

Exercise 22-A

CALCIUM CHLORIDE PROCEDURE FOR MAKING COMPETENT CELLS

Introduction

Although **transformation**, the transfer of DNA from dead donor cells to live recipients occurs in nature, the efficiency with which this process occurs appears to be quite low. In order to use transformation in genetic manipulation, investigators needed to develop techniques to increase the efficiency of the process. It was discovered that cells suspended in the early middle portion of their **logarithmic growth phase** (mid-log phase) were more likely to take in DNA than cells in other phases of growth. It was also found that cells treated with ice-cold **calcium chloride** (or other divalent cationic solutions) and subjected to a brief heat shock at 42° C were better able to take in DNA. Such cells were said to be **competent** for transformation. The cold temperature of the calcium chloride is essential, as this causes the fluid cell membrane to stabilize. The cations present then bind with the phosphate groups of phospholipid molecules, shielding these negative charges. Since DNA is a negatively charged particle, it is now attracted to the cell surface, and can enter the cell. When a population of cells is treated in the manner described above, only about 1/10 of all the viable cells present become competent. The limiting factor in transformation then is the number of cells available to take up DNA rather than the quantity of DNA present. In fact, excess plasmid DNA tends to decrease the efficiency of the transformation process.

In this laboratory, students will be provided with cells in the early mid-log phase of growth and will make them competent using the calcium chloride procedure. To obtain the required amount of mid-log culture, samples of the *E. coli* host strains (JM83 and DH5 α) are inoculated into tubes of tryptic soy broth (TSB) and allowed to grow over night. Approximately 2.5 to 3 hours prior to the start of the laboratory, 1 ml samples of these cultures are aseptically transferred into flasks containing 50-100 ml samples of sterile TSB, and the flasks are placed on an orbital shaker in the 37° C incubator. Cells being prepared for the transformation exercise are transferred directly from these flasks into centrifuge tubes.

Materials:

- Mid-log cultures of *E. coli* host strains JM83 and DH5 α .
- 50 mM calcium chloride (CaCl₂) pre-measured into 5 ml aliquots
- Sterile 15ml plastic centrifuge tubes
- Sterile 10ml pipettes and pipette bulbs
- 100-1000 μ L digital pipettes and tips (blue)
- Clinical centrifuge
- Ice buckets and crushed ice

Procedure: (Students will work in pairs or groups of three.)

1. Obtain two or three tubes containing 50 mM calcium chloride (CaCl₂) and place these on ice. Note that the calcium chloride solution has been pre-measured into 5ml aliquots.
2. Obtain two or three sterile centrifuge tubes and **Aseptically** add 10ml of the mid-log culture provided to each one. Students on tables 1 and 3 will use *E. coli* strain JM83, and students on tables 2 and 4 will use *E. coli* strain DH5 α .

3. Make certain the tube caps are closed securely and then label the tops with a permanent marker to indicate table # and culture identity (do not use tape).
4. Place these tubes in the centrifuge provided being careful to **balance the rotor**. The samples will be centrifuged at approximately 2,000 rpm for 10 minutes. This will pellet the cells at the tube bottoms.
5. When centrifugation is complete, remove your tubes from the rotor and pour off the supernatant (TSB) into the flask of disinfectant provided (front bench). Be careful not to disturb the pellet of cells at the tube bottom (do not tap tubes against the flask mouth).
6. Pour **one** of your 5ml aliquots of ice-cold calcium chloride into each centrifuge tube and then re-suspend the cells by vortex mixing. Be careful to break up any cell clumps as only cells exposed directly to the calcium chloride can be made competent.
7. Place all centrifuge tubes in the ice bath provided and allow them to remain there for at least 20 minutes.
8. Return the tubes to the centrifuge (balance the rotor). This time the samples will be centrifuged for 5 minutes at 2000 rpm. The treated cells will again form a pellet at the bottom of each tube.
9. Pour off the supernatant (CaCl_2) into the flask of disinfectant provided (front bench), being careful not to disturb the pellet of cells.
10. **Aseptically** add 1ml (1000 μL) of fresh, ice-cold calcium chloride to each centrifuge tube using a digital pipette and sterile tip (the same pipette and tip can be used for all tubes as long as the tip is not allowed to touch any contaminated surface between measurements).
11. Vortex mix the cells to re-suspend them in the fresh CaCl_2 . Again it is important that the cells be thoroughly mixed with the calcium chloride and any cell clumps broken up.
12. Return the centrifuge tubes to the ice bucket, as they are now competent for transformation. If the cells are not going to be used immediately they can be stored on ice in the refrigerator. Transformation efficiency may actually increase if the cells are stored in this manner for up to 24 hours.

**** STOP POINT ****

Exercise 22-B

TRANSFORMATION OF *E. coli* WITH PLASMID DNA

Introduction

In natural settings, bacteria are able to exchange genetic material and thus increase their genetic variability in a number of ways. One method of genetic exchange is called **transformation** (DNA mediated transformation), and involves essentially the transfer of "naked" DNA from dead donor cells to living recipient cells. Genetic exchange as it occurs in nature is restricted to bacteria that are closely related to one another (usually within the same species), and does not usually occur at high frequencies. Cells that are "**competent**" (in the logarithmic phase of growth) undergo transformation most readily.

Scientists have developed techniques that greatly increase the frequency with which bacteria will pick up "naked" DNA and so undergo transformation. These techniques are employed by microbiologists and other investigators involved in recombinant DNA research and in genetic manipulation for industrial purposes. Since bacteria that are competent will readily pick up plasmid DNA, and because plasmid DNA can easily be removed from cells and manipulated, it is frequently used in transformation procedures.

The plasmids used for this exercise (pUC19, pGEM and pGLO) are all the products of recombinant DNA technology, and do not occur in nature. These plasmids carry genes called **marker genes** that encode resistance to the antibiotic ampicillin (among other things), and so allow for the detection of transformed cells on media containing this antibiotic. The gene providing ampicillin resistance (*Bla*) encodes **β -lactamase**, an enzyme catalyzing the hydrolysis of β -lactam antibiotics (penicillins and cephalosporins).

The pGLO plasmid also carries portions of an inducible operon involved in regulating the utilization of arabinose. The promoter and operator sites of this operon retain their normal function, but the structural genes have been replaced with a gene encoding **green fluorescent protein** (GFP) originally produced by the fluorescent jellyfish, *Aequoria victoria*. Cells carrying the pGLO plasmid will grow on media containing ampicillin, but when placed on media containing ampicillin plus arabinose, will also produce green fluorescent protein. Colonies composed of cells carrying pGLO plasmids can readily be distinguished from colonies composed of cells carrying pGEM, by exposing plates to ultraviolet light. The colonies expressing green fluorescent protein will appear to be glowing green.

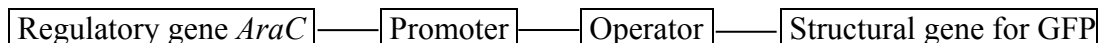


Fig. 22B.1 - Operon controlling arabinose utilization and expression of GFP.

Regulation of the operon shown above involves a regulatory gene (*AraC*) that encodes a **repressor protein** (*AraC* protein). When this protein binds to the **operator** site of the arabinose utilization operon, transcription of the GFP gene is repressed (transcription is OFF). When arabinose is present, it serves as an **inducer**, inactivating the repressor (*AraC* protein), and allowing transcription to proceed (the operon is ON). Since the expression of GFP is strongly regulated by the repressor (*AraC* protein), green fluorescent protein can only be produced when colonies have formed on media containing arabinose. The genes encoding β -lactamase and *AraC* protein are both controlled by **constitutive promoters**, i.e., they are not repressible. Genes encoding the enzyme **L-arabinose permease** (required for the transport of arabinose through cell membranes) are associated with more than one operon in *E. coli* cells, so the absence of this gene from the operon shown does not prevent arabinose uptake.

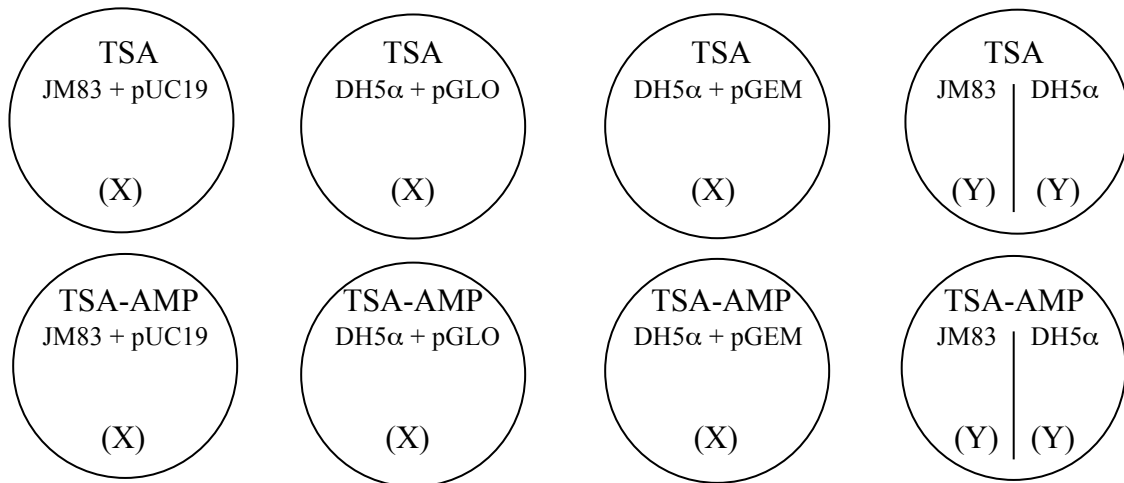
In this exercise students will add plasmid DNA to samples of calcium chloride treated (competent) cells, and then expose the cell suspensions to a brief heat shock (42° C for 90 seconds). The heat shock is reported to make competent bacterial cells take up DNA more efficiently, and thus increase the success of transformation. Samples of cultures that have been subjected to transformation will then be spread on the surfaces of agar plates containing ampicillin, an antibiotic that will inhibit the growth of non-transformed cells. Incubation of the plates at 37° C will allow colonies to form, and transformed cells to be detected. Plates containing arabinose and inoculated with cells carrying pGEM and pGLO plasmids will be exposed to ultraviolet light, allowing any green fluorescent protein present to be observed.

Materials:

Competent *E. coli* cells: (JM83 and DH5 α)
Plasmid samples (pUC19, pGEM and pGLO)
Luria, Nutrient or Trypticase Soy agar plates containing Ampicillin (for selection of transformants)
Luria, Nutrient or Trypticase Soy agar plates and broth (without ampicillin)
42° C temperature block or water bath
10-100 μ L (yellow) and 100-1000 μ L (blue) micropipettes and sterile tips
Ice buckets and ice

Procedure: (Students should work in pairs or groups of three.)

1. Obtain two tubes of competent cells, one containing *E. coli* strain JM83 and the other *E. coli* strain DH5 α . This will require a tube exchange between tables 1 and 2 and between tables 3 and 4. **Make certain all competent cells are maintained within the ice baths provided.**
2. Obtain your samples of plasmid DNA (pUC19, pGEM and pGLO) prepared during the mini-screen exercise, and place these tubes on ice.
3. Obtain **two** clean, sterile centrifuge tubes, one blue and the other green or orange (class choice). Close these tubes by pushing each cap against the tube mouth, and mark the top of each cap Y (no plasmid).
4. Aseptically transfer one 200 μ l sample of JM83 competent cells into the blue microfuge tube containing your pUC19 plasmid DNA, and mark the top of this tube X (plasmid). Transfer another 200 μ l sample of JM83 competent cells into your blue microfuge tube labeled Y (no plasmid). Place these tubes on ice.
5. Aseptically transfer one 200 μ l sample of DH5 α competent cells into your green tube containing pGLO and another 200 μ l sample into your orange tube containing pGEM. Mark the tops of these tubes X (plasmid). Transfer another 200 μ l sample of DH5 α competent cells into your orange or green microfuge tube labeled Y (no plasmid). Place these tubes on ice.
6. Obtain four tryptic soy agar (TSA) plates, four TSA-ampicillin (TSA-AMP) plates and one TSA-ampicillin plus arabinose (TSA-AMP-Arab) plate. Divide the eight TSA and TSA-AMP plates into four pairs each containing one TSA plate and one TSA-AMP and label them as indicated below.



7. Label the TSA-AMP-Arabinose plate with the appropriate medium label and indicate that it will be inoculated with cell samples carrying the plasmids pGEM and pGLO (X).
8. Remove all tubes containing competent cells from the ice and "heat shock" the cells by **immediately** placing the tubes in a 42° C water bath for 90 sec. (It is essential that the cells be given a sharp and distinct shock, so move the tubes directly from the ice bucket to the hot water bath.) After the heat shock, return all tubes to the ice.
9. Inoculate each TSA and TSA-AMP plate labeled (X) with 50µl of the cell and plasmid type indicated on the plate label. Be sure to use a new tip for each different plasmid type.
10. Inoculate each half of the plates labeled (Y) with 25µl of the cell and plasmid type indicated on the plate label. Be careful to keep your pipette vertical during inoculation so the inoculum does not roll across the surface to the wrong side of the plate.
11. Inoculate the TSA-AMP-Arabinose plate with 25µl of DH5α with pGEM and 25µl of DH5α with pGLO. These samples will be mixed on the plate surface, so it is not necessary to divide the plate in half or to keep the samples separated.
12. Using a glass spreader as instructed, spread the inoculum on each plate so that the entire surface is covered. **Important note** – The spreader must be sterilized between each pair of TSA and TSA-AMP plates containing different cell and plasmid types. Be careful not to overlap the two cell strains along the center of the two (Y) plates. Avoid exposing cells to hot glass.
13. Place all nine plates in a stack (bottom-side-up), tape them together, and place them in the 37° C incubator until the next laboratory session.
14. During the next laboratory session, observe and compare your plates, count colonies if present, and look for phenotypic variation (satellite colonies). Record your data and results.
15. Expose the *E. coli* DH5α colonies carrying pGLO plasmids (TSA-AMP and TSA-AMP-Arabinose plates) to ultraviolet light of a wavelength appropriate for making green fluorescent protein visible (this observation will occur in the prep lab). Compare the colonies present on these two plates, and record the data and results.
16. Compare colonies expressing GFP genes with colonies expressing lux genes.

Questions:

1. What are competent cells?
2. Why does treatment with ice-cold calcium chloride increase the efficiency of transformation?
3. What percentage of cells in a bacterial population are usually made competent by treatment with ice cold calcium chloride?
4. What is transformation? Why is plasmid DNA frequently used in transformation procedures?
5. The plasmids pUC19, pGEM and pGLO carry marker genes that encode resistance to what type of antibiotic?
6. Were more bacteria able to grow on the TSA with ampicillin plates, or on the TSA plates? Why would you have expected these results?
7. If a 100 μ L sample of log phase *E. coli* cells typically contains about one million (1×10^6) cells, approximately what percentage of your competent cells were transformed?
8. How do *E. coli* colonies expressing GFP genes differ from *Photobacterium* colonies expressing lux genes? What optical characters are being displayed by these colonies?

Name _____

Lab Section _____

WORKSHEET
Exercise 22A and 22B
Competent Cells and Transformation

Goals: _____

Materials & Methods:

A. Calcium Chloride Procedure for Making Competent Cells

Date: _____ *E. coli* strain used: _____

Cells were made competent (circle one):
A) immediately before use or B) prior to my class time

If you chose B, how long have they been on ice? _____

B. Transformation of *E. coli* with plasmid DNA

Transformations were set up as follows:

	<i>E. coli</i> strain	Plasmid
Tube 1		
Tube 2		
Tube 3		
Tube 4		

Following the transformation procedure, 50µl of each were plated as follows:

	Type of Medium	Incubation Temperature	Duration of Incubation
Tube 1			
Tube 2			
Tube 3			
Tube 4			

Data & Results:

Transformation	Type of Medium	Number of Colonies	Were any colonies green in UV?

Conclusions:

Based on your data, are *E. coli* JM83 or DH5 α (without plasmids) resistant to ampicillin? _____ Explain.

Were the transformations successful? _____ Explain. _____

Did any *E. coli* become transformed with pGLO? _____ Explain. _____
