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ENZYMES IN MODIFICATION
LIGASES, POLYNUCLEOTIDE KINASE, RNASE
AND THEIR MECHANISM OF ACTION

1. Ligases:

- DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.
- DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.
- *E.coli* and other bacterial DNA ligase utilizes NAD^+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.
- The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.
- This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.
- The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from *E. coli* uses cofactor NAD. Except this, the catalysis mechanism is somewhat similar for both the ligases. The role of cofactor is splitting and forming an enzyme-AMP complex which further aids in formation of

phosphodiester bonds between hydroxyl and phosphate groups by exposing them.

1.1 Mechanism of Action of DNA Ligases:

- ATP, or NAD+, reacts with the ligase enzyme to form a covalent enzyme–AMP complex in which the AMP is linked to $_{\varepsilon}$-amino group of a lysine residue in the active site of the enzyme through a phosphoamide bond.
- The AMP moiety activates the phosphate group at the 5´-end of the DNA molecule to be joined. It is called as the donor.
- The final step is a nucleophilic attack by the 3´-hydroxyl group on this activated phosphorus atom which acts as the acceptor. A phosphodiester bond is formed and AMP is released.
- The reaction is driven by the hydrolysis of the pyrophosphate released during the formation of the enzyme–adenylate complex. Two high-energy phosphate bonds are spent in forming a phosphodiester bond in the DNA backbone with ATP serving as energy source.
- The temperature optimum for T4 DNA ligase mediated ligation *in vitro* is 16˚C.

  However ligation is also achieved by incubation at 4˚C by incubating over night or at room temperature condition by incubating for 30 minutes.
- Adenylate and DNA-adenylate are the important intermediates of the phosphodiester bond forming pathway.

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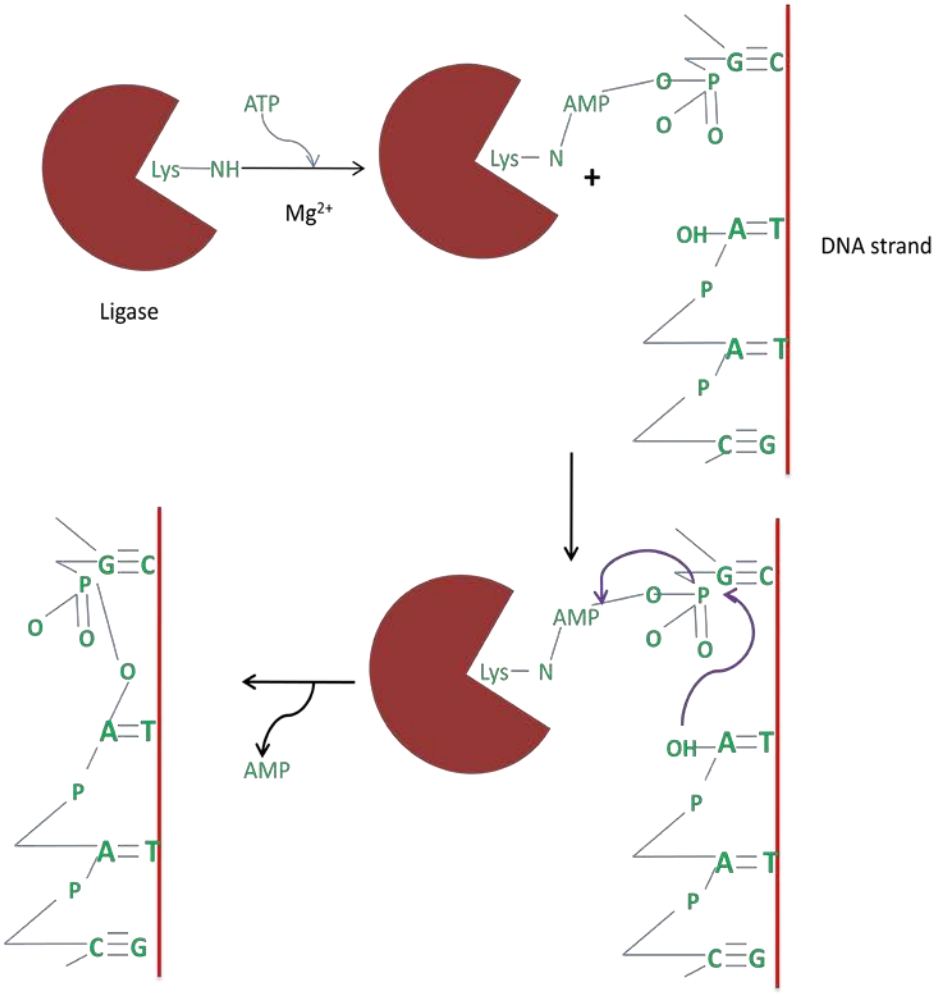

Fig: The mechanism of DNA joining by DNA ligase.

1.2 Application:

- DNA ligase enzyme is used by cells to join the "Okazaki fragments" during DNA replication process. In molecular cloning, ligase enzyme has been routinely used to construct a recombinant DNA. Followings are some of the examples of application of ligase enzyme in molecular cloning.Joining of adapters and linkers to blunt end DNA molecule.

- Cloning of restricted DNA to vector to construct recombinant vector.

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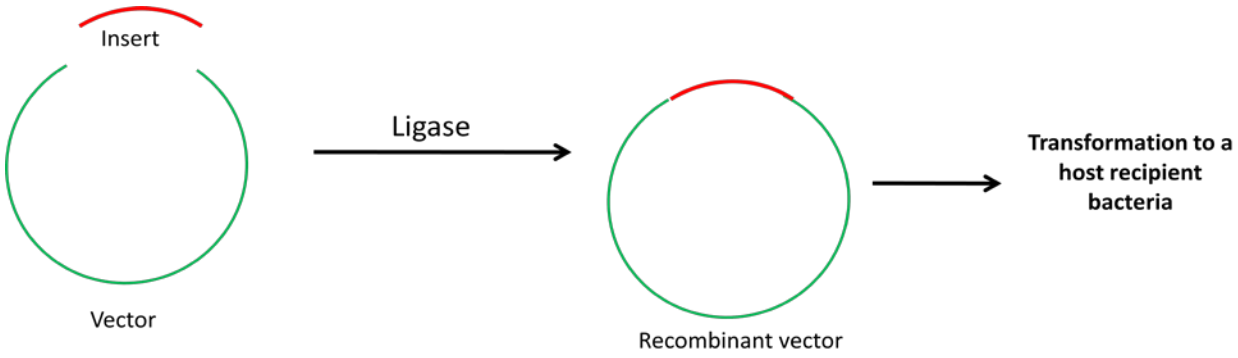

Fig: Ligation of a gene fragment into the vector and transformation of the cell.

2. Polynucleotide Kinase:

- 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 (PO_4^{-2}) from γ position of ATP to the 5' end of either DNA or RNA and nucleoside monophosphate.
- PNK can convert 3' PO_4/5' OH ends into 3' PO_4/5' PO_4 ends which blocks further ligation by ligase enzyme.
- PNK is used to label the ends of DNA or RNA with radioactive phosphate group.

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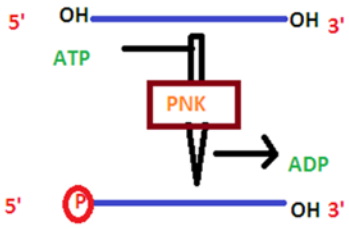
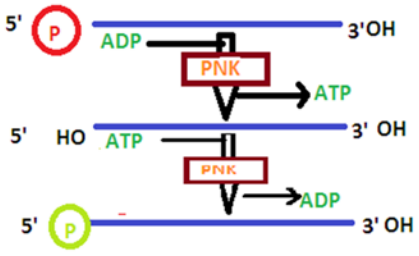
- T4 polynucleotide kinase is the most widely used PNK in molecular cloning experiments, which was isolated from T4 bacteriophage infected *E.coli.*

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.



A. FORWARD REACTION



B. EXCHANGE REACTION

Fig 2-4.2: Polynucleotide kinase reaction (A) forward (B) exchange.
(Adapted from http://www.vivo.colostate.edu/hbooks/genetics/biotech/)

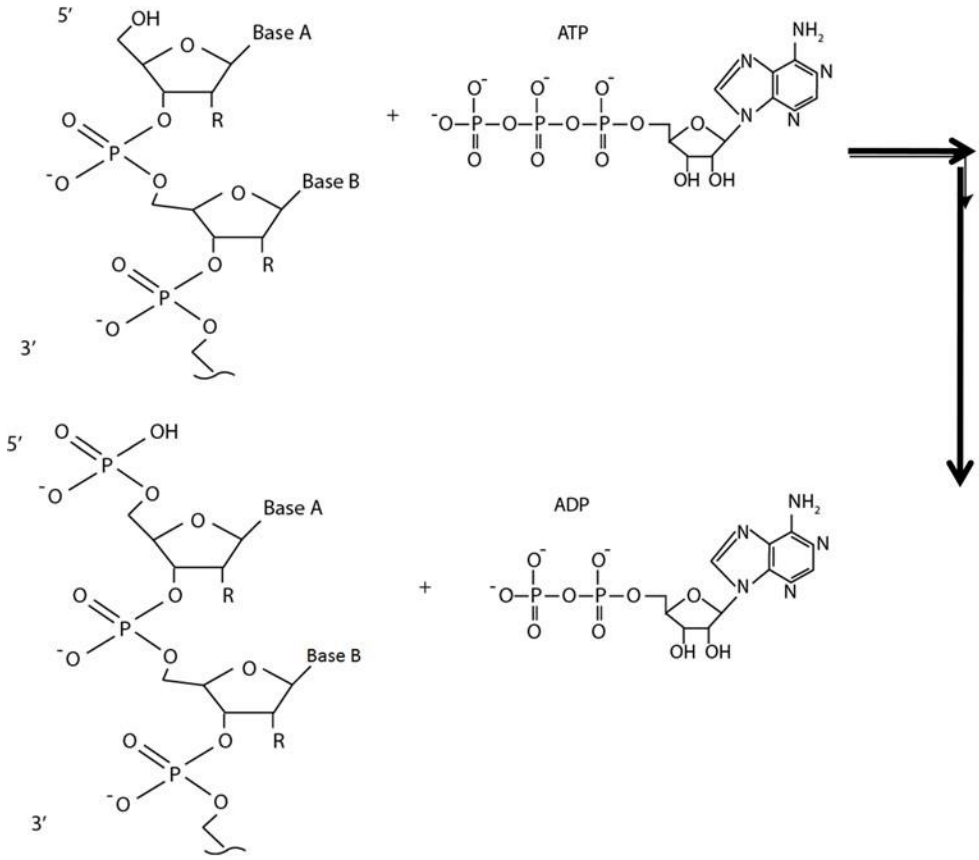


Fig.: Conversion of 5’ dephospho-(deoxy) ribonucleic acid to 5’ phospho-(deoxy) ribonucleic acid by the action of PNK.

The efficiency of phosphorylation is less in exchange reaction compared to forward reaction. Along with the phosphorylating activity, PNK also has 3' phosphatase activity.

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 ^{32}P for preparing hybridization probes.

PNK is inhibited by ammonium ions, so ammonium acetate cannot be used to precipitate nucleic acids before phosphorylation. Sometimes phosphate ions or NaCl of greater than 50 mM concentration can also inhibit this enzyme.

3. Ribonuclease (RNase):

- Nuclease that can catalyze hydrolysis of ribonucleotides from either single stranded or double stranded RNA sequence are called ribonucleotides (RNase).
- RNase are classified into two types depending on position of cleavage, i.e. endoribonuclease (cleave internal bond) and exoribonuclease (cleave terminal bond).
- RNase is important for RNA maturation and processing.
- RNaseA and RNaseH play important role in initial defence mechanism against RNA viral infection.

Two common types of ribonucleases are discussed below:

3.1 RibonucleaseA (RNaseA):

- An endo-ribonuclease that cleaves specifically single-stranded RNA at the 3' end of pyrimidine residues.
- The RNA is degraded into 3'-phosphorylated mononucleotides C and U residues and oligonucleotides in the form of 2', 3'-cyclic monophosphate intermediates.

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- Optimal temperature for RNaseA is 60˚C (activity range 15-70˚C) and optimal pH is 7.6.
- RNaseA has two histidine residues in its active site (His12 and His119). In the first step, His12 acts as a base; accepting proton forming a nucleophile which then attacks positively charged phosphorus atom. His119 acts as an acid in this case, donating a proton to oxygenated P-O-R' bond. The imidazole side chain acts as base in His 12 here.
- The side chain of Lys41 and Phe120 further stabilize the transition state. Nitrogen of the main chain of Phe120 donates hydrogen, thus bonding with the unbound oxygen atom.
- In the second step the acid base activities get reversed and His119 accepts proton from water causing hydroxyl attack on cyclic intermediate.
- Activity of RNaseA can be inhibited by alkylation of His12 and His119 residue essential for activity of the enzyme.

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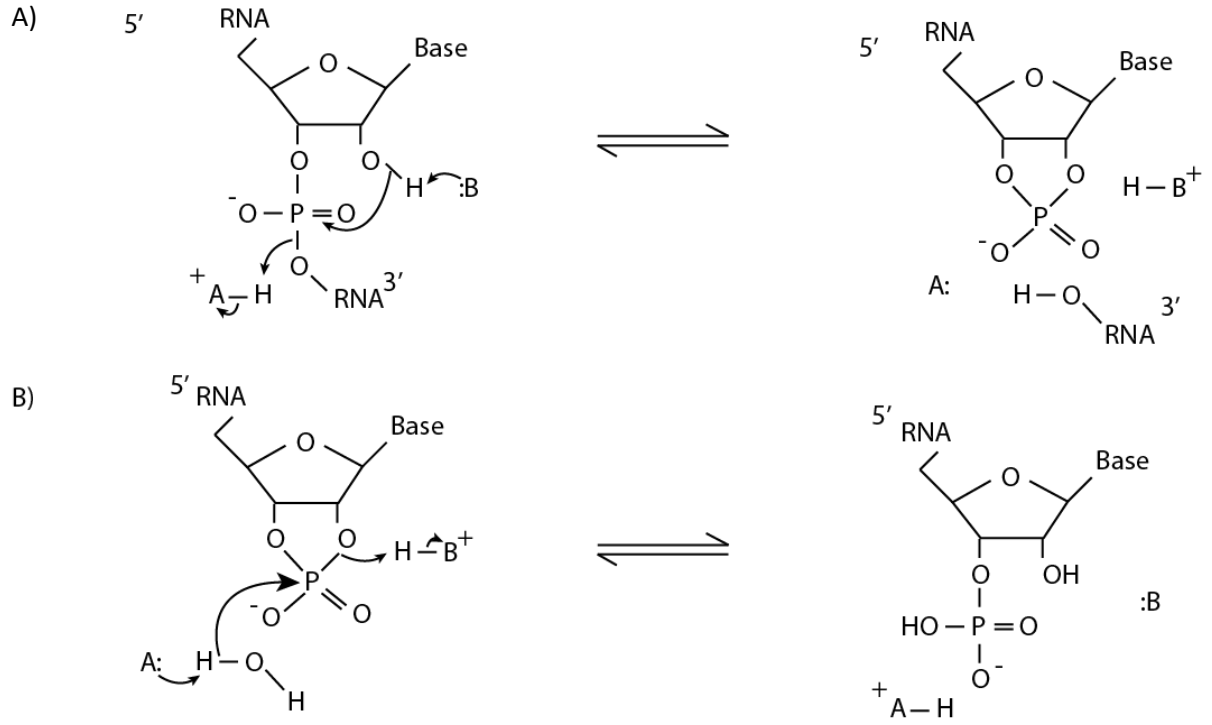

Fig : (A) Transphosphorylation reaction by RNase A
(B) Hydrolysis reaction catalyzed by RNase A

["B" → His 12
"A" → His 119]

Fig.: Mechanism of action of RNase A

Application:

- It is used to remove RNA contamination from DNA sample.

3.2 Ribonuclease H:

- Non-specific endoribonuclease that degrades RNA by hydrolytic mechanism from DNA/RNA duplex resulting in single stranded DNA.
- Enzyme bound divalent metal ion is a cofactor here. The product formed is 5’ phosphorylated ssDNA.

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- During cDNA library preparation from RNA sample, RNaseH enzyme is used to cleave RNA strand of DNA-RNA duplex.

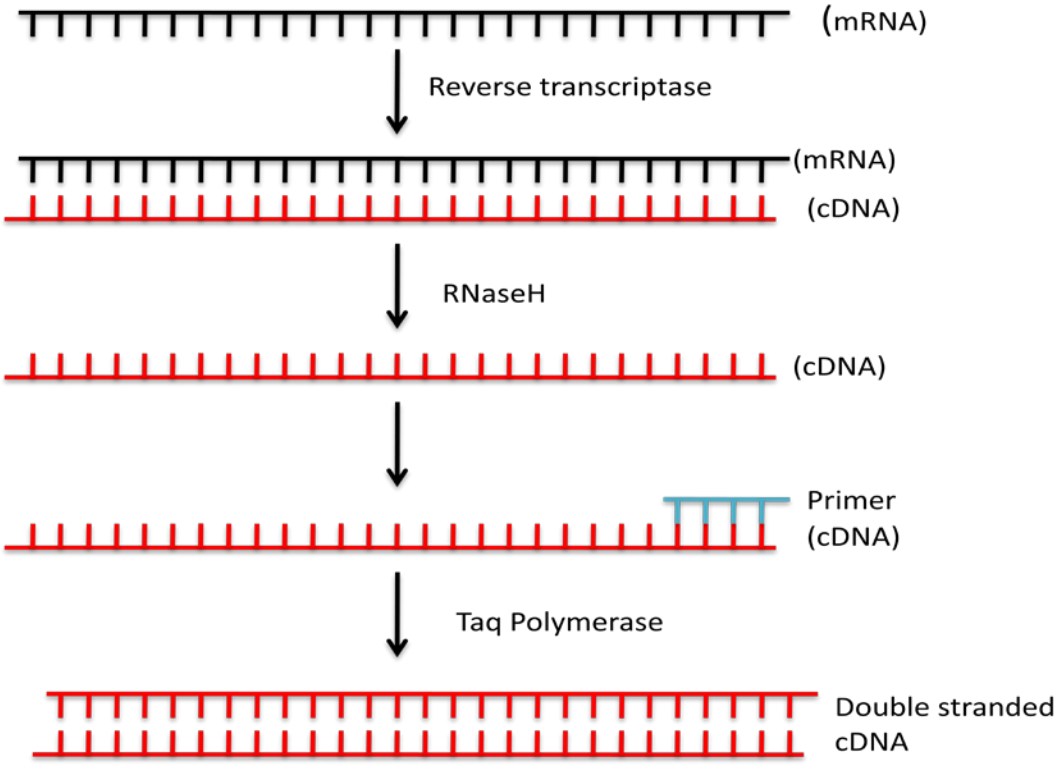

Fig: Schematic representation of cDNA preparation from mRNA