**Preparation of Chemically Competent E.coli – Inoue Buffer (IB) method.**

This protocol is based on the Calcium CaCl2 and MgCl2 method but uses Inoue transformation buffer (IB) instead, for higher-efficiency results.

250mL bacterial culture will yield 1mL of competent cells (20 x 50uL aliquots).

**You will need:**

40-50mL sterile centrifuge tubes (e.g. Beckman JA-17 rotor)  
500mL-1L sterile conical flask

Shaking 37oC incubator  
OD600-capable spectrophotometer and spectrophotometer cuvettes  
Ice-cold (0oC) Inoue Buffer (IB)\*

Ice-cold IB\* containing 15% v/v glycerol

**Method:**

1. Prepare a fresh overnight culture of desired strain grown in 5mL Luria-Bertani (LB) broth or similar.
2. Subculture bacterial overnight culture 2.5mL🡪 250mL (can supersize: 10mL🡪 1L).
3. Grow at 37oC with vigorous shaking. Measure the OD600 every hour until OD600 reaches 0.2. then every 15-20 minutes until OD600 = 0.4
4. Chill the culture in ice for 20-30 minutes, swirling occasionally to ensure even cooling. Place centrifuge tubes on ice to cool.

IMPORTANT NOTES:

• It is important not to let the OD600 get any higher than 0.4. The OD should be carefully monitored and checked often, as the cells grow exponentially. It usually takes about 3 hours to reach an OD of 0.35

• 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.

1. **Spin 1:** Harvest the cells by centrifugation at 3000 g (~4000 rpm in the Beckman JA-10 rotor) 15 minutes at 4°C
2. Decant the supernatant and gently resuspend pellet in 100 mL of ice cold IB.
3. **Spin 2:** Harvest the cells by centrifugation at 2000 g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C
4. Decant the supernatant and gently resuspend pellet in 50 mL of ice cold IB. Keep this suspension on ice for 20 min – 1h.
5. **Spin 3:** Harvest the cells by centrifugation at 2000 g (~3000 rpm in the Beckman JA-10 rotor) for 15 minutes at 4°C
6. Decant the supernatant and gently resuspend pellet in 12.5 mL of ice cold IB with 15% glycerol.
7. **Spin 4:** Harvest the cells by centrifugation at 1000 g for 15 minutes at 4°C
8. Decant the supernatant and gently resuspend pellet in 1 mL of ice cold IB with 15% glycerol. The final OD600 of the suspended cells should be ~100-125.
9. Aliquot 50 μL into sterile 1.5 mL microfuge tubes and snap freeze with liquid nitrogen or dry ice-ethanol bath. Store frozen cells in the -80°C freezer.

\*Inoue Buffer: Prepare buffer and chill to 0 °C (ice slurry) before use.

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| Innoe Transformation buffer [final] | 1L |
| 55mM MnCl2 | 10.88g |
| 15mM CaCl2 | 2.20g |
| 250mM KCl | 18.65g |
| 10mM PIPES | 20mL of 0.5M, pH 6.7 stock solution |
| H2O | To 1L |

Buffer preparation: Organic contaminants in the H2O used to prepare transformation buffers can reduce the efficiency of transformation of competent bacteria. H2O obtained directly from a well-serviced Milli-Q filtration system usually gives good results. If problems should arise, treat the deionized H2O with activated charcoal before use.

a. Prepare 0.5 M PIPES (pH 6.7). Adjust the pH of the solution to 6.7 with 5 M KOH, and then add pure H2O to bring the final volume to 100 ml. Sterilize the solution by filtration through a disposable prerinsed Nalgene filter. Divide into aliquots and store frozen at -20 °C

b. Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H2O and then add 20 ml of 0.5 M PIPES (pH 6.7). Adjust the volume of the Inoue transformation buffer to 1 liter with pure H2O.

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