TECHNIQUES IN MOLECULAR BIOLOGY – BACTERIAL TRANSFORMATION

The uptake of exogenous DNA by cells that alters the phenotype or genetic trait of a cell is called transformation. For cells to uptake exogenous DNA they must first be made permeable so the DNA can enter the cells. This state is referred to as competency. In nature, some bacteria become competent due to environmental stresses. We can purposely cause cells to become competent by treatment with chloride salts of metal cations such as calcium, rubidium or magnesium and cold treatment. These changes affect the structure and permeability of the cell wall and membrane so that DNA can pass through. However, this renders the cells very fragile and they must be treated carefully while in this state. Competent cells vary in how well they take up DNA. We can express this The amount of cells transformed per 1 µg of DNA is called the **transformation efficiency**. Too little DNA can result in low transformation efficiencies, but too much DNA also inhibits the transformation process. Transformation efficiencies generally range from 1×10^4 to 1×10^7 transformed cells per µg of added DNA.

E. coli bacteria are normally poisoned by the antibiotic **ampicillin**. Ampicillin inhibits synthesis of the bacterial cell wall (in bacteria like *E. coli* , found between the inner and outer cell membranes), resulting in bacteria that are very structurally weak. In the hypotonic media in which these cells grow, cells exposed to ampicillin will swell and burst or not grow at all. For cells to survive, they must include a means to break down the ampicillin. The plasmid has an additional gene coding for an enzyme, **β-lactamase**, that is secreted by cells and in a local area will hydrolyze the ampicillin. Therefore, by adding ampicillin, only bacteria that contain the plasmid will survive. We also need to be sure not to allow our transformed *E. coli* to become overgrown. If the colonies on the LB plates are large they will break down enough ampicillin so that bacteria without the plasmid will survive and form satellite colonies in the surrounding region of inactivated antibiotic. Another commonly used antiobiotic is **kanamycin**. This drug blocks protein synthesis by binding irreversibly to the ribosome and preventing translation.

An Important Point When Transforming cells – Depending on which strain of bacteria you are using (for simplicity we either us a strain to maintain DNA or a strain(s) better suited for protein expression) and how they were made competent, there may be slight but important differences in using the cells for transformation. Therefore it is important to first check that you are using the proper protocol. DO NOT LET CELLS WARM UP! This will allow the holes in the cells to seal and your experiment will not work!

Antibiotic	Stock Concentration	Storage	Working Conc (dilution)
Ampicillin (Sodium Salt)	50 mg/ml in water (500X)	-20° C	100µg/ml (2 µl of stock/ml)
Chloramphenicol	34 mg/ml in EtOH (200X)	-20° C	170 µg/ml (5 µl of stock/ml)
Kanamycin	25 mg/ml in water (500x)	-20° C	50 µg/ml (2 µl of stock/ml)
Streptomycin	10 mg/ml in water (200X)	-20° C	50 µg/ml (5 µl of stock/ml)
Tetracycline HCl	5 mg/ml in EtOH (100X)	-20° C	50 µg/ml (10 µl of stock/ml)

Most transformation protocols can be divided into these four major steps:

1. ***Preincubation***: Cells are suspended in a solution of cations (such as Ca^{++}) and incubated at 0° C. The cations are thought to complex with the negatively-charged phosphates in membrane lipids in the bacterium. The low temperature congeals the cell membrane, stabilizing the distribution of charged phosphates and allowing them to be more effectively shielded by the cations.
2. ***Incubation***: DNA is added, and the cell suspension is incubated further at 0° C. The cations again are thought to neutralize charged phosphates – this time in the DNA backbone – allowing DNA to adhere or at least be close to the *E. coli* cell membrane.
3. ***Heat shock***: The cell-DNA mix is rapidly heated to 37°– 42° C for a short period and then returned to 0° C. The rapid temperature change creates a thermal imbalance on either side of the cell membrane, creating pores and a draft that sweeps the plasmid into a small percentage of cells.
4. ***Recovery/Outgrowth***: Nutrient broth is added to the transformation mix which is then incubated at 37° C (ideally with shaking) before plating on selective media. Transformed cells recover from the treatment, amplify the transformed plasmid, and begin to express the plasmid's antibiotic resistance gene before encountering the antibiotic.

Other Methods of Transformation

E. coli can also be transformed (replacing the heat shock) with a brief but intense electrical pulse in what is termed ***electroporation***. [For example: brief – 5 millisec, intense – 10,000 V/cm.] Very high transformation efficiency can be achieved using specialized equipment (an ***electroporator***), typically with disposable cuvettes

outfitted with electrodes. Preparation of **'electro-competent' cells** is quite different from that of classically prepared 'chemically competent' cells, although mid-log phase cells are still typically used. These cells must be in a very low conductivity medium (few ions) such as 1 mM buffer with glycerol. Electroporation apparently does not depend on DNA binding to the cell surface. The recovery period is particularly important following electroporation.

Variants of Competent Cell Preparation and Transformation

The 'rapid colony method' of competent cell preparation and transformation omits using mid-log phase cells and a preincubation. Colonies on a plate are picked and resuspended in $CaCl_2$, and DNA added immediately. Depending on the antibiotic used for selection, a prolonged recovery period can also be omitted. Therefore, the procedure is fast, and virtually foolproof for transformations not requiring high efficiency. Transformation efficiencies achieved with the rapid colony method (5×10^3 – 5×10^4 transformants per μg supercoiled plasmid DNA), however, can easily be ~200 times less than those with cells prepared by more labor-intensive classical methods (5×10^4 – 10^6 transformants per μg DNA). The rapid colony method is perfectly adequate for transformation with purified, intact plasmid DNA at high concentration. The method is wholly inadequate for transformations with manipulated DNA (such as following ligations of linearized vector and insert DNAs in a traditional cloning experiment). These molecules, consisting of relaxed circular DNAs, are 5 – 100 times less efficient at transforming cells than the same quantity of intact, supercoiled plasmid DNA.

Transformation Parameters are Important

When performing a transformation, it is important to follow directions very carefully, as transformation efficiency can be affected by many parameters. This is particularly true of the heat shock, which is typically optimized taking into account the cell type, volume of transformation mix, tube type (shape, material, thickness), length and temperature of the shock. Heat must be transferred rapidly throughout the cell mix to deliver a sharp shock. Cells should be kept on ice at all times prior to the heat shock and transferred directly from ice to the water bath, and back to ice during the procedure.

Antibiotic Selection

Ampicillin (**Amp**) is a practical, inexpensive antibiotic for routine selection of transformed cells, although some prefer a sturdier (and more expensive) equivalent antibiotic such **carbenicillin**. Amp interferes with construction of the peptidoglycan layer in the cell wall but kills only replicating cells that are assembling new cell membranes. Therefore, if necessary, a recovery step can be omitted, although it is better to include one. When using other antibiotics, such as **Kanamycin** (**Kan**), a recovery step is required before plating transformed cells. Kanamycin – which blocks bacterial translation by irreversibly binding the 30S subunit of the ribosome – can kill both replicating and non-replicating cells that are not expressing the resistance protein.

Satellite Colonies

If plates are incubated too long following a transformation (particularly when using an antibiotic like Amp), small 'satellite' colonies will appear that surround larger transformed colonies. In time, these can grow as large as the original transformed colonies, and can arise more quickly on plates with many transformants. These are non-transformed, nonresistant cells that grow in the 'antibiotic shadow' where Amp has been broken down by the large resistant transformed colonies (the Amp resistance protein is secreted by the cells). Initially, these colonies will be tiny, but will grow with time. Do not include tiny colonies (particularly surrounding a large colony) in your count of transformants. It is best to score transformation plates in the morning following transformation before satellite colonies appear or become large.

<u>Materials & Equipment</u>

Ice bath	Bacterial loop or hockey stick	Bunsen burner
Plasmid DNA (0.01 - 1.0 mg/ml)	SOC liquid growth media	Tube racks
37°C and 42°C water baths	Ethanol in beaker (1 cm deep)	LB agar/Amp/Carb/Kan plates
37°C incubator	tube floaties	

General Workflow / Protocol for traditional competent cells

- 20 - 100 μl of competent cells (ICE ICE ICE) into prechilled, labeled tubes.
- QUICKLY, mix 1-5 μl of plasmid DNA (10 pg to 100 ng) and mix by tapping of "flicking" the tube with your finger.
- Incubate the mixture on ice for 20-30 min.
- Heat shock at 42° C for 45 sec, immediately place back in ice bucket and incubate for 2 min
- Add 250 μl of SOC and incubate at 37° C with shaking for 45 min. This is called the outgrowth step.
- Plate cells onto appropriate antibiotic containing LB Agar plates. Because it is difficult to predict the level of transfection (cell health, plasmid quality and quantitity, overall technical handling and size of plasmid) it is best to spread two plates with a low volume (10-50 μl) and a high volume (200-250 μl) of outgrown cell culture.
- Spread with hockey stick, beads or inoculating loops. Ensure the entire culture is evenly spread over plate. You are NOT trying to isolate streak in this procedure. Give plates a few min for liquid to seep into agar.
- Incubate plates at 37° C overnight with the agar upside down.
- After ~12-16 hours, remove plates from incubator, seal with parafilm and store at 4°C upside-down.

Lab experimental design:

Plasmid Preparation

- If the plasmid concentration is known, dilute plasmid with water (mol bio grade) to 0.05 mg/ml (aka 0.05 μg/μl).
- Use 1 μl of diluted DNA for each transformant. Calculate and record the mass (ng) of DNA used for the transformation.
- If the plasmid concentration is unknown, use 1 μl of DNA.
- Record the antibiotic resistance and other information on your plasmid in your notebook.

Tranformation

- Record the type of compenent cells in the notebook - what cell strain, level of competent cells, if commercial name of company and information on the preparation of cell strain.
- For this class, you will conduct two transformants - one transformant plated for the appropriate antibody (two volumes - you decide how much for each) and the other will be divided equally onto an LB Agar plate without antibioitic and another LB plate with an incorrect antibiotic.

Your Procedure/Experimental Design.

- Using the information in this handout and the recorded information on your cell strain, outline a protocol in your lab book. Check with your instructor before beginning the experiment.

Notes:

SOB is Super Optimal Broth - a rich broth supporting cell growth that contains glucose. SOC is an SOB broth with glucose. Full name is Super Optimal broth with Catabolite repression. In this case, glucose serves to ensure the cells use glucose for metabolism leaving other energy sources (amino acids and lipids) for protein expression. Use of SOC will ensure the antibiotic selective protein can be expressed in transformed cells. Without SOC, much of the cell resources needed for protein expression will be used for metabolism instead

Outgrowth (aka Recovery) step - important for antibiotic resistance. Transformed cells must express protein to survive in antibiotic medium/agar plate. In a pinch, Amp or Carb resistant cells can be directly plated onto antibiotic containing agar. This is because Amp and Carb act by inhibiting enzymes which create the cell wall but will not stop the cell from making protein needed to degrade the antibiotic. HOWEVER, Kan acts by inhibiting translation and thus cells will not produce any protein. Cells transformed with Kan resistance plasmids thus need the outgrowth/recovery period to produce the protein neomycin phosphotransferase II (encoded by the *neo* gene). This is the enzyme that degrades Kan. BUT the cell must make the protein from the newly transformed plasmid before the degredation of Kan can happen. Thus outgrowth is more important for Kan than Amp/Carb resistant plamids. ... the more you know!!!

Some competent cell preparations (lots of variety on the commercial market) will allow you to skip a heat shock, ice or even outgrowth step (be careful with Kan). You must read the individual protocol for all commercial steps to know what is going on and what can be safely skipped.

No transformants? Check the antibiotic resistance. Double check the DNA concentration and quality. Degraded or non-supercoiled plasmid will not transform as efficiently. If the amount of DNA used was high (0.1 to1 μg or higher), the transformation can be less efficient than if using a lower amount of cells. Also if new or not sure what is happening, use a positive control - a plasmid that works well, often supplied with commercial preparations.

Post-Lab work

1. Count colonies on transformation plates the next day and calculate the transformation efficiency (see below). Score your plates in the morning - the later you check, the more satellite colonies will grow and make it difficult to distinguish between transformants and satellites. If for some reason you do not have time in the morning, at least remove the plates from the incubator and leave at room temperature until you can count them. (The colonies will grow more slowly.) Place plates in a drawer rather than on the bench since Amp is light-sensitive.

To aid counting large numbers of colonies (>100), use a Sharpie pen to mark colonies with a dot on the bottom of the plate as they are counted. Be careful not to miss colonies that are obscured by writing on the bottom of the plate or are at the edges of the plate.

For plates that appear to have >200 colonies, assuming a fairly even spread of colonies, divide the plate in half (or quarters) with a ruler and a Sharpie. Count one half (or one quarter) of the plate and multiply by 2 (or 4) to estimate the total number of colonies. If you use this method, indicate that you have done so in your report.

2. Check your positive and negative control plates for the expected results. For example, a control plate with competent cells on LB plate (no selection) should be a "lawn." This is especially important if your transformation did not work as expected. If so, these plates may provide clues as to what went wrong.

3. After counting colonies on your plates, discard all plates in the autoclave bag.

Calculating Transformation Efficiency

a. Determine the *total mass* (in μg) of plasmid DNA used in the transformation:

concentration of DNA (μg/μl) x volume (μl) = mass (μg)

b. Determine fraction of transformation mix spread on the plate

volume of mix spread / total volume of transformation mix = fraction

[Do not include any volume of LB you added at the last minute simply to help spread the cells on the plate.]

c. Determine mass of DNA in volume of mix spread on plate

total mass of DNA x fraction

d. Transformation efficiency is expressed in scientific notation as the number of colonies (transformants) per μg of supercoiled plasmid DNA.

Complete formula

$$\text{T.E.} = \frac{\text{no. of colonies (transformants)}}{\text{Fraction (of transf. mix vol spread)} \times \text{total mass of DNA } (\mu g)}$$

or

$$\frac{\text{no. of transformants}}{[\text{ vol spread } (\mu l) / \text{ total vol of mix } (\mu l)\ ] \times \text{ conc of DNA } (\mu g/\mu l) \times \text{ vol of DNA } (\mu l)}$$