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Polymerase Chain Reaction (PCR)

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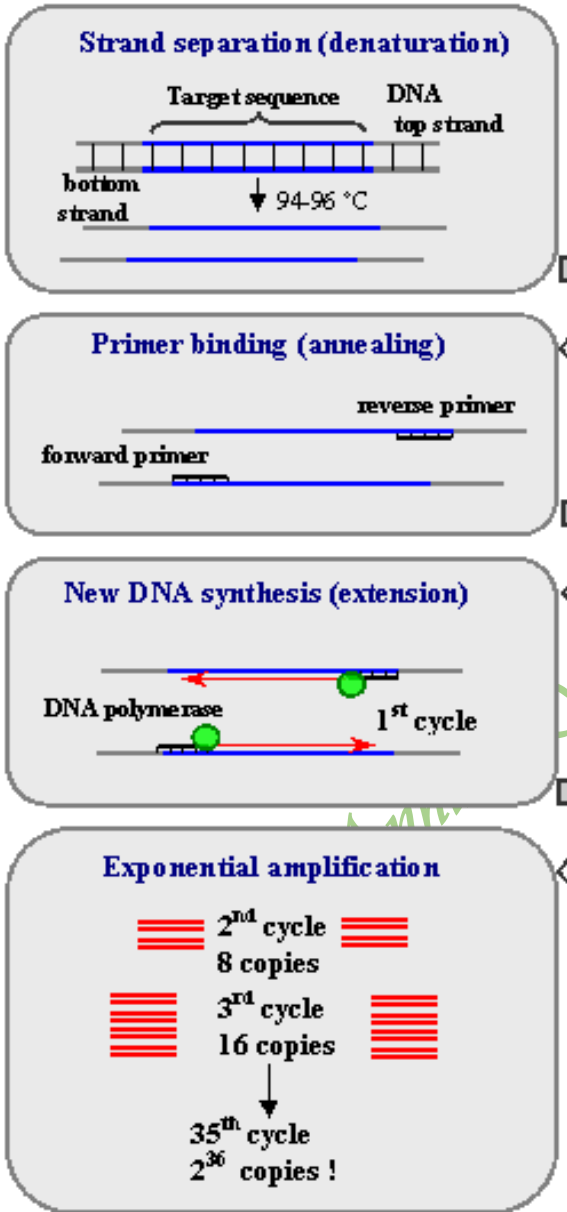
Polymerase Chain Reaction (PCR)

- 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

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.

Polymerase Chain Reaction (PCR)

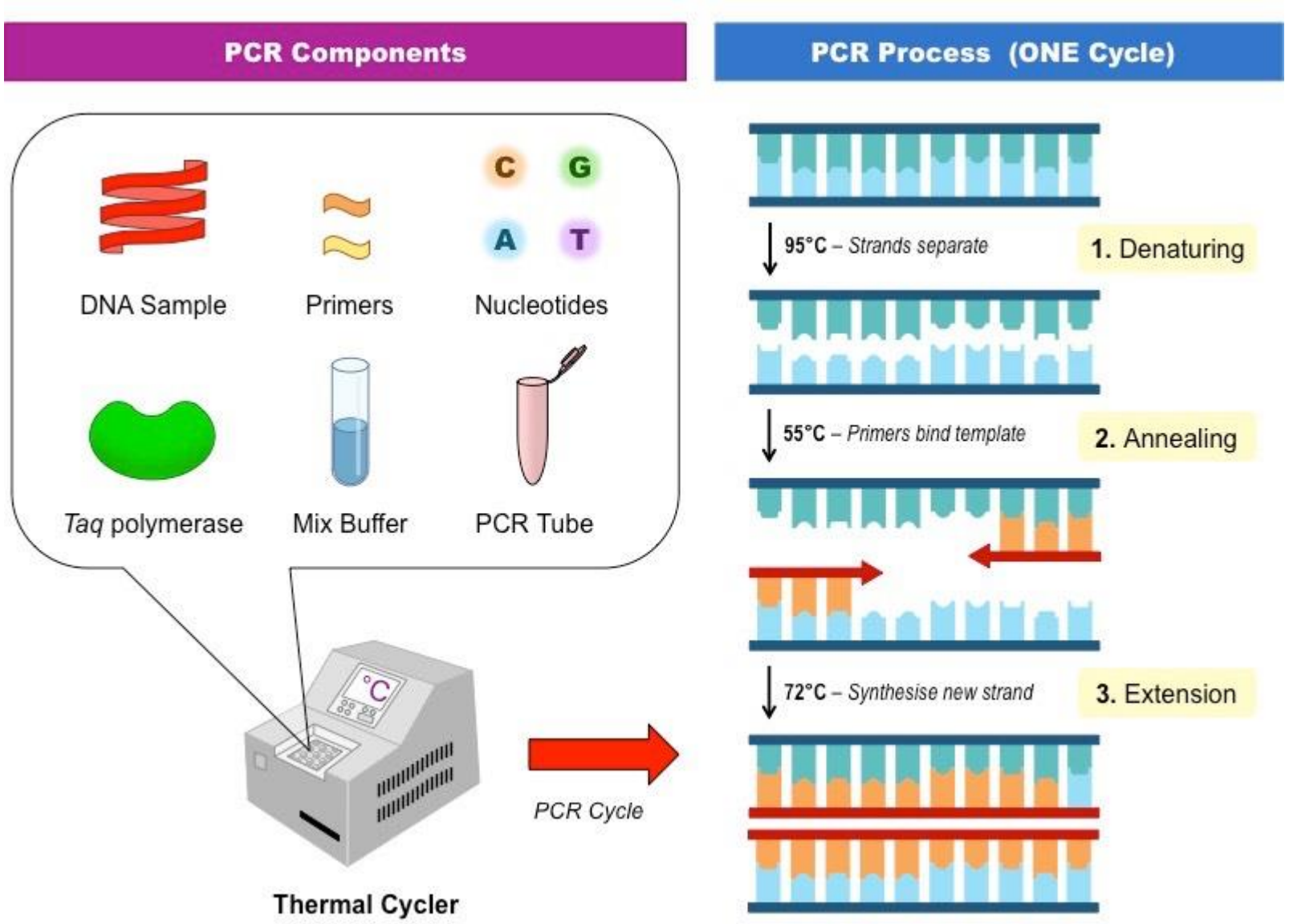
- *Final elongation*: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
- *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.

Polymerase Chain Reaction (PCR)

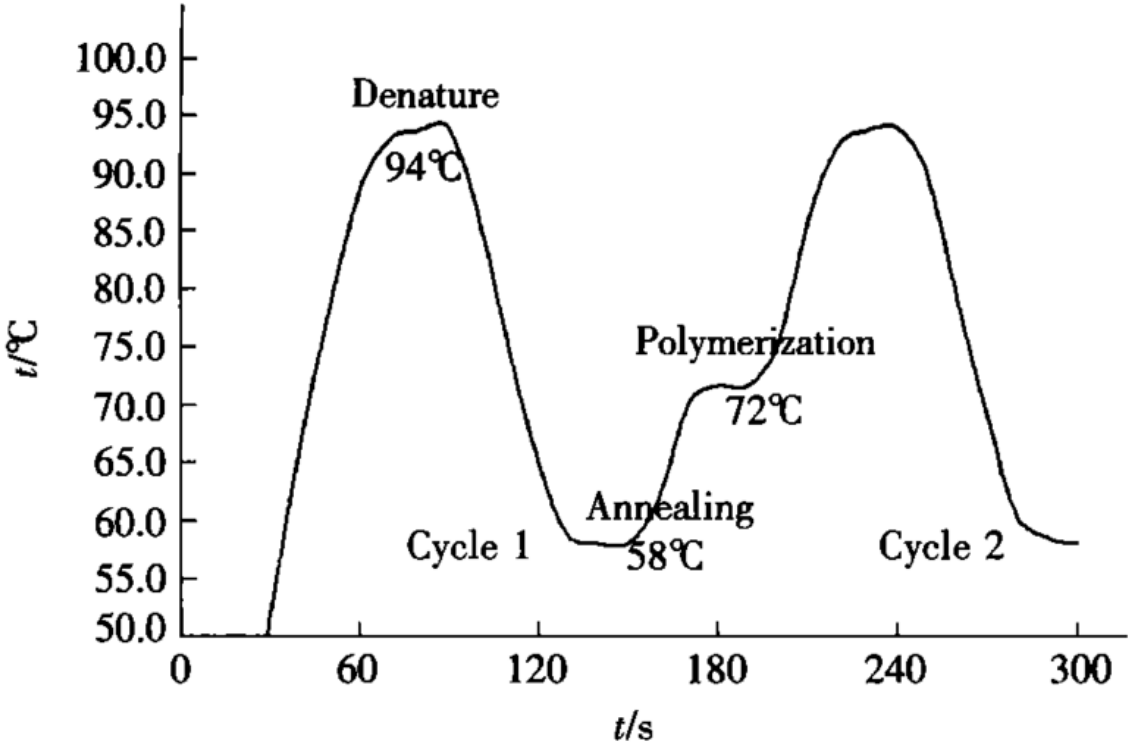
Components of PCR A simple PCR reaction consists of

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

Polymerase Chain Reaction (PCR)



Polymerase Chain Reaction (PCR)



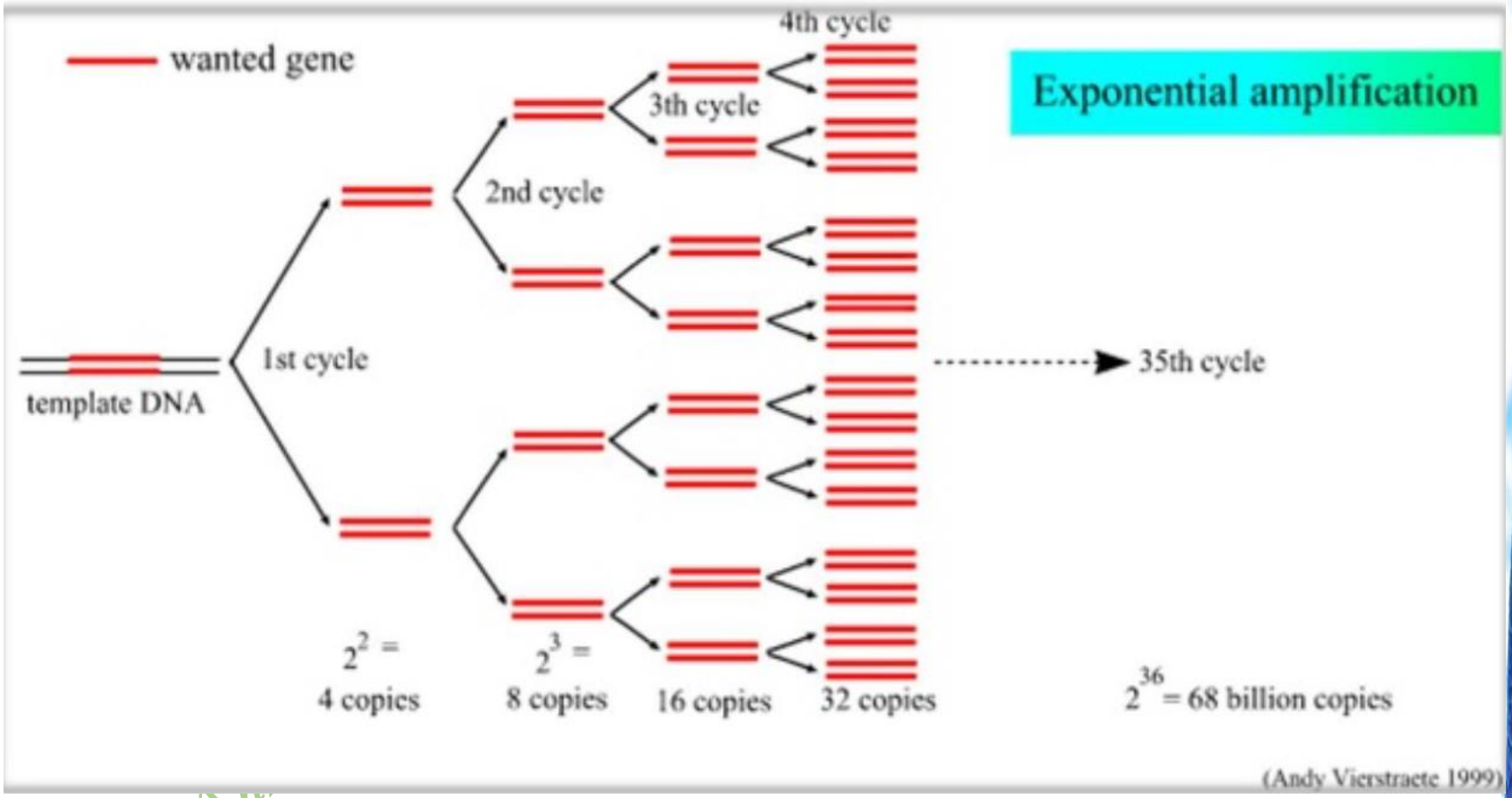
$$T_m = (4 \times [G + C]) + (2 \times [A + T])^{\circ}C$$

Primer sequence: 5' AGACTCAGAGAGAACCC 3'

4 Gs 5 Cs 7 As 1 T

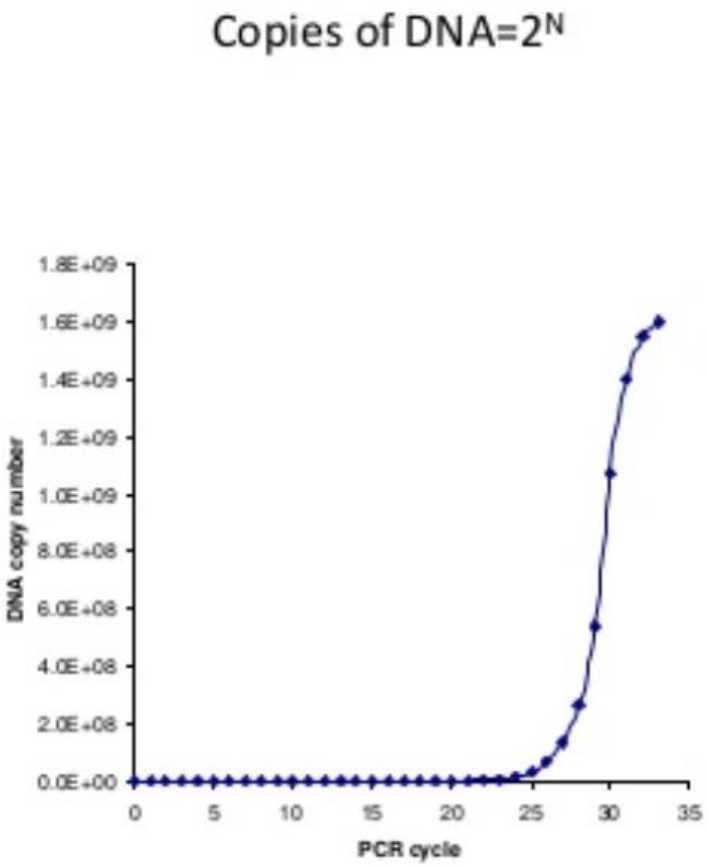
$T_m = (4 \times 8) + (2 \times 8)$
$= 36 + 16$
$= 52^{\circ}C$

Polymerase Chain Reaction (PCR)



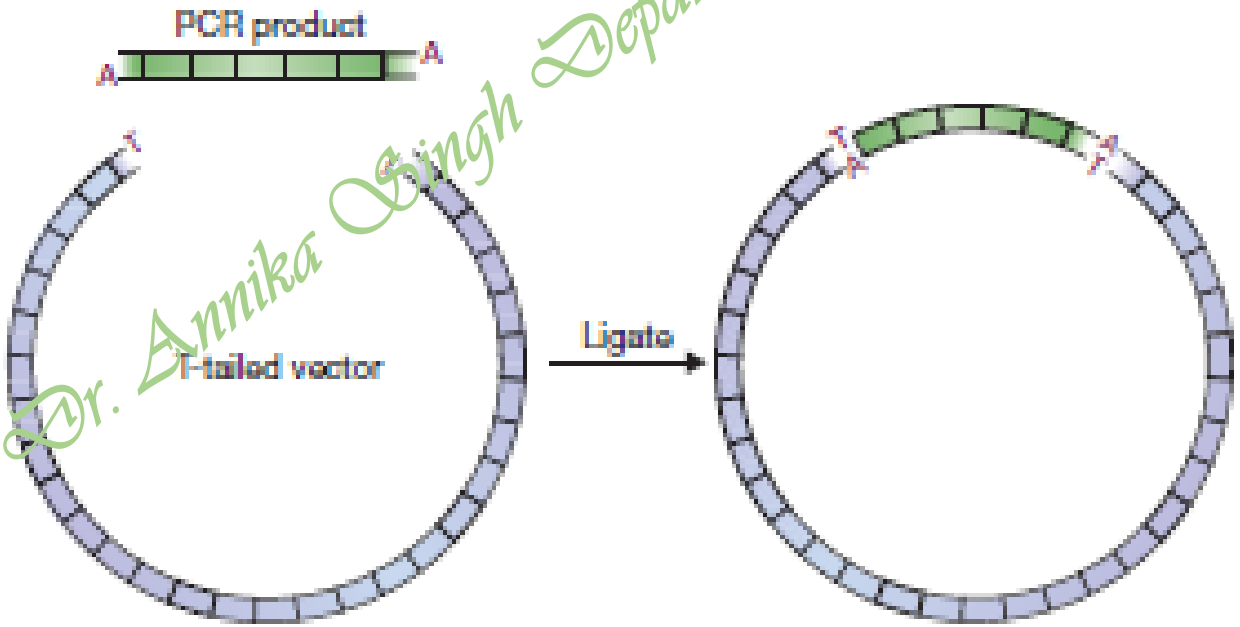
Polymerase Chain Reaction (PCR)

CYCLE NUMBER	DNA copy number
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1,024
11	2,048
12	4,096
13	8,192
14	16,384
15	32,768
16	65,536
17	131,072
18	262,144
19	524,288
20	1,048,576
21	2,097,152
22	4,194,304
23	8,388,608
24	16,777,216
25	33,554,432
26	67,108,864
27	134,217,728
28	268,435,456
29	536,870,912
30	1,073,741,824
31	1,400,000,000
32	1,500,000,000
33	1,550,000,000
34	1,580,000,000



Cloning PCR products

- PCR products can be ligated into a vector and examined by any of the standard methods used for studying cloned DNA.
- *Taq* polymerase tends to add an additional nucleotide, usually an adenosine, to the end of each strand that it synthesizes.
- **Hence double stranded PCR product 3′ termini have a single nucleotide overhang**
- **Cloning vector with thymidine (T) overhangs can be used to ligate a PCR product**



Cloning PCR products

- Short PCR amplicons are generally blunt-ended, therefore could be inserted into a cloning vector by blunt-end ligation
- PCR products could be provided with sticky ends by the attachment of linkers or adaptors

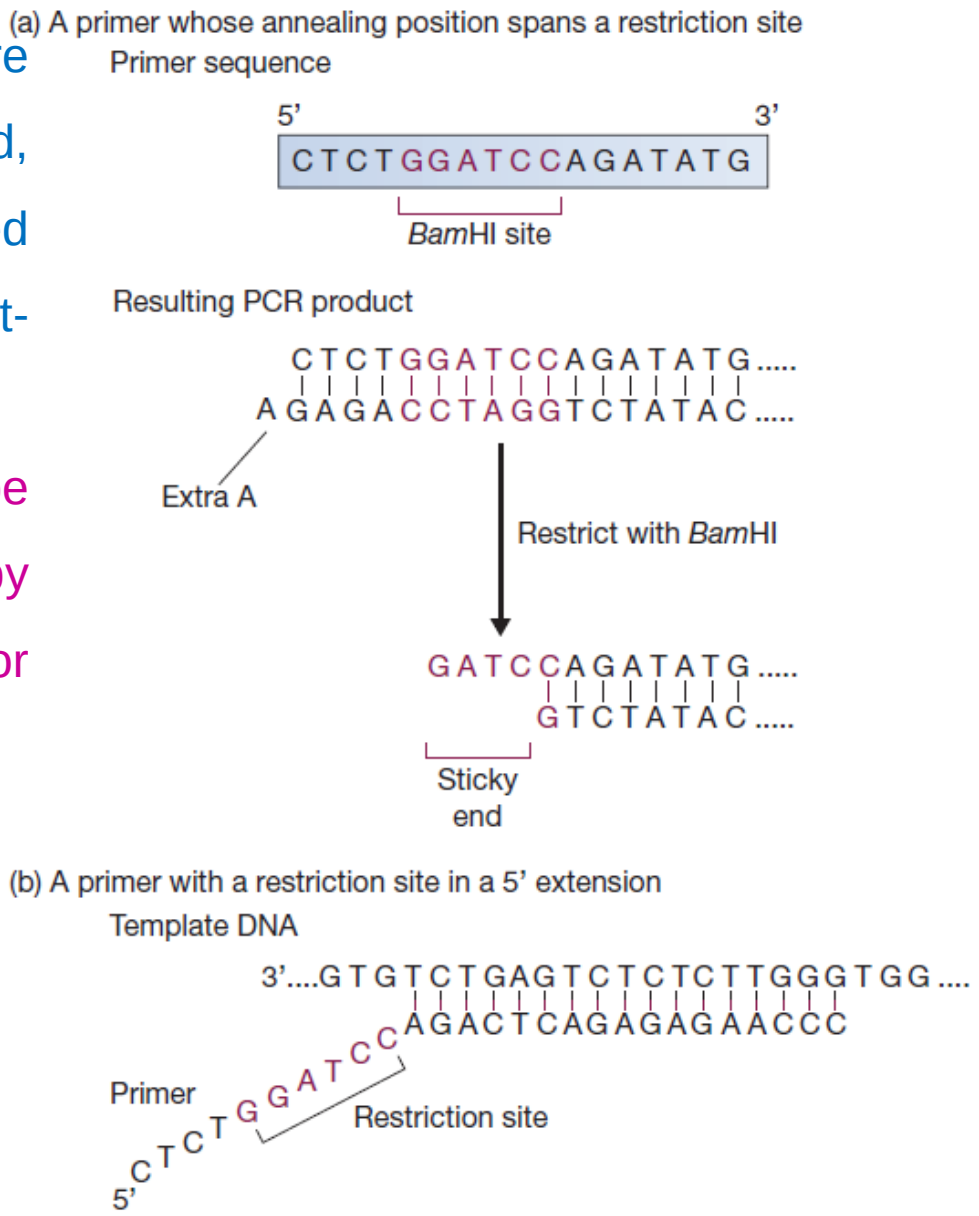


Fig. Obtaining a PCR product with a sticky end through use of a primer whose sequence includes a restriction site.

DNA polymerases used in PCR

Successful PCR depends on two crucial components, an optimized reaction buffer, and a high-quality, thermostable DNA polymerase (such as Taq DNA polymerase).

Four basic properties of DNA polymerases can help you define the best enzyme for your particular research needs:

1. **Thermal stability.** DNA polymerase must be robust enough to tolerate high-temperature cycles without compromising activity, a factor dependant on buffer composition and pH.
2. **Extension rate.** This refers to the speed at which nucleotides are added, per second, per molecule of DNA polymerase, a factor determined by extension temperature, DNA template sequence and buffer composition.
3. **Fidelity.** Fidelity is an inherent DNA polymerase property defining the frequency of insertion of an incorrect nucleotide per kb of DNA. High-fidelity polymerases are more accurate because of the ability to "proofread" and excise incorrectly incorporated mononucleotides, replacing them with the correct base.
4. **Processivity.** The probability that a polymerase will detach from DNA during extension, indicating the average number of nucleotides the enzyme adds in a single binding event, is known as its processivity.

DNA polymerases used in PCR

- ***Taq DNA Polymerase*** is a thermostable DNA polymerase used in PCR, originally isolated from *Thermus aquaticus*, a thermophilic bacteria, optimum activity temperature of 75-80°C, high processitivity replicating 1 kb of DNA within 30-60 seconds during PCR
- Taq is unable to proofread as it lacks 3'-5' exonuclease activity, therefore giving low replication fidelity (1 error in 9000 base pairs)
- Produces single adenine overhang at the 3' ends of both strands, producing DNA with sticky ends hence, suitable forTA cloning
- ***Pfu DNA Polymerase*** is a thermostable enzyme originally isolated from *Pyrococcus furiosus*, a hyperthermophilic species of archaea. it operates optimally at 90°C and is not denatured by the heating steps. Pfu DNA polymerase displays 3'-5' exonuclease activity, and therefore, giving very high replication fidelity (1 error in 1.3 million base pairs)
- Low Processivity it requires up to 2 minutes to amplify 1 kb of DNA during a PCR cycle Pfu DNA polymerase also produces DNA products with blunt ends
- **KOD DNA polymerase** is a recombinant form of DNA polymerase derived from the thermophilic solfatara bacterium *Thermococcus kodakaraensis* KOD1 type strain. KOD DNA polymerase functions optimally at 85°C and displays 3'-5' exonuclease proofreading activity,
- Producing blunt-ended DNA products

DNA polymerases used in PCR

- ***Bst DNA Polymerase*** isolated from *Bacillus stearothermophilus* species displays helicase-like activity to unwind DNA strands in addition to its polymerase activity. Bst polymerase functions optimally at 60-65°C, but denatures above 70°C, making it more suitable for loop-mediated isothermal amplification (LAMP) which does not undergo the high-temperature denaturation steps and thermocycling used in PCR it cannot proofread as it lacks 3'-5' exonuclease activity
- **Tth polymerase** Tth polymerase is derived from *Thermus thermophilus*, a thermophilic thermal vent bacterium
- Tth DNA polymerase functions optimally at 75°C with high processivity, but lacks proofreading 3'-5' exonuclease activity
- **Pwo DNA polymerase** is derived from the ultra-thermophilic archaeon *Pyrococcus woesei* found in deep marine environments
- Pwo polymerase functions optimally at 100-103°C, and displays high proofreading 3'-5' exonuclease activity, giving it 18-fold higher fidelity than Taq polymerase
- Pwo polymerase creates blunt-ended products

Primer Designing

Some general guidelines for primer design:

- Binding region should be 17-27 bp
- Binding region Tm's in the range of 50 to 65°C
- Moderate G+C content
- Avoid repetitive poly-Nucleotides regions
- Avoid secondary structure (hairpins, self dimerisation or primer dimer)
- Primer pairs should have similar Tm's (±4°C)

Degenerate Primers.

•These are mixtures of primers that are similar, but not identical.

These may be convenient when amplifying the same gene from different organisms as the sequences are probably similar but not identical.

This technique is useful because the genetic code itself is degenerate, meaning several different codons can code for the same amino acid.

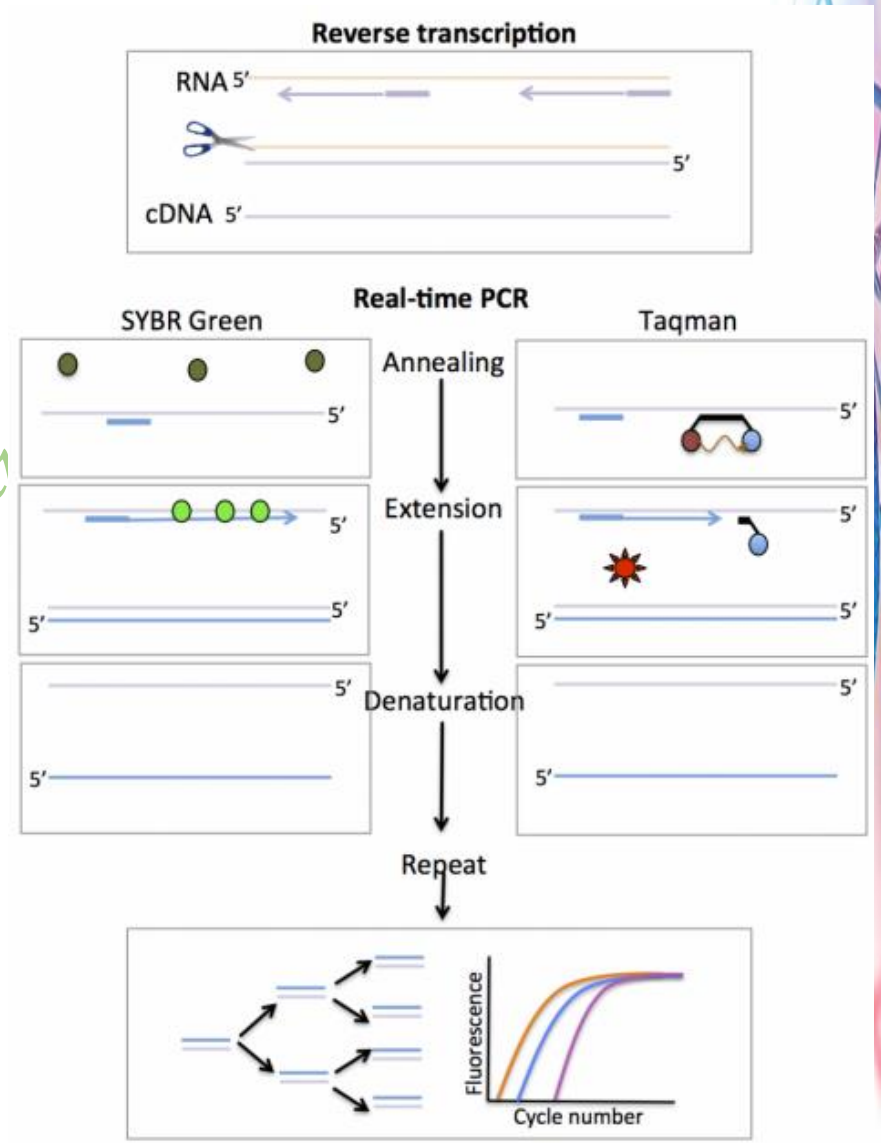
This allows different organisms to have a significantly different genetic sequence that code for a highly similar protein.

Variants (Types) of PCR

1. AFLP PCR
2. Allele-specific PCR
3. Alu PCR
4. Assembly PCR
5. Asymmetric PCR
6. COLD PCR
7. Colony PCR
8. Conventional PCR
9. Digital PCR (dPCR)
10. Fast-cycling PCR
11. High-fidelity PCR
12. High-Resolution Melt (HRM) PCR
13. Hot-start PCR
14. In situ PCR
15. Intersequence-specific (ISSR) PCR
16. Inverse PCR
17. LATE (linear after the exponential) PCR
18. Ligation-mediated PCR
19. Methylation-specific PCR (MSP)
20. Miniprimer PCR
21. Multiplex-PCR
22. Nanoparticle-Assisted PCR (nanoPCR)
23. Nested PCR
24. Overlap extension PCR
25. Real-Time PCR (quantitative PCR or qPCR)
26. Repetitive sequence-based PCR
27. Reverse-Transcriptase (RT-PCR)
28. Reverse-Transcriptase Real-Time PCR (RT-qPCR)
29. RNase H-dependent PCR (rhPCR)
30. Single cell PCR
31. Single Specific Primer-PCR (SSP-PCR)
32. Solid phase PCR
33. Suicide PCR
34. Thermal asymmetric interlaced PCR (TAIL-PCR)
35. Touch down (TD) PCR
36. Variable Number of Tandem Repeats (VNTR) PCR

Real-time polymerase chain reaction

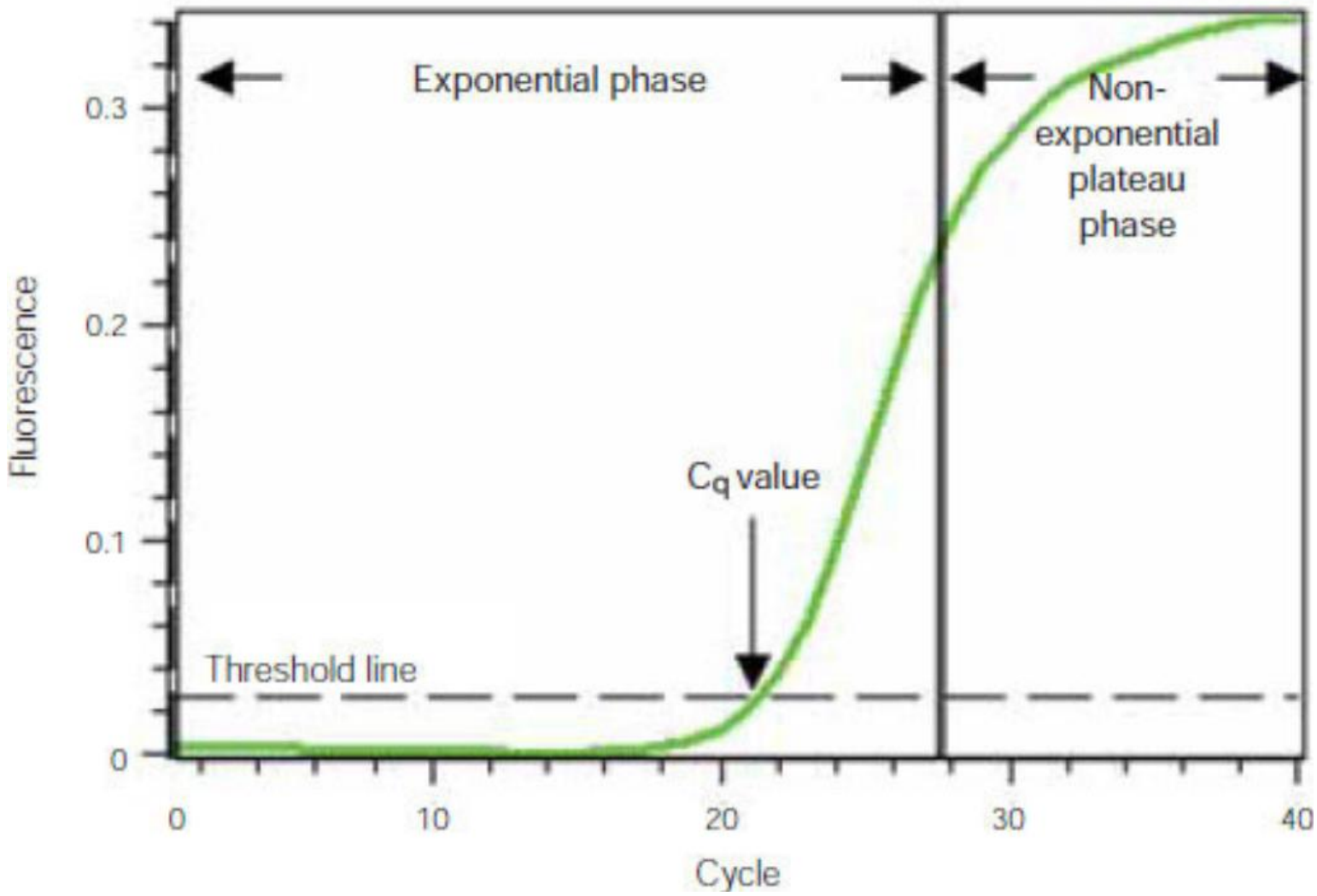
- **Real-time polymerase chain reaction** In real-time PCR, the accumulation of amplification product is measured as the reaction progresses, in real time, with product quantification after each cycle.
- The qPCR workflow below delineates the steps in real-time PCR. First, amplification reactions are 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 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.

- 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.
-

- 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.


Exponential phase
Non-exponential plateau phase
Fluorescence
0.3
0.2
0.1
0
C_q value
Threshold line
0
10
20
30
40
Cycle

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.

Colony PCR, bacterial colonies are screened directly by PCR, for example, the screen for correct DNA vector constructs. Colonies are sampled with a sterile pipette tip and a small quantity of cells transferred into a PCR mix. To release the DNA from the cells, the PCR is either started with an extended time at 95 °C (when standard polymerase is used), or with a shortened denaturation step at 100 °C and special chimeric DNA polymerase.

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

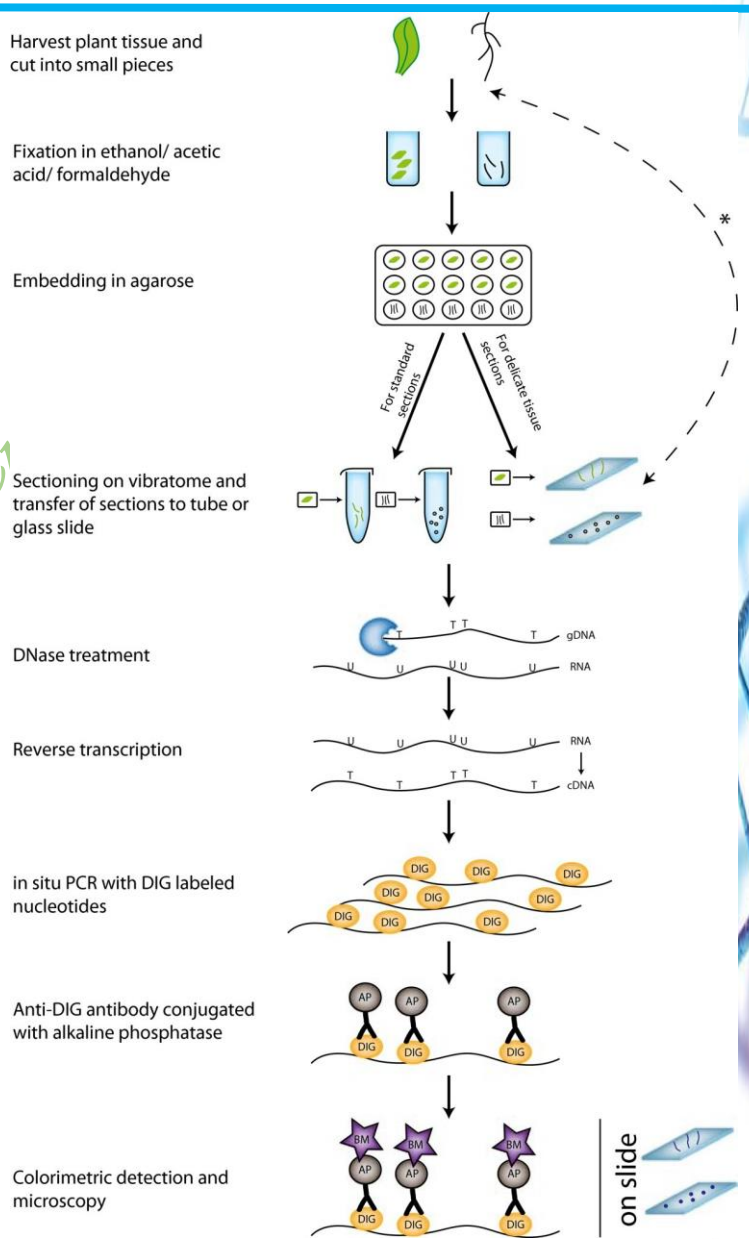
•Higher throughput

•Cost effective — fewer dNTPs, enzymes, and other consumables

•Time saving

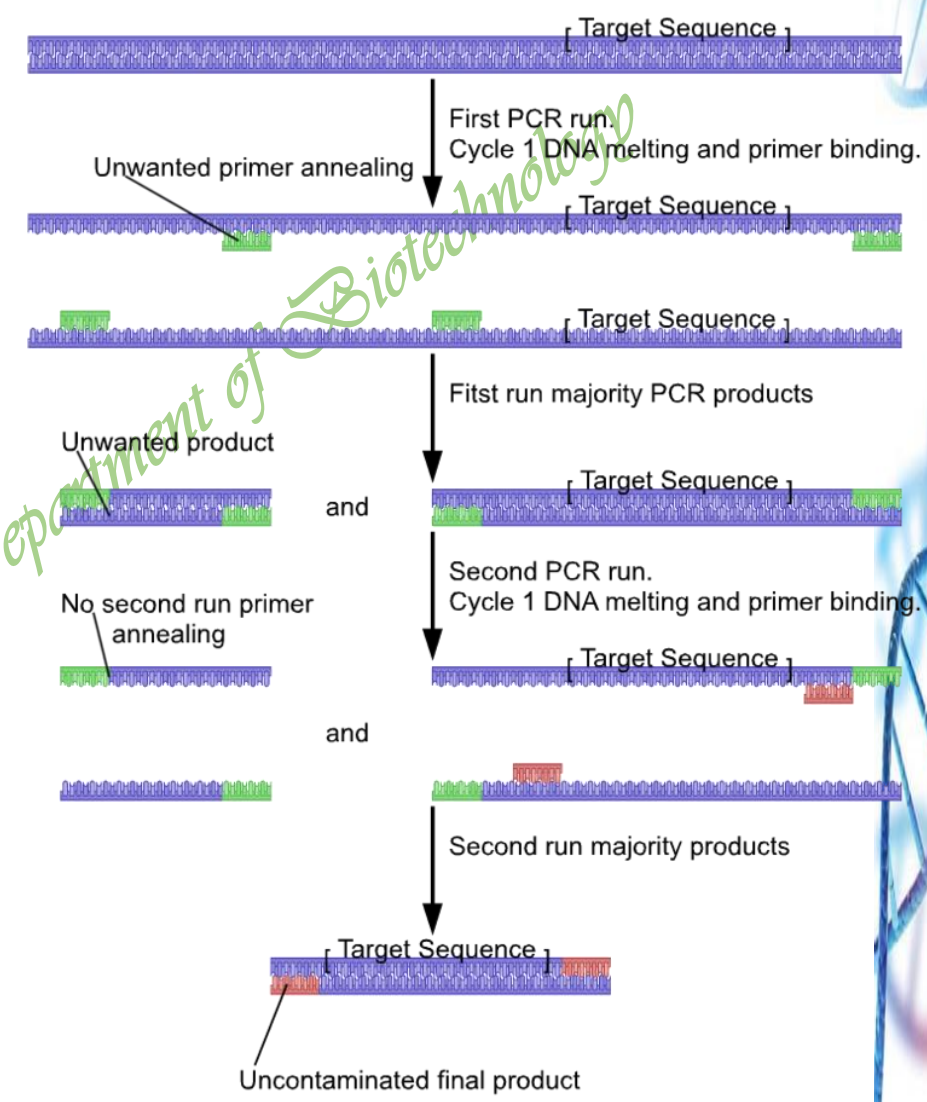
***In Situ* Polymerase Chain Reaction** (*In situ* PCR) is a powerful method that detects minute quantities of rare or single-copy number nucleic acid sequences in frozen or paraffin-embedded cells or tissue sections for the localization of those sequences within the cells. The target sequences are amplified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



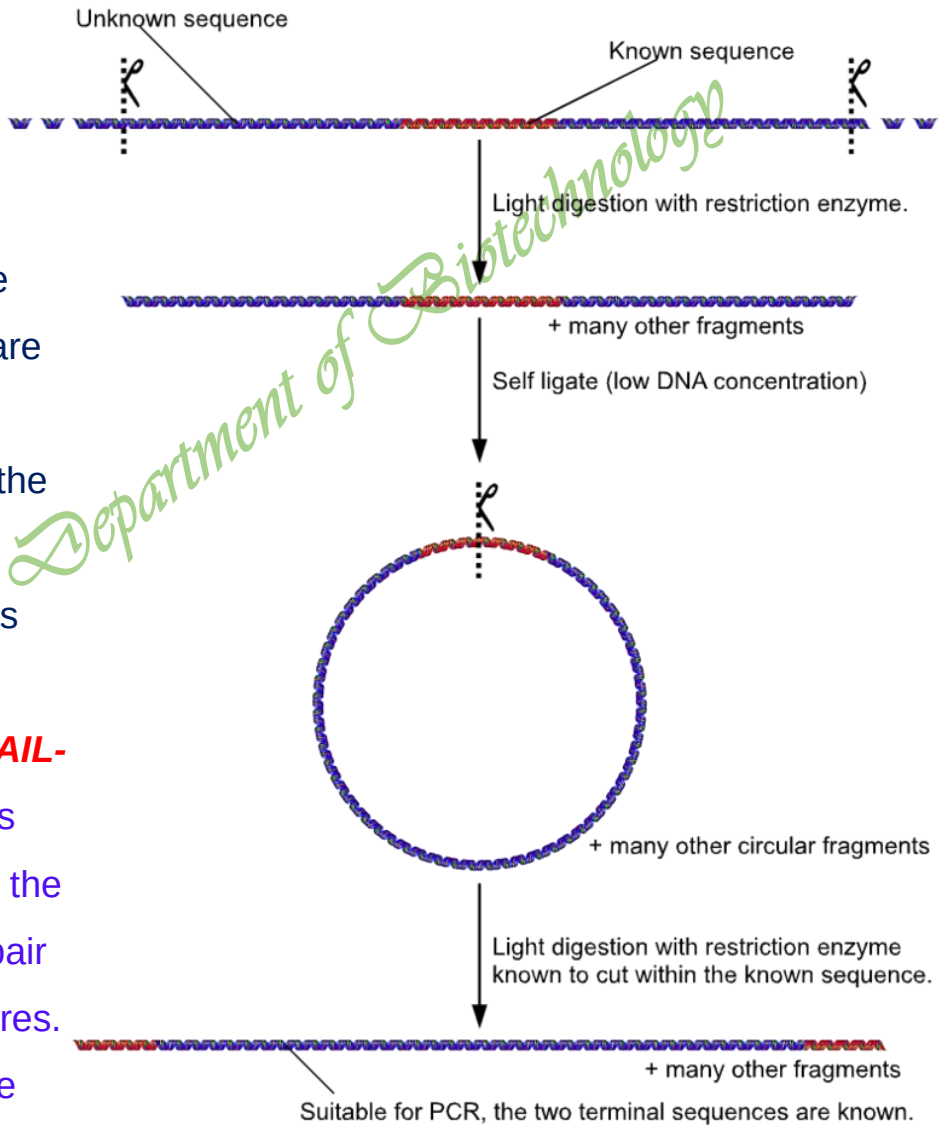
Nested polymerase chain reaction (Nested PCR) is a modification of polymerase chain reaction intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites.

Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product.



Inverse PCR allows amplification and sequencing of DNA that surrounds a known sequence. It involves initially subjecting the target DNA to a series of restriction enzyme digestions, and then circularizing the resulting fragments by self ligation. Primers are designed to be extended outward from the known segment, resulting in amplification of the rest of the circle. This is especially useful in identifying sequences to either side of various genomic inserts.

Thermal asymmetric interlaced PCR **(or *TAIL-PCR*)** is used to isolate unknown sequences flanking a known area of the genome. Within the known sequence, TAIL-PCR uses a nested pair of primers with differing annealing temperatures. A 'degenerate' primer is used to amplify in the other direction from the unknown sequence.



B.Sc. Syllabus Biotechnology
B.Sc.III[rd] Year

BBT-301
Paper First
Recombinant DNA Technology **(Max. marks: 100)**

Unit I: Basics Concepts- Restriction Enzymes; DNA ligase, Klenow enzyme, DNA polymerase, Polynucleotide kinase, Alkaline phosphatase; Cohesive and blunt end ligation; Linkers; Adaptors; Introduction to probes; Hybridization techniques: Northern and Southern blotting.

Unit II: Cloning Vectors - Introduction to cloning and expression vectors; Plasmids; Bacteriophages; PUC19 and Bluescript vectors, Phagemids; Lambda vectors; Insertion and Replacement vectors; Cosmids; Artificial chromosome (YACs; BACs); GST; pET-based vectors; Yeast vectors, Shuttle vectors.

Unit III: Cloning Methodologies- Insertion of Foreign DNA into Host Cells- Introduction of DNA into mammalian cells; Transfection techniques; Transformation; Construction of libraries; Isolation of mRNA and cDNA libraries; genomic DNA libraries; cDNA and genomic cloning.

Unit IV: PCR and Its Applications- Fidelity of thermostable enzymes; DNA polymerases; Types of PCR – multiplex, nested, reverse transcriptase, real time PCR, PCR in molecular diagnostics.

Unit V: Sequencing methods; Enzymatic DNA sequencing; Chemical sequencing of DNA; Automated DNA sequencing; Pyrosequencing; Applications of recombinant DNA technology; Genome editing tools and techniques; Gene silencing techniques; Introduction to siRNA; Micro RNA.

Text/References

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2. J. Sambrook and D.W. Russel; Molecular Cloning: A Laboratory Manual, Vols 1-3, CSHL, 2001.
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