Making Calcium Competent Cells

Day 1

1. Streak out frozen glycerol stock of bacterial cells (Top10, DH5α, etc.) onto an LB plate (**no antibiotics** since these cells do not have a plasmid in them). Work sterile. Grow plate overnight at 37°C.

Day 2

1. Autoclave:
   1 L LB (or your preferred media)
   1 L of 100 mM $CaCl_2$
   1 L of 100 mM $MgCl_2$
   100 mL of 85 mM $CaCl_2$, 15% glycerol v/v
   4 centrifuge bottles and caps
   Lots of microfuge tubes

   这些溶液不需要新鲜配置，如果冰箱里面有，可直接使用，如果担心细菌已污染，可以直接高压锅灭菌后冷却再使用。

2. Chill overnight at 4°C:
   100 mM $CaCl_2$
   100 mM $MgCl_2$
   85 mM $CaCl_2$, 15% glycerol v/v
   Centrifuge rotor

3. Prepare starter culture of cells

   不需要挑单克隆，直接取菌种摇

   Select a single colony of E. coli from fresh LB plate and inoculate a 10 mL starter culture of LB (or your preferred media – no antibiotics). Grow culture at 37°C in shaker overnight.

   Notes:
   - You will have extra $CaCl_2$ and $MgCl_2$. These solutions can be saved and re-autoclaved for the next time you make competent cells.
   - You can also substitute other media like SOB, 2xYT, etc. for the LB if you prefer.
   - All glassware should be detergent free. Presence of detergent reduces competency of cells. 摇菌的锥形瓶需要微波炉加热灭菌后使用，或者其它方法灭菌后使用。

Day 3

1. Inoculate 1 L of LB media with 10 mL starter culture and grow in 37°C shaker. Measure the OD_{600} every hour, then every 15-20 minutes when the OD gets above 0.2.

   细菌不要太老，对数生长期即可，可目测，可用LB培养基

2. When the OD_{600} reaches 0.35-0.4, immediately put the cells on ice. Chill the culture for 20-30 minutes, swirling occasionally to ensure even cooling. Place centrifuge bottles on ice at this time.

IMPORTANT NOTES:

- It is important not to let the OD get any higher than 0.4. The OD should be carefully monitored and checked often, especially when it gets above 0.2, as the cells grow exponentially. It usually takes about 3 hours to reach an OD of 0.35 when using a 10 mL starter culture.

- It is also very important to keep the cells at 4°C for the remainder of the procedure. The cells, and any bottles or solutions that they come in contact with, must be pre-chilled to 4°C.

离心时低温状态，不要离心太长时间，3-4分钟大部分细菌离心下来即可。离心时间太长，后面不好打散。

3. (Spin #1) Split the 1 L culture into four parts by pouring about 250 mL into ice cold centrifuge bottles. Harvest the cells by centrifugation at 3000*g* (~4000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 离心速度不要太高，后面不好打散
4. Decant the supernatant and gently resuspend each pellet in about 100 mL of ice cold $MgCl_2$. Combine all suspensions into one centrifuge bottle. Make sure to prepare a blank bottle as a balance.
5. (Spin #2) Harvest the cells by centrifugation at 2000*g* (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. 离心速度和时间和前面一样，不要太高，不要太长，细菌离心下来，损失不大就行。保存低温，很关键
6. Decant the supernatant and resuspend the pellet in about 200 mL of ice cold $CaCl_2$. Keep this suspension on ice for at least 20 minutes. Start putting 1.5 mL microfuge tubes on ice if not already chilled. 充分打散细菌颗粒，保证细菌都接触到化学药品的浸润
7. (Spin #3) Harvest the cells by centrifugation at 2000*g* (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C. At this step, rinse a 50 mL conical tube with ddH2O and chill on ice. 离心方法和前面一样，
8. Decant the supernatant and resuspend the pellet in ~50 mL of ice cold 85 mM CaCl2, 15% glycerol. Transfer the suspension to the 50 mL conical tube. 建议不要做步骤8，在有甘油的状态下，细菌较难离心下来。
9. (Spin #4) Harvest the cells by centrifugation at 1000*g* (~2100 rpm in the Beckman GH-3.8 rotor) for 15 minutes at 4°C.
10. Decant the supernatant and resuspend the pellet in 2 mL of ice cold 85 mM CaCl2, 15% glycerol. The final OD_{600} of the suspended cells should be ~ 200-250. 最后感受态的浓度不需要很精确，目测是浑浊的，不要太澄清那种就可以，浓些稀些都不影响使用
11. Aliquot 50 µL into sterile 1.5 mL microfuge tubes and snap freeze with liquid nitrogen. Store frozen cells in the -80°C freezer.

步骤8和步骤10是一样的，设计这么一个重复步骤的目的是为了防止步骤7中管内残留的液体将冻存液即15%甘油稀释了，也就是说第8步时，最终的冻存液浓度可能因为稀释低于15%，所以把它离心倒掉，再加入新鲜的15%的冻存液，感受态冻存更好。如果在步骤7时，尽量吸干净里面的残留液，就只需要将冻存液加入后直接分装保存就好了，不需要重复一次。

感受态制备很容易，关键点就是两个：一是过程中一直保持低温，包括最后分装感受态，都是将离心管放在冰上预冷了再分装，保持放在冰上，分装完成后放入盒中，立即存入-80度冰箱。第二个要点是化学药品处理细菌时，保证细菌是完全打散，没有颗粒聚集的，保证化学药品可以对所有细菌充分接触和处理。

除了上面两点以外，可能会碰到产率很低的问题，大部分情况是对细菌的离心没有把握好，如果离心速度太低的话，每次离心处理后，上清液都会有大量细菌残留，造成大量损失；如果离心太长，速度太高，细菌就粘在一起，很难打散。每次离心后可以目测一下，如果上清液太混，就再离心一下，调速度。