Experiment (1): Plasmid Isolation and Purification

Aim:

- To isolate pure plasmid DNA from E. coli using alkaline lysis method.

Introduction:

The DNA of most bacteria is contained in a single circular molecule, called the bacterial chromosome. In addition to the chromosome, bacteria often contain an extra hereditary genetic element called plasmids which are small, double stranded, closed circular DNA molecules that replicate independently from a bacterial chromosome. Every plasmid has its own origin of replication (replicon) and use the enzymes and proteins that encoded by their host for their replication and transcription. Plasmid found in a wild variety of bacterial species and they are not essential for the bacterium but benefit the survival of the organism. There are three general classes for plasmids which can be advantageous for host cell: i. Virulence plasmids encoding toxin genes, ii. Drug-resistance plasmids that confer resistance to antibiotics and iii. Plasmids encode gene required for bacterial conjugation.

- PAUSE AND THINK ➔ How plasmid could contribute to drug resistance?

Plasmids have become used in different applications including molecular cloning, gene therapy, drug production and making a large amount of proteins. Plasmids are widely used as **vectors** in molecular cloning, serving to drive the replication of recombinant DNA sequences within host organisms (It is used to provide a "vehicle" in which to insert a desired DNA fragment). In the laboratory, the modified plasmids (recombinant DNA) are usually reintroduced into a host cell for replication via process called *transformation*.

- PAUSE AND THINK ➔ Is there another method than molecular cloning used for DNA amplification?

Generally, plasmid vectors should contain three important parts: origin of replication (Ori), antibiotic resistance gene and cloning site. The Ori is a DNA sequence which allows initiation of replication of the plasmid by cellular enzymes. Antibiotic resistance gene allows for selection of plasmid-containing bacteria. Cloning site is a short segment of DNA which contains several restriction sites allowing for the easy insertion of DNA (A place to insert foreign DNAs).

Isolation of plasmid DNA from bacterial cells is an essential step for many molecular biology procedures. In general, plasmid purification involved three steps: 1. Growth of the bacterial culture, 2. Harvesting and lysis of bacteria and 3. Purification of plasmid DNA. Depending upon nutritional status, bacteria exhibit different growth patterns which include:

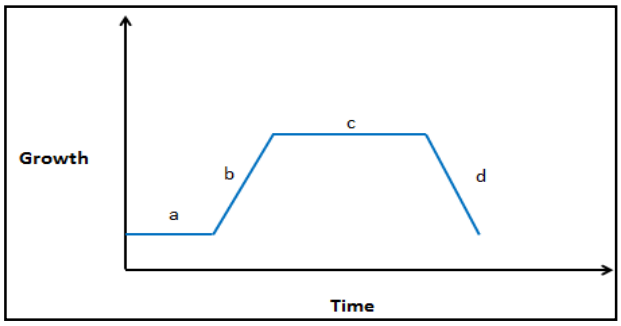

Figure.1. Bacterial culture growth curve.

a) **Lag phase:** in this phase bacteria adapt themselves to growth conditions and synthesis its own DNA, RNA and proteins.
b) **Log phase:** it is exponential phase, the bacterial cells divide and the production of new cells is proportion to increased time.
c) **Stationary phase:** the growth rate slows as nutrients become limited, waste products accumulate and the rate of cell division equals the rate of death.
d) **Death phase:** due to continuous accumulation of toxic metabolites and the lack of nutrients, death occurs of the bacteria.

PAUSE AND THINK ➔ In which phase should the bacteria take for plasmid isolation?

In the second step, the bacteria are recovered by centrifugation and lysed by any one of many methods, including treatment with detergents, alkali, organic solvents, and heat. The choice among these methods depends on three factors: the size of plasmid, the bacterial strain and the technique used to subsequently purify the plasmid DNA.

The plasmid purification procedures, unlike the procedures for purification of genomic DNA, should involve removal of not only protein but also another major impurity - bacterial chromosomal DNA. There are basic methods of plasmid preparation: chemical base lysis methods, and application of affinity matrixes for plasmid or proteins.

Principle:

In the alkaline lysis method, cells are lysed and DNA denatured by SDS and alkaline pH. The SDS will lyse the bacterial cell membrane and denature the proteins. While the alkaline pH will denature the genomic DNA and the proteins too. Neutralization of the solution results in a fast reannealing of covalently closed plasmid DNA due to the interconnection of both single-stranded DNA circles. Much more complex bacterial chromosomal DNA cannot reanneal in this short time and forms a large, insoluble DNA network, largely due to interstrand reassociation at multiple sites along the long linear molecules. At the next step of the procedure, lowering the temperature results in precipitation of protein-SDS complexes. Subsequently both complexes, DNA and protein, are removed by centrifugation leaving native plasmid molecules in the supernatant.

Alkaline lysis purification method performing steps:

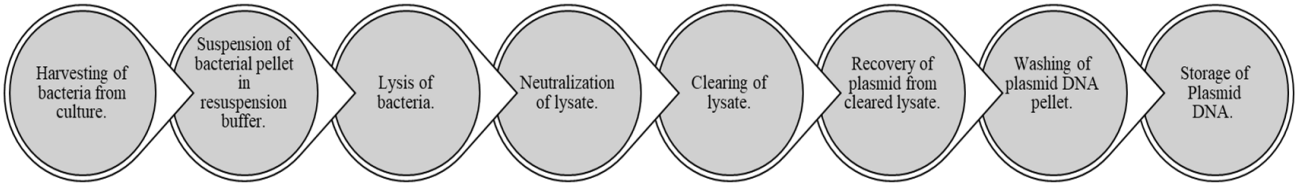

Figure.2. A typical steps of isolation procedure by alkaline lysis method.

Materials:

Chemical

LB medium, Ethylene diamine tetra acetate (EDTA), NaOH, Tris-HCl, glucose, potassium acetate, acetic acid, Sodium dodecyl sulphate (SDS), NaCl, Tryptone, Yeast extract, Tris-Cl, Ethanol.

Preparation of solutions

1) LB medium

To 950 ml of deionize H_2O add 10g Tryptone, 5g yeast extract, and 10g NaCl. Shake until the solution dissolve. Adjust pH to 7.0 with 5N NaCl. Adjust the volume to 1L with deionize H_2O. Sterilize by autoclave for 20 minutes at 15 psi on liquid cycle.

2) Alkaline lysis solution I

50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), deionized water.

3) Alkaline lysis solution II

0.2 N NaOH, 1% (w/v) SDS,deionized water.

4) Alkaline lysis solution III

5 M potassium acetate, acetic acid, deionized water.

Equipment and Glassware

Microfuge centrifuge, electronic balance, microcentrifuge tube, centrifuge tube, Pasteur pipette, micropipette, tips.

Protocol:

1. Centrifuge the bacterial samples at 4 °C, maximum speed for 5 minutes, using microcentrifuge device.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Resuspend the bacterial pellet in 100 µl of ice cold alkaline lysis solution I then vortex vigorously.
4. Add 200 µl of freshly prepared alkaline lysis solution II to the bacterial suspension. Invert the tube rapidly 5 times. Store the tube on ice for 1 min.
5. Add 150 µl ice cold alkaline lysis solution III to the microcentrifuge tube. Invert the tube 3-5 times, then incubate the tube on ice for 3-5 minutes.
6. Centrifuge the bacterial lysate at maximum speed for 3 minutes.
7. Transfer the supernatant to a new labelled microcentrifuge tube.
8. To the tube, add 2 volumes of 95% ethanol.
9. Vortex and allow the tube to stand at room temperature for 2 minutes.
10. Centrifuge at maximum speed for 5 minutes.
11. Remove the supernatant by gentle aspiration.
12. Stand the tube in an inverted position over a paper towel to allow all fluid to drain away.
13. Add 20 µl of 70% ethanol, then invert the closed tube several times.
14. Centrifuge at maximum speed for 5 minutes.
15. Remove the supernatant by gentle aspiration.
16. Remove any beads of ethanol from the sides of the tube. Leave tube open at room temperature until residual ethanol has evaporated.
17. Dissolve the pellet in 25-50 µl sterile water or TE buffer and vortex the solution gently for few seconds.
18. The plasmid DNA can be stored at -20 °C.

Results:

- Concentration of plasmid DNA (ng/µl) = _____
- Plasmid purity: A_{260}/A_{280} = _____

References:

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