

Exercise 5

Bacterial Transformation and Plasmid Recovery

INTRODUCTION

When Frederick Griffith observed the conversion of *Pneumococcus* colonies from 'rough' to 'smooth', he did not realize that the change was due to the uptake of DNA from the dead 'smooth' cells by the live 'rough' cells. Even Avery and his co-workers, when they showed that the transforming agent was DNA, did not foresee the significance of this technique. This process, transformation, has become one of the key techniques in genetic engineering. Transformation can be defined as the uptake of exogenous, or foreign, DNA by a recipient cell and the insertion of that DNA into the recipient cell's genome, resulting in a new and heritable trait.

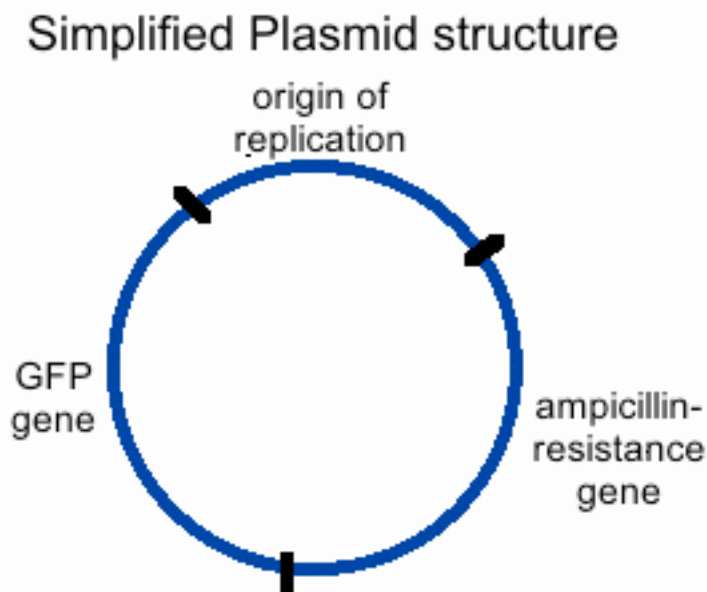
Bacterial cells must be in a particular physiological state before they can be transformed. This state is referred to as competency. Competency can occur naturally in certain species of *Haemophilus* and *Bacillus* when the levels of nutrients and oxygen are low. Competent *Haemophilus* express a membrane-associated transport complex, which binds and transfers certain DNA molecules from the medium into the cell where they are incorporated and their genes are expressed. In nature, the source of the external DNA is cells that have died and released their DNA.

Much of the current research and experimentation in molecular biology involves the transformation of *E. coli*. However, this organism does not enter a stage of natural competency. *E. coli* can be artificially induced to enter competency by treating the cells with the chloride salts of the metal cations calcium, magnesium and rubidium. In addition, sudden cycles of heat and cold help to bring about competency. The metal ions and temperature changes affect the structure and permeability of the cell wall and membrane so that DNA molecules can pass through. Competent *E. coli* cells are fragile and must be treated carefully.

The number of cells transformed per 1 microgram of DNA is called the transformation efficiency. In practice, much smaller amounts of DNA are used (5 to 100 nanograms) since too much DNA inhibits the transformation process. In research laboratories, transformation efficiencies generally range from 1×10^4 to 1×10^7 cells per microgram of DNA. There are special procedures that can produce cells having transformation efficiencies approaching 10^{10} . Transformation is never 100% efficient. Approximately 1 in every 10/000 cells successfully incorporates the DNA in preparations having average competency. However, there is such a large number of cells

in a sample (typically 1×10^9) that only a small fraction needs to be transformed to obtain colonies on a plate. These ideas can be demonstrated by plating the same volume of recovered cells on selective and non-selective agar media. The nonselective media will have many more growing cells. In this case, all the untransformed cells also survive. The bacterial agar plates will be covered heavily with untransformed cells forming a "lawn", in contrast to individual colonies. Because such a small percentage of even competent cells ever take up DNA, one of the problems facing geneticists is how to identify and keep the rare transformed cells, while getting rid of the rest.

The most common solution to this problem is to use plasmids. A plasmid is an extrachromosomal, circular piece of DNA that usually has very specific, and useful, properties. Plasmids naturally exist as supercoiled molecules. The two strands of DNA in the supercoiled molecule are wound and folded around each other in a way that produces a condensed, entangled structure when compared to relaxed (non-supercoiled) DNA. Competent *E. coli* are sensitive to the conformation of the DNA they will accept. Supercoiled DNA gives the highest transformation efficiencies. Most plasmids used nowadays have been genetically engineered to contain some very useful features:



The origin of replication tells the bacterial cell that this piece of DNA should be copied. In most plasmids this causes the cell to make dozens or even hundreds of copies of the plasmid. This type of plasmid is called a 'high-copy-number' plasmid.

The ampicillin-resistance gene makes the bacterium resistant to the antibiotic. The gene codes for the enzyme β -lactamase that destroys ampicillin. The enzyme is secreted into the cell's environment, so that the antibiotic is destroyed before it even enters the cell. Cells that do not take up the plasmid remain sensitive to ampicillin. After transformation the cells are grown in the presence of ampicillin. Only those cells that contain the plasmid can grow, all the remaining cells are killed by the antibiotic. (Note: there may be an exception to this. If a cell secretes enough β -lactamase, then it creates an ampicillin-free zone around itself. If so, then cells that are sensitive to the antibiotic may grow in this area. These are called 'satellite' colonies.)

The plasmid used in today's exercise contains another gene, one that codes for the production of 'green fluorescent protein (gfp) produced by the bioluminescent jellyfish *Aequorea victoria*. For more information, check <http://faculty.washington.edu/cemills/Aequorea.html>. A bright burst of light is observed when energy is transferred to the green fluorescent protein, which is located in specialized photogenic cell located in the base of the jellyfish umbrella. Although this family of proteins had been known for some time and significant research in this area had been reported, it is only recently that fluorescent proteins have been cloned and expressed both in prokaryotic and eucaryotic cells. These proteins do not require substrates, other gene products, or cofactors. When exposed to long or short U.V. light they will emit a bright green light that is clearly visible in bacteria that are transformed by plasmids that contain gfp. Likewise, purification of gfp from crude proteins extracts is simplified by their detection based on fluorescence. There are many examples of chimeric proteins that are fusion products using the gfp fluorescent proteins as biological tags. Such fusions are at either the N- or C- terminal and often result in no biological activity loss in the heterologous partner or in fluorescence. These new biotechnology tools have made possible studies that deal with protein localization and trafficking within cells. The green fluorescent protein (gfp) has 238 amino acid residues and a molecular weight of approximately 40,000 daltons.

In today's exercise you will first isolate a plasmid from a culture of *E. coli* that was previously transformed with a gfp-containing plasmid. The plasmid DNA will then be run on an agarose gel, and visualized using ethidium bromide. The second part of the experiment is to infect (transform) *E. coli*

cells with a gfp-containing plasmid. After they have grown up for a few days, you will estimate the efficiency of the transformation.

PLASMID MINIPREP

ALKALINE SDS METHOD

Cells that already contain the gfp plasmid have been grown in nutrient broth containing ampicillin. A culture of this is available for the next step: Isolating plasmids from transformed cells and identifying the plasmid DNA on an agarose gel.

The protocol used to extract plasmid DNA from bacterial cell suspensions is based on the alkaline lysis procedure developed by Birnboim and Doly (Nucleic Acids Research 7:1513, 1979). The procedure takes advantage of the fact that plasmids are relatively small, supercoiled DNA molecules while bacterial chromosomal DNA is much larger and less supercoiled. This difference in topology allows for selective precipitation of the chromosomal DNA and cellular proteins from plasmids and RNA molecules. The cells are lysed under alkaline conditions, which denatures both nucleic acids and proteins, and when the solution is neutralized by the addition of potassium acetate, chromosomal DNA and proteins precipitate because it is impossible for them to renature correctly (they are so large). Plasmids renature correctly and stay in solution, effectively separating them from chromosomal DNA and proteins.

Procedure

Note: The procedure below is used to make duplicate minipreps. This provides balanced tubes for the centrifuge as well as twice as much product when you are finished. Each group should follow this method.

1. Gently swirl the contents of the culture tube to resuspend the cells.
2. Pipet 1000 μ L of the cell suspension into each of two 1.5 mL Eppendorf tubes.
3. Close the caps and place the tubes in a centrifuge (remember to balance the centrifuge by putting the tubes opposite one another) and spin at maximum speed for 90 s.

In this step, the bacterial cells will form a pellet at the bottom of the tube.

4. Withdraw and discard the supernatant using a pipettor, being careful not to disturb the cell pellet. Discard the supernatant in a waste container.

5. Add 100 μL of Buffer 1 (50 mM Tris-HCl, 10 mM EDTA, 100 $\mu\text{g}/\text{mL}$ RNAase A, pH 8.0) to each tube and resuspend the cells by vortexing. It's very important that the cell suspension is homogenous and no clumps are visible.

The cells are now resuspended in a buffered solution with RNAase. When the cells are lysed in the next step, the RNAase will catalyze hydrolysis of all RNA molecules into nucleotides, but the DNA will not be affected.

6. Add 200 μL of Buffer 2 (1% SDS, 0.2 M NaOH) to each tube. Close the caps and mix the solutions by rapidly inverting them a few times. DO NOT VORTEX since the chromosomal DNA released from the broken cells could be sheared into small fragments and contaminate your plasmid prep.

SDS is an acronym for Sodium Dodecyl Sulfate. It is an ionic detergent, which disrupts cell membranes and destabilizes all hydrophobic interactions holding various macromolecules in their native conformation. The high pH of the 0.2 M NaOH also denatures macromolecules by changing the condition of ionizable groups (ionizing certain groups and deionizing others). The clearing you see is because the cells are lysing. The viscosity of the solution is increased by the increase in concentration of macromolecules in solution (a result of the cell lysis).

7. Let tubes stand on ice for 5 minutes (it's OK if they go longer).

8. Add 150 μL of ice-cold Buffer 3 (3.0 M potassium acetate, pH 5.5) to each tube. Then add 2 μL of 'DNA pellet paint'. Close the caps and mix the solutions by rapidly inverting them a few times. A white precipitate will form.

This is really the key step in the alkaline lysis procedure. The low pH of the potassium acetate solution neutralizes the NaOH and when the pH returns to near-neutrality then the macromolecules renature. The proteins and large DNA molecules do not renature correctly however. They form hydrophobic, ionic and hydrogen bonds with each other nonspecifically because the correct conformation of the molecule was not maintained during denaturation. The plasmid DNA molecules, however, never really fully denatured because they are small circular molecules, which are supercoiled. Even though the hydrogen bonds between base pairs were broken by the high pH, they reform correctly when the pH is lowered. The large DNA molecules (chromosomal DNA) and proteins form precipitates because they

bind to each other in a large aggregate but the plasmids don't precipitate because they renature correctly and don't become part of the large multi-molecule aggregates. Thus plasmid DNA remains in solution while proteins and other DNA molecules precipitate.

9. Let tubes stand on ice for 5 minutes (it's OK if they go longer).
10. Place the tubes in a centrifuge (balanced) and spin at maximum speed for 5 minutes. The precipitate will form a white pellet along the side of the tube.
11. Transfer the supernatants to clean 1.5 mL Eppendorf tubes, being careful not to pick up any of the precipitate. **Discard** the tubes with the precipitate and **KEEP the tubes with the supernatant**.
12. Before you do this step, make sure there is a centrifuge available. To each tube of supernatant add an equal volume (about 400 μ L) of isopropanol to precipitate the nucleic acids. Close the caps and mix vigorously. Let the tubes stand at room temperature for 2 minutes, place them, with their hinges pointing outward from the center, in a centrifuge (balanced) and spin at maximum speed for 5 minutes. This step pellets the nucleic acids but if you leave it around too long, proteins remaining in solution will begin to precipitate as well.

This concentration of isopropanol will cause the DNA to become insoluble. Since DNA molecules are ionic (uniform negative charge due to the phosphates) they are highly soluble in water but not soluble in organic solvents. Isopropanol and ethanol are commonly used to precipitate DNA from aqueous solution.
13. The reason that you oriented the tubes with the hinges outward is because the plasmid DNA pellet may be difficult to see. It will be at the bottom and along the hinge side of the tube. The addition of 'DNA paint' in step 8 (above) should color the pellet pink and make it readily visible. Carefully remove and discard the supernatant without disturbing the DNA pellet.
14. Add 200 μ L of 95% ethanol to each tube and mix by inversion several times.
15. Spin the tubes at maximum speed in a centrifuge for 2-3 minutes (hinges out).

This "ethanol wash" will speed up the process because the next step is to evaporate the alcohol used in the precipitation step. Ethanol evaporates much faster than a solution of 50% isopropanol.

16. Carefully remove and discard the supernatant. Try to get as much out as possible without dislodging the pellet of plasmid DNA.

17. Leave the tubes with the caps open for 15-20 minutes to dry off the last traces of ethanol. To speed up the evaporation of the ethanol, the tubes can be aerated using a plastic squeeze-bulb pipet as a bellows.

18. When the ethanol is gone (you can check this by smelling the tube) add 20 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to dissolve the pellet. Pipette the 20 μL in and out, up the side of the tube to ensure that all of the plasmid DNA comes into contact with the TE buffer.

TE buffer is commonly used to redissolve DNA because it contains EDTA. The EDTA will chelate magnesium ions, which are a cofactor for most nucleases (enzymes which degrade nucleic acids). If your DNA prep becomes contaminated with a nuclease (like the ones produced by the cells in your skin) then the nuclease will be inactivated by the fact that the magnesium cofactor is unavailable in the solution (because it is chelated by the EDTA).

19. Pool the two 20 μL solutions into one labeled tube

20. Add 10 μL loading buffer to the sample and mix. When ready, load 20 μL of the mixture into two of the wells prepared in the next part.

Loading buffer contains four ingredients. 1:TE. 2:glycerol, to make the solution dense, so it will sink into the well. 3/4: two dyes that will move fast, and separate, so you can see how far the run has progressed.

DNA ELECTROPHORESIS

1. Seal the ends of the gel tray with the rubber dams and insert the comb.

2. Add 0.24 g agarose to 30 mls TAE buffer (40mM Tris-acetate pH 7.6, 1 mM EDTA) and microwave on high power for 30 seconds. Allow the flask to cool for a minute or two, then repeat until the agar is fully dissolved.

3. Allow the agarose to cool to 50-55°C, then pour the mixture into the gel tray. Let the agar solidify for ~15-20 min.
4. Carefully remove the rubber ends from the gel tray and remove the comb. Place gel tray in the buffer reservoir, and add TAE buffer until both wells are filled and gel is covered with buffer. Be sure the gel doesn't slide off the tray.
5. Load plasmid DNA-loading buffer samples (20 μL) into two wells. Load 5-10 μL of the DNA molecular weight markers in a separate well.
6. Run the gel at 70 volts for 45 minutes. Transfer the gel to a Ziploc bag containing 10 mls (0.1mg/ml) ethidium bromide solution which binds to the DNA and which will fluoresce bright orange in UV, thus showing you where the DNA is in the gel. Leave the bag on the rocker for 15 minutes. Pour the ethidium bromide solution into the labeled waste and add 10 mls distilled water to the bag. Agitate gently for 15 minutes.
7. Examine the gel under UV light. Note the number of bands in your plasmid prep and measure the distance they have traveled. Use the standard molecular weight markers to estimate the size of the plasmid DNA.

TRANSFORMATION WITH PGFP

- 1) Label two Eppendorf centrifuge tubes as '+DNA' and 'Control'.
- 2) Place 1 mL of the cell culture into each of two tubes and spin at 14,000 x g for 5 minutes. Pour off all the supernatant.
- 3) Add 250 μL 50mM CaCl_2 to each of the tubes. Resuspend the pellets completely and place the tubes on ice for several minutes.
- 4) Using a sterile tip, add 10 μL of the plasmid DNA solution to the '+DNA' tube.
- 5) Incubate the two tubes on ice for 10 minutes (longer is OK).

6) While the cells are on ice: label three plates as follows:

LB / Control

LB-AMP / Control

LB-AMP / + DNA

7) Place the tubes with the bacteria into a 42°C water bath for 50 seconds.

8) Return the tubes to the ice for 2 minutes.

9) Add 250 µL LB broth to each tube and leave them at room temperature for 15 minutes.

10) Pipette 100 µL from the control tube onto the two plates labeled 'Control' and spread the liquid evenly over the surface with a sterile loop.

11) Pipette 100 µL from the +DNA tube onto the plate labeled +DNA and spread the liquid evenly over the surface with a sterile loop.

12) When the plates are dry, invert them, tape them together and label with your group's initials. Incubate plates overnight at 37°C.

Questions to be considered: Hand in your answers next week

1) Assuming you used 1 ng of plasmid DNA, what was the transformation efficiency?

2) Provide two explanations for the growth of cells on the LB-AMP/+DNA plate but not showing fluorescence. How would you determine which explanation is correct?

3) While ampicillin-resistance by production of the β -lactamase enzyme is the commonest selective marker for transformation success, there are several others that are used. Describe one of these and explain how it works at the molecular level

4) all of the plasmid molecules you extracted are the same size, yet sometimes several bands are seen in the gel. How can this be explained

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