Department of BSBE
Indian Institute Of Technology Guwahati

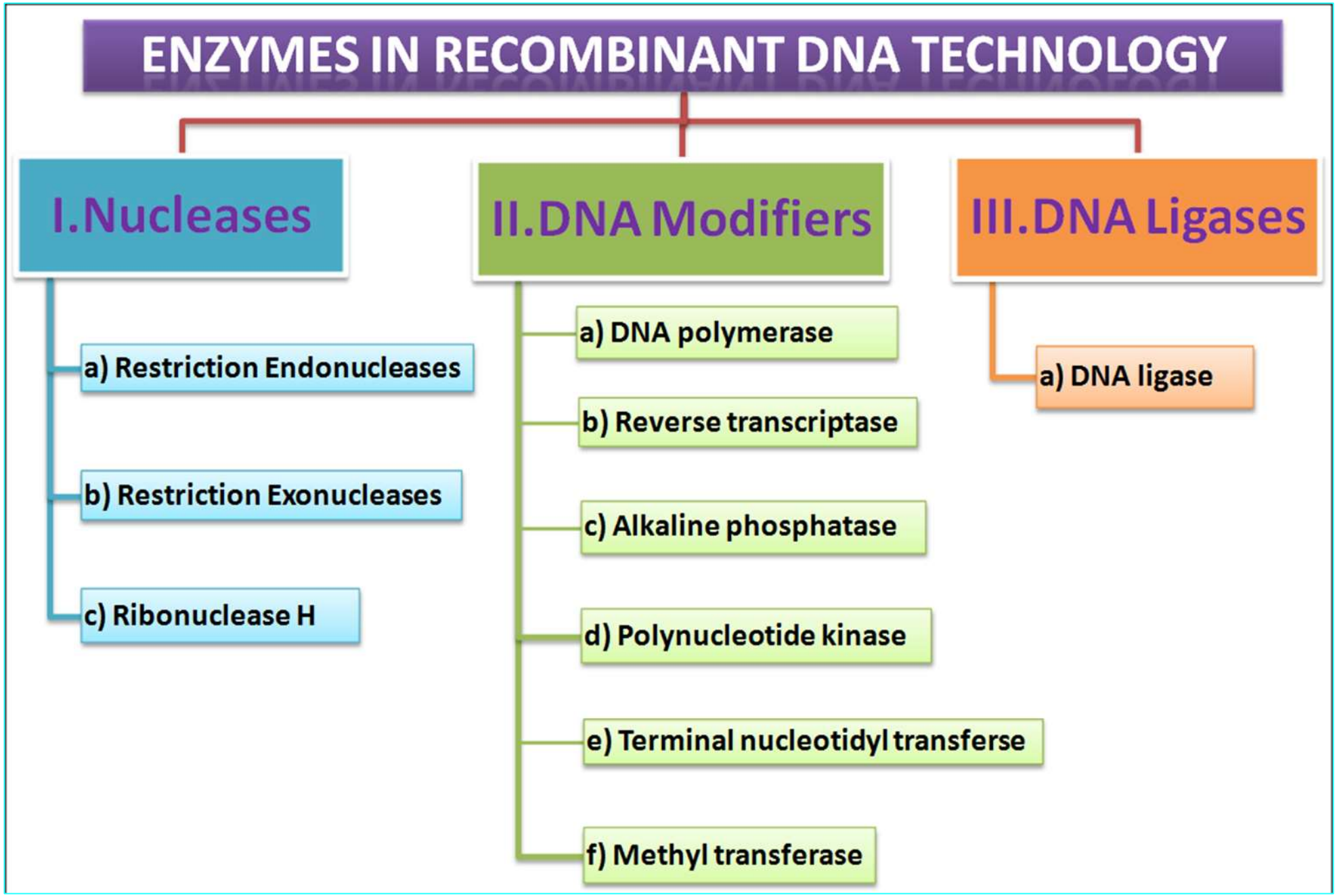
Enzymes in recombinant DNA technology
DNA modifiers

Dr. Sanjukta Patra

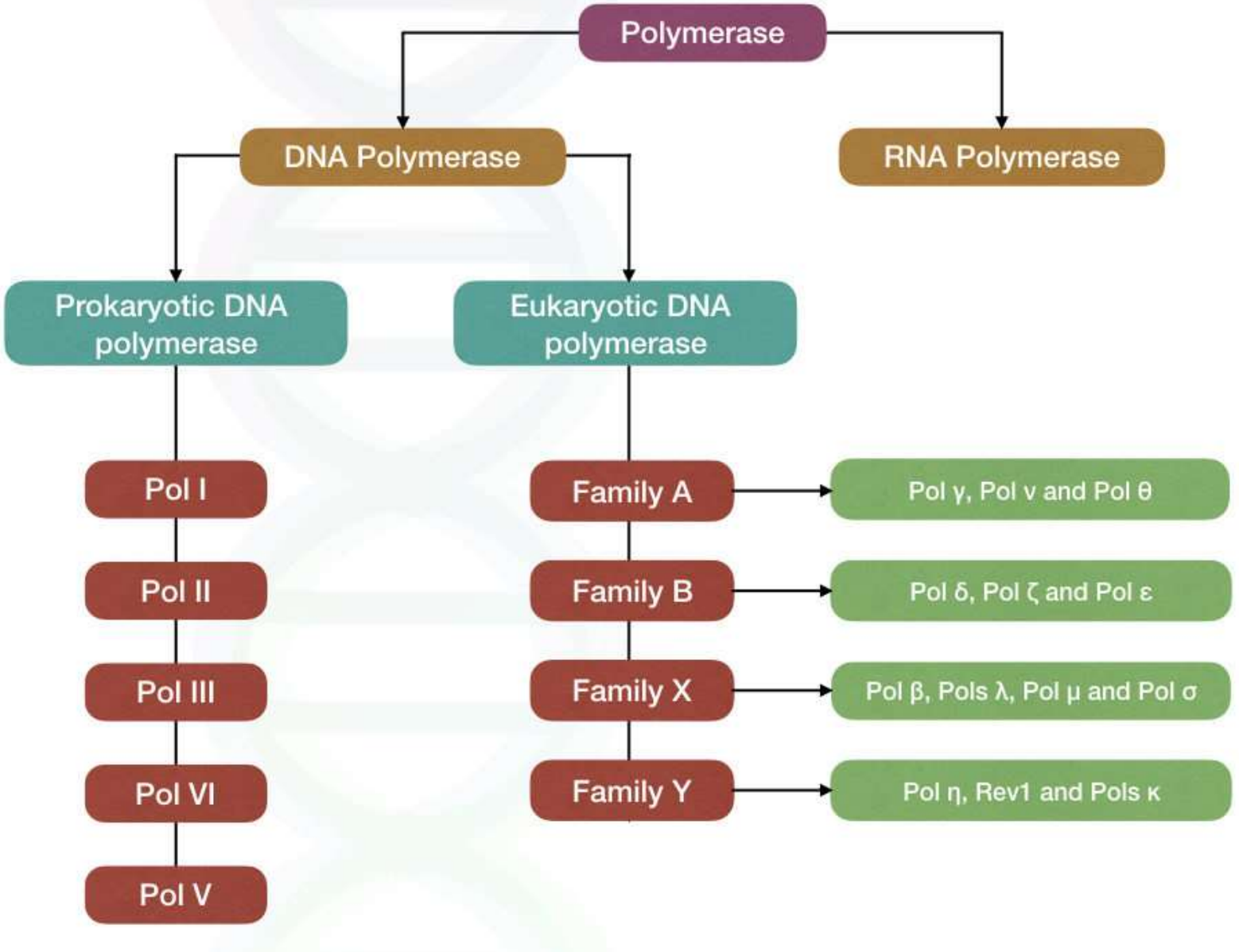
BT 207

Genetic Engineering

Jan-April 2023


ENZYMES IN RECOMBINANT DNA TECHNOLOGY
I.Nucleases
a) Restriction Endonucleases
b) Restriction Exonucleases
c) Ribonuclease H
II.DNA Modifiers
a) DNA polymerase
b) Reverse transcriptase
c) Alkaline phosphatase
d) Polynucleotide kinase
e) Terminal nucleotidyl transferse
f) Methyl transferase
III.DNA Ligases
a) DNA ligase

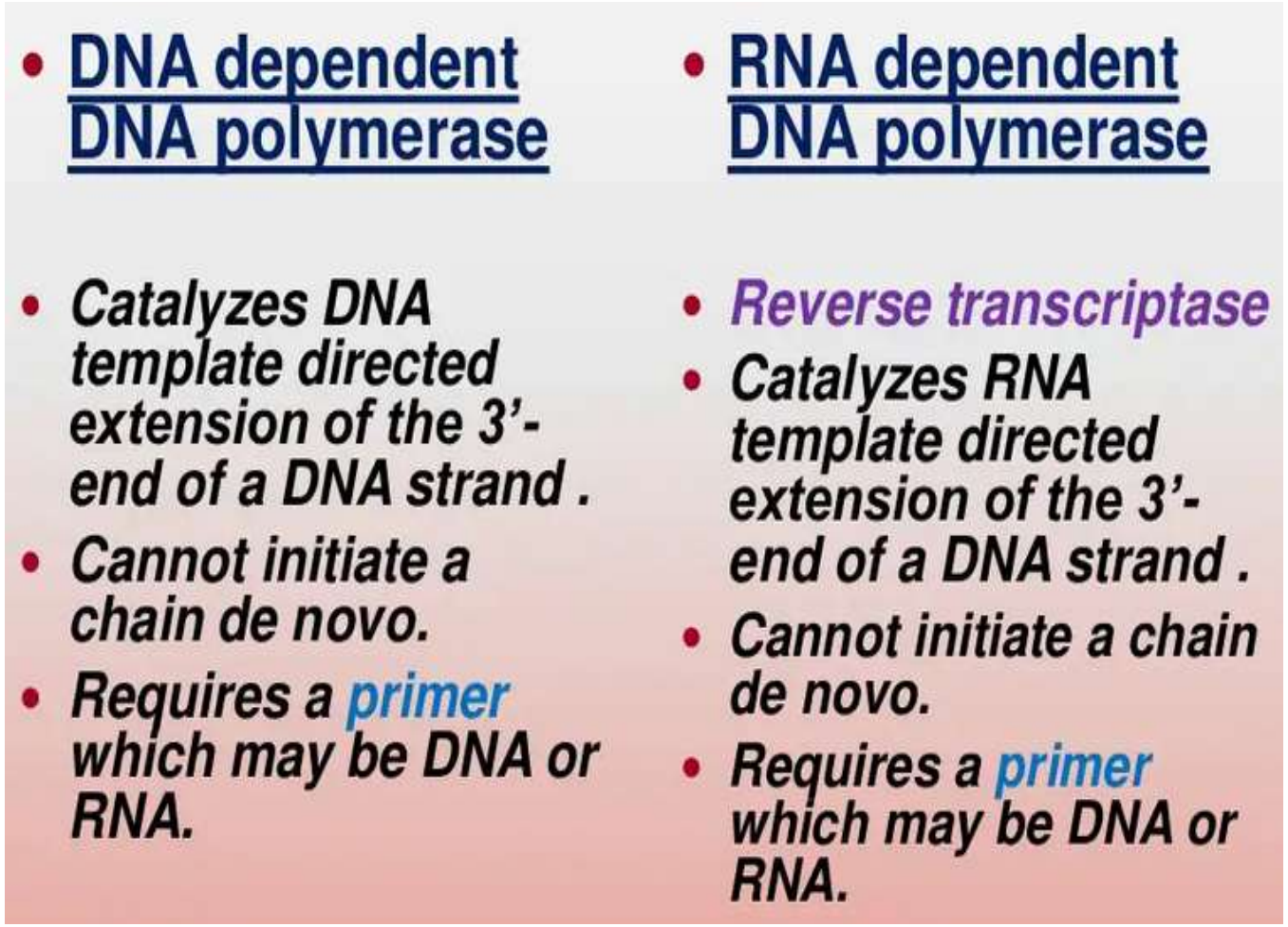
Polymerases

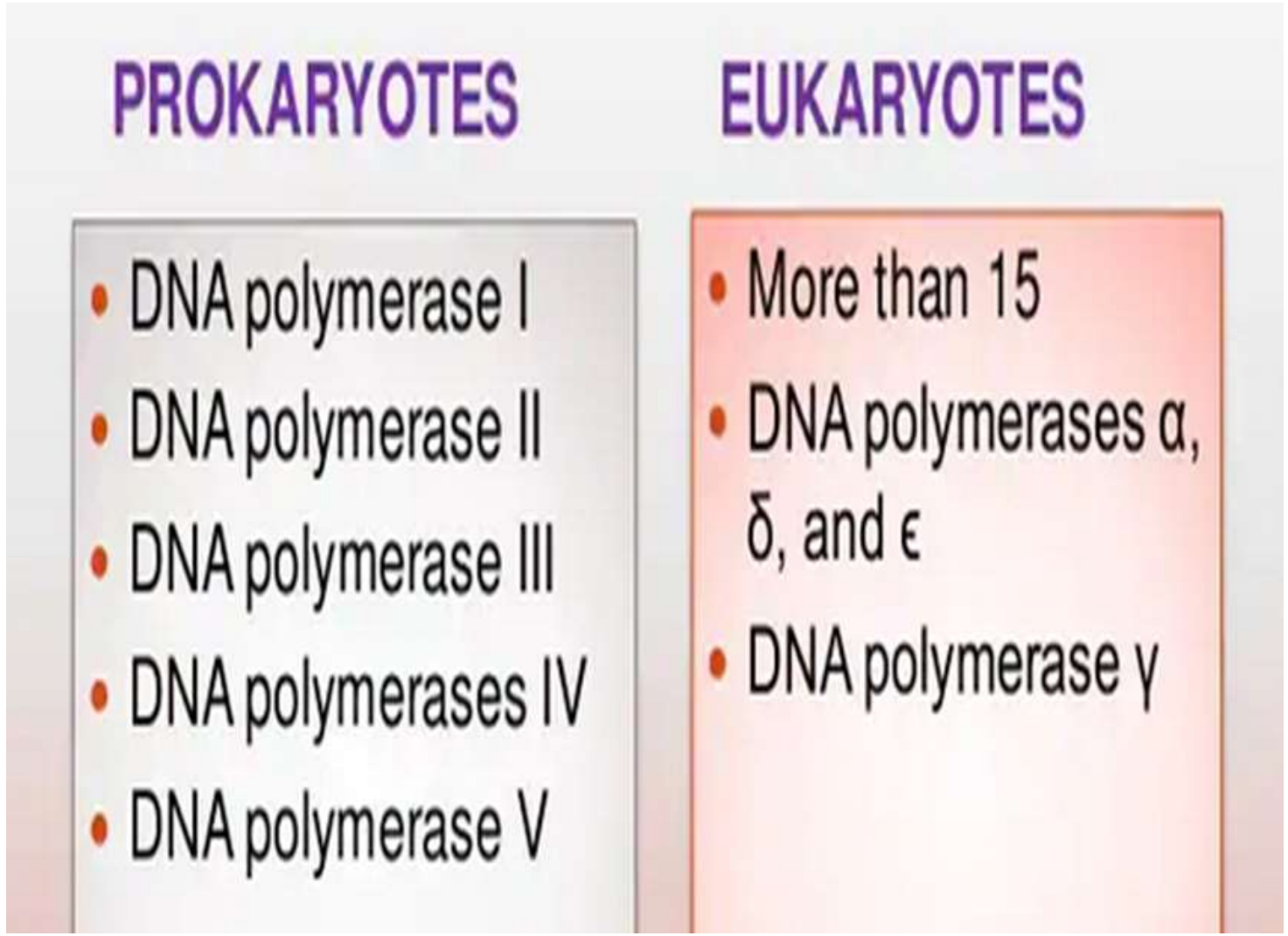


What is the role of DNA Polymerase in living cells?

DNA Polymerases

DNA polymerase: synthesizes DNA by linking together deoxyribonucleoside monophosphates in an order directed by the complementary sequences of nucleotides in a template strand. DNA polymerases add nucleotides only on to a pre-existing 3'-OH group.




PROKARYOTES
EUKARYOTES
DNA polymerase I
DNA polymerase II
DNA polymerase III
DNA polymerases IV
DNA polymerase V
More than 15
DNA polymerases α, δ, and ϵ
DNA polymerase γ

DNA polymerases in Prokaryotes

DNA polymerase I

- This is a repair polymerase and is involved in excision repair with 3'-5' and 5'-3' exonuclease activity and processing of Okazaki fragments generated during lagging strand synthesis.
- Most abundant polymerase accounting for >95% of polymerase activity in *E. coli.*
- Cells lacking Pol I have been found suggesting Pol I activity can be replaced by the other four polymerases.
- Pol I adds ~15-20 nucleotides per second.

DNA polymerase II

- Pol II has 3'-5' exonuclease activity and participates in DNA repair.
- Pol II is also thought to be a backup to Pol III as it can interact with holoenzyme proteins and assume a high level of processivity.
- The main role of Pol II is thought to be the ability to direct polymerase activity at the replication fork and help stalled Pol III bypass terminal mismatches.

DNA polymerase III

▪Primary enzyme involved in DNA replication in *E. coli* and belongs to Family C polymerases.

▪The core consists of three subunits - α, the polymerase activity hub, ε, exonucleolytic proofreader, and θ, which may act as a stabilizer for ε.

▪The holoenzyme contains two cores, one for each strand, the lagging and leading.

▪The beta sliding clamp processivity factor is also present in duplicate, one for each core, to create a clamp that encloses DNA allowing for high processivity.

DNA polymerase IV

▪An error-prone DNA polymerase involved in non-targeted mutagenesis.

▪ During SOS induction, Pol IV production is increased tenfold and one of the functions during this time is to interfere with Pol III holoenzyme processivity.

▪This creates a checkpoint, stops replication, and allows time to repair DNA lesions via the appropriate repair pathway.

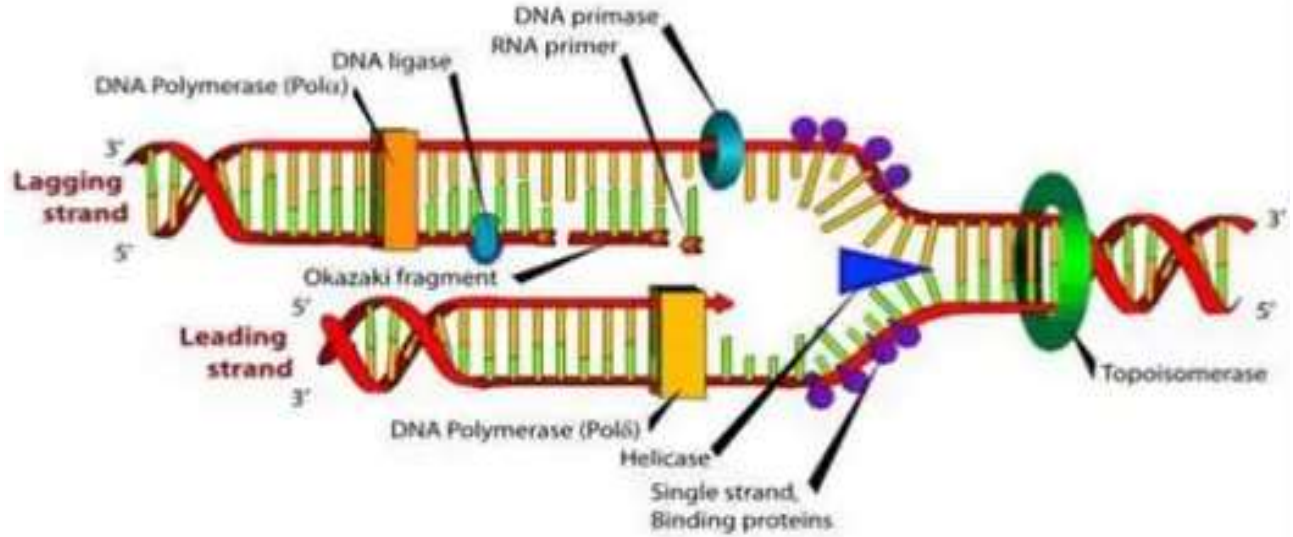
DNA polymerase V

Pol V is a Y-family DNA polymerase that is involved in SOS response and translesion synthesis DNA repair mechanisms.

DNA polymerases in Eukaryotes

- Semi-conservative replication of DNA.
- Use of single stranded DNA chain as a template and four deoxynucleotides(TTP, dCTP, dGTP, dATP)as precursors for DNA synthesis.
- Assembly of the precursor nucleotides on the template to form a complementary DNA strand, selecting the incoming nucleotide using the base pair rules A.T and G.C.
- To start synthesis on a single stranded DNA molecule, DNA polymerases need a primer.

Primer : length of RNA or DNA that is annealed to the single-stranded template.



APPLICATIONS

- DNA cloning
- The polymerase chain reaction (PCR)
- DNA sequencing
- Single nucleotide polymorphism (SNP) detection
- Whole genome amplification (WGA)
- Synthetic biology
- Molecular diagnostics.

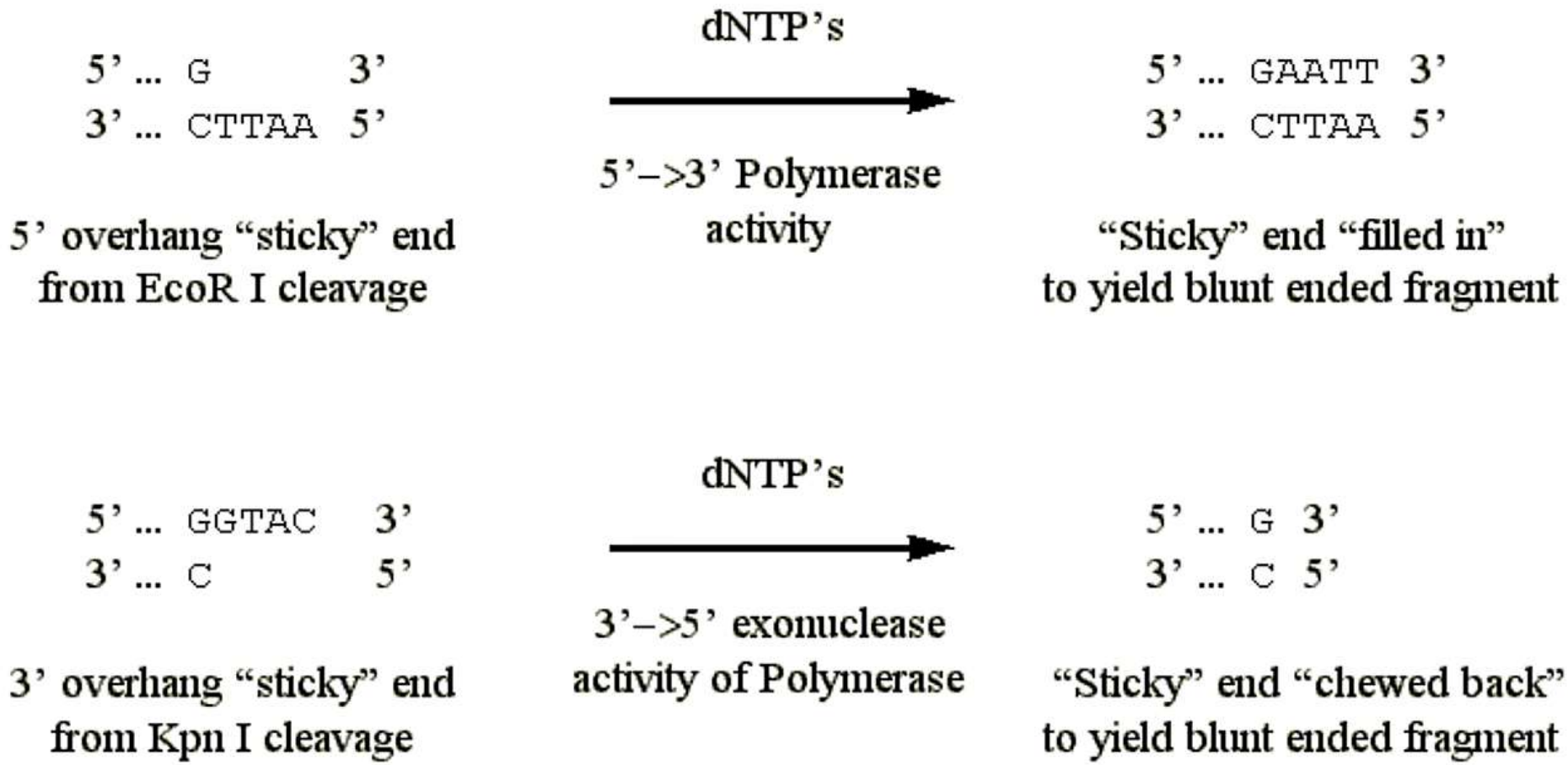
PCR: USE

Thermostable DNA polymerase

- The thermophilic DNA polymerases catalyze template-directed synthesis of DNA from nucleotide triphosphates.
- *Several thermostable polymerase enzymes are used in PCR*

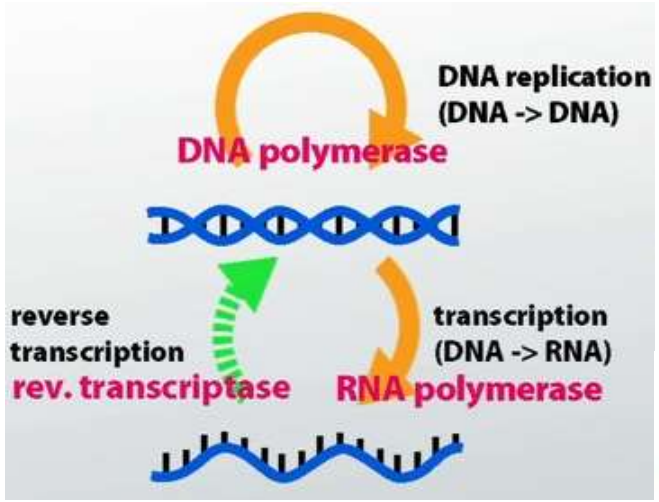
❖Pfu DNA polymerase- *Pyrococcus furiosus*
❖Vent polymerase- *Thermococcus litoralis*
❖Taq polymerase- *Thermus aquaticus*

Combined effect of restriction and polymerase enzymes to modify DNA

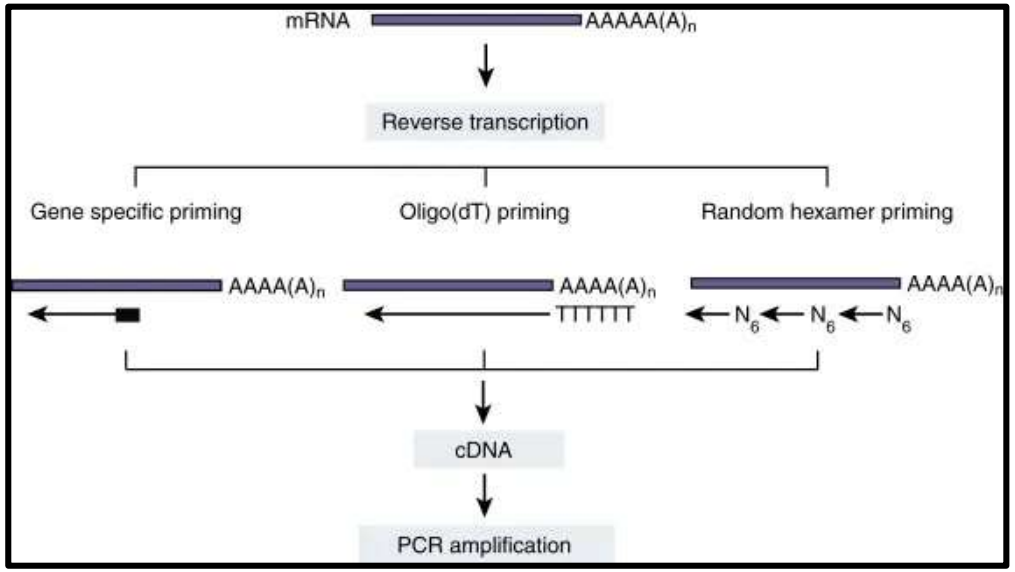


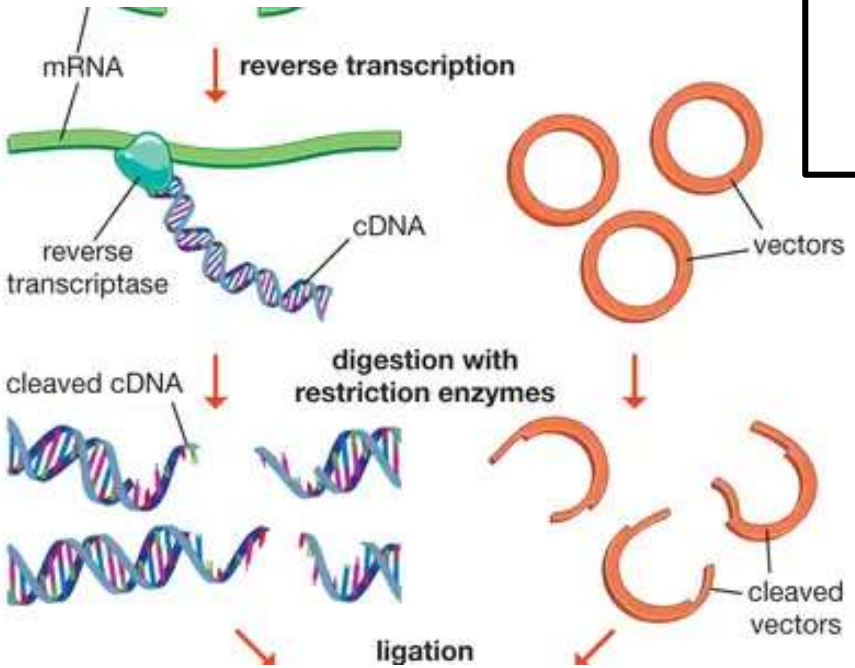
Reverse transcriptase

Reverse transcriptase, also called **RNA-directed DNA polymerase,** an enzyme encoded from the genetic material of retroviruses that catalyzes the transcription of retrovirus RNA (ribonucleic acid) into DNA (deoxyribonucleic acid). This catalyzed transcription is the reverse process of normal cellular transcription of DNA into RNA, hence the names reverse transcriptase and retrovirus.



Application in cDNA synthesis (RT-PCR) & cDNA library construction





PHOSPHATASES

• Phosphatase catalyses the cleavage of a phosphate group from substrate by using a water molecule (hydrolytic cleavage).

• This reaction is not reversible.

•On the basis of their activity there are two types of phosphatase i.e acid phosphatase and alkaline phosphatase. In both forms the alkaline phosphatase are most common.

• Special class of phosphatase that remove a phosphate group from protein, called "Phosphoprotein phosphatase

Commercial enzymes:
Bacterial alkaline phosphatase (BAP)
Calf intestinal alkaline phosphatase (CIP)
Shrimp alkaline phosphatase (SAP)

Alkaline phosphatase

- **Alkaline phosphatase removes 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH**

- Calf-intestinal alkaline phosphatase (CIAP/CIP) is a type of alkaline phosphatase that is used in RDT as CIP removes 5′- and 3′- phosphates from DNA, RNA and dNTPs.

- **Used in cloning, dephosphorylation prevents religation of linearized plasmid DNA.**

- The enzyme acts on 5′ protruding, 5′ recessed and blunt ends. CIP may also be used to degrade unincorporated dNTPs in PCR reactions to prepare templates for DNA sequencing or SNP analysis

The scheme above is part of the cloning strategy for maximising recombinant plasmids; and it is achieved by ligating the insert (blue) to the linearlised vector (red). In this strategy, we needn't worry about background clones of the re-ligated vector because…

(A) The vector can't religate to itself.

(B) The equimolar mix of inserts and vectors ensures that background ligations will not happen.

(C) The vector ends lack 5-prime phosphates and can't ligate to themselves.

(D) Only the insert can self-ligate.

(E) Recombinants may be easily identified on transformation plates by the blue-while colony system.

Methylases

Methyltransferase or methylase catalyzes the transfer of methyl group (-CH3) to its substrate. The process of transfer of methyl group to its substrate is called methylation.

• Methylation is a common phenomenon in DNA and protein structure.

• Methyltransferase uses a reactive methyl group that is bound to sulfur in S - adenosyl methionine (SAM) which acts as the methyl donor.

1. Methylation normally occurs on cytosine (C) residue (**methylation at the C5 position)** or adenine (A) in DNA sequence **(methylation at the N^6 position of the adenine)**.
2. In protein, methylation occurs on nitrogen atom either on N -terminus or on the side chain of protein.

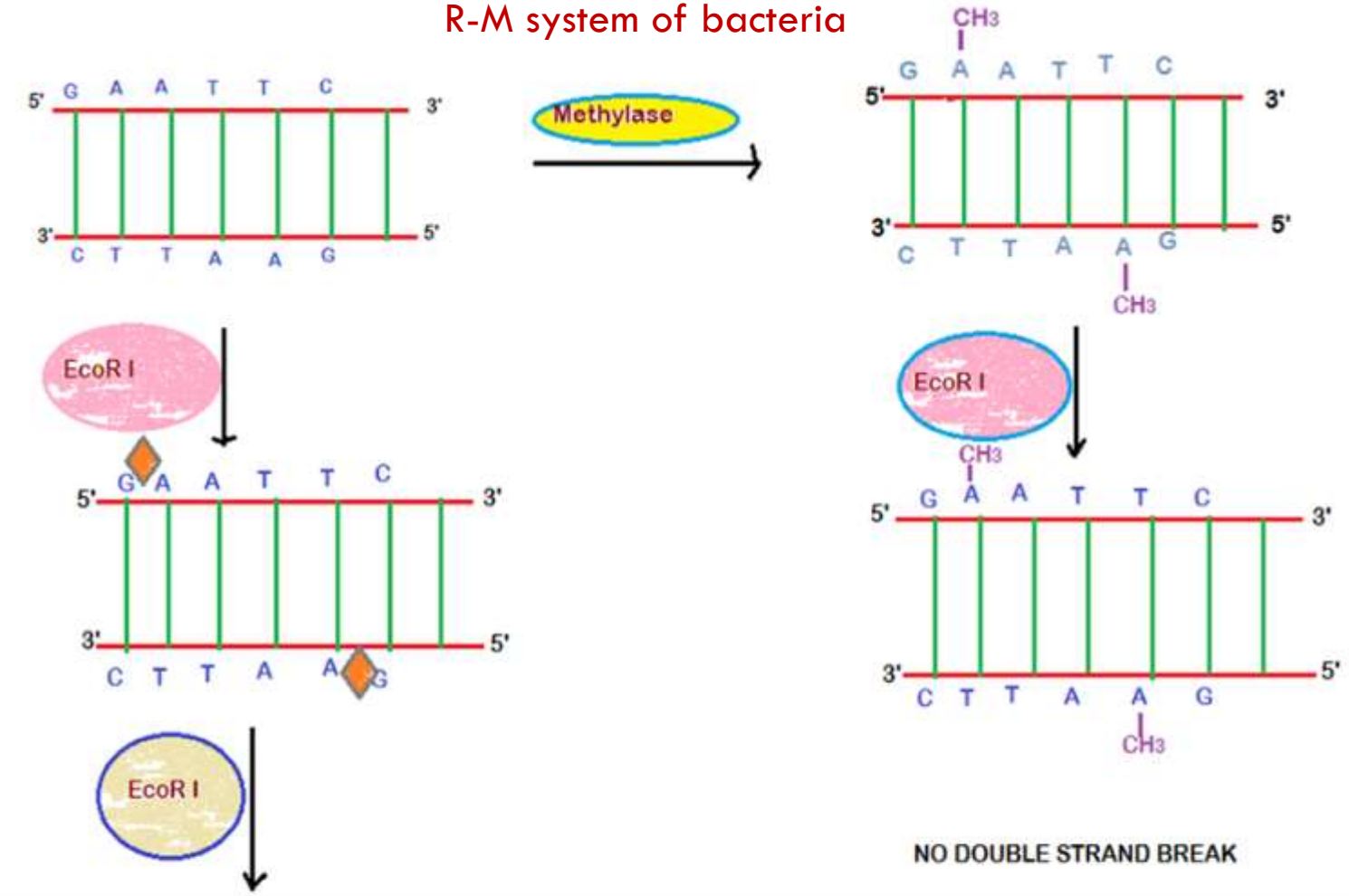
Dam methylase
Dcm methylase

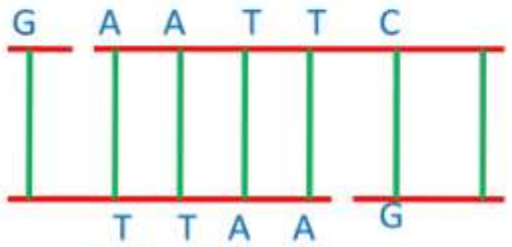
• DNA methylation regulates gene or silence gene without changing DNA sequences, as a part of epigenetic regulation.

• In bacterial system, methylation plays a major role in preventing their genome from degradation by restriction enzymes. It is a part of restriction – modification system in bacteria.

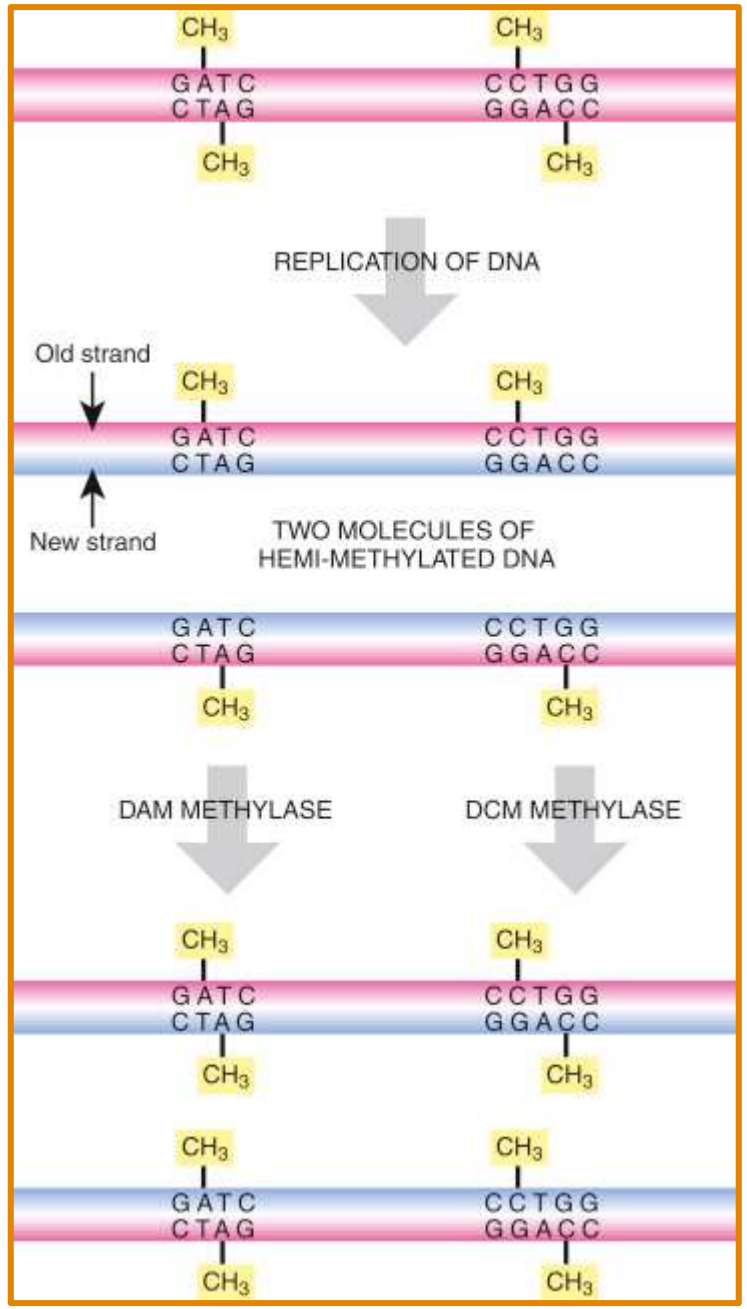
Methylase

R-M system of bacteria



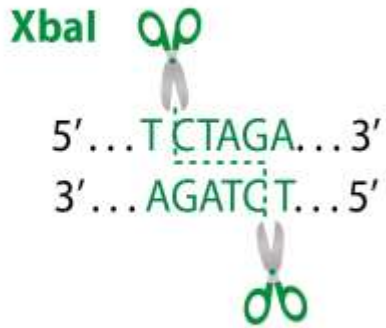


Dam methylase–**methylation at the N^6 position of the adenine in** the sequence GATC. Dcm methyltransferases–methylation at the C5 position of the second cytosine in the sequences CCAGG and CCTGG



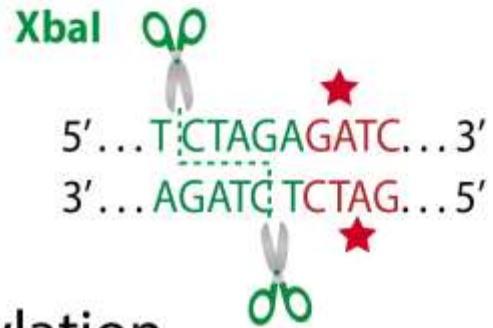
Dam vs Dcm methylase

Restriction endonucleases (REases) and Methyltransferases (MTases) both use code words

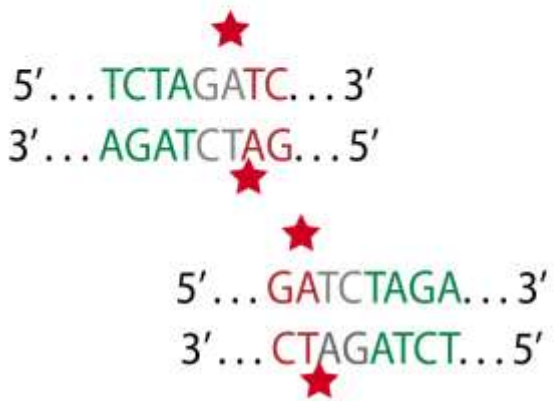


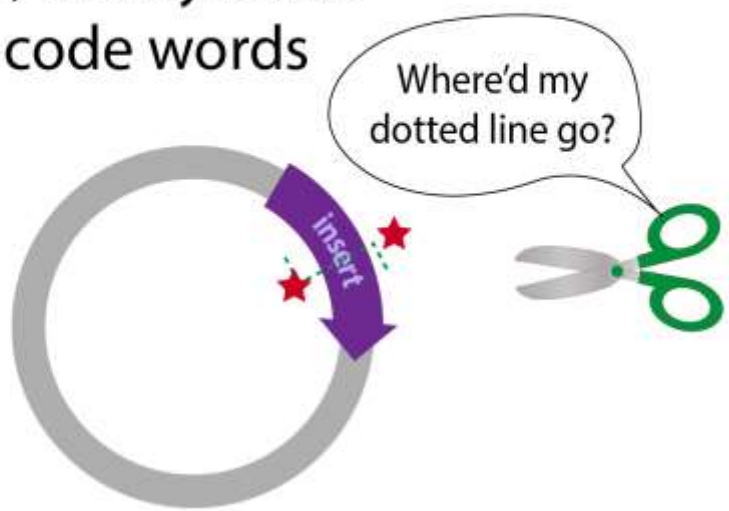
N6-methyladenine

if they don't overlap, they can each still do their thing



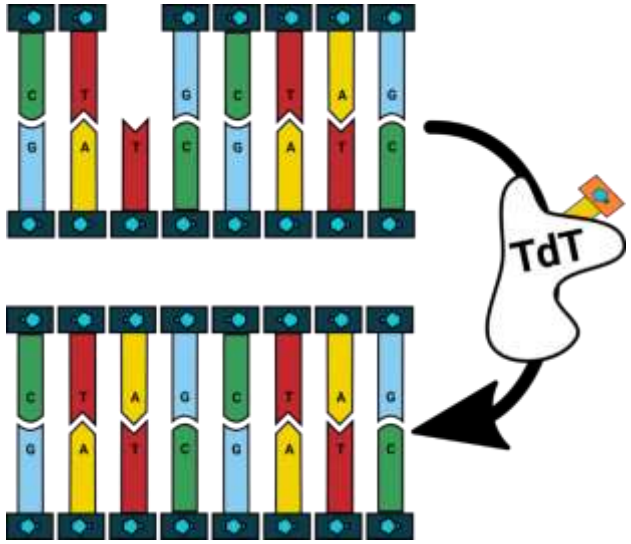
but when they overlap, methylation can hide the REases' code words





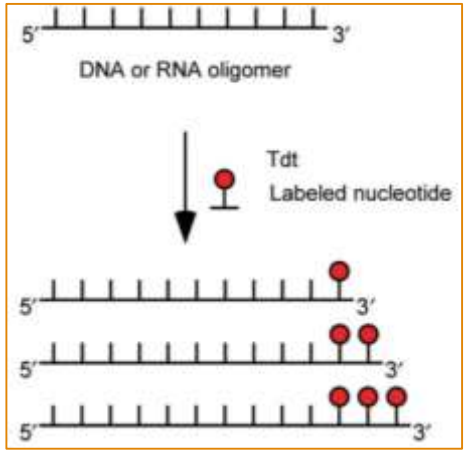
Terminal transferase

In a template-dependant manner, TdT can incorporate nucleotides across strand breaks in double-stranded DNA in a manner referred to as in trans in contrast to the in cis mechanism found in most polymerases. This occurs optimally with a one base-pair break between strands and less so with an increasing gap



APPLICATION:

- It can be used in **RACE(rapid amplification of cDNA ends) to add nucleotides that can then be used as a template for a primer in subsequent PCR.**
- It can also be used to add nucleotides labeled with radioactive isotopes, for example in the TUNEL assay (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) for the demonstration of apoptosis (which is marked, in part, by fragmented DNA).
- It is also used in the immunofluorescence assay for the diagnosis of acute lymphoblastic leukemia.

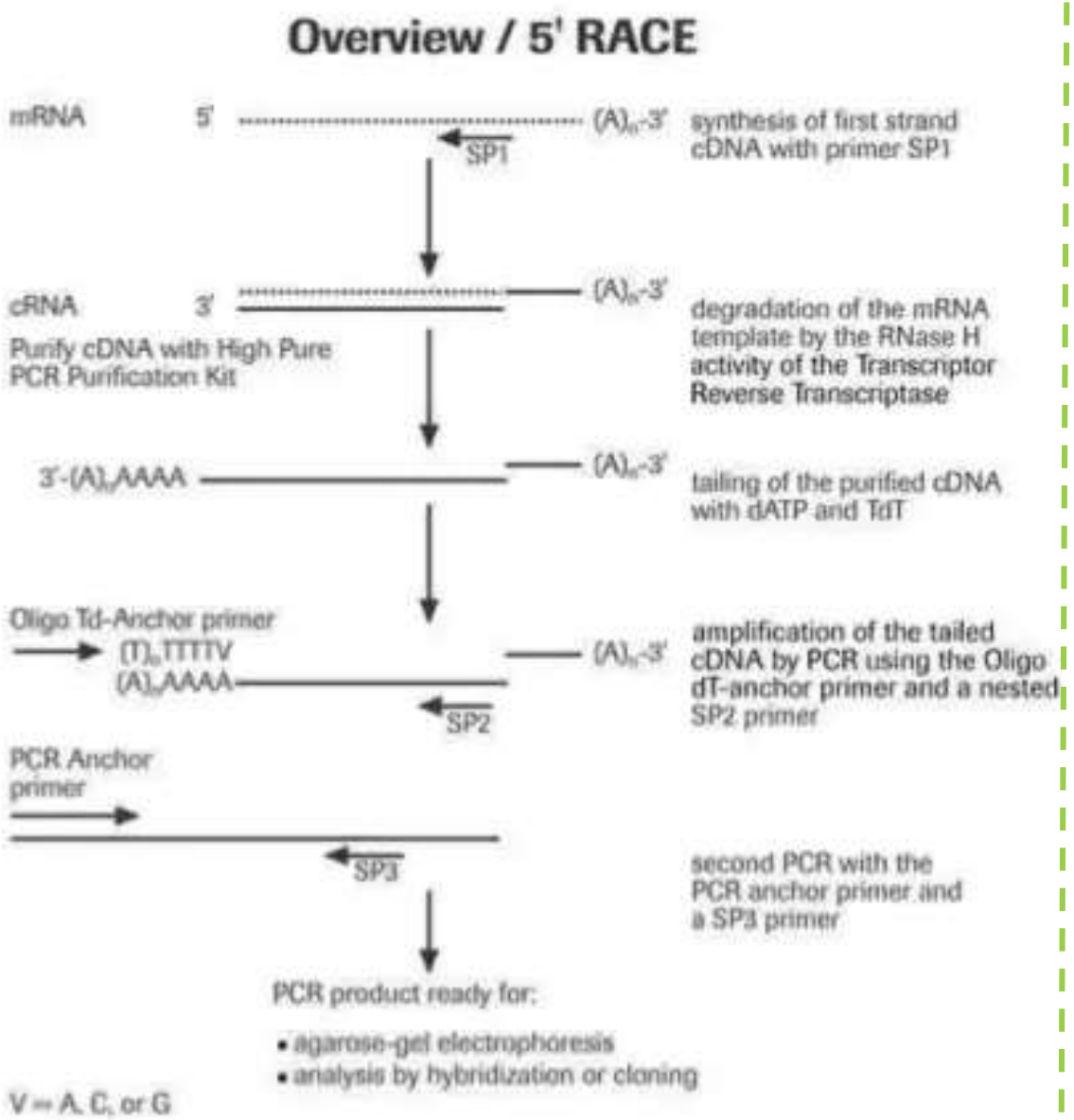


3′-end labeling of DNA and RNA oligomers using Terminal deoxynucleotidyl transferase

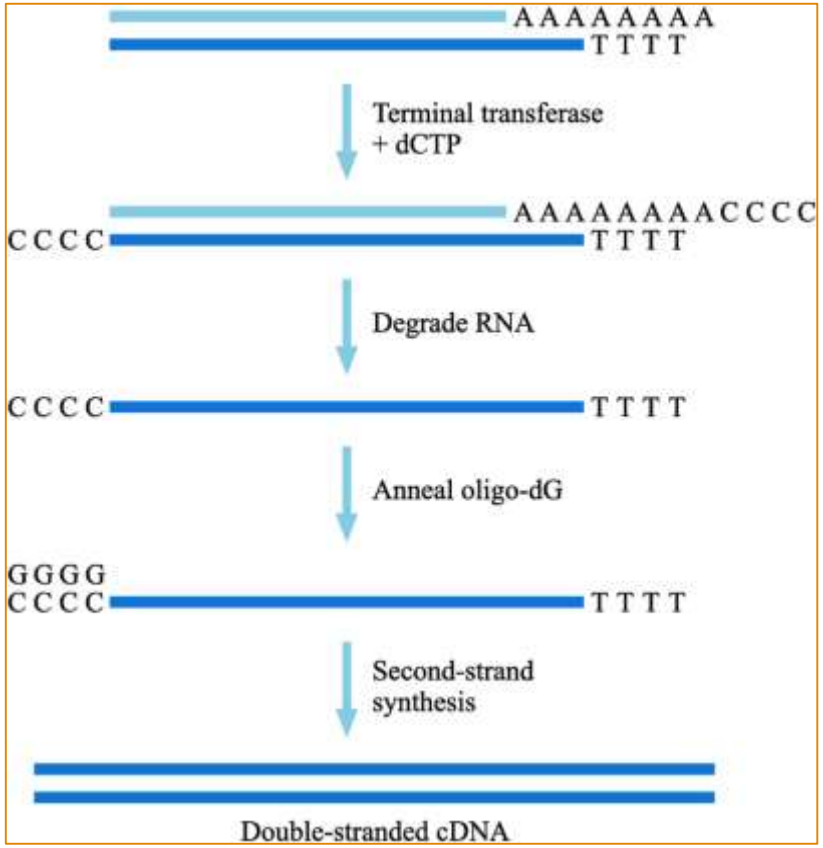
TdT has also seen recent application in the De Novo synthesis of oligonucleotides, with TdT-dNTP tethered analogs capable of primer extension by 1 nt at a time. In other words, the enzyme TdT has demonstrated the capability of making synthetic DNA by adding one letter at a time to a primer sequence.

RACE PCR

Rapid amplification of cDNA ends (RACE) is a technique used in molecular biology to obtain the full length sequence of an RNA transcript found within a cell.



Concept of TdT homopolymer tailing



3’ Homopolymer tailing to provide a priming site for second-strand synthesis. Dark blue shaded material is DNA; the light blue shaded material is RNA.

Polynucleotide kinase

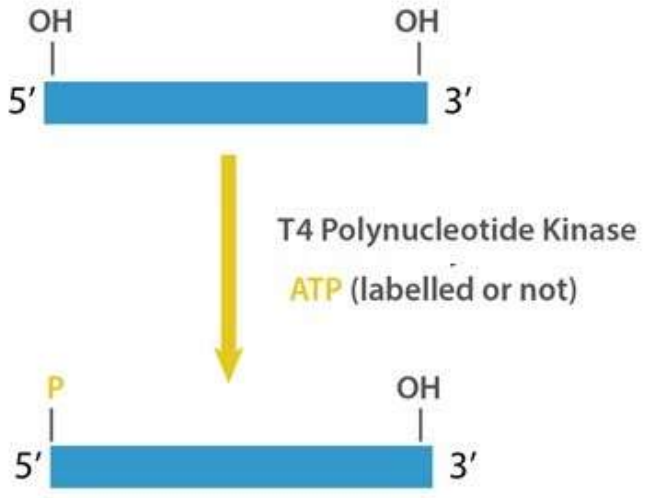
Polynucleotide 5'-hydroxyl-kinase is an enzyme that catalyzes the reversible chemical reaction:

$$\text{ATP} + \text{5'-dephospho-DNA} \rightleftharpoons \text{ADP} + \text{5'-phospho-DNA}$$

Thus, the two substrates of this enzyme are ATP and 5'-dephospho-DNA, whereas its two products are ADP and 5'-phospho-DNA. Polynucleotide kinase is a T7 bacteriophage (or T4 bacteriophage) enzyme that catalyzes the transfer of a gamma-phosphate from ATP to the free hydroxyl end of the 5' DNA or RNA. The resulting product could be used to end-label DNA or RNA, or in a ligation reactions.

Other names in common use include:

ATP:5'-dephosphopolynucleotide 5'-phosphatase
polynucleotide 5'-hydroxyl kinase (phosphorylating),
5'-hydroxyl polynucleotide kinase,
5'-hydroxyl polyribonucleotide kinase,
5'-hydroxyl RNA kinase,
DNA 5'-hydroxyl kinase,
DNA kinase,
polynucleotide kinase, and
polynucleotide 5'-hydroxy-kinase



Polynucleotide Kinase also catalyzes the removal of 3′-phosphoryl groups from 3′-phosphoryl polynucleotides, deoxynucleoside 3′-monophosphates and deoxynucleoside 3′-diphosphates

End-labeling DNA or RNA for probes and DNA sequencing can be done by PNK

Exchange reaction: target DNA or RNA having a 5' phosphate is incubated with an excess of ADP - where PNK transfers the phosphate from the nucleic acid to an ADP, forming ATP. PNK then performs a forward reaction and transfer a phosphate from ATP to the target nucleic acid. Exchange reaction is used to label with radioactive phosphate group.

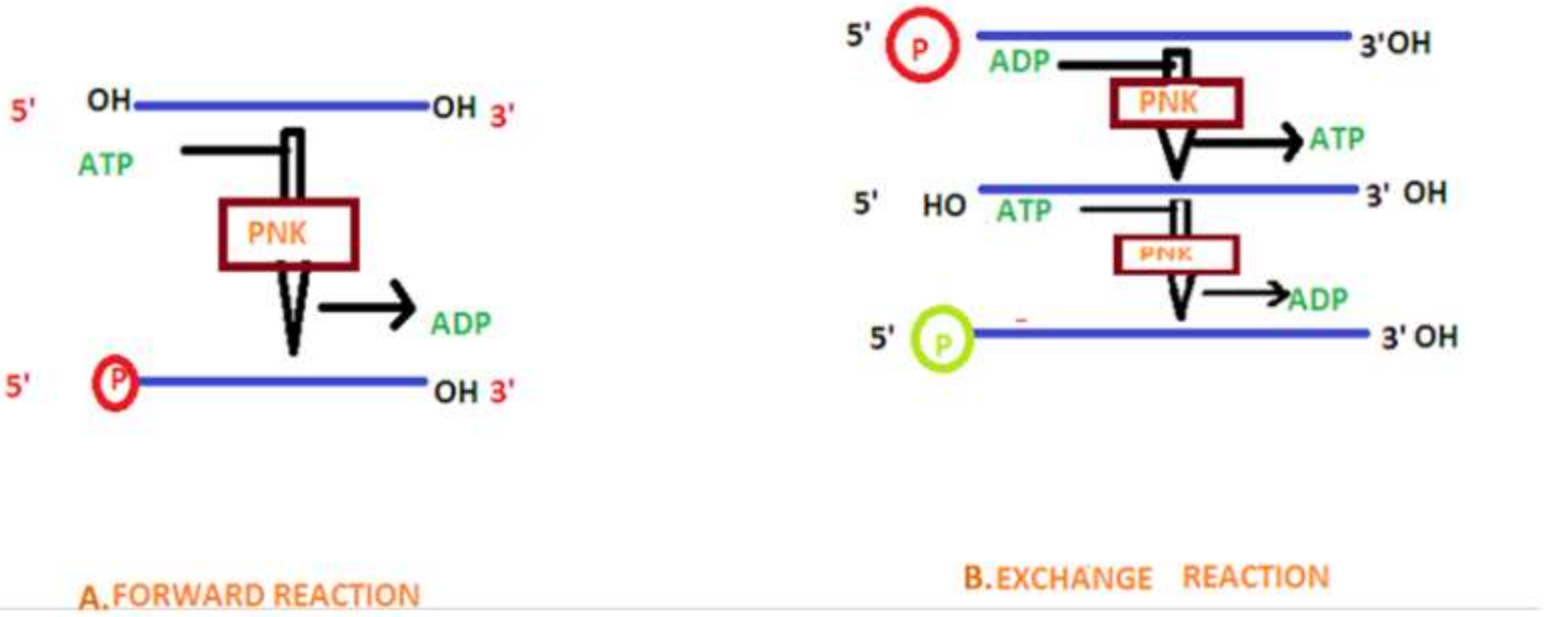


Fig 2-4.2: Polynucleotide kinase reaction (A) forward (B) exchange.

There are two major uses of PNK:

- The linkers and adopters are phosphorylated a long with the fragments of DNA before ligation, which requires a 5' phosphate. This includes products of polymerase chain reaction, which are generated by using non -phosphorylated primers.
- PNK is also used for radio labelling oligonucleotides, generally with 32P for preparing hybridization probes.

Department of BSBE
Indian Institute Of Technology Guwahati

Enzymes in recombinant DNA technology

Ligase

Dr. Sanjukta Patra

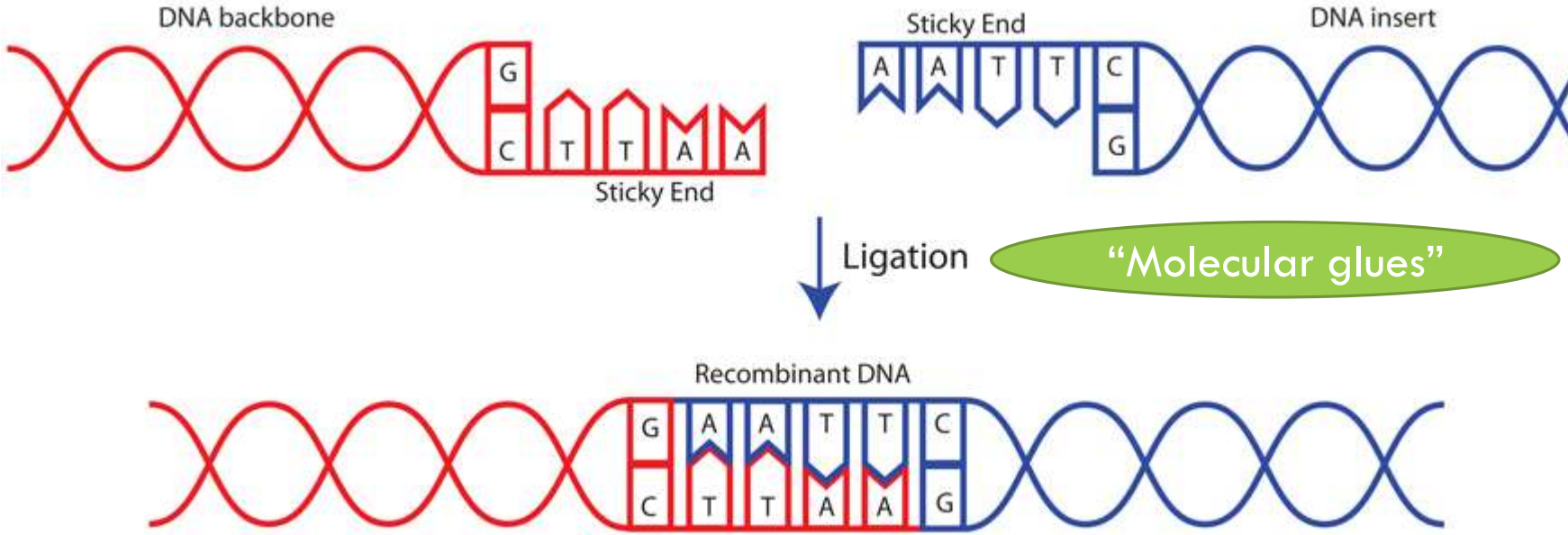
BT 207

Genetic Engineering

Jan-April 2023

Ligase

ligase is an enzyme that can catalyze the joining (ligation) of two large molecules by forming a new chemical bond. ... Ligase can join two complementary fragments of nucleic acid and repair single stranded breaks that arise in double stranded DNA during replication

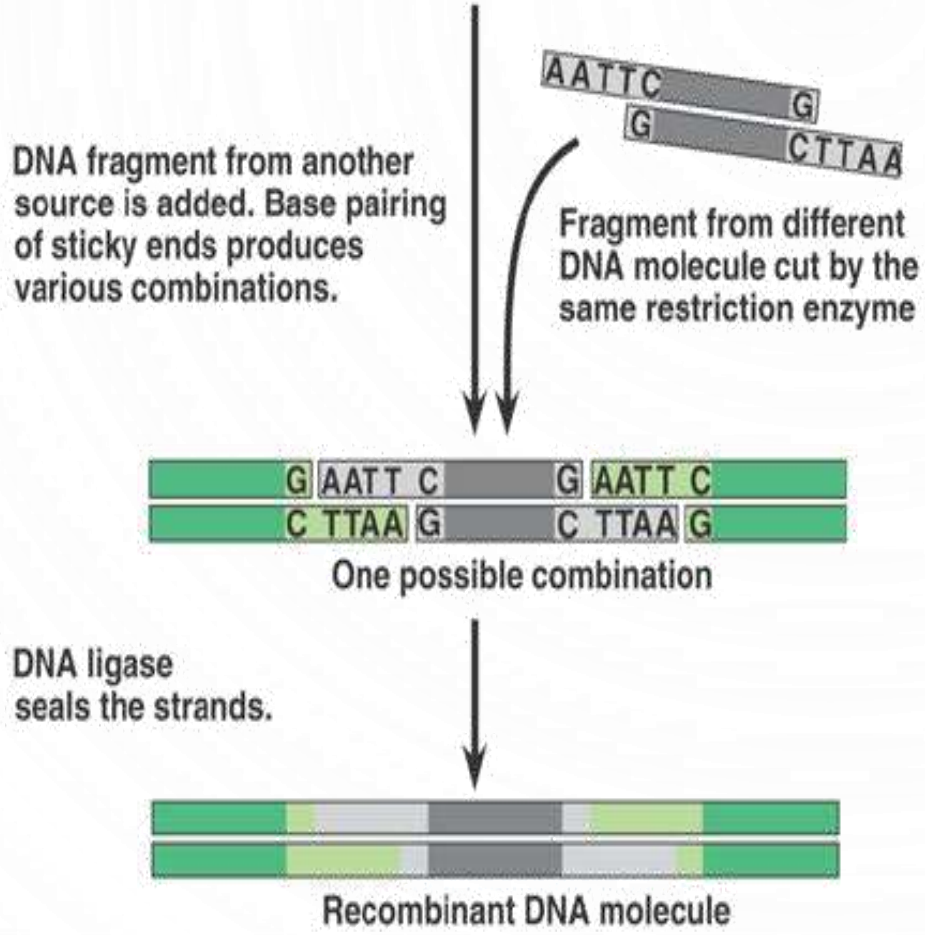


Application

- Joining linkers and adapters to blunt-ended DNA
- Cloning of DNA fragments generated by restriction enzyme digestion
- Adding linkers or adapters to dsDNA
- Circularization of linear DNA
- Nick-sealing in dsDNA
- Site-directed mutagenesis

Thermostable ligases are engineered for incorporation of phosphorylated oligonucleotides during PCR and Ligase Chain Reaction.
Can be used for mutagenesis by incorporation of a phosphorylated oligonucleotide during primer extension amplification

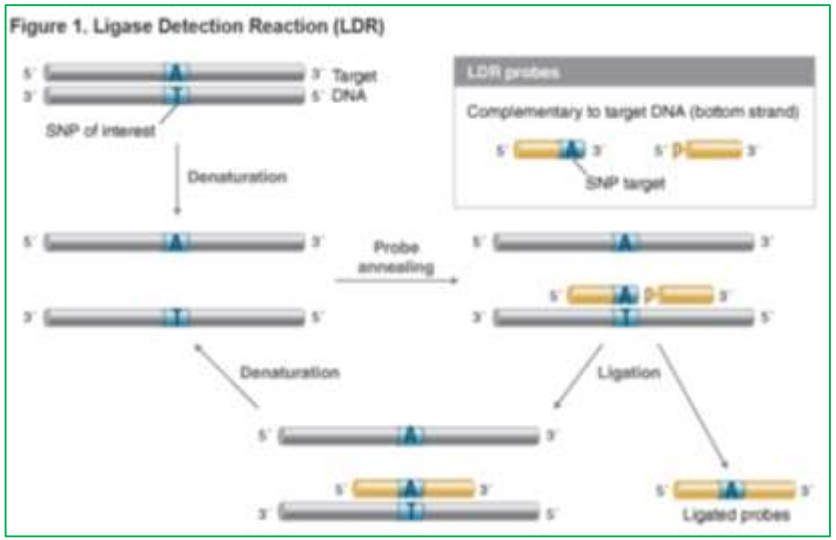
Examples: T4 DNA ligase, T7 DNA ligase, E.coli DNA ligase, Taq DNA ligase

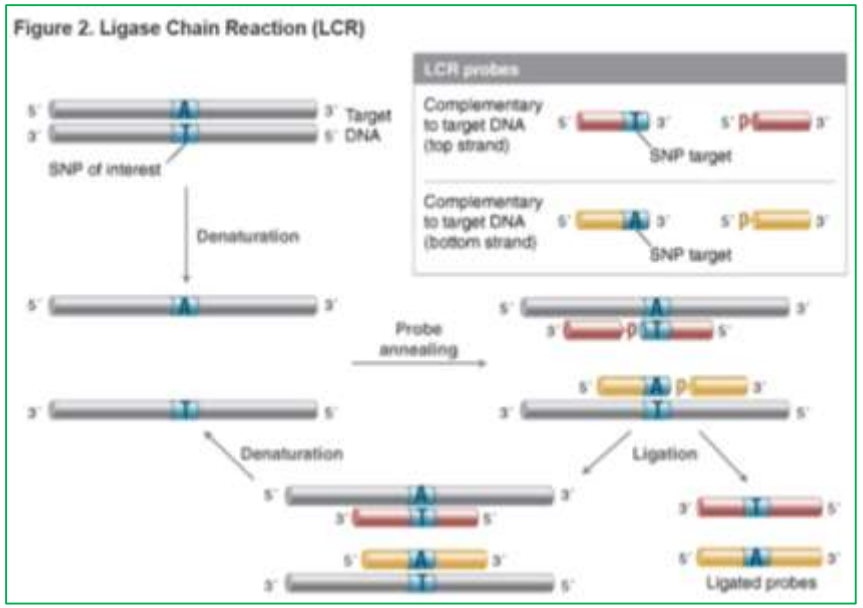


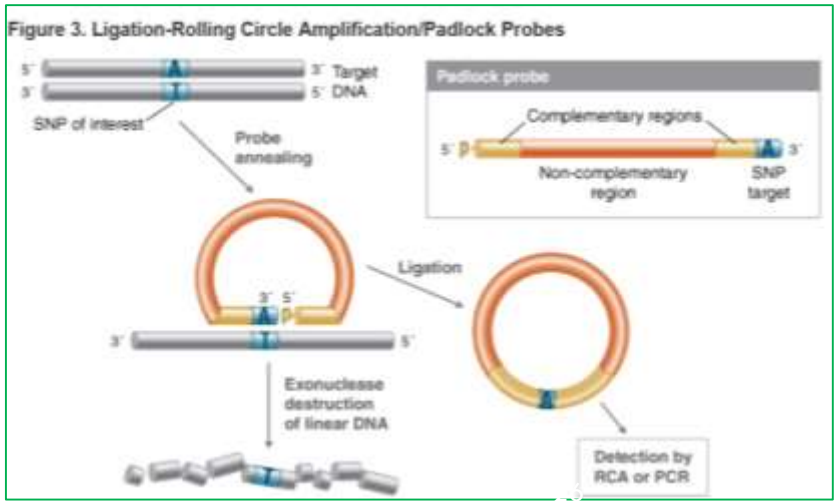
- Complementary ends (sticky ends) H-bond
- Ligase forms phosphodiester bond to seal strands together.

Application in SNP detection/ genotyping

- Ligases also vary in their type of activity. The specificity and accuracy of the ligation depends upon ligase selection and careful optimization of reaction conditions. With the right ligase, conditions and probes, even single-base variations in sequence can be reliably detected.
- However, ligases can ligate some mismatches to a significant degree, and very active ligases, such as T4 DNA Ligase, can ligate nicks containing one or more mismatches near the ligation junction with high efficiency.
- In the Ligase Detection Reaction **(LDR)**, a set of probes complementary to the sequence of interest are annealed to target DNA (genomic DNA, or a PCR amplified fragment) and treated with a high-fidelity thermostable DNA ligase **(Figure 1)**
- The closely related Ligase Chain Reaction **(LCR)** takes the LDR method and makes it amplifiable in an exponential fashion. In LCR, four probes are used, one pair complementary to one target strand, and a second pair of probes complementary to the other strand **(Figure 2)**
- Additional detection-by-ligation technologies have been devised to take advantage of high-fidelity ligation events by generating circular templates that can be detected in a secondary reaction. In the **"padlock"** probe design, a single-stranded probe is devised where the 5′ and 3′ ends are both complementary to a target sequence **(Figure 3)**







Combined application of alkaline phosphatase & ligase enzymes in cloning