**COMPETENT CELL PRODUCTION PROTOCOL**

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**Here is an overview of where the bugs are and what reagents you need:**

1. Stbl3 and Top10 bugs are kept in EFS stock box in -80C 🡪 cryovials 🡪 not labeled 🡪 blue box
2. Stbl3 are in a black lid cryovial (grown in LB media)
3. Top10 are in a purple lid cryovial (grown in LB media or sometimes TYM)
4. Follow recipe for CaCl2 (competent) final pH 7.0 with NaOH to adjust 🡪 the recipe is near the last page (this is kept at 4C in main lab fridge)

🡪 this recipe requires PIPES (may have to make yourself, but you can look up recipe on internet)

1. Note: all LB broth used and all glass flasks used must be autoclaved and extremely clean 🡪 getting contamination of plasmids during competent cell production will ruin every experiment that uses bugs (Maxi prep, ligations, transformations for checking DNA, and everything) SO PLEASE TAKE CAUTION
2. Additionally you should bake an entire box of the 1.5mL Eppendorf tubes in 2 flasks to be sure they are not contaminated (they are where you will be storing the final bugs so it is also important)

**Start**

1. Get the Stbl3 or Top10 bugs from -80C (keep on ice) and pick a colony via the inoculating loop (this is to be done in the bacterial hood)
2. Inoculate the picked colony into 20mL of LB in a 300mL flask
3. Shake this flask overnight at 37C shaker at 250rpm (this should be done between 3-5pm) (culture is best left to grow for 18hrs; if you have to 24 is the absolute max, anymore then this and it will not yield fruitful product
4. At 9am the next day, check your culture; it should be thick and cloudy and a yellowish white colour
5. In the bacterial hood, take 5mL of O/N culture and place into 500mL of LB broth (this is done in a 2L flask)
6. Swirl the flask around gently with your hand and take an initial reading of Optical Density (OD) @ the beginning of your shake
7. Using the spec in the lab, for to test, and choose cell concentration or cell growth
8. For this reading, on the spec, use a regular clear cuvette- blank with 1mL of LB and then run your sample with 1mL of the bugs culture that you just placed in the 2L flask
9. This will give you an OD reading 🡪 to give a rough guideline; the OD doubles every 30 min 🡪 the bugs grow logarithmically so this will give you an idea of around what time you should take the next reading with out going over the OD you want
10. The best OD reading is 0.38 (0.4 is the absolute cut off; may even be pushing it)
11. While you are taking the initial reading, the 2L flask should already be shaking at 37C at 250 rpm
12. Now would be a good time to place your bottle of CaCl2 solution on ice. It needs to be very cold
13. ASIDE: you started with your 20mL morning culture from which you took 5mL to grow up further🡪 this should leave you with 15mL left over. There are a couple of options for the remaining culture. (A) You can save 5mL incase when you are growing the bugs up you go over the optimal OD. (B) you can make glycerol stocks of the bugs to keep for next time you make them, that way you don’t have to inoculate and grow them up overnight again, you can simply start at 9am and begin at step 4 of this protocol
14. Glycerol Stocks can be made as with the remaining 10 or 15mL of the O/N culture. The ratio to follow is basically 70% glycerol (50% glycerol in 1X PBS) to 30% bugs. Can make a “master mix and then aliquot this) Janice usually does 100uL total in a Eppendorf tube. 1mL total volume is actually quite a bit . NOTE: keep the culture on ice if you are making these stocks so that they will remain viable after freezing. These can be stored at -80C.
15. Again make sure you have a lot of baked Eppendorf tubes, running out part way through aliquoting is a huge inconvenience and may compromise the integrity and competency of the bugs if you keep them on ice too long
16. When you reach the OD of 0.38, get 10 **brand new** 50mL conical tubes.
17. Pour 50mL of the 0.38OD culture into each tube and centrifuge at 3000rpm for 7min and 4C.
18. Pour off LB supernatant and add 10mL of CaCl2 solution to the pellet
19. The pellet will be hard to resuspend. DO NOT vortex. It is best if you shake vigorously by hand until resuspended. This may take a little while, best to have 2 or 3 people shaking, so all tubes can be done unanimously
20. Once fully resuspended, spin again at 2500rpm for 5 min at 4C
21. Remove CaCl2 supernatant 🡪 keep pellet 🡪 resuspend pellet once more in 10mL of ice cold CaCl2 and leave this on ice for 30 min. THIS IS A VERY IMPORTANT STEP; this is where the pores are made in the membrane of the bugs. You need LOTS of ICE and Very cold (it is best to use a big tall bucket or one of the big autoclaving bins filled with ice) IF you run out of ice, there is another machine on the third floor by the genetics lab
22. Once 30 min is up, immediately centrifuge the bugs for 5 min at 2500rpm at 4C
23. Discard the supernatant
24. Resuspend each pellet with an automatic pipetor 🡪 use ice cold CaCl2 as your solution and try to collect all 10 pellets in the same 1mL of solution 🡪 this should create a thick white sticky substance
25. If it becomes too hard to resuspend you can use 1mL to resuspend 5 pellets and another 1mL to resuspend the remaining 5 pellets
26. Now, your pooled pellet can be diluted total 10mL total resuspension if they are large pellets. However if your pellets are just average size, then a 7mL final resuspension volume is best (keep in mind, the more volume you resuspend in, the more aliquoting that is later on)
27. You resuspension should still be thick and opaque, yellow or white. If your pellet is translucent this is not good
28. Now you have grown the bugs and made them competent, so you can aliquot them and flash freeze them
29. Using the repetitive pipet 🡪 Janice and Jessica have this one 🡪 you can do 50uL aliquots or 100uL 🡪 50uL is great for experiments and is better because the lab will waste less and you wont have to make the bugs as frequently
30. Set up 10 or 20 eppendorf tubes at a time in a tube rack, aliquot into them and then put them immediately on ice (BE SURE THAT THE LIDS ARE CLOSED WELL, you don’t want them to explode, it could be dangerous for you)
31. If there is someone else to help, it would be a good idea to have them fill the calorimeter with liquid nitrogen while you are aliquoting the bugs to save time and have the bugs remain more viable
32. Once the bugs are on ice, you can use the ladel to put them into the liquid N2, put as many that will fit so they are still covered to freeze (10 seconds is enough)🡪 once flash frozen 🡪 ladel out onto ice 🡪 be sure not to burn yourself on the liquid nitrogen, and wear the big glove to be extra careful!!
33. Usually we store the bugs in a Ziploc bag at -80C until someone can test them to be sure they are good!
34. ☺ GOOD JOB on completing the competent cells, it is a big job and an important one! Woo Bam!!

**REAGENTS**

**CaCl2 solution**

60mM CaCl2 (6mL of 1M)

15% glycerol (15mL of 100% glycerol stock ) (stored in flammable cabinet)

10mM PIPES, pH 7.0 (10mL of 0.1M)

diH2O up to 100mL

solution needs to be filtered with 0.2uM filter or autoclaved

OR

30mL of 1M CaCl2

75mL glycerol from stock

50mL (0.1M PIPES)

345mL diH2O

also needs to be filter sterilized or baked

ASIDE

CaCl2 1M 🡪 use 4.6g in 40mL diH2O (MW=115.02)

PIPES 0.1M 🡪 use 1.2g in 40mL diH2O (MW=302.4) … go to pH 7.0 with NaOH