerase Chain Reaction (RT PCR)



References Open Access

Real-time polymerase chain reaction

- **Real-time polymerase chain reaction** In real-time PCR, the accumulation of amplification product is measured as the reaction progresses, in real time, with product quantification after each cycle.
- The qPCR workflow below delineates the steps in real-time PCR. First, amplification reactions are set up with PCR reagents and unique or custom primers. Reactions are then run in real-time PCR instruments and the collected data is analyzed by proprietary instrument software.
- real-time qPCR data can be evaluated without gel electrophoresis, resulting in reduced bench time and increased throughput. Finally, because real-time qPCR reactions are run and data are evaluated in a unified, closed-tube qPCR system, opportunities for contamination are reduced and the need for postamplification manipulation is eliminated in qPCR analysis.



Real-time polymerase chain reaction

- (real-time PCR) is commonly used to measure gene expression. It is more sensitive than microarrays in detecting small changes in expression but requires more input RNA and is less adaptable to high-throughput studies . real-time PCR can only be used for studying **known genes**.
- **Real-time PCR steps**
- The first step in a real-time PCR reaction is the conversion of RNA to complementary DNA (cDNA) - this process is known as reverse transcription. The next step uses fluorescent reporters and a PCR reaction to amplify and detect specific genes.
- Two types of fluorescent reporters are commonly used; these are SYBR green and Taqman probes.
- **SYBR green and Taqman probes**
- **SYBR green** is a dye that fluoresces only when bound to double stranded DNA (i.e the PCR product).
- **Taqman probes** are made of a gene-specific nucleic acid probe, joined to reporter and quencher molecules.
- The probe binds to the DNA between the forward and reverse primer. While the reporter and quencher are bound to the probe, the quencher absorbs the fluorescence emitted by the reporter. During the extension phase of the PCR reaction the probe is degraded, releasing the reporter and allowing its fluorescence to be detected.
- The advantage of the Taqman method is that probes with different coloured reporters can be combined in multiplex assays.

References

Real-time polymerase chain reaction

- For both SYBR green and Taqman methods, the amount of fluorescence in a sample is detected in 'real-time' and plotted against the cycle number. The amount of fluorescence is proportional to the amount of PCR product.
- The number of PCR cycles is shown on the x-axis, and the fluorescence from the amplification reaction, which is proportional to the amount of amplified product in the tube, is shown on the y-axis.
- The amplification plot shows two phases, an exponential phase followed by a non-exponential plateau phase.
- During the exponential phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, however, reaction components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase.
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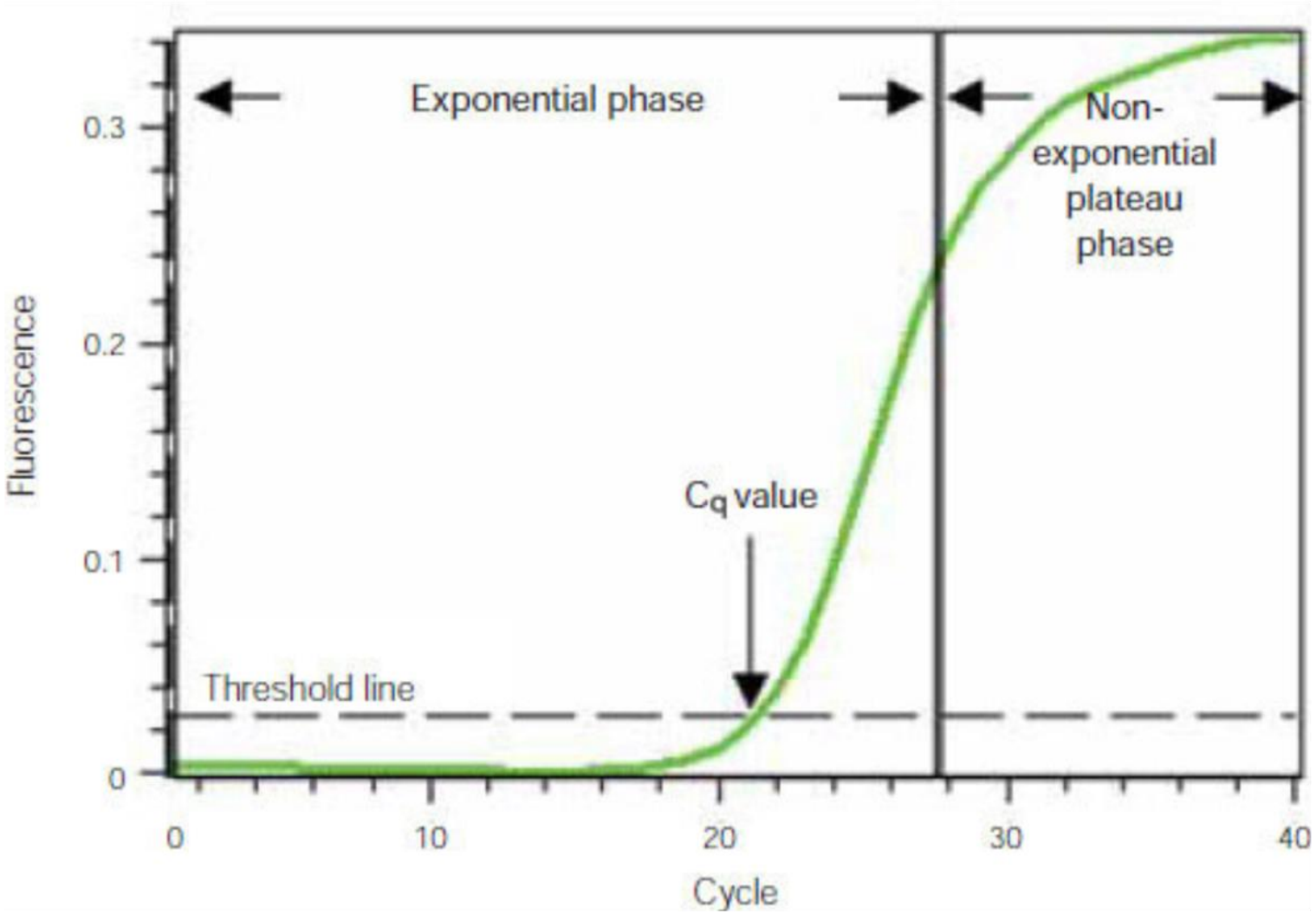
References

Real-time polymerase chain reaction

- Initially, fluorescence remains at background levels, and increases in fluorescence are not detectable even though product accumulates exponentially. Eventually, enough amplified product accumulates to yield a detectable fluorescence signal. The cycle number at which this occurs is called the quantification cycle, or C_q.
- The C_q of a reaction is determined mainly by the amount of template present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescence signal above background.
- Thus, the reaction will have a low, or early, C_q. In contrast, if a small amount of template is present at the start of the reaction, more amplification cycles will be required for the fluorescence signal to rise above background. Thus, the reaction will have a high, or late, C_q. This relationship forms the basis for the quantitative aspect of real-time PCR.

References

Real-time polymerase chain reaction

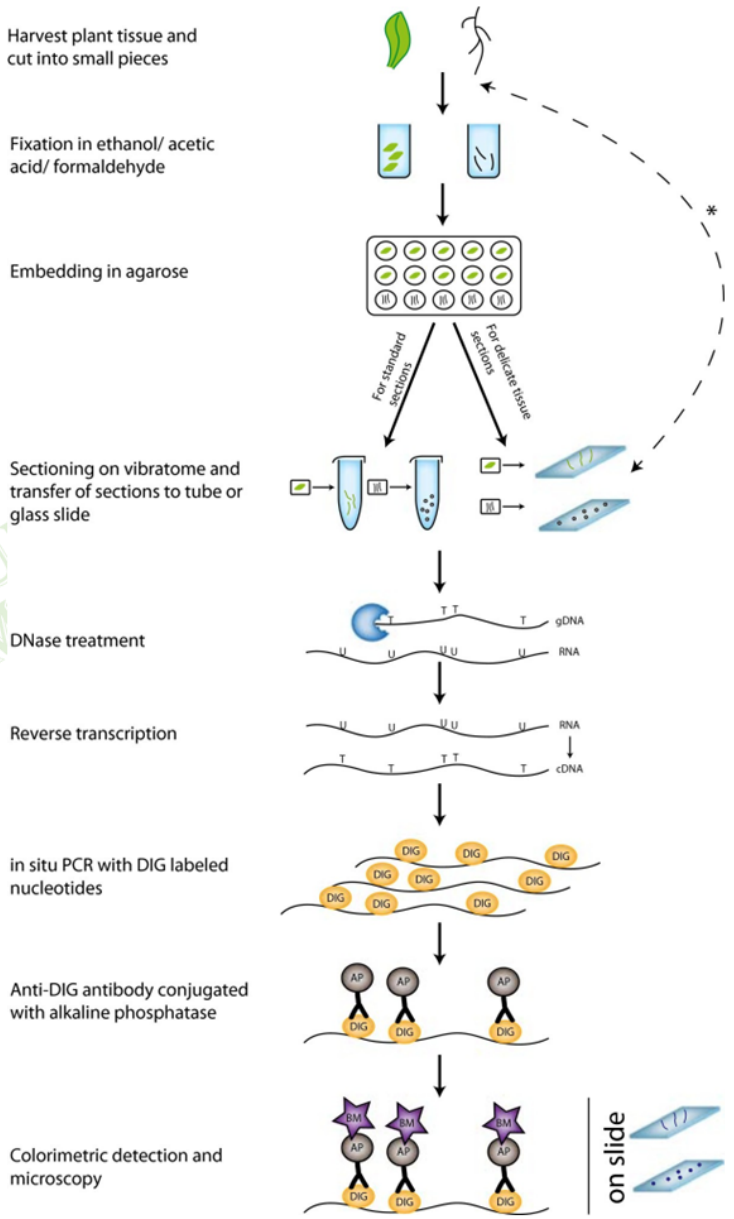


References

Polymerase Chain Reaction (PCR)

***In Situ* Polymerase Chain Reaction** (*In situ* PCR) is a powerful method that detects minute quantities of rare or single-copy number nucleic acid sequences in frozen or paraffin-embedded cells or tissue sections for the localization of those sequences within the cells. The target sequences are amplified by those reagents and then detected by standard immunocytochemical protocols.

In situ PCR combines the sensitivity of PCR or RT-PCR amplification along with the ability to perform morphological analysis on the same sample, and thus it is an attractive tool in diagnostic applications. One of the most prominent applications is the detection of infectious disease agents including HIV-1, HBV, HPV, HHV-6, CMV, and EBV.



The amplification-refractory mutation system (ARMS)

- The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base changes or small deletions.
- ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample.
- Presence or absence of a PCR product is diagnostic for the presence or absence of the target allele.
- Multiplex ARMS for the analysis of two or more mutations.
- The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base change

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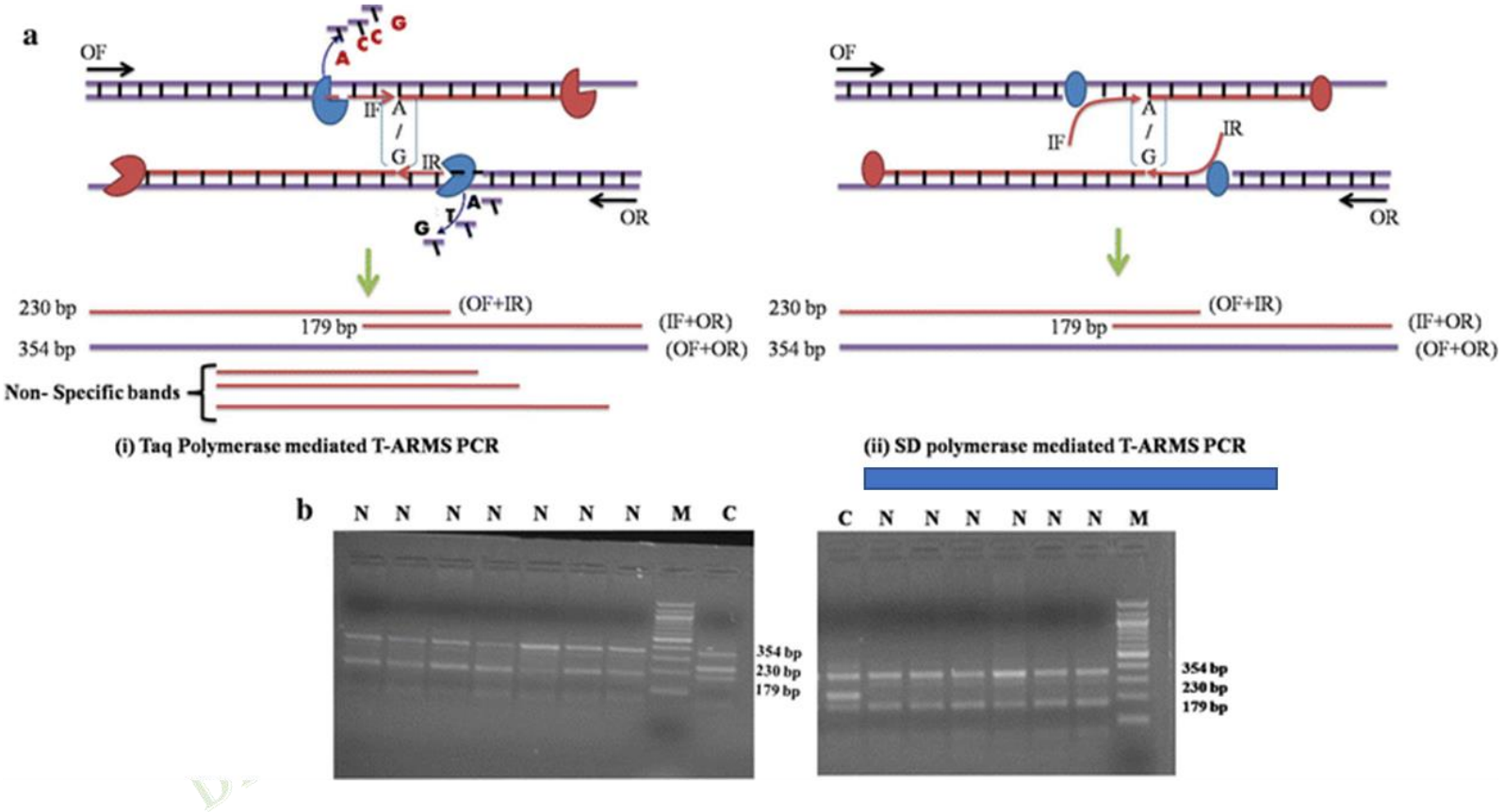
The amplification-refractory mutation system (ARMS)



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