Quadrant II – Transcript and Related Materials

Programme: Bachelor of Science (Third Year)

Subject: Botany

Course Code: BOC 109

Course Title: Molecular Biology and Genetic Engineering

Unit: Methods of Gene Transfer

Module Name: Selection of Transformants

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Notes

Introduction:

After introducing recombinant DNA into the host cells, it is important to identify those cells which receive recombinant DNA molecule. This process is called screening or selection. This selection of recombinant DNA cells depends on expression or non -expression of certain characters. The vector or foreign DNA present in the recombinant cells expresses certain characters or traits, while non-recombinants do not express the traits. The methods for screening or selection of recombinant clones include:

1. Direct selection method

Direct Selection is when the desired gene specifies antibiotic resistance. Consider an experiment to clone the gene for ampicillin (amp^R) resistance from plasmid. If the cloned DNA itself codes for resistance to the antibiotic (amp^R) the recombinants are allowed to grow on minimal medium containing ampicillin. The recombinants which contain amp^R on its plasmid vector will grow and produce colony on the medium. After this procedure one cannot tell whether the recombinants growing on such medium contain religated plasmid vector or recombinant plasmid and foreign DNA fragment. Because amp^R gene is present in both the recombinants.

2. Insertional inactivation selection method

Insertional inactivation is the inactivation of a gene after insertion of another gene in its place or within its coding sequence. In this method, one of the genetic traits is disrupted by inserting foreign DNA. Antibiotic resistance gene serves as a good insertion system. Plasmid pBR322 contains two antibiotic resistance genes, one for ampicillin (*amp*^{R} gene) and the other for tetracycline (*tet*^{R} gene) If the target DNA is inserted into *tet*^{R} gene using an enzyme BamHI, the property of resistance against tetracycline will be lost. When recombinants containing target DNA in *tet*^{R} gene are grown on the medium containing tetracycline, they will not grow because their *tet*^{R} gene has been inactivated. But these recombinants will be resistant to ampicillin because *amp*^{R} gene is functional.

Self-ligated recombinants show resistance against tetracycline and ampicillin and therefore, they can grow on medium containing tetracycline as well as ampicillin. In order to confirm the presence or absence of *tet*^{R} gene in the inserted DNA fragment in plasmid a replica plating is done from the master plate. Bacterial colonies on master plate are gently pressed with sterile velvet, a few cells of each colony may adhere on it which is then pressed on other plate containing the nutrient medium amended with tetracycline. Plates are then incubated for the growth of bacterial colonies. The appearance of the colonies is compared with the master plate and the colonies which fail to grow on replica plate is considered to possess a plasmid which contains insert DNA in the *tet*^{R} gene of plasmid which destroyed *tet*^{R} gene and therefore, *tet*^{R} gene became non-functional.

3. Blue-white screening method

It is a powerful method for screening recombinants. In this method a reporter gene *lacZ* is inserted in the vector. The *lacZ* encodes enzyme β-galactosidase which contains several recognition sites for restriction enzymes. β-galactosidase breaks a synthetic substrate, X-gal (5-bromo-4-chloro-indodyl- β -D-galacto-pyranoside) into an insoluble blue coloured product. If a foreign gene is inserted into *lacZ*, this gene will be inactivated. Therefore, no blue colour will develop, β-galactosidase is not synthesised due to inactivation of *lacZ*. The host cells containing recombinant DNA will form white coloured colonies on the medium containing X-gal. The host cells which contain non recombinant DNA will form

the blue colored colonies. On the basis of colony colour the recombinants can be selected from the agar plate.

4. Colony hybridization technique

This technique was developed by M. Grustein and S. Hogness in 1975. The transformed cells grow on nutrient medium, but it is possible that among several thousand cells, very few cells may have specific DNA as desired. To pick up those cells which has desired DNA the "colony hybridization technique" is used. This technique is suitable when plasmids are used as vectors.

a. DNA probes method: The colony hybridization technique is based on the availability of a radiolabelled DNA probe. A DNA probe is the radio-labelled ($^{32}PO_4$) small fragment of DNA molecule (20-40 bp) which is complementary to atleast one part of desired DNA. Probe is labelled with $^{32}PO_4$ or sometimes with ^{125}I. The $^{32}PO_4$ liberates β – particles and ^{125}I liberates λ- rays. In addition to radioactive isotopic elements, isotopic sulphur or fluorescent molecules can also be used. Therefore, a DNA probe recognises A, T, G and C nucleotides and combines with the complementary sequences of the target DNA. A and T of the probe combine with T and A of target DNA and *vice versa*. Similarly, G and C of probe combine with C and G of target DNA and *vice versa*. The small sized DNA probe easily catches target DNA for hybridization than the long-sized probe. High rate of hybridization is achieved by maintaining higher concentration of ^{32}P-DNA than the target DNA molecules. The DNA probe is very specific to target DNA and this specificity is called stringency. The probe may be partially pure mRNA, a chemically synthesized oligonucleotide or a related gene, which identifies the corresponding recombinant DNA.

b. Colony or Plaque Hybridization Technique:

Colony hybridization is applied to DNA or RNA released from blotted microbial colonies. In this technique master plates of microbial colonies are prepared. Replica plating of colonies is done onto a nitrocellulose filter disc which is placed on the gelled nutrient medium. Master plate and replica disc are incubated to develop colonies. Cells growing on nitrocellulose filter disc gets nourishment through the diffusion of nutrients from gelled nutrient medium. The filter disc is removed and put on blotting paper soaked with 0.5 N NaOH solution. The cells

are lysed to release the nucleic acids. It is neutralised by tris (hydroxymethyl) aminomethane-HCl buffer by maintaining high salt concentrations. Then the filter is dipped in proteinase K solution which removes proteins from the filter. Due to this binding of DNA with nitrocellulose disc takes place in the same pattern as the bacterial colonies. In order to fix the cDNA properly, the disc is baked at 80^{o}C.

After that it is incubated with a solution containing radioactive chemical labelled probe (P^{32} DNA) at suitable conditions. The probe will hybridise any bound DNA which contains sequences complementary to the probe. Blocking reagent may be added prior to the probe to prevent unspecific binding. Excess probe is washed away and the membrane is visualized by autoradiography of the nitrocellulose filter disc. Colonies which develop positive x-ray image are compared with master plate and picked up, and are multiplied on the nutrient medium.

c. Plaque lifting method:

This technique was devised by W.D. Benton and R.W. Davis in 1977 to screen bacteriophages from plaque forming units. The recombinant phage particles are isolated and used to construct library. A lawn of *E. coli* is prepared on agar medium and allowed to get infected by recombinant phage particles. They infect *E. coli*, multiply inside cells and lyse them forming plaques. Replica plates are prepared from the master plates containing plaques by using nitrocellulose filter paper. The filter is treated with alkali solution to denature phage DNA. Then filter is baked at 80^{o}C to fix denatured phage DNA and it is transferred in a solution containing ^{32}P-DNA probe. The probe hybridises the desired DNA when complementary bases are present. The filter paper is washed with standard saline citrate solution to remove unhybridized probe and autoradiographed. Dark spots are formed where probes have hybridised the desired DNA. Then the recombinant phage particles can be isolated from the plaques.

5. Immunological tests

Instead of radio-labelling of DNA molecules, antibodies are used to identify the colonies or plaques developed on master plates which synthesize antigens encoded by the foreign DNA present in plasmids of the bacterial clones. A special

vector, known as expression vector, is designed where foreign DNA is transcribed and translated within bacterial cell. The growth medium containing specific anti-serum help in detection of viable immunoprecipitate. The method consists of following steps:

a) replica plating of bacterial colonies of master plate on nutrient agar,

b) lysis of cells after their growth by exposure to chloroform vapour or treatment with high temperature.

c) Making gentle contact with a solid support (cellulose filter paper) containing immobilized antibody to solid support with the lysed cells within the colonies to allow absorption of antigen to antibody.

d) detection of antigen antibody complex by incubating the cellulose filter paper with a radio-labelled second antibody.

e) the antibodies which do not react are washed off.

f) the determination of position of the antigen-antibody complex by passing the filter paper through X-ray. It gives the signal of those bacterial cells which synthesize antigen on the master plate.