

Protocol: Producing Competent Cells and Plasmid Transformation

SAFETY NOTE: before you begin, remember that we are handling live bacteria and all of our sterile technique and safety procedures **MUST** be followed. In addition, we will be using alcohol and open flame in this lab. *Be very careful!* Keep the alcohol and the flame separated and keep extra paper and other materials away from your work area.

PROCEDURAL NOTE: It is very important to keep the competent cells **cold** (except during heat shock). Chill the tubes before adding the bacteria and remove the tubes from ice only long enough to do your tasks.

A. Producing Competent Cells

1. Before lab, bacteria were grown to log (or exponential) phase and 10 mL of bacteria were aliquotted into 15 mL sterile plastic tubes. Each student will use one tube to prepare competent cells. For this experiment, we are using the DH5-alpha strain of *E. coli*.
2. Label the tube with your initials. Pellet the cells using the tabletop centrifuge. The caps must be removed from the tubes because the lid of the centrifuge will not close otherwise. Spin the cells at 7000 x g for 10 minutes. This should correspond to ~4000 rpm.

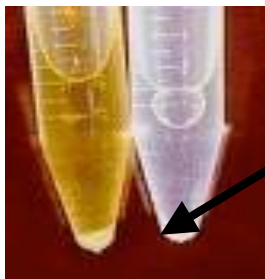


Figure 4.3:

A cell pellet similar to the one that we will obtain.

NOTE: A small but firm beige cell pellet should be visible after centrifugation. If the pellet appears loose then spin the tube for another 5 minutes.

3. Carefully pour off the supernatant into the liquid waste container. Hold your tube with the pellet up and pour away from the pellet. Use a quarter of a KimWipe to catch the last drops of liquid and dispose in solid waste. Recap the tube. **NOTE:** Watch the cell pellet carefully! Stop pouring and spin again if the pellet starts to move with the supernatant.
4. Sharply tap the tube several times on the bench top to loosen the cell pellet. Use a sterile 10 mL pipette and an automatic pipettor to add 5 mL of cold CaCl₂ to the pellet. Follow your instructor's directions for using the automatic pipettor.
5. Thoroughly resuspend the cells in the cold CaCl₂ solution. You should have no visible cell clumps when finished. Repeated pipetting with a

P1000 or using the vortex mixer will help to break up the cells. *The finished cell suspension should be homogeneous with no visible particles.*

6. Put the tube on ice and incubate for at least 20 minutes. Your instructor may direct you to work on other parts of the lab during the incubation.
7. After incubation, spin the cells again for 5 minutes at 5000 x g (2500 rpm). Carefully take your tube out of the centrifuge and examine the cell pellet. The cells are now coated with Ca^{+2} and are *fragile*. Note how the pellet is more diffuse and moves up the side of the tube. The cells *must* be treated more gently now.
8. Carefully pour off the supernatant into the liquid waste container as we did in step 4. Again, hold your tube with the pellet up and pour away from the pellet. Use a quarter of a Kim Wipe to catch the last drops of liquid.
9. Use a P1000 to add 1 mL of cold CaCl_2 or “magic slime” solution. Gently wash the sides of the tube with the solution to loosen all of the cells. Again, we must thoroughly resuspend the cells but this time we cannot be rough – NO vortex.
10. The cells are now competent. Keep them on ice while you prepare the glass test tubes for transformation.
11. DO NOT throw away the competent cells you do not use. Everyone has made more cells than they will use. These cells will be used by the next lab section or frozen for use next semester, so put all the extra competent cell tubes into one designated ice bucket.
12. **New concepts:** Seasoning; when freshly-made competent cells are kept in an ice bucket in the refrigerator (4°C) for 12-24 hours, they *increase* in competence 5- to 10-fold. After that, they begin to lose competence and eventually die. Magic slime; this solution contains glycerol and CaCl_2 . Cells can be frozen at -80°C and then used for up to 6 months. They “come back to life” when thawed – it’s magic!

B. Plasmid Transformation

1. Label a glass test tube with $\text{DH5}\alpha/\text{pLIG}$ (our cloning experiment). You may be directed by your instructor to also perform one of the control transformations:
 - plasmid control (labeled $\text{DH5}\alpha/\text{pGEM}$ or your control plasmid)
 - competent cells only (labeled $\text{DH5}\alpha$ only)
2. Using a P200, add 200 μL of competent cells to each tube. Add the cells directly to the bottom of the tube. Then put the tubes immediately on ice.

3. Use a P20 to add 10 uL of the ligation mix (pLIG) directly into the cell suspension in the glass test tube with DH5 α /pLIG.
4. Close the cap and tap the tube lightly with your fingers to mix the solution. Be gentle, no bubbles. Put the tubes on ice for at least 20 minutes.
5. During the incubation times, label plates as follows depending on the samples you are assigned:

experiment, use LB-Amp plates

DH5 α /pLIG 50 uL, your initials, the date
DH5 α /pLIG 100 uL, your initials, the date

control plasmid, use LB-Amp plates

DH5 α /pGEM3 (or your control plasmid), 50 uL, your initials, the date
DH5 α /pGEM3 (or your control plasmid), 100 uL, initials, date

competent cells, use LB and LB-Amp plates

DH5 α only, 50 uL, your initials, the date - on an LB plate
DH5 α only, 50 uL, your initials, the date - on an LB-Amp plate

6. Spread X-gal solution on the plates. Add 40 uL of 20 mg/mL X-gal solution to the plate with a sterile tip. Read Section C, #2-6, "Spreading Plates".
7. Use a sterile spreader to evenly distribute the X-gal over the entire surface of the agar. This is important! Mark the plate with a blue stripe (for X-gal).
8. After the incubation on ice, prepare the cells for transformation. *The cells must receive a sharp and distinct **heat shock**.*
9. Verify that the water bath is registering 42°C. Gather all of the glass test tubes into one test tube rack.
10. Take the test tubes in the rack and an ice bucket to the 42°C water bath. Put the entire rack into the water bath for EXACTLY 1 1/2 minutes and then IMMEDIATELY put the test tubes in the ice bucket.
11. The tubes must remain on ice for 2 minutes. Then sterilely add 800 uL of LB broth to the cell suspension. Tap the tubes lightly with your fingers to gently mix the solution.
12. Incubate the tubes at 37°C with gentle shaking for at least 45 minutes.
13. **New concepts:** X-gal used as the substrate for the blue-white screen. Recovery period; during the incubation period after the heat shock, the cells repair the damage done to the cell membrane. Cells that have taken

up a plasmid have time to *express* the antibiotic resistance gene on the plasmid.

C. Spreading Plates

1. Use a P200 with a sterile tip to add the indicated amount of cell suspension from the proper test tube to the center of the plate. *Do not let the cell suspension sit on the plates for long before spreading.* The will be absorbed into the agar as concentrated spots.

cell/plasmid	plate	volume
DH5 α /pLIG	LB-Amp-X-gal	50 uL
DH5 α /pLIG	LB-Amp-X-gal	100 uL
DH5 α /pGEM3 (or your control plasmid)	LB-Amp-X-gal	50 uL
DH5 α /pGEM3 (or your control plasmid)	LB-Amp-X-gal	100 uL
DH5 α comp. cells	LB-Amp-X-gal	50 uL
DH5 α comp. cells	LB-X-gal	50 uL

2. Sterilize the cell spreader in the beaker of ethanol, and then **carefully** burn off the alcohol *away from the flame*. **DO NOT ignite the alcohol with the flame.** Review the procedures to smother an alcohol fire and fire safety.
3. Lift the lid like a clamshell wide enough to use the spreader. Cool the spreader by touching it to the agar.
4. Touch the spreader to the cell suspension and gently spread the suspension around the plate. You must spread until **all** of the liquid is *absorbed* into the agar, or you will not get isolated colonies.
5. Continue until all the plates have been spread. If the spreader collects soot, it can be wet with alcohol and the soot removed with a Kim Wipe.
6. Sterilize and flame the spreader when finished with spreading. Collect the completed plates and incubate at 37°C with agar side up.
7. Assist in *lab cleanup*: dispose of solid waste, wipe down your bench area with Confligt disinfectant. Put the tubes with bacteria on the rack by the sink and squirt in 30% bleach. **Do not** bleach the competent cells. Wash your hands with antibacterial soap before leaving lab.
8. **New concept:** Spreading a plate; The spreader is sterilized by submerging in alcohol. The flame is used to burn off the alcohol so the *alcohol does not kill the E. coli cells*. The spreader can overheat in the flame and so should be cooled on the agar surface before spreading. The most important thing when spreading is to spread the liquid *evenly* over the ENTIRE SURFACE of the plate.