The words in black are original content from *Molecular Cloning*, the ones in red are remarks modifying this original protocol to suits our own needs.

## The Inoue Method for Preparation of Competent E.Coli: " Ultra-Competent" Cells

### Buffers /Solutions/Other Materials:

DMSO
Inoue transformation buffer( please see Step 1)
Chilled to 0°C before use.
LB medium for initial growth of culture
Liquid nitrogen
EP tubes,autoclaved, chilled in ice.
paper towels, autoclaved
Shaking incubator(18°C)

Method:

1. Prepare Inoue transformation buffer ( chilled to 0°C before use). ( Inoue buffer can be made in a large quantity then aliquoted and stored in -20 °C for future use.)

a. Prepare 0.5M PIPES(pH 6.7) ( piperazine-1, 2-bis{2-ethanesulfonic acid})by dissolving 15.1 g of PIPES in 80 ml of pure $H_2O$ (Milli-Q or equivalent). Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure $H_2O$ to bring the final volume to 100 ml. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter(0.45-um pore size).Divide into aliquots and store frozen at -20 °C.

b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800ml of pure $H_2O$ and then add 20 ml of 0.5M PIPES(pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure $H_2O$.

| Reagent | Amount per liter | Final concentration |
|---|---|---|
| $MnCl_2$. 4 $H_2O$ | 10.88 g | 55mM |
| $CaCl_2$. 2$H_2O$ | 2.20 g | 15mM |
| KCl | 18.65 g | 250mM |
| PIPES(0.5M,pH 6.7) | 20ml | 10mM |
| $H_2O$ | to 1 liter | |

c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-um Nalgene filter. Divide into aliquots and store at -20 °C.

2.**Do this during the first half of the day:**
Pick a single bacterial colony (2-3mm in diameter) from a plate that has been incubated for 16-20 hours at 37 °C( Do not have to be a single colony, can be multiple mixed together, also since we use tube rather than flasks, it is better to shake multiple tubes each with a small volume(e.g. 1.5mL to 2mL ) of bacterial culture). Transfer the colony into 25 ml of LB broth or SOB medium in a 250 ml flask( add antibiotics accordingly if the strain comes with a certain antibiotic resistance as a selection marker). Incubate the culture for 6-8 hours at 37 °C with vigorous shaking(250-300 rpm).

3. At about 6 o'clock in the evening(the starting time is not rigid but generally should not be earlier than 6 p.m.) use this starter culture to inoculate three(2 for our lab) 1-liter (500 ml for our lab)flasks, each containing 250 ml(125ml for our lab) of SOB. The first flask receives 10ml of starter culture, the second receives 4ml, and the third receives 2ml. Incubate all three flasks overnight at 18-22 °C ( we do not have a low-temperature shaker but any shaker put in the 22 °C in the environmental room will also works as fine） with moderate( around 200rpm） shaking( we only need to add 2mL and 1mL of the starter culture to two flasks respectively, no need for OD measuring for this step).

4.The following morning, read the $OD_{600}$ of all three ( 2 for our lab)cultures. Continue to monitor the OD every 45 minutes( this interval time can vary according to the actual situation).

5. When the $OD_{600}$ of one of the cultures reaches 0.55 （any value between 0.4 to 0.6 is well acceptable), transfer the culture vessel to an ice-water bath for 10 minutes. Discard the two other cultures.

6.Harvest the cells by centrifugation at 2500g for 10 minutes at 4 °C( We can use plastic centrifuge tubes and the low-temperature centrifuge from the Tumer Lab).

7. Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 minutes. Use a vacuum aspirator to remove any drops of remaining medium adherng to walls of the centrifuge bottle or trapped in its neck( The vacuum step is not a solid requirement, but you do have to make the liquid leftover as little as possible, i.e. no flowing liquid visible inside).

8. Resuspend the cells gently in 80ml ( if for a 125mL culture, use 40 ml in total )of ice-cold Inoue transformation buffer.
(The cells are best suspended by swirling rather than pipetting or vortexing)

9. Harvest the cells by centrifugation at 2500g for 10 minutes at 4 °C.

10. Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 minutes . Use vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube or trapped in its neck( same notes as above).

11. Resuspend the cells gently in 20 ml( if for a 125 ml culture then use 10mL in total) of ice-cold Inoue transformation buffer. **( NOTE: the iced Inoue buffer is particularly hard to melt under room temperature, so you need to either take them out of the -20 °C really early in the morning or you have to warm it up to accelerate the melting process on a 65 °C dry bath surface.**

12. Add 1.5ml ( 0.75ml in total if for a 125 ml culture) of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 minutes.

13. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes. Immediately snap-freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen. Store the tubes at -70 °C until needed. **NOTE: tubes on the ice can be chilled down quickly but since you may need lots of chilled tubes you'd better put them in the ice during one of the previous steps when you have some spare time.**

Final words:

1. If you have prepared the transformation buffer and autoclaved things you need ahead of time, the real work for "making competent cells" actually starts from step 6. If work efficiently enough, all things can be done within an hour. Even work loosely things can be perfectly finished within 1.5 hr. However, since quick work can also significantly improve the transformation efficiency of the cells made( from high to terribly high...), you'd better be a quick worker.

2.The key to this method is keeping cells under low temperature ( below 4 °C after starting working )whenever you can- just keep an ice box at hand.

3.Don't forget incubating the cell culture in ice during steps that require you doing so.

4.DMSO is for preserving the competent cells( similar function as glycerol for other kinds of stock), it cannot be omitted.

5.Snap-freezing in liquid nitrogen can greatly increase transformation efficiency so you'd better do this.

6.DO NOT re-freeze any competent cells that has thawed once after it is made. So if you accidentally take more competent cells than necessary, just throw away the unneeded.

7. There are already a lot of transformation buffer made and stored in our -20 °C. Plus, also lots of PIPES already made and adjusted to the desirable pH. So please do not try to make any new. For the sake of saving time and resource, just use what we already have.