Course: M.Sc. Biotechnology

Paper: BIOT4009: Genetic Engineering and Gene Therapy

UNIT – III

POLYMERASE CHAIN REACTION-8

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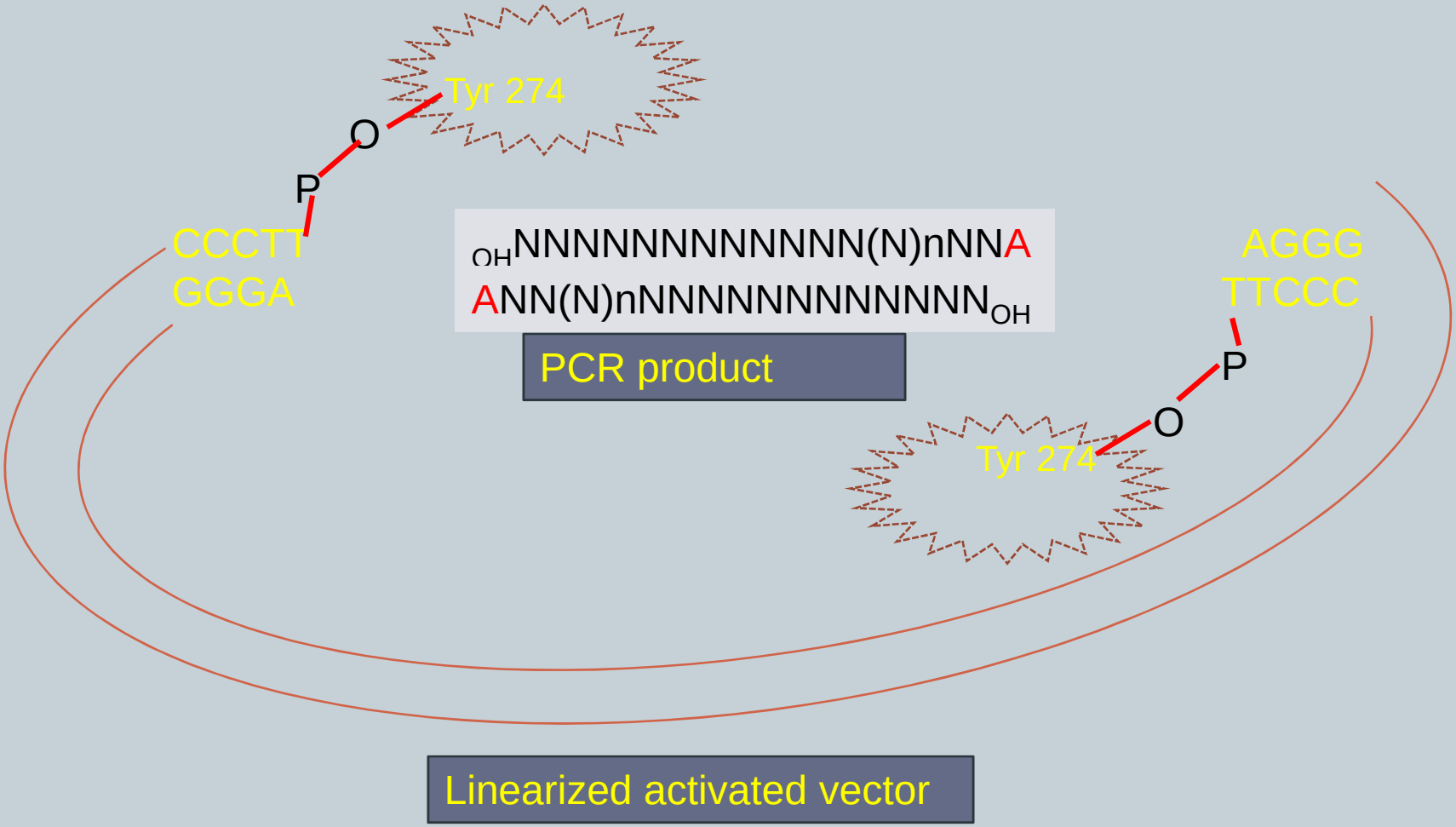
TOPO Cloning

- It is a special PCR based cloning method which does not require separate ligase enzyme
- Method depends upon
- Terminal extendase/ terminal transferase activity of *Taq* DNA Polymerase
- PCR based amplification of target gene
- Use of primers which are dephosphorylated at 5' end
- Activity of Topoisomerase I

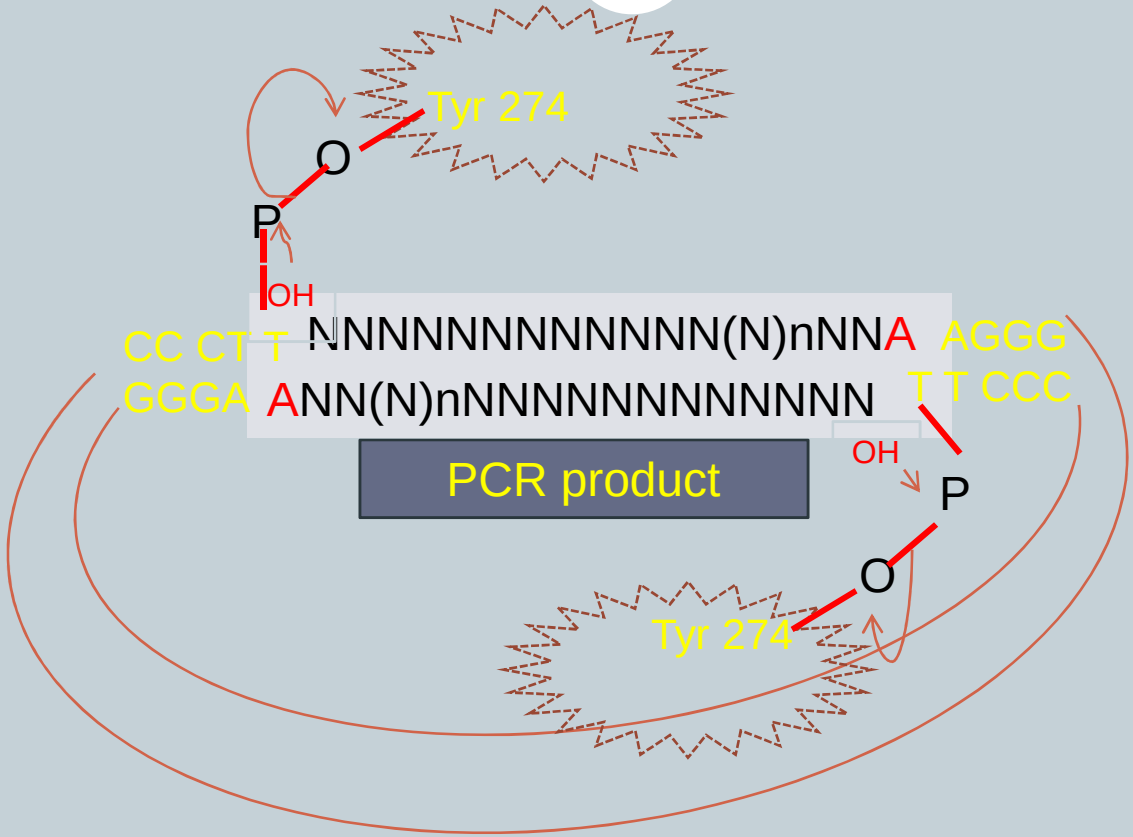
TOPO Cloning contd.

- It is also called TOPO T/A cloning
- One step cloning technique
- PCR product has additional A due to Taq DNA Polymerase
- Does not require post PCR procedures
- It is direct insertional cloning in specialized activated vector (TOPO vector)
- Linearized vector has additional T
- Vector is linked to Topoisomerase I (activated vector)

TOPO Cloning contd.



TOPO Cloning contd.



Mechanism of joining the vector and insert

Mechanism of cloning

- Topoisomerase I from Vaccinia virus interacts to dsDNA at specific sites and break the phosphodiester backbone after 5′-CCCTT in one
- The released energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I.
- The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5′ hydroxyl of the PCR product joining the vector and insert (PCR product) releasing the topoisomerase

Regulatory aspects and precautions

- Inclusion of salt (200mM NaCl and 10 mM CaCl2) prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA.
- Primers used to generate insert DNA in PCR must not have 5'phosphate
- 5'OH in insert is must for cloning in this method

TOPO Cloning Contd.

- Recombinant vector is transferred to host cell
- Transformed cells are screened on solid plate with selection pressure
- Transformed colonies are isolated and used to grow cells in broth
- Large scale culture is grown and plasmid is isolated
- A part of culture is converted to glycerol stock and another pat is used to isolate plasmid
- Presence of insert is confirmed
- Insert may be subjected to sequencing or used for further study

References

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and WileyInterscience).
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Automation in cloning and expression

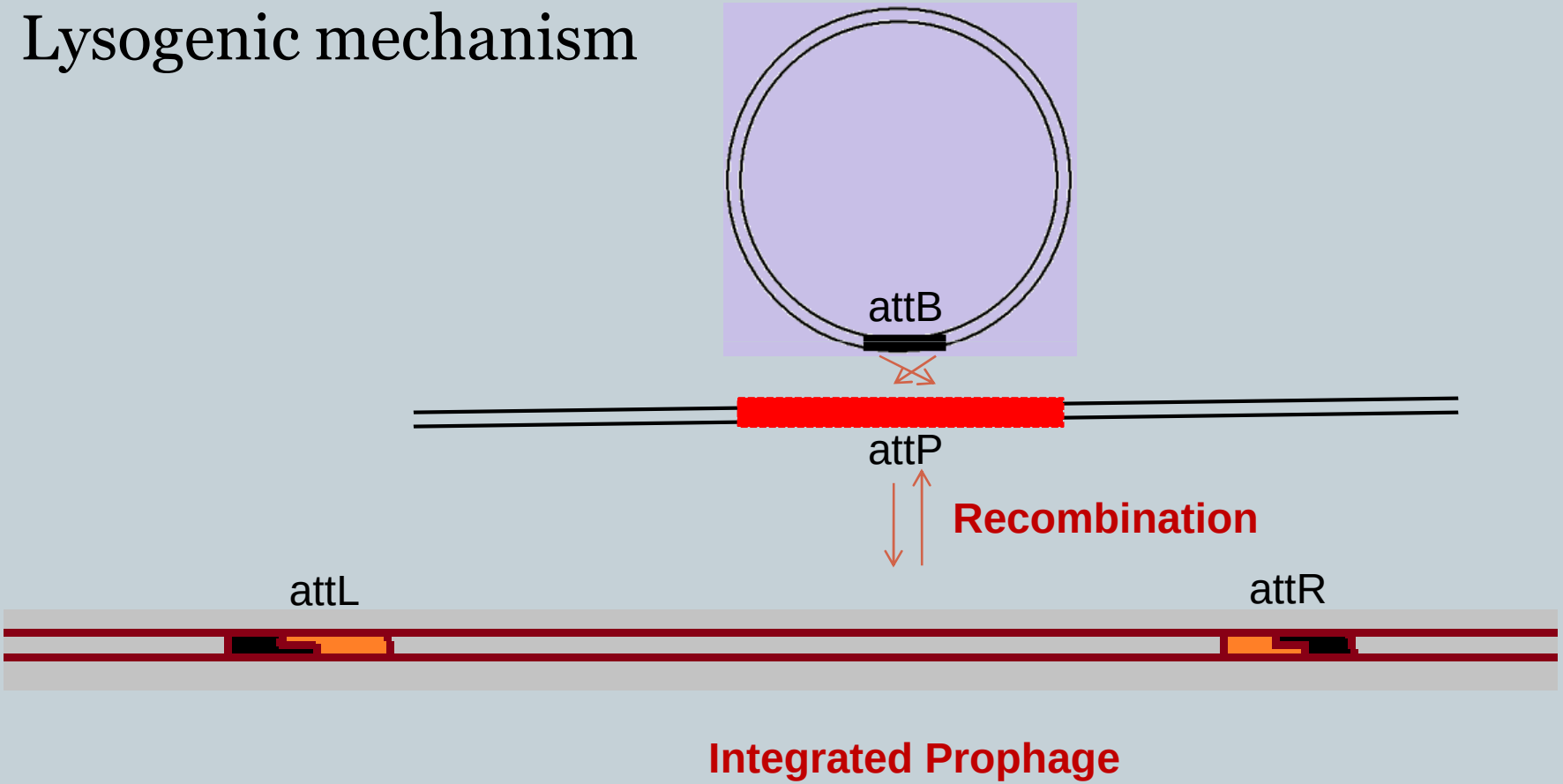
Gateway Cloning system

Gateway Cloning system: Basics

- It relies on mechanism of lysogenic integration of λ Phage genome in *E. coli* bacterial genome.
- It is result of site specific recombination between attP (243 nt) site in λ Phage and attB (25 nt) site in *E. coli* bacteria
- Lysogenic integration of λ Phage in to bacterial genome leads to development of new sites aatL (100 nt) and attR (168 nt) that flanks the λ Phage in bacterial genome

Basic integration mechanism

Lysogenic mechanism



Basic integration mechanism contd.

- **Two sequences are involved attP and attB**
- **Two proteins are involved**
  - **Phage coded Integrase (Int)**
  - **Bacteria coded IHF (Integration host factor)**
- **Two new sites aaL and attR are produced after integration**
- **The process of integration is reversible and excision may also occur**
- **Excision is catalysed by Int, IHF and Phage coded X protein**

Gateway cloning : improvements

- **The enzymes collectively c/d clonase (BP clonase and LR clonase) are sequence specific**
- To make the reactions directional two and specific site *att*1 and *att*2 (with slight changes) were developed, for each recombination site
- These sites react very specifically with each other. E.g., in the **BP Reaction** *att*B1 only reacts with *att*P1 resulting in *att*L1 and *att*R1
- *Similarly* *att*B2 reacts only with *att*P2 giving *att*L2 and *att*R2.
- The reverse reaction (**LR Reaction**) shows the same specificity.

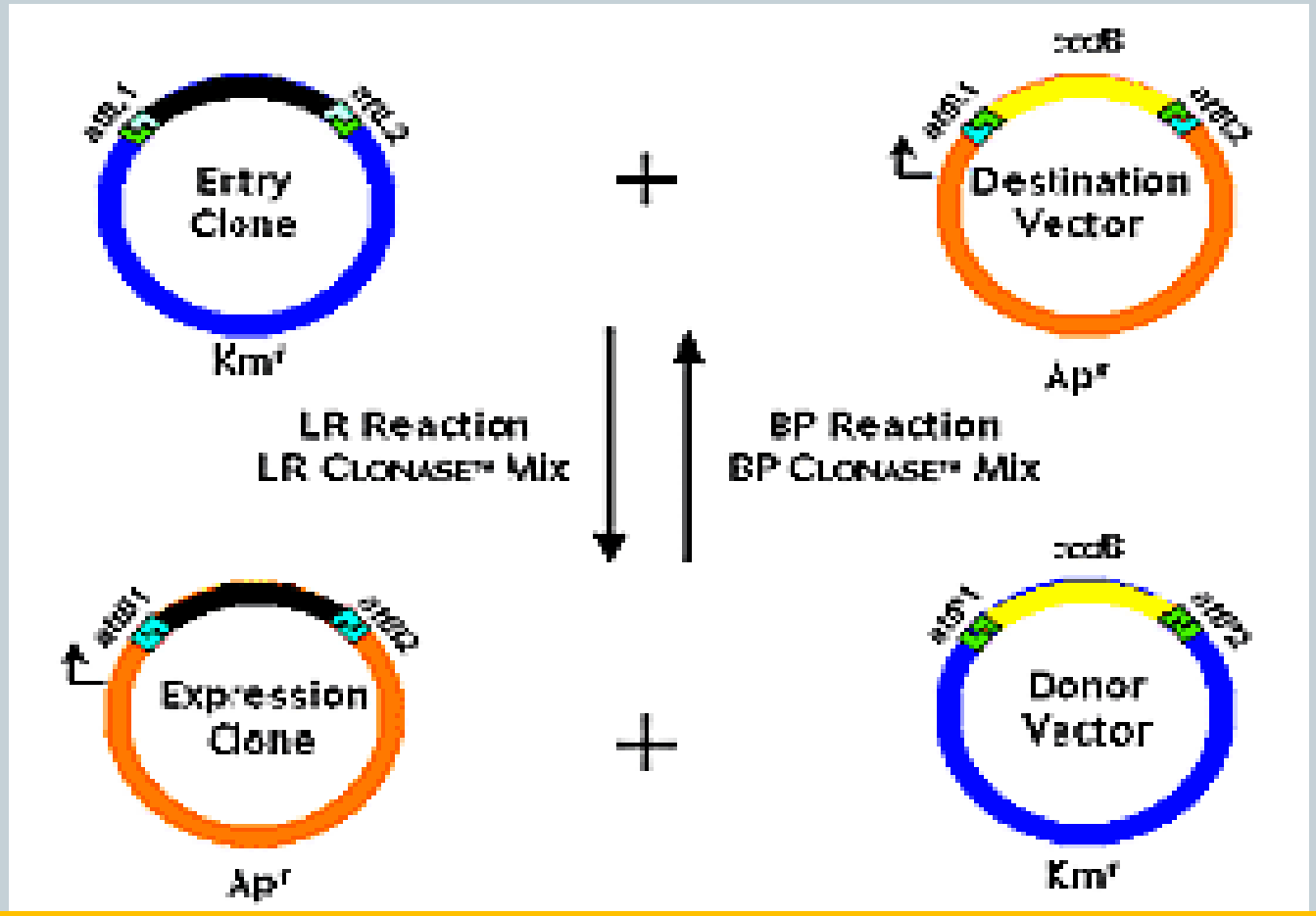
In vitro objectives and methods

Ultimate objective is hassle free cloning in expression system

Step 1 involves : Cloning the gene of interest into an *Entry Vector* using the **BP Reaction**.

Step 2 involves: Subcloning the gene of interest from the Entry Clone (Step 1) into a *Destination Vector* using the **LR Reaction** producing the *Expression Clone*.

Gene transfer mechanism: Clonase mediated



https://www.embl.de/pepcore/pepcore_services/cloning/cloning_methods/recombination/gateway/

Advantages

- Once amplified with requisite sites, can be easily cloned in entry vector
- Transfer to destination, expression vector with desired promoter, regulator is easy and hassle free
- Tiring screening events at each level are omitted.
- It facilitates shuttling of gene between different vectors of different properties for regulation of expression

References

- Weisberg, R. A., and Landy, A. (1983) Site-Specific Recombination in Phage Lambda. In Lambda II, R. A. Weisberg, ed. (Cold Spring Harbor, NY: Cold Spring Harbor Press), pp. 211-250.
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Thanks

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AND SUPPLEMENTARY STUDY MATERIAL FOR DETAILS