Transfection of DNA into Rat 2 cells to produce stable cell lines

Calcium Phosphate Method

Plasmid DNA is introduced into monolayers of Rat 2 cells via a precipitate that adheres to the cell surface. The method uses calcium chloride in a HEPES buffered solution to form a calcium phosphate precipitate that the DNA binds to, which is then directly layered onto the cells.

Solutions:

2X HeBS (HEPES Buffered Saline to make 50 mL	
280mM NaCl	2.8mL 5M NaCl
10mM KCl	0.12mL 4M KCl
12mM dextrose	0.1gm dextrose
50mM HEPES	0.5gm HEPES
1.5mM Na2HPO4	75uL 1M Na2PO4

Adjust pH to 7.05* with 1M NaOH, filter sterilize through a 0.22um filter. Store at room temperature.

* the pH is extremely critical and directly affects the formation of the precipitate, make sure the pH meter is properly calibrated.

2.5M CaCl2 use the dihydrate salt, make up 50ml and filter through a 0.22um filter. Store at room temperature.

Method

For optimum precipitate formation the DNA should be at a concentration of 20ug/ml and you will need 1ml precipitate for one 10cm dish of cells. If you do not want to use 20ug of your test plasmid (and 1ug is often enough) then the quantity of DNA can be made up to 20ug by adding carrier DNA, either salmon sperm DNA or some inert plasmid DNA.

It is also important to include a positive control: usually the expression vector without your gene inserted as a check that the transfection did work; and a negative control: carrier DNA with no expression plasmid to ensure that the selection criterium you are using will kill all the cells that are not expressing the antibiotic resistance gene.

Day1:

1. Split confluent dishes of Rat 2 cells: 1dish into 3 so that the cells will be near confluence when the precipitate is added.

2. Mix your DNA samples (plasmid + carrier) and ethanol precipitate overnight at -20oC. eg 20ul DNA + 200ul 0.3M NaOAc + 500ul ethanol. If you are unsure of how much DNA to put into the transfection try 2 or 3 different amounts, eg 1ug, 5ug, 10ug.

Day2: (a.m.)

1. Check that the cells are OK before proceeding further.
2. Spin down the DNA samples, wash with 70% ethanol and place in the laminar flow hood to air dry. All manipulations from here are done in the hood to maintain sterility
3. Dissolve the DNA pellets in 450ul sterile 18mê water, pipette up and down but don't vortex.
4. Dispense 500ul 2 X HeBS solution into sterile 5ml polycarbonate tubes (Falcon #2058).
5. Add 50ul 2.5M CaCl to the DNA samples, mix by pipetting up and down.
6. With a P1000 pipetteman draw up the DNA solution and add it dropwise to the 2 X HeBS. Mix by blowing air from the pipette tip and by pipetting up and down.
7. Let sit at room temperature for 15 to 20 mins. a fine precipitate should be visible at this point.
8. Using a pasteur pipette add the precipitate dropwise over the surface of the dish. Do not remove the medium. Swirl very gently to distibute the precipitate over the dish. Place in the incubator for 4 to 5 hours.

Day3: (p.m.)

1. After 4 hours look at the cells. The precipitate should be visible, stuck to the cells. If they still look OK leave them for another 30 mins and check again. Rat 2 cells are very susceptible to the toxic effects of the calcium phosphate precipitate, but the longer the precipitate stays on the better the transfection efficiency although obviously too much cell death leads to lousy transfection efficiency. I have found 4.5 hours to be a good length of time for our Rat 2 cells,
2. Remove the medium, wash twice with 5mls PBS, twice with 5mls PBS + 1mM EGTA (this helps to chelate the calcium).
3. Add back 10mls complete medium but without seletion antibiotic, incubate overnight so the cells can recover somewhat.

Day4:

To select for cells that have taken up and integrated a plasmid with a selectable marker the medium must now be changed to complete medium including the selection antibiotic eg G418 (Geneticin, Gibco-BRL) at 400ug/ml. If you have suffered significant cell loss from the transfection procedure and the cells are about 50% confluent or less then just change the medium. If, however, the cells are still almost confluent it is advantageous to trypsinize the cells (see TC - 5) and replate the whole dish onto 2 fresh dishes with complete medium + G418. This ensures that resultant colonies grow up well spaced and therefore easier to pick.

The selection medium is changed every 3 to 4 days for 2 to 3 weeks until large colonies of cells are clearly visible with the naked eye. Then they can be picked, expanded and assayed (see TC - 12)