

Linker, Adaptor, Homopolymeric Tailing & Terminal Transferase



Introduction



- Ligation efficiency depends on the ends of DNA in the reaction.
- Mainly two types of end.
 1. “sticky” ends:
 - Ligation is efficient
 - annealing of complementary overhangs brings 5’P and 3’OH into close proximity.
 2. “Blunt” ends:
 - Ligation is less efficient
 - need high concentrations of ligase and DNA

Blunt end ligation



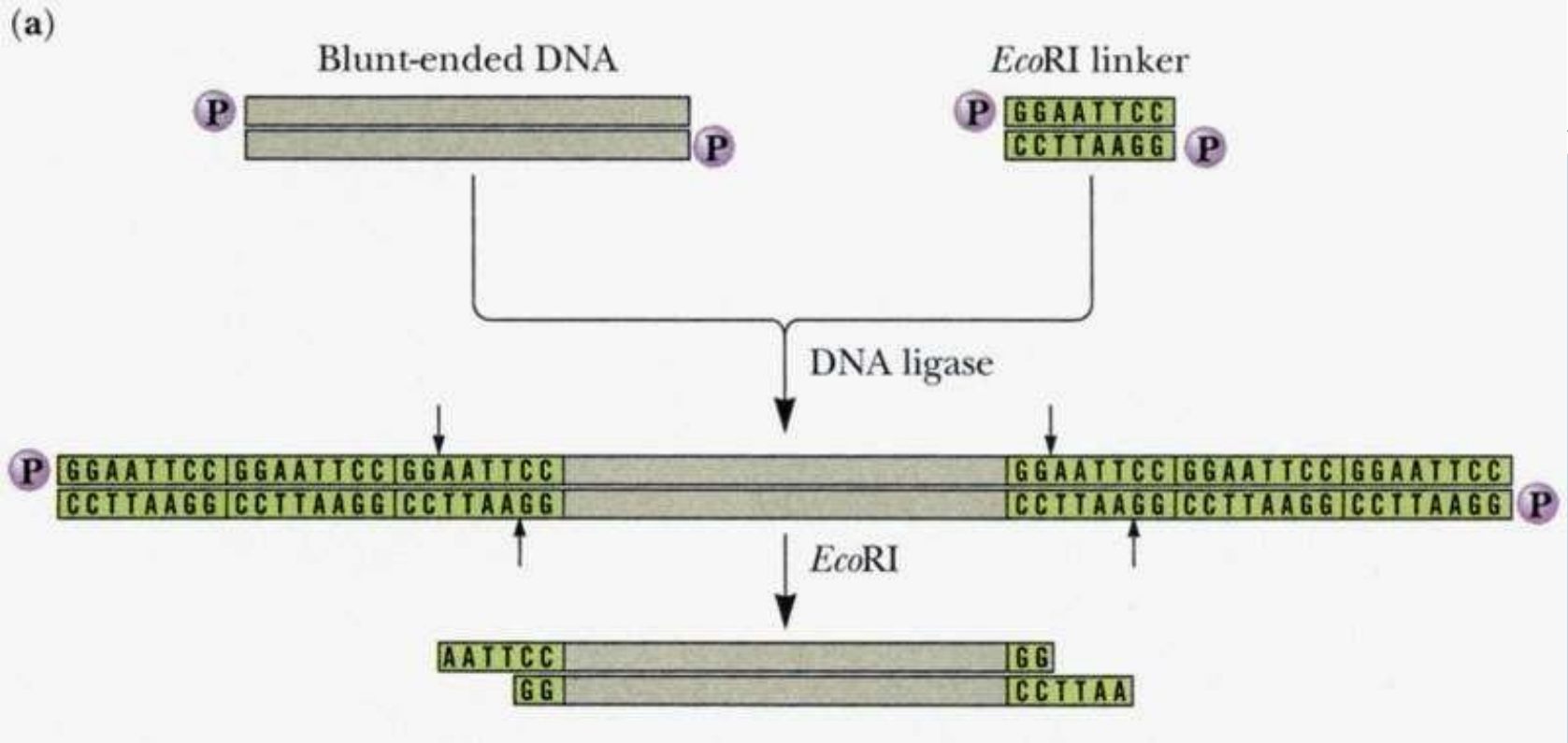
□ Mainly **three** methods can be used to put the correct sticky ends onto the DNA fragments-

1. Cloning foreign DNA by adding **linkers**
2. Cloning foreign DNA by adding **adaptors**
3. **Homopolymeric tail** adding by using **Terminal transferase** enzyme.

1. Linker



- Linkers are the **chemically synthesized double stranded DNA oligonucleotides** containing on it **one or more restriction sites** for cleavage by restriction enzymes, e.g. Eco RI, Hind III, Bam HI, etc.
- Linkers are ligated to blunt end DNA by using DNA ligase.
- Both the vector and DNA are treated with restriction enzyme to develop sticky ends.
- The staggered cuts i.e. sticky ends are then ligated with T4 DNA ligase with very high efficiency to the termini of the vector and recombinant plasmid DNA molecules are produced.



Before cloning of a particular sequence in PCR, a primer, associated with a linker is used. This type of primer is called as **linker-primer**. Now-a-days, **two different linkers** are used which has **different RE sites** with F/R primer. This strategy helps in **directional cloning**.

Limitations



- It may be the case that the restriction enzyme used to generate the cohesive ends in the linker **will also cut the foreign DNA** at internal sites.

Solution: CHOOSE ANOTHER RESTRICTION ENZYME

But there may not be a suitable choice if the foreign DNA is large and has sites for several restriction enzymes.

Methylation of internal restriction sites with the appropriate modification methylase for example EcoRI methylase.

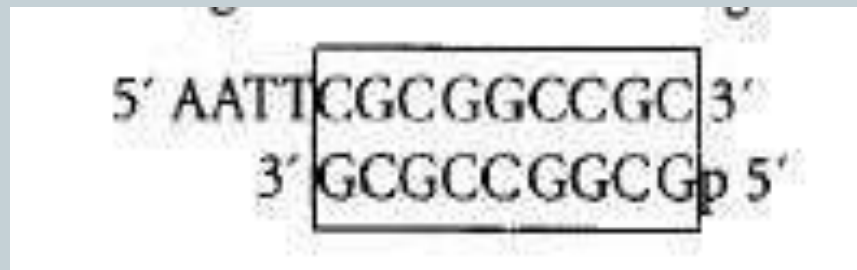
2. Adaptors



- They are also short double stranded oligonucleotides that carry an **internal RE sites** and **single stranded tails at one or both ends**.
- This protruding sequences can be ligated to DNA fragments containing a **complementary single stranded terminus**.
- After ligation, the DNA can be cleaved with appropriate RE to **create new protruding terminus**.

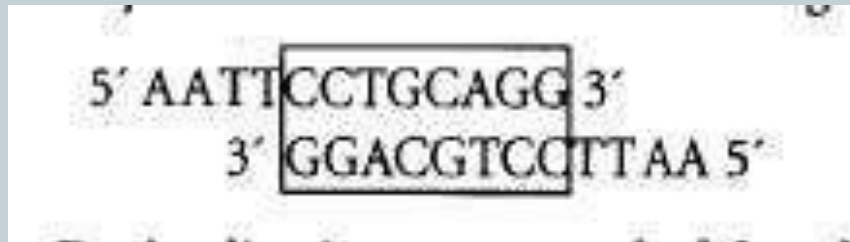


- Adaptors are available in two basic designs and a variety of specifications.
- **1.** Some consists of a partial duplex formed between two oligonucleotides of different length; for example, the **EcoR1-Not1 adaptor**.





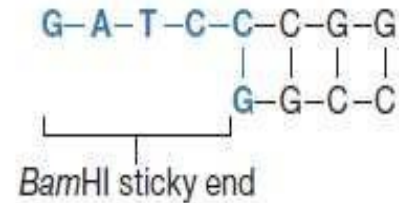
- 2. Another class of adaptor is supplied as an unphosphorylated single oligonucleotide whose sequence is partially self complementary. As an example- **EcoR1-Pst1 adaptor**.



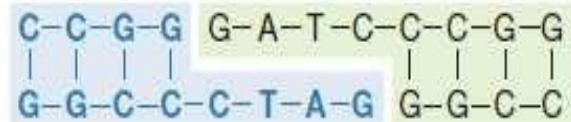
Problems

□ The sticky ends of individual adaptors could **base pair with themselves** to form dimers and the new DNA molecule remains blunt-ended.

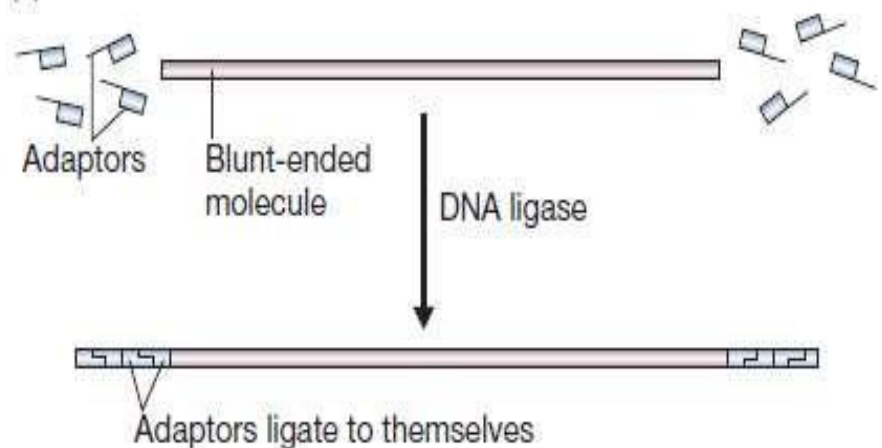
(a) A typical adaptor



(b) Adaptors could ligate to one another



(c) The new DNA molecule is still blunt-ended



Solution

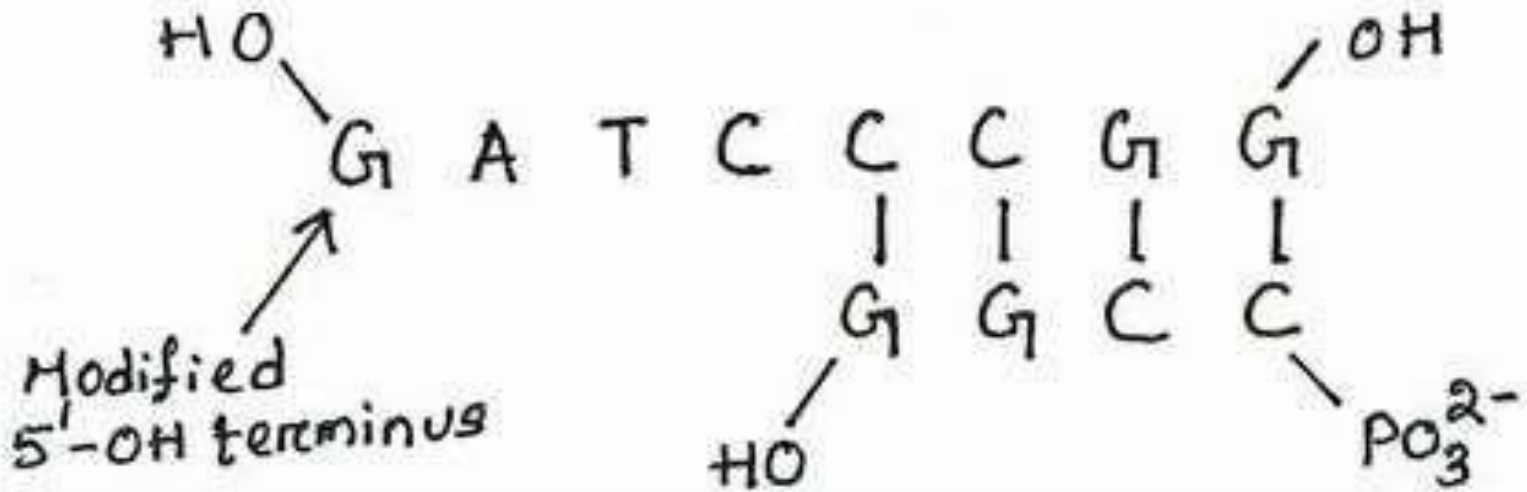


Figure 7: Structure of an adaptor showing the modified 5'-OH terminus

- Adaptor molecules alter their 5' terminus (From 5'-P to 5'-OH) by an enzymatic treatment of the enzyme **Alkaline phosphatase** to prevent self ligation.
- Afterwards, they can be treated with **Polynucleotide kinases** to restore it & ligate to vectors.

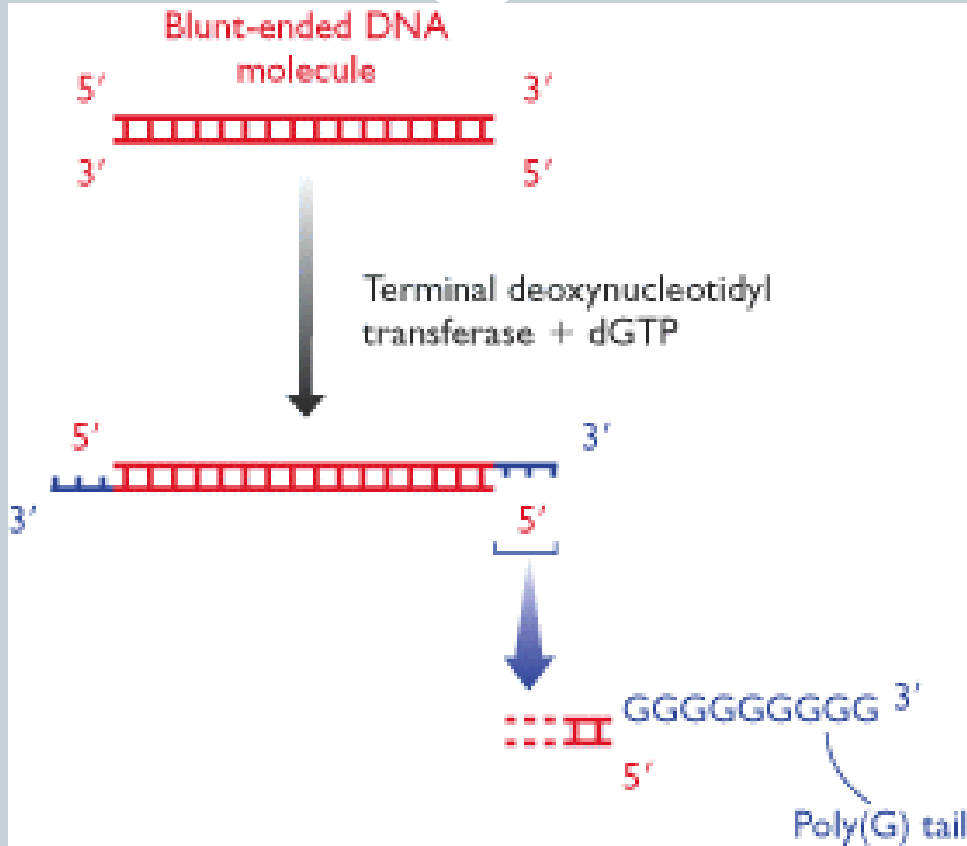


- However, H-Bonds can form between the complimentary bases of 2 adaptor.
- The adaptor molecules can be heated **at 90°C for 3 minutes** before use.
- 30X more adaptor is given (in terms of molar) in reaction than insert to ensure proper binding.
- This strategy **eliminates the need of methylation** of cDNA or to digest it with RE before insertion into vector.

3. Homopolymeric tailing

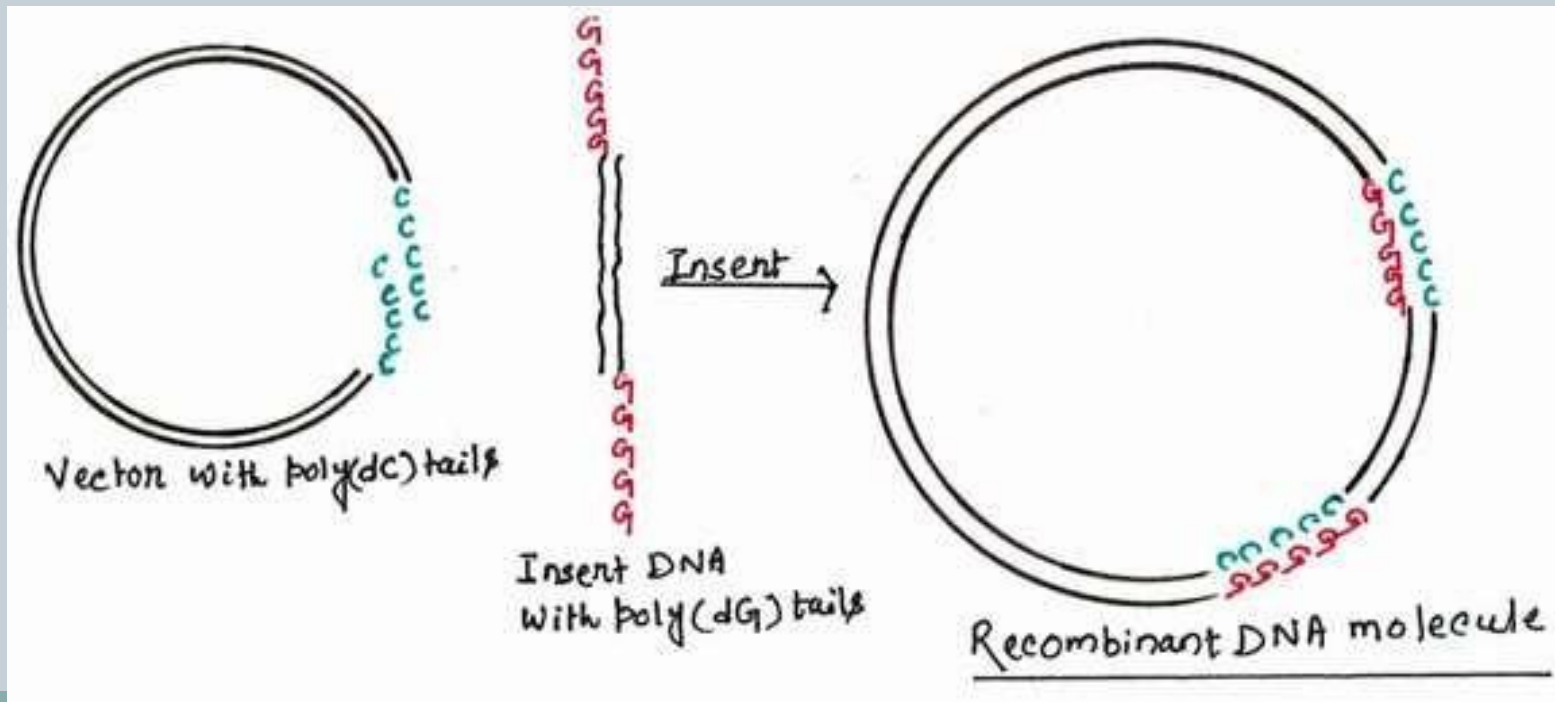


- It is a technique by which **sticky ends can be produced** on a **blunt-ended DNA molecule**.
- In a homopolymer, all the subunits are same. A DNA strand made up **entirely of deoxyguanosine** is an example of homopolymer, and is referred to as **polydeoxyguanosine or poly(dG)**.
- Tailing involves using the enzyme **terminal deoxynucleotidyl transferase** to add a series of nucleotides **on to the 3'-OH termini** of a double-stranded DNA molecule.
- The reaction when carried out in the presence of just one deoxynucleotide, then a homopolymer tail will be produced.

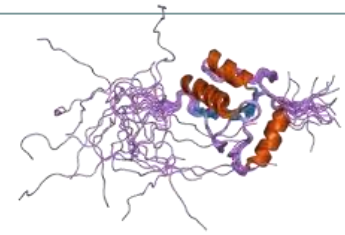




- For ligation of two tailed molecules, **the homopolymers must be complementary**. Frequently poly(dc) tails are attached to the vector and poly(dg) to the DNA to be cloned.



Terminal transferase



- Terminal deoxynucleotidyl transferase (TdT) is a template independent DNA polymerase.
- It is expressed in **immature, pre-B, pre-T lymphoid** cells, and **acute lymphoblastic leukemia/lymphoma** cells.
- In humans, terminal transferase is encoded by the ***DNTT*** gene.

Function



- TdT catalyses the **addition of nucleotides** to the 3' terminus of a DNA molecule. Unlike most DNA polymerases, it **does not require a template**.
- The preferred substrate of this enzyme is a **3'-overhang** but it can also add nucleotides to blunt or recessed 3' ends.
- **Cobalt** is a necessary cofactor, however the enzyme catalyzes reaction upon Mg and Mn administration *in vitro*.

Regulation

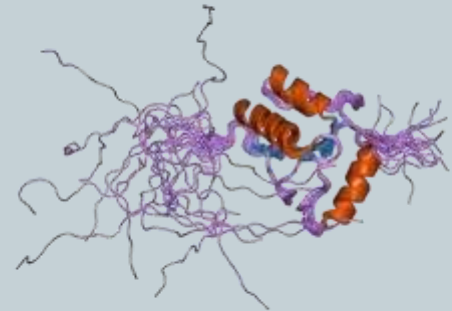


- TdT is expressed mostly in the primary lymphoid organs, like the **thymus and bone marrow**.
- Regulation of its expression occurs via multiple pathways. These include protein-protein interactions, like those with **TdIF1**.
- TdIF1 is another protein that interacts with TdT to inhibit its function by masking the DNA binding region of the TdT polymerase

Structure



- Monomeric
- Mol. Wt.- 58000 Da
- Amino acids- 508 to 529 (depending upon source)
- A high degree of **sequence homology** (>80%) in TdT between different species



Reaction buffer



- Activity is strongly inhibited by the **ammonium** ion as well as **chloride**, **iodide** and **phosphate** anions.
- Potassium or sodium **cacodylate** (dimethyl arsenic acid) buffers are preferred- shown to be optimal for polypurine and polypyrimidine synthesis
- Certain drawbacks with cacodylate such as **toxicity**, **contamination by metal** etc

Divalent cation



- Polymerization requires presence of divalent cations.
- Order of efficiency for damp addition to oligonucleotide is as following
 $Mg > Zn > Co > Mn$
- For dGTP- Magnesium ion
- For pyrimidine- Cobalt ion



Heat Inactivation

75°C for 20 min

5' - 3' Exonuclease

No

3' - 5' Exonuclease

No

Source

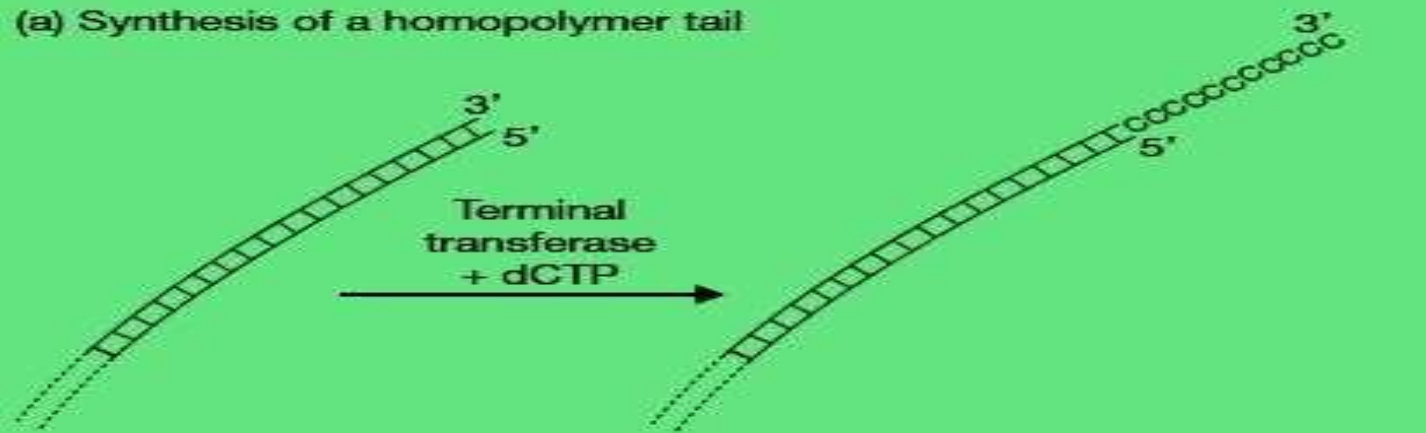
An *E. coli* strain that carries the cloned Terminal Transferase gene from calf thymus.

Applications

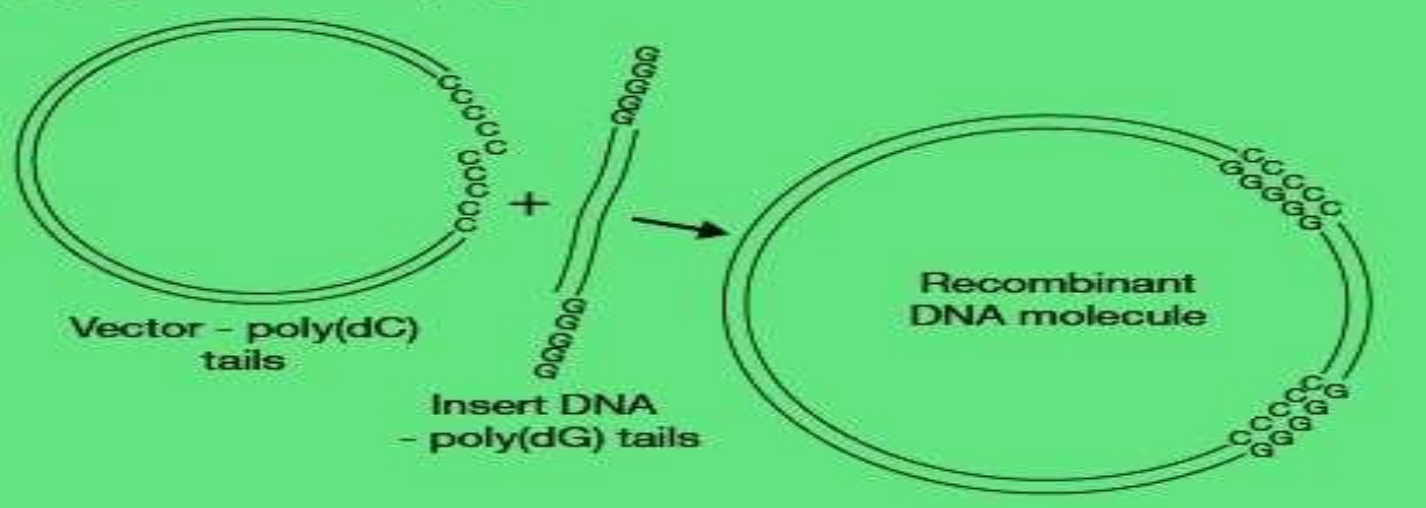


- Addition of homopolymeric tails to plasmid DNA and to cDNA.
- Double- or single-stranded DNA 3' -termini labeling with radioactively labeled or non-radioactively labeled nucleotides.
- Addition of single nucleotides to the 3' ends of DNA for in vitro mutagenesis.
- Production of synthetic homo- and heteropolymers.
- RACE (Rapid Amplification of cDNA Ends).
- TUNEL assay (*in situ* localization of apoptosis).

(a) Synthesis of a homopolymer tail



(b) Ligation of homopolymer tails





THANK YOU