Preparation of electrocompetent cells of *Pseudomonas* or *E.coli*

1. Inoculate a 50 ml LB broth with several colonies from a freshly-grown LB agar plate culture (1-7 days old), grow overnight with shaking at 30°C (for *Pseudomonas*) or 37°C (for *E.coli*)

2. In the morning, add the entire 50 ml culture to 500 ml LB broth in a large Erlenmeyer flask. Measure the optical density (OD_{600}) – this should be ~0.1 - 0.2. Record the value.

3. Incubate the 500 ml culture with shaking at 30°C. Check the OD600 approximately every 30 minutes. When the value reaches 0.4-0.6 (*E.coli*) or 0.8-1.0 (*Pseudomonas*), take it off the shaker, and pour culture aseptically into 2 x 500 ml sterile Nalgene centrifuge bottles which have been chilled on ice. (note that these centrifuge bottles should only be half full so they don't leak in the centrifuge. Resist the temptation to just use 1 x 500 ml bottle!).

4. Ensure tubes are balanced (weigh them, and top up the lighter one with the appropriate amount of buffer). Centrifuge in large floor centrifuge for 15 minutes at 7500 g (=6700 RPM for Sorvall GS3 or equivalent rotor) at 4°C.

5. Pour off the supernatant liquids into culture waste, and resuspend each pellet in 30 ml of cold electroporation buffer *. Put caps on tight and shake vigorously to resuspend the cells. (approx 10 seconds), then pour into 2 x 50 ml Falcon tubes.

6. Ensure tubes are balanced (matching volumes by eye is OK this time). Centrifuge at top speed (~4500 rpm, or ~ 3000 g) for 15 minutes in Centaur/Centurion type centrifuge at 4°C.

7. *Gently* pour off supernatants into culture waste –the pellets may be quite soft, and are easily lost if you are not gentle! You need to retrieve the tubes immediately from the centrifuge when the spin is finished, and also handle the tubes gently in order to maintain the pellet at the bottom of the tube.

8. Resuspend each pellet in 30 ml of cold electroporation buffer, and spin again.

9. Pour off supernatant, resuspend each pellet in 30 ml cold electroporation buffer, and spin again.

10. Resuspend one pellet in 5 ml of cold electroporation buffer, resuspend cells by vortexing (~10 sec). Pour this cell suspension aseptically into the other tube, and vortex again. Place on ice.

11. Pipette the cell suspension into multiple small aliquots in sterile 1.5 ml Eppendorf tubes. For example, 25 x 200 µl aliquots would be typical. Each of these single 200 µl aliquots has enough cells for four electroporation cuvettes. Label the tubes with the strain name before you start. Although 'correct practice' here would involve setting up your 25 Eppi tubes opened, and on ice, I woudn't do this due to the potential for contamination (melted ice getting in the tubes). But you should aim to do this step quickly, and get the cell aliquots into the -80C freezer ASAP.

12. Put the cell aliquots into a plastic bag or box and freeze at -80 C. Label the bag or box in addition to the individual tubes. Don't label the bag surface directly with permanent marker – this will come off–better to write with marker in large clear text on a piece of paper, and put this in the bag.

* Electroporation buffer: For *E.coli* and *Ps.putida*, you can use 10% glycerol, but for *Ps.stutzeri*, you need a more complex buffer containing 10% sucrose, 1 mM HEPES (pH 7), 1 mM MgSO4. This is because the cells are more fragile. Both types of buffer need to be sterilised by autoclaving, and should be chilled on ice before use (at least 30 min on ice; or keep this buffer in the cold room).

Electroporation of electrocompetent cells of E.coli or Pseudomonas

1. Retrieve the appropriate number of cell aliquots from the -80°C freezer and allow to thaw. You can do this by putting them directly on ice and waiting a long time (~20 min?) or you can put them in room-temperature water in a floatie, then put on ice as soon as they are fully thawed (~5 min?). In the latter case, don't leave them too long at room temperature, keep an eye on them ! Occasional mixing by flicking helps to speed the thawing process.

<<<note ! don't try to electroporate chemically-competent cells, these will spark! >>>

2. Divide up the cells into multiple 50 µl aliquots in sterile 1.5 ml Eppi tubes, on ice. Also put on ice the same number of sterile electroporation cuvettes (2 mm gap size). These need to chill for at last 10 min before use. Label the cuvettes on the cap before use.

3. Add your plasmid DNA or ligation mixture to the cells. For purified plasmid, 1 µl is heaps ! (and may even be too much). For ligation mixture, it would be typical to use 2-3 µl. In both cases more is not better, because the more DNA you add, the more salt you are also adding, and it doesn't take very much salt to make the electroporation procedure fail (short circuit = sparks!). Make sure you change pipette tips for each DNA sample. Even 1uL of TE can have a noticeable effect on efficiency and you may actually get more colonies using less DNA.

4. For all electroporation experiments, you should include a negative control (no DNA added) – this will let you know firstly whether your competent cells are OK (not too salty), and secondly whether your aseptic technique is good. If your negative control makes a spark, your cells are no good (need more wash steps!) or the electroporation machine is not set up correctly. If the electroporation procedure works (no spark), but you get colonies on the plates from the negative control, this means you have contamination, either in your competent cells, or getting in somewhere in the electroporation procedure.

5. In the case of electroporation experiments with ligation mixtures, make sure you also include a positive control, which is 1 µl of a purified plasmid of known good quality with the correct antibiotic resistance. This should give you thousands of colonies or a confluent lawn of growth on the appropriate antibiotic agar plate – if it doesn't, this could mean your cells are not competent, or the plates are bad, or the electroporator is not set up properly, or your 'good quality' plasmid stock is no longer good quality.

6. Once all your cell aliquots have the appropriate DNA samples added, transfer each mixture individually to the corresponding electroporation cuvette (again make sure everything is labelled before starting!). Give each cuvette a few taps on the bench to make sure the cell mix is at the bottom of the cuvette.

7. Take your esky, a p1000 pipette, box of blue tips, some sterile LB broth, and some paper towel over to the electroporation machine (You need ~1 ml LB per sample). Turn on the electroporator, and choose "Exponential" protocol. Adjust the settings on the electroporator to 2500 V, 25 µF, and 200 Ω. These settings will work with a wide variety of Gram-negative bacteria, but note that for Gram-positives you need to increase the ohms to 800 Ω (see next protocol). Also note that if you use cuvettes with a different gap size you need to adjust the electroporator settings.

8. Take your first cuvette off ice, and wipe down the outside and underneath briefly with paper towel. Place this firmly into the cuvette holder on the machine, then close the lid.

9. Press the “Pulse” button. After a few seconds, the machine will beep to let you know the pulse has been delivered. Note down the time constant displayed on the screen (ms= milliseconds). This tells you how long the pulse lasted, and a higher number here is better. Retrieve your cuvette and immediately add 1 ml of sterile LB to it. Return to ice.

10. Repeat steps 8 and 9 for all your samples. When finished, turn off electroporation machine, and return to your bench.

11. As the sample becomes more salty with increasing amounts of DNA, the time constant will decrease until eventually you will get a spark (short circuit). If any of your samples make a spark, this means either your competent cells are not prepared properly (the negative control will spark too), or that you have added too much DNA and/or the DNA is of low quality. It is possible to sometimes get transformants from a cuvette which as sparked, but don’t count on it! Its better to repeat the experiment using less DNA (or a dilution of the DNA in sterile MQ water) and/or a different batch of electrocompetent cells. Take note of the time constant for each electroporation and compare it to your “no DNA” control. Even if it doesn’t spark, a salty sample can lead to a lower efficiency, and a reduced time constant. This is useful information if you need to troubleshoot later.

12. Aseptically pour each transformed cell mixture into a sterile 1.5 ml Eppi tube, as follows. First, label all the Eppi tubes, then open them all, positioned in a rack.(they don’t need to be in ice). Take the cap off the first cuvette and briefly pass the top of the cuvette through the Bunsen flame a few times (if you flame it too much it will melt!), then pour into the first Eppi tube. Tap the cuvette on the tube to ensure you get all the sample out. Repeat for other tubes.

13. Ensure all the Eppi tubes are tightly closed and labelled, and then incubate with shaking for 1 hour at 37°C (E.coli) or 1.5 hours at 30°C (Pseudomonas). Put the tubes lying on their side in a beaker or other container in the shaker, or you can attach them to the shaker with elastic bands or masking tape. Lying horizontal is important for good shaking action in these small tubes.

14. Retrieve tubes from shaker. Prepare 2x the number of appropriate agar plates as you have samples (double check you are using the correct antibiotic(s)!!!), and label all these plates before going any further. In addition to the usual information (antibiotic, plasmid, strain, date), label half of the plates “100 µl” and the other half “pellet”

15. Spread plate 100 µl of each sample onto the appropriate antibiotic agar. Centrifuge the remaining culture (2 min at 10,000 g in Eppi centrifuge), pour off most of supernatant, and resuspend the pellets by vortexing in the drop of liquid that remains. Plate these resuspended cells on a second set of plates of the same antibiotic type. The reason for doing two plates for each sample is to give us the best chance of getting countable and well-isolated colonies on at least one of these plates.

16. Don’t throw out the used electroporation cuvettes. These can be recycled. (repeated rinses with water then 80% ethanol, see elsewhere for this protocol). Don’t let the cuvettes dry out with cell mix still in them, you should give these a couple of rinses with WATER as soon as you finish the experimental part of the work. (discard rinsate into culture waste).