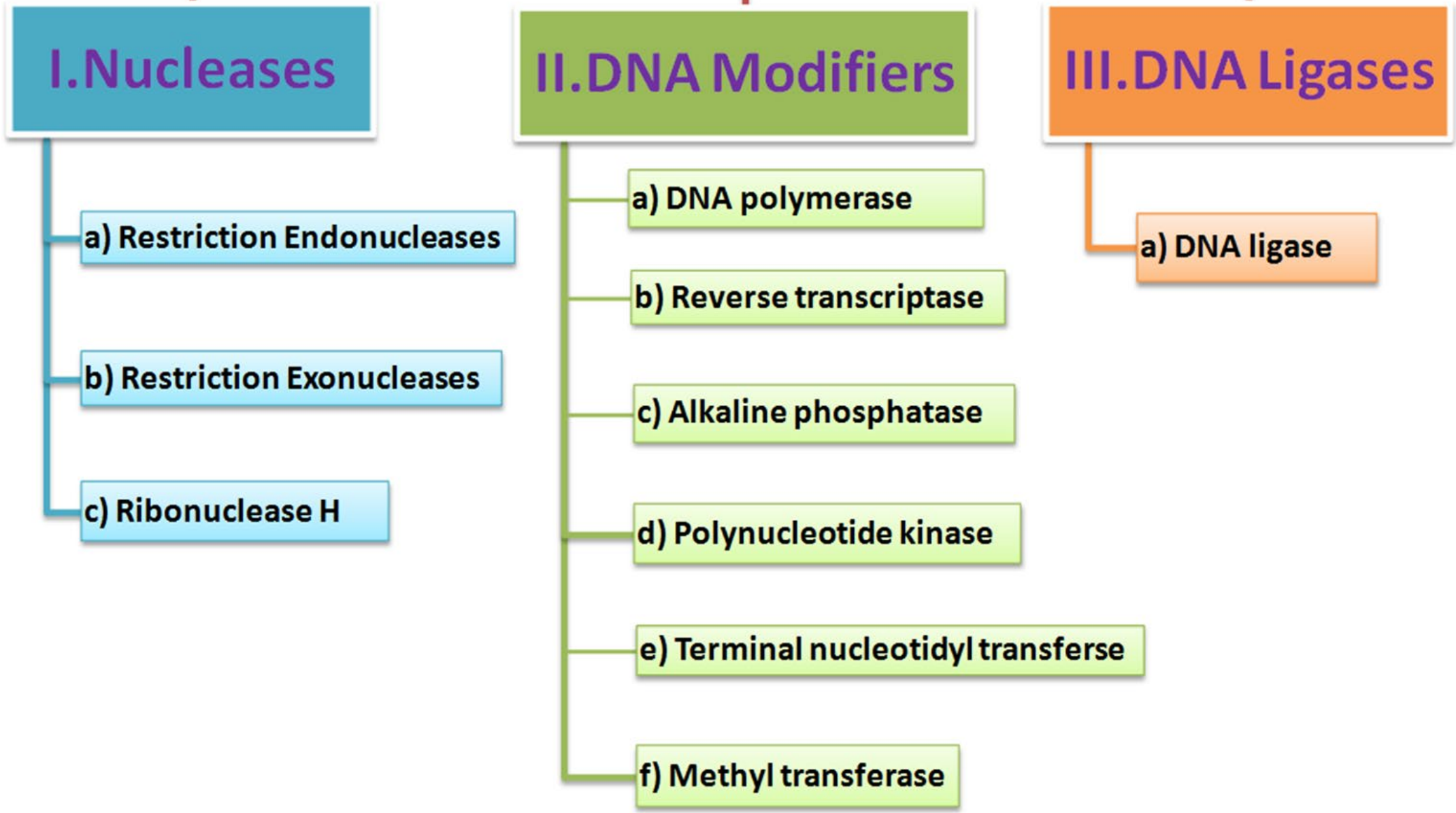
MSc III Sem – Biotechnology

Course – Principles of Genetic Engineering

Enzymes in Genetic Engineering

ENZYMES IN RECOMBINANT DNA TECHNOLOGY



Most Important Enzymes used in Molecular Biology

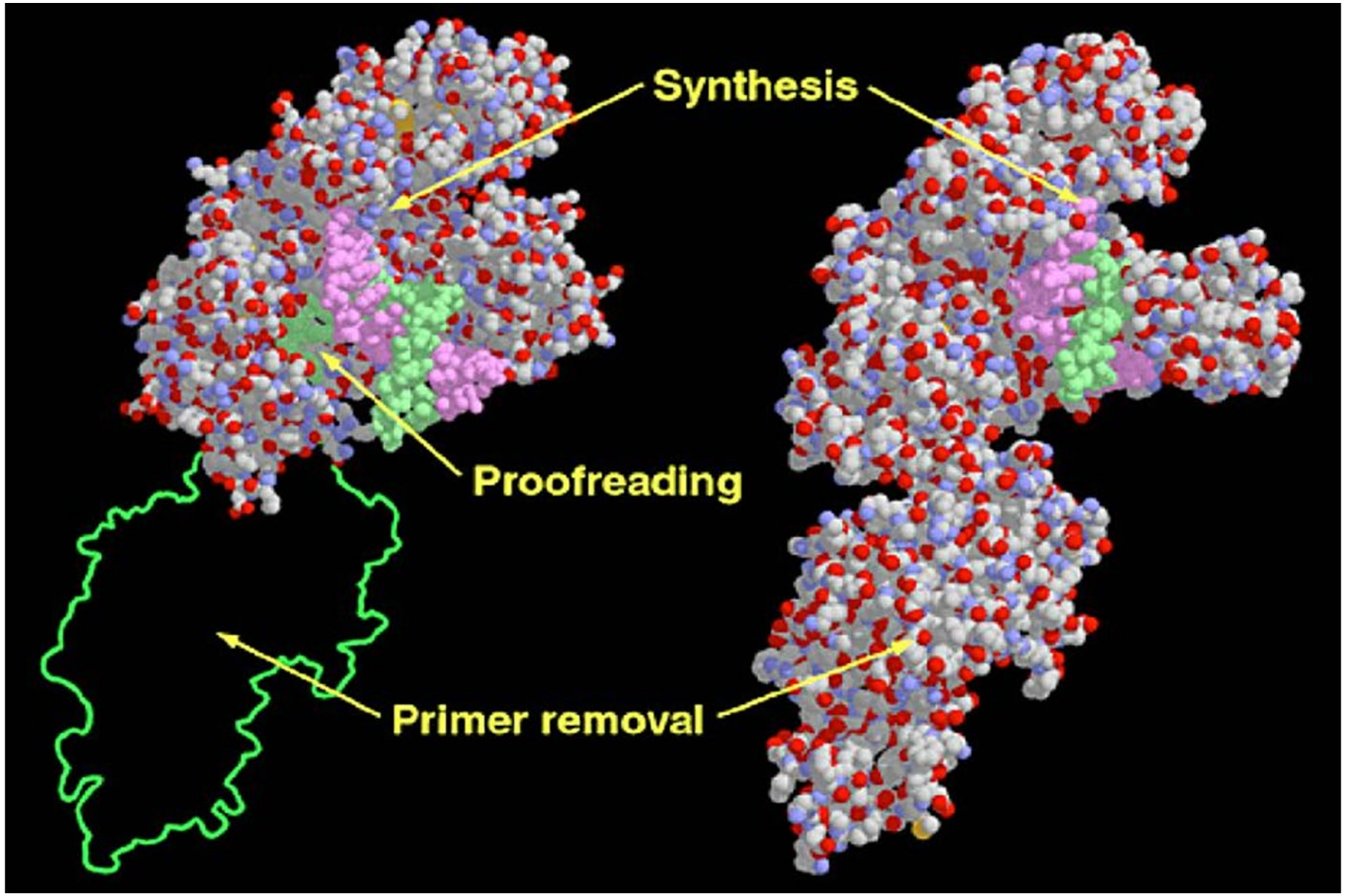
Alkaline phosphatase	Removes phosphate groups from 5' ends of DNA (prevents unwanted re-ligation of cut DNA)
DNA ligase	Joins compatible ends of DNA fragments (blunt/blunt or complementary cohesive ends). Uses ATP
DNA polymerase I	Synthesises DNA complementary to a DNA template in the 5'-to-3' direction. Starts from an oligonucleotide primer with a 3' OH end
Exonuclease III	Digests nucleotides progressiviely from a DNA strand in the 3' -to-5' direction
Polynucleotide kinase	Adds a phosphate group to the 5' end of double- or single-stranded DNA or RNA. Uses ATP
RNase A	Nuclease which digests RNA, not DNA
Taq DNA polymerase	Heat-stable DNA polymerase isolated from a thermostable microbe (*Thermus aquaticus*)

Klenow Enzyme

- The **Klenow fragment** is a large protein fragment produced when DNA polymerase I from *E. coli* is enzymatically cleaved by the protease subtilisin.
- It retains the 5' → 3' polymerase activity and the 3’ → 5’ exonuclease activity for removal of precoding nucleotides and proofreading, but loses its 5' → 3' exonuclease activity.
- The other smaller fragment formed when DNA polymerase I from *E. coli* is cleaved by subtilisin retains the 5' → 3' exonuclease activity but does not have the other two activities exhibited by the Klenow fragment (i.e. 5' → 3' polymerase activity, and 3' → 5' exonuclease activity).

The Klenow fragment is extremely useful for research-based tasks such as:

- Synthesis of double-stranded DNA from single-stranded templates
- Filling in receded 3' ends of DNA fragments to make 5' overhang blunt
- Digesting away protruding 3' overhangs
- Preparation of radioactive DNA probes



Functional domains in the Klenow Fragment (left) and DNA Polymerase I

T4 DNA Polymerase

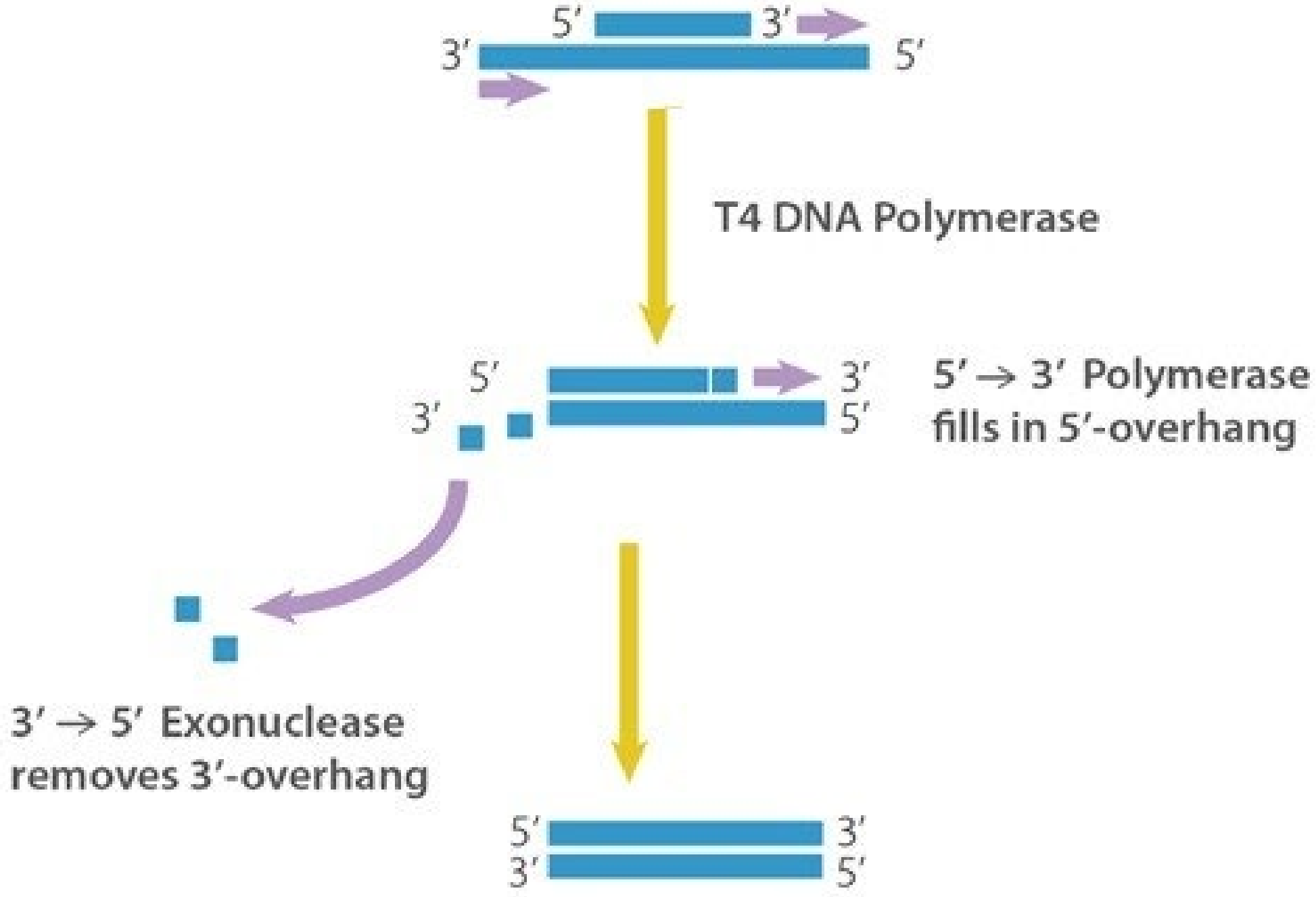
T4 DNA Polymerase is a template-dependent DNA polymerase that catalyzes 5'-3' synthesis from primed single-stranded DNA. The enzyme has a 3'-5' exonuclease activity, but lacks 5'-3' exonuclease activity.

Highlights

- **Stronger 3'-5' exonuclease activity** on single-stranded than on double-stranded DNA and greater (more than 200 times) than DNA polymerase I, *E. coli,* and Klenow fragment
- Active in Thermo Scientific restriction enzyme, PCR, RT and T4 DNA Ligase buffers

Applications

- Blunting of DNA ends: fill-in of 5'-overhangs or/and removal of 3'-overhangs
- Blunting of PCR products with 3'-dA overhangs
- Synthesis of labeled DNA probes by the replacement reaction(*see* Reference3)
- Oligonucleotide-directed site-specific mutagenesis(*see* Reference4)
- Ligation-independent cloning of PCR products


5′
3′
3′
5′
T4 DNA Polymerase
5′
3′
3′
5′
5′ → 3′ Polymerase
fills in 5′-overhang
3′ → 5′ Exonuclease
removes 3′-overhang
5′
3′
3′
5′

Polynucleotide Kinase (PNK)

- PNK is a homotetramer with phosphatase activity at 3’ end and kinase activity at 5’ end with a tunnel like active site. The active site has side chains which interact with NTP donor’s beta-phosphate and 3’ phosphate of acceptor with an acid which activated 5’ –OH.
- Lys-15 and Ser-16 are important for the kinase activity of the enzyme.
- The basic residues of active site of PNK interact with the negatively charged phosphates of the DNA.
- Polynucleotide kinase (PNK) catalyzes the transfer of a phosphate group ($PO4^{-2}$) from γ position of ATP to the 5' end of either DNA or RNA and nucleoside monophosphate.
- PNK can convert 3' PO4/5' OH ends into 3' PO4/5' PO4 ends which blocks further ligation by ligase enzyme.
- PNK is used to label the ends of DNA or RNA with radioactive phosphate group.
- T4 polynucleotide kinase is the most widely used PNK in molecular cloning experiments, which was isolated from T4 bacteriophage infected *E.coli.*

PNK carries out two types of enzymatic activity:

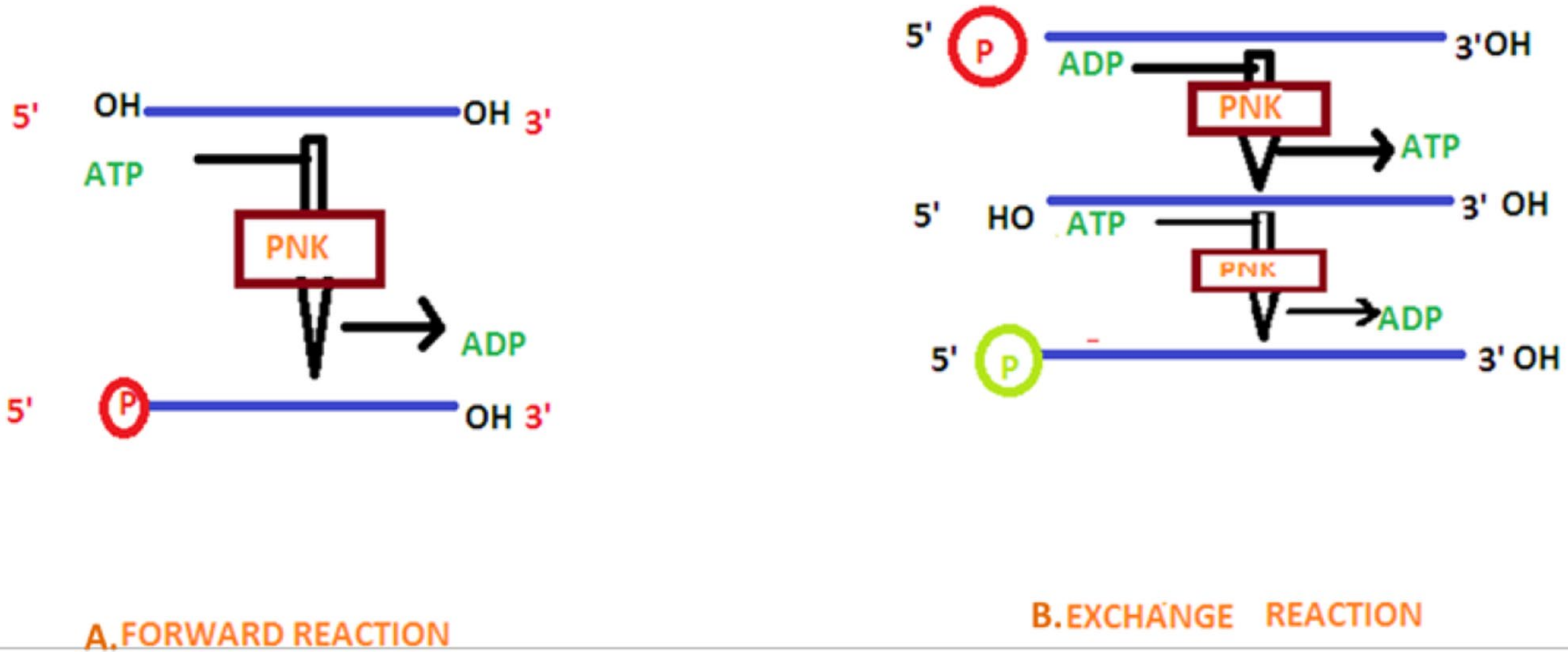
Forward reaction*: γ-phosphate is transferred from ATP to the 5' end of a polynucleotide (DNA or RNA). 5' phosphate is not present either due to chemical synthesis or dephosphorylation. The 5' OH nucleophile is activated by abstraction of the proton. Asp35 of PNK forms the co-ordinate bond with 5' OH and attacks γ phosphorus forming an intermediate.*

• **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.

There are two major uses of PNK:

• The linkers and adopters are phosphorylated along 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.



Polynucleotide kinase reaction (A) forward (B) exchange.

Alkaline Phosphatase

- Homodimeric enzyme which catalyzes reactions like hydrolysis and transphosphophorylation of phosphate monoester.
- They show their optimal activity at pH of about 10.
- Alkaline phosphatase was the first zinc enzyme discovered having three closed spaced metal ion. Two Zn+2 ions and one Mg+2 ion, in which Zn+2 ions are bridges by *Asp* 51. The mechanism of action is based on reaction where a covalent serine – phosphate intermediate is formed to produce inorganic phosphate and an alcohol.
- In human body it is present in four isoforms, in which three are tissue specific isoform i.e. placental, germ cell, intestinal and one is non tissue specific isoform. The genes that encode for tissue specific isoforms are present on chromosome -2 p37-q37, while the genes for one non tissue specific are present on chromosome -1 p34- p36.1.
- During post-translational modification, alkaline phosphatase is modified by N-glycosylation. It undergoes a modification through which uptake of two Zn+2 ion and one Mg+2 ion occurs which is important in forming active site of that enzyme. Alkaline phosphatases are isolated from various sources like microorganisms, tissue of different organs, connective tissue of invertebrate and vertebrate, and human body.

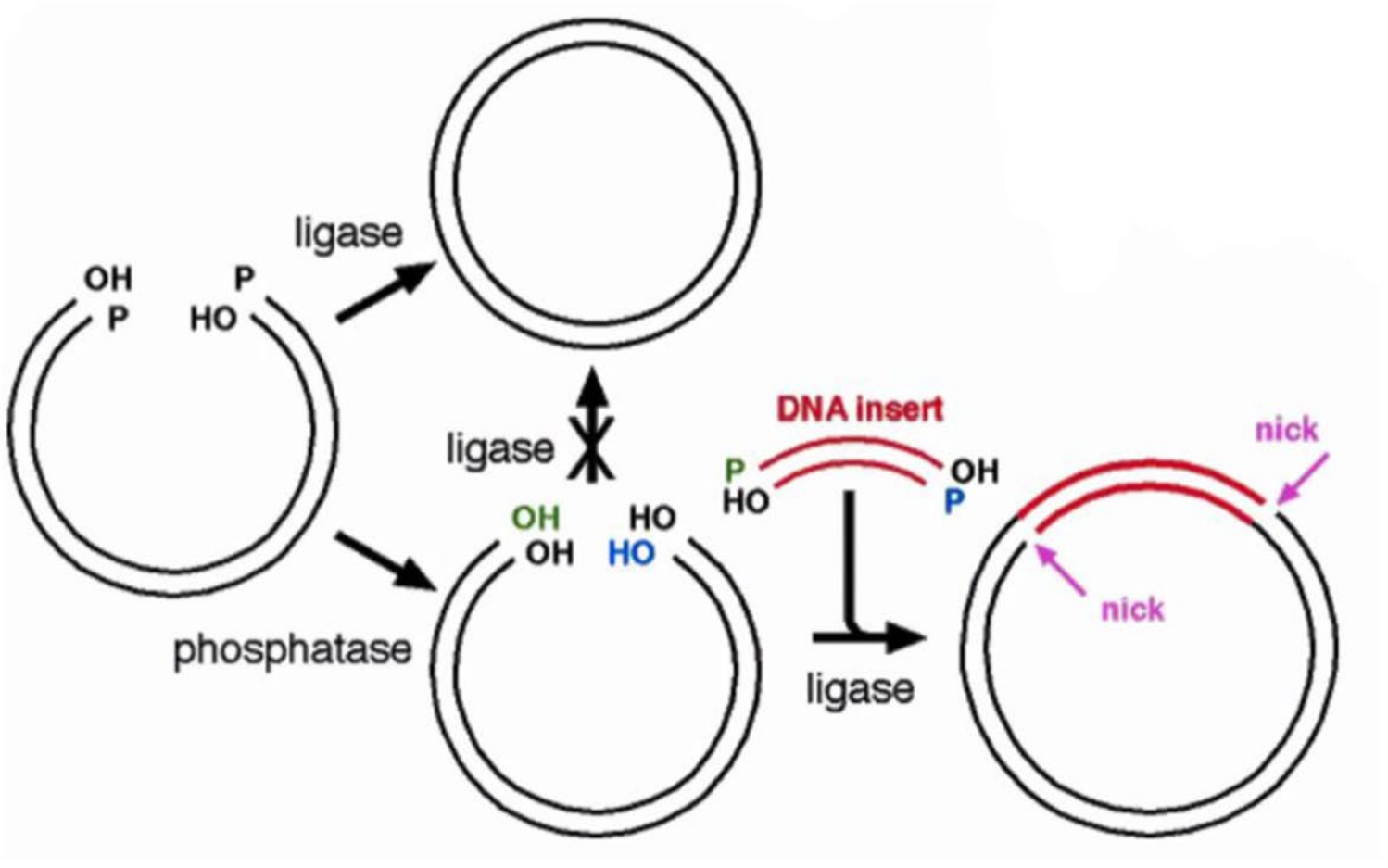
Calf intestinal alkaline phosphatase (CIP) – It is isolated from calf intestine, which catalyzes the removal of phosphate group from 5’ end of DNA as well as RNA. This enzyme is highly used in gene cloning experiments, as to make a construct that could not undergo self-ligation. Hence after the treatment with CIP, without having a phosphate group at 5’ ends a vector cannot self ligate and recircularise. This step improves the efficiency of vector containing desired insert.

Two primary uses for alkaline phosphatase in DNA modification:

- Removing 5’ phosphate from different vector like plasmid, bacteriophage after treating with restriction enzyme. This treatment prevents self ligation because unavailability of phosphate group at end.
- It is used to remove 5’ phosphate from fragment of DNA prior to labeling with radioactive phosphate.

DNA fragment with 5'phosphate

DNA fragment and separated PO_4^{-2} group



Action of alkaline phosphatase

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