Lecture 36: Basics of DNA Cloning-II

Note: Before starting this lecture students should have completed Lecture 35

Sequential steps involved in DNA cloning using plasmid DNA as vector: Molecular cloning using a plasmid vector involves five major steps as shown in Fig. 1 and Fig. 2

Step 1: ***Isolation of DNA (gene of interest and vector):***

The first initial step in cloning a DNA fragment is to isolate foreign DNA containing gene of interest and bacterial plasmid. If the sequence of the gene of interest is known it is isolated by PCR amplification using gene specific primers which include restriction sites selected from the multiple cloning site of the plasmid selected for cloning. When the sequence of the gene is not known degenerate primers are used for PCR amplification. Most of the time people generate genomic DNA library and screen for the gene using southern hybridization technique. According to the result of southern hybridization, the DNA is sequenced and the gene was confirmed by BLAST analysis. Now the gene is amplified by PCR and cloned. There are many plasmids available commercially for cloning.

Step 2: ***Treatment of plasmid and foreign DNA with the same restriction enzyme and ligation:***

The gene of interest and the plasmid are modified using same restriction enzymes. Plasmid vectors are engineered to contain a specific antibiotic resistance gene and a multiple cloning site (also called the polylinker region) which contain many unique target sites for restriction endonucleases. When the circular plasmid is cut with one of the restriction enzyme whose restriction site is present in the plasmid, it results the linearization of plasmid. A fragment of DNA molecule, referred to as the "insert," is treated with the same restriction enzyme, and then can be joined to the plasmid DNA in a ligation reaction. The chance for recombinant clones in ligations of the insert to vector will not be 100% as there is more possibility of self-ligation of two ends of the plasmid. To decrease the degree of self-ligation, enzyme phosphatase is used which removes the terminal 5′-phosphate and prevents self-ligation. Another strategy to overcome self-ligation is by using two different restriction enzymes cutting sites with non-complementary sticky ends. In this way self-ligation is inhibited and also promotes correct

orientation of the insert DNA within the plasmid. The ligation of the digested insert and the plasmid is performed by pooling both in a single reaction tube and adding DNA ligase enzyme which catalyses the formation of phosphodiester bond between insert and plasmid DNAs, there by forming the recombinant DNA molecule.

Step 3: ***Transformation: transfer of recombinant plasmid DNA to a suitable host:***

The ligation reaction mixture of recombinant DNA described in the step 2 is introduced into bacterial cells in a process called transformation. The traditional method to prepare cells for transformation process is to incubate the cells in a concentrated calcium salt solution to neutralize the negative charge of membrane (due to salicylic acid), so that the negatively charged DNA molecules can come close to bacterial membrane and during heat shock (method of transformation) can easily enter in the cells. These "competent" cells are then mixed with ligation product to allow entry of the DNA into the bacterial cell. An alternative mode of transformation is electroporation method which is used to drive DNA (comparatively larger size) into cells by a strong electric current. This method is not very common due to less percentage of survival of transformed cells.

As mentioned earlier bacterial species use restriction enzymes to degrade foreign DNA lacking the methylation pattern, including the plasmids, then why don't they degrade the transformed recombinant DNA. The answer is that molecular biologist have cleverly engineered and developed the bacterial strains that lake restriction-modification system. The best example is common lab strain *E.coli* DH5α. A transformed bacterial cell may carry either recombinant or non- recombinant plasmid DNA. The plasmid DNA multiplies within each transformed bacterial cell. Each transformed bacterial cell when plated to the solid agar media (nutrient media) can multiply to form a visible colony made of millions of identical cells. As the transformed cell divides, the plasmids are passed on to progeny, where they continue to replicate. Single transformed bacteria undergo numerous cell divisions results in clones of a cell (single bacterial colony) from a single parental cell. From this step the name "cloning" is given. From the colony of bacterial cells the cloned DNA can now be isolated.

Step 4: Screening for transformed cells:

To avoid the growth of the untransformed bacterial cells, plasmid vectors are engineered with selectable marker gene for resistance to the antibiotics (Table 1). The media in which the transformed bacterial cells are grown is supplied with that antibiotic whose resistance gene is present in the plasmid. Due to this only transformed cells show antibiotic resistance will grow in the media supplied with antibiotic and untransformed cells cannot grow as they do not carry antibiotic resistance gene. Transformed bacterial cells may contain either recombinant plasmid DNA (vector containing foreign DNA insert) or non-recombinant plasmid DNA (self ligated vector only). Both type of transformed bacterial cells will show antibiotic resistance and grow on the agar media plate.

Table 1: Some commonly used antibiotics and antibiotic resistance genes.

antibiotic	Mode of action	Resistance gene
Kanamycin	Inactivates translation by interfering with ribosome function	Neomycin or aminoglycoside phosphotransferase (*neo*r) gene product inactivates kanamycin by phosphorylation
Ampicillin	Inhibits bacterial cell wall synthesis by disrupting peptidoglycan cross-linking	β-Lactamase (*amp*r) gene product is secreted and hydrolyzes ampicillin
Tetracycline	Inhibits binding of aminoacyl tRNA to the 30S ribosomal subunit	*tet*r gene product is membrane bound and prevents tetracycline accumulation by an efflux mechanism

Blue-white screening or "*lac* selection" (also called α-complementation) can be used to distinguish between recombinant transformants and non- recombinant transformants. Bacterial colonies are allowed to grow on selective media containing antibiotic and X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside), a colorless chromogenic compound. Not all plasmid vectors are engineered for "*lac* selection"; the plasmid that are engineered for blue-white screening carry a MCS site in between gene that encodes for amino acids for enzyme β-galactosidase which cleaves β-glycosidic bond in D- lactose. X-gal mimic D-lactose and β-galactosidase enzyme acts on X- gal and produces a blue color complex.

A successful ligation of the desired gene disrupts the *lac Z* gene, hence no functional β-galactosidase is produced resulting in white colonies. Hence successful recombinant transformed colonies can be easily identified by its white coloration from unsuccessful blue ones. pUC19, pBluescript, pGem-T are few example of cloning vectors used for this test and it also requires the use of specific *E. coli* host strains such as DH5α which carries the mutant *lacZΔM15* genes.

Step 5: Amplification and purification of recombinant plasmid DNA

The final step in DNA cloning is the isolation of the cloned recombinant DNA. A positive colony containing recombinant plasmid is identified and it is aseptically transferred to liquid medium and cell are allowed to grow exponentially overnight. A fully grown culture contains trillions of identical cells, which is harvested for the isolation of the plasmid DNA. The plasmid DNA is purified from harvested bacterial cell lysates. The purified plasmid DNA is dissolved in an appropriate buffer solution and can be used for further confirmation of the clone by restriction digestion and sequencing the plasmid DNA.

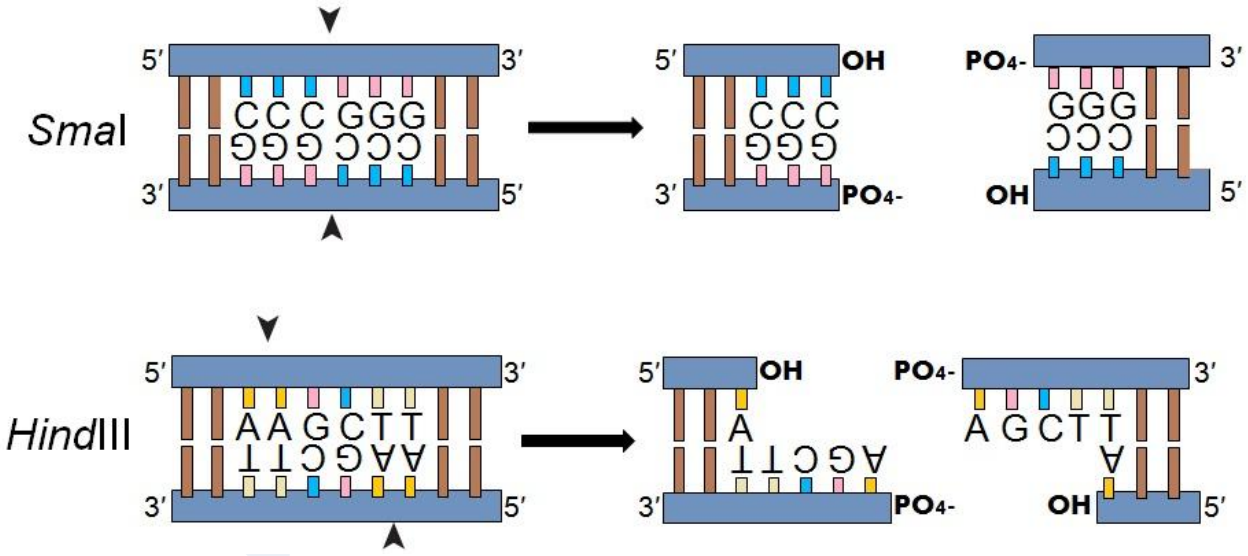

Fig 1. Cleavage patterns of *Hind*III and *Sma*I restriction endonucleases. The recognition sites and cleavage patterns of *Hin*dIII and *Sma*I are shown. Restriction endonucleases catalyze the hydrolysis of phosphodiester bonds in palindromic DNA sequences to produce double-strand breaks, resulting in the formation of 5′-PO_4− and 3′-OH termini with "sticky" ends (*Hin*dIII) or "blunt" ends (*Sma*I).

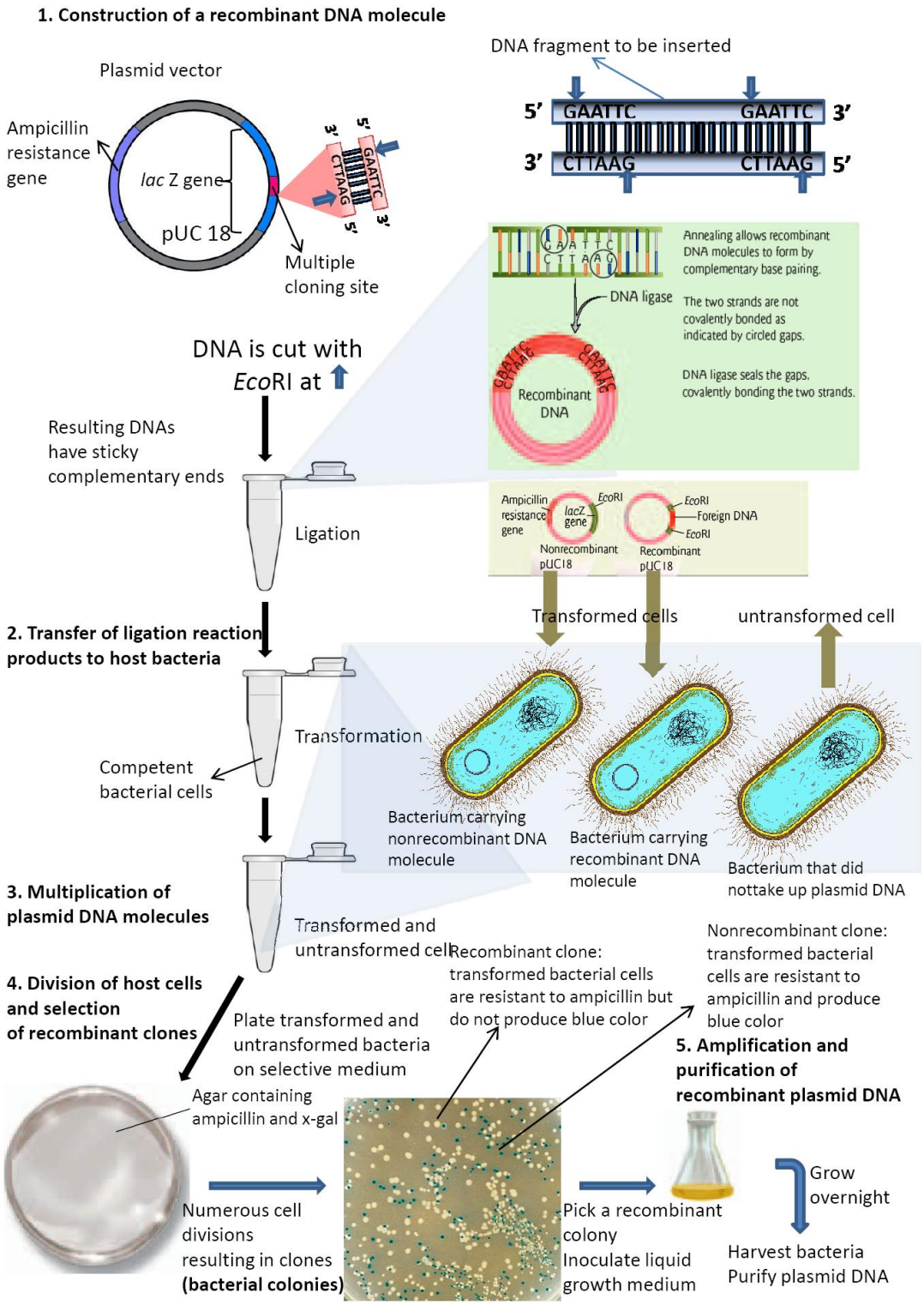

Figure 2: DNA cloning using a plasmid vector. Molecular cloning using a plasmid vector involves five major steps.

DNA cloning using a plasmid vector: Molecular cloning using a plasmid vector involves five major steps as already shown in Fig. 1 and Fig. 2

(1) Construction of a recombinant DNA molecule.

In the above figure, vector DNA (the plasmid pUC18) and the foreign DNA insert are cleaved with *Eco*RI and mixed together in a ligation reaction containing DNA ligase. Plasmid pUC18 carries the ampicillin resistance gene and has a large number of restriction sites comprising a multiple cloning site within a selectable marker gene.

(2) Transfer of ligation reaction products to host bacteria.

Competent *E. coli* are transformed with ligation reaction products

(3) *Multiplication of plasmid DNA molecules*.

Within each transformed host bacterium, there is autonomous multiplication of plasmid DNA. Each bacterium may contain as many as 500 copies of pUC18. Some bacteria in the mixture will be untransformed (not carrying either recombinant or non-recombinant plasmid DNA).

(4) *Division of host cells and selection of recombinant clones by blue-white screening.*

Bacterial cells are plated on a selective agar medium containing the antibiotic ampicillin and X-gal. If foreign DNA is inserted into the multiple cloning site, then the *lacZ′* coding region is disrupted and the N-terminal portion of β-galactosidase is not produced. Since there is no functional β-galactosidase in the bacteria, the substrate X-gal remains colorless, and the bacterial colony containing recombinant plasmid DNA appears white, thus allowing the direct identification of colonies carrying cloned DNA inserts. If there is no insertion of foreign DNA in the multiple cloning site, then the *lacZ′* gene is intact and enzymatically active β-galactosidase is produced and X-gal is degraded. The bacterial colonies containing non-recombinant plasmid DNA thus appear blue.

(5) Amplification and purification of recombinant plasmid DNA.

A recombinant colony is used to inoculate liquid growth medium. After growing the bacteria overnight, the culture is harvested, bacterial cells are lysed, and the plasmid DNA is purified from other cellular components.

Few applications of gene cloning

- *Production of recombinant proteins:* Gene of a given sequence may be expressed in bacteria. Desired affinity tag may be added with protein for simpler purification (please recall our discussion during affinity chromatography).
- *Agricultural utility*: Making transgenic crop (expression foreign gene) to boost food production.
- *Transgenic* organisms: Cloned genes may be inserted into organisms, generating transgenic species, producing pharmaceuticals and other commercially useful compounds.
- *Gene Therapy:* Gene therapy involves supplying a functional gene to cells lacking that function for correcting a genetic disorder or acquired disease.