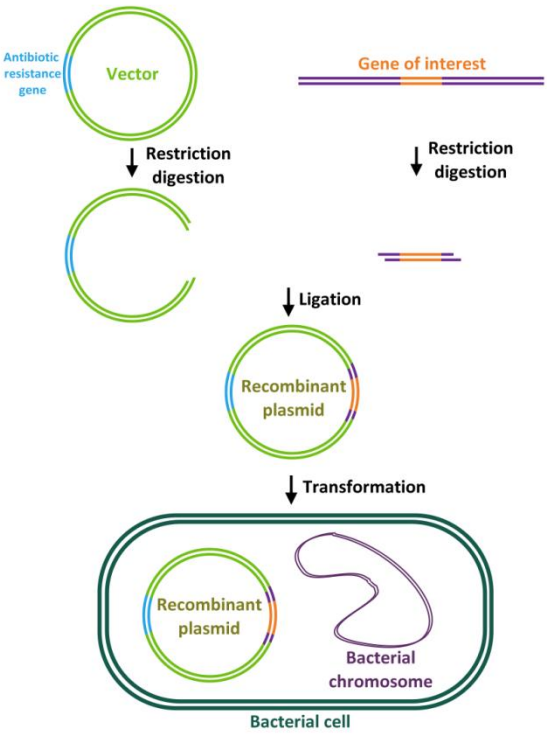
4. Ligation, Transformation, Spread plate and Site-directed mutagenesis

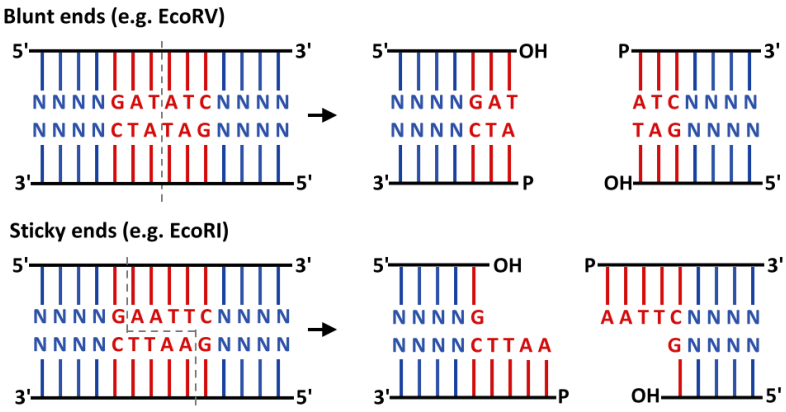
A. *T4 DNA Ligation*

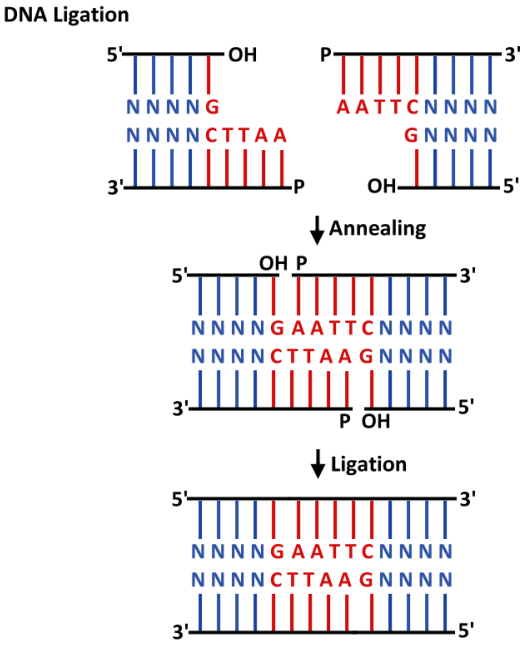


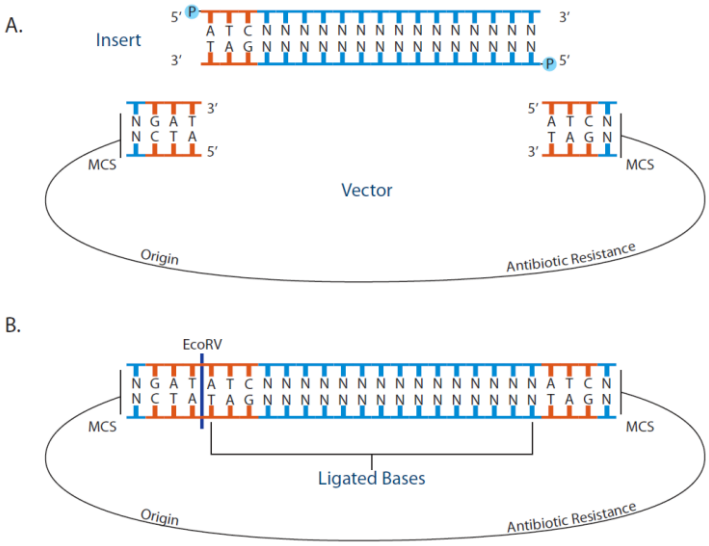
With the production of DNA fragments with joining sites from restriction digestion, a clone can be made by ligating the gene of interest with the vector (antibiotic resistant gene-containing). The reaction mixture is then transformed into competent cells. Heat shock is thought to generate pore on cell membrane and calcium ions is thought to facilitate intake of DNA due to chare-charge interaction.

T4 ligase catalyses the formation of phosphodiester bond between 3'-hydroxyl group and 5'-phosphate group with ATP (adenosine triphosphate) as energy source. However, it is non-specific to DNA sequence, choice of restriction enzymes in previous steps is important. Blunt end ligation is non-directional, while sticky end ligation after double digestion is directional. The reaction follows collision theory that the higher concentration of DNA, the higher the reaction rate. Unfortunately, self-ligation is a serious problem. The more DNA population, the more impure the products are and also the lower the yield of your target DNA.

To solve this problem, people use excess DNA insert than linear vector to lower the chance of self-ligated empty vector and to increase the yield. Cell population transformed with self-ligated inserts are removed by antibiotic selection. In reality, since sticky ends are susceptible to damage and DNA fragments may be digested if the sample is not clean, colonies may be found to have flaws or even fake (empty vector). Therefore, controls including self-ligation of single digested and double digested vector are essential as a quality check.


Blunt ends (e.g. EcoRV)
5'
3'
NNNNGATATCNNNN
NNNNCTATAGNNNN
OH
P
ATCNNNN
TAGNNNN
Sticky ends (e.g. EcoRI)
NNNNGAATTCNNNN
NNNNCTTAAGNNNN
NNNNG
NNNNCTTAA
AATTCNNNN
GNNNN


DNA Ligation
NNNNG
NNNNCTTAA
AATTCNNNN
GNNNN
Annealing
NNNNGAATTCNNNN
NNNNCTTAAGNNNN
Ligation


A.
Insert
MCS
Vector
Origin
Antibiotic Resistance
B.
EcoRV
Ligated Bases

Materials

T4 DNA ligase

10X T4 DNA ligase buffer

ddH_2O (deionized, sterile, DNase-free)

DNA with restriction cut

16 ^oC dry bath/ 4 ^oC fridge

Procedure

1. Place the reagents on ice.
2. Prepare the reaction mixture with the steps in the following table (recommended by NEB)

Total volume	**10 µl**	**20 µl**
DNA (10-200 ng) + ddH_2O	8.5	17
10 × T4 DNA Ligase Reaction Buffer	1	2
T4 DNA Ligase (400,000 units/mL)	0.5	1

* Ligation can be performed in any NEB restriction buffers if it is supplemented with 1 mM ATP. ATP decomposes quickly at room temperature. It is preferable to aliquot the buffer into 10 µl/tube, and do not re-freeze the aliquot tubes after used.

* One unit is defined as the amount of enzyme required to give 50% ligation of HindIII fragments of λ DNA (5′ DNA termini concentration of 0.12 µM, 300-µg/ml) in a total reaction volume of 20 µl in 30 minutes at 16°C in 1× T4 DNA Ligase Reaction Buffer.

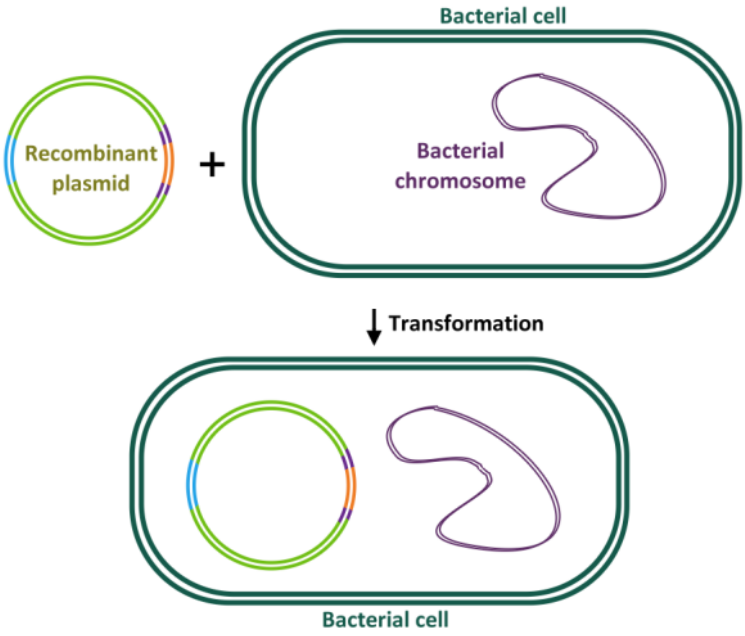
3. Allow the ligation to take place at 4 ^oC over-weekend; or 16 ^oC overnight; or 22-25 ^oC for 10 minutes.
4. 5 µl of the ligated sample should be used for agarose gel electrophoresis to confirm whether ligation has occurred.

Notes

- Insert/'Vector mass' ratio varies with protocols (1:1, 3:1, 6:1). The basic principle is the amount of insert should be more than that of vector, and the total amount should be <100 ng (Too many DNA leads to self-ligation/ inhibition of ligation).

(This video shows the procedures of setting up a ligation reaction)

B. *Bacterial Transformation*



Materials

Competent bacterial cell (*DH5α*)

42 ^{o}C water bath/ dry bath

LB medium/ SOC medium

37 ^{o}C incubator

LB plate with appropriate antibiotics

Plasmid DNA

Procedures

1. Thaw a tube of competent cells (usually 100 µL) on ice, and use as soon as possible.
2. Pipette 50 ~ 100 ng DNA (<10 µL) to the solution surface of competent cells. (The amount varies according to the efficiency of the cells)
3. Put the tube on ice for 15 – 30 min.
4. Heat shock: Put the tube at 42 ^{o}C for 45 s to 2 min.
5. Put the tube in ice for 5 min.
6. Transfer the cells to a 1.5 mL or 2 mL microfuge tube
7. Add 1 ml LB broth or SOC medium.
8. Incubate the tube at 37 ^{o}C shaker for 45 – 90 min with shaking (~ 250 rpm).
9. Spread 3 dilution of cells (10-fold serial dilution) onto the pre-warmed agar plate (with suitable antibiotics), each in 50 – 100 µl.
10. Incubate the agar plates upside down at 37 ^{o}C incubator overnight (~ 12- 16 h).
11. Wrap the plates with parafilm and store at 4 ^{o}C for further use.

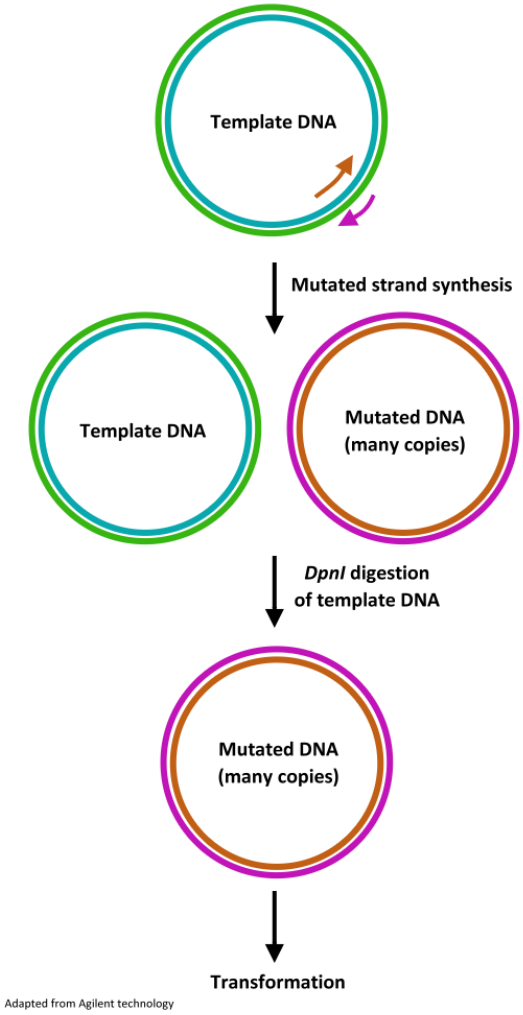
Notes

- The cells should be in 0.5 mL microfuge tube before recovery due to better heat transfer across thin wall.
- Heat shock temperature must be accurate.
- During spread plate, cool down the hot spreader to prevent it from killing the cells or degrading the antibiotics on plate.
- The plates can be stored up to two weeks without loss of plasmid DNA.

(This video shows the procedures of transformation and spread plate)

C. *Site-directed mutagenesis*

When there are illegal restriction sites in your target gene, you may want to remove it by introducing silent mutation; or one may want to make a point mutation to make a mutant protein. Traditional method Quikchange makes use of a mutant primer pair overlaps to produce mutant plasmid under thermal cycles. However, unlike PCR where the product increases exponentially, the mutant plasmid cannot be used as template for further PCR since it is nicked at different positions on the two strands, therefore the product increases linearly. It is followed by *Dpn*I digestion to remove template plasmid, which is methylated during propagation in bacterial host.



Adapted from Agilent technology

Another method developed recently by NEB is called Q5 mutagenesis kit. The magical part is on back-to-back primer design, generating nicks at the same position on both strands for exponential increase in product. By manipulating the primers, one can do mutation, insertion and deletion using this kit. The idea is to PCR nicked mutant plasmid with Q5 polymerase, then phosphorylate the 5'-end of primers, ligate them and digest out the template plasmid by *Dpn I* in a KLD reaction mixture, finally take a small portion of the product and transform.