The Inoue Method for Preparation and Transformation of Competent *E. Coli*: "Ultra-Competent" Cells

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INTRODUCTION

This protocol reproducibly generates competent cultures of *E. coli* that yield 1 x 10^8 to 3 x 10^8 transformed colonies/µg of plasmid DNA. The protocol works optimally when the bacterial culture is grown at 18°C. If a suitable incubator is not available, a standard bacterial shaker can be set up in a 4°C cold room and regulated to 18°C.

MATERIALS

DMSO

Inoue transformation buffer (please see Step 1)

Chilled to 0°C before use.

Plasmid DNA (recombinant plasmid)

Construct using one of the methods described in Directional Cloning into Plasmid Vectors, Attaching Adaptors to Protruding Termini, Blunt-ended Cloning into Plasmid Vectors, Dephosphorylation of Plasmid DNA, Addition of Synthetic Linkers to Blunt-ended DNA and Ligating Plasmid and Target DNAs in Low-melting-temperature Agarose.

SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic

SOB medium for initial growth of culture

SOB medium, for growth of culture to be transformed

SOC medium

Approximately 1 ml of this medium is needed for each transformation reaction.

DMSO

Purchase a high grade of DMSO (dimethylsulfoxide, HPLC grade or better). Divide the contents of a fresh bottle into 1-ml aliquots in sterile tubes. Close the tubes tightly and store at -20°C. Use each aliquot only once and then discard.

SOB

NaCl, 0.5 g

tryptone, 20 g

deionized H_2O, to 950 ml

For solid medium, please see Media Containing Agar or Agarose.

yeast extract, 5 g

Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized H_2O.) Adjust the pH of the medium to 7.0 with 5 N NaOH (approx. 0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm ^{2}) on liquid cycle. Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. (This solution is made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized H_2O. Adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by autoclaving for 20 minutes at 15 psi [1.05 kg/cm^2] on liquid cycle.)

SOC

yeast extract, 5 g

deionized H_2O, to 950 ml

For solid medium, please see Media Containing Agar or Agarose.

NaCl, 0.5 g

tryptone, 20 g

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less. Add 20 ml of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of deionized H_2O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by passing it through a 0.22-µm filter.)

METHOD

1. Prepare Inoue transformation buffer (chilled to 0°C before use).
Prepare 0.5 M 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-µm pore size). Divide into aliquots and store frozen at -20°C.

Prepare Inoue transformation buffer by dissolving all of the solutes listed below in 800 ml of pure H_2O 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 H_2O.

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

Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-µm Nalgene filter. Divide into aliquots and store at -20°C.
2. Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 hours at 37°C. Transfer the colony into 25 ml of SOB medium (LB may be used instead) in a 250-ml flask. 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, use this starter culture to inoculate three 1-liter flasks, each containing 250 ml of SOB. The first flask receives 10 ml of starter culture, the second receives 4 ml, and the third receives 2 ml. Incubate all three flasks overnight at 18-22°C with moderate shaking.

4. The following morning, read the OD_{600} of all three cultures. Continue to monitor the OD every 45 minutes.

5. When the OD_{600} of one of the cultures reaches 0.55, transfer the culture vessel to an ice-water bath for 10 minutes. Discard the two other cultures.

6. Harvest the cells by centrifugation at 2500*g* (3900 rpm in a Sorvall GSA rotor) for 10 minutes at 4°C.

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 adhering to walls of the centrifuge bottle or trapped in its neck.

8. Resuspend the cells gently in 80 ml of ice-cold Inoue transformation buffer.

9. Harvest the cells by centrifugation at 2500*g* (3900 rpm in a Sorvall GSA rotor) 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 a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube or trapped in its neck.

11. Resuspend the cells gently in 20 ml of ice-cold Inoue transformation buffer.

12. Add 1.5 ml 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 microcentrifuge 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.

14. When needed, remove a tube of competent cells from the -70°C freezer. Thaw the cells by holding the tube in the palm of the hand. Just as the cells thaw, transfer the tube to an ice bath. Store the cells on ice for 10 minutes.

15. Use a chilled, sterile pipette tip to transfer the competent cells to chilled, sterile 17 x 100-mm polypropylene tubes. Store the cells on ice.
Include all of the appropriate positive and negative controls.

16. Add the transforming DNA (up to 25 ng per 50 µl of competent cells) in a volume not exceeding 5% of that of the competent cells. Swirl the tubes gently several times to mix their contents. Set up at least two control tubes for each transformation experiment, including a tube of competent bacteria that receives a known amount of a standard preparation of superhelical plasmid DNA and a tube of cells that receives no plasmid DNA at all. Store the tubes on ice for 30 minutes.

17. Transfer the tubes to a rack placed in a preheated 42°C circulating water bath. Store the tubes in the rack for exactly 90 seconds. Do not shake the tubes.

18. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 minutes.

19. Add 800 µl of SOC medium to each tube. Warm the cultures to 37°C in a water bath, and then transfer the tubes to a shaking incubator set at 37°C. Incubate the cultures for 45 minutes to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

20. Transfer the appropriate volume (up to 200 µl per 90-mm plate) of transformed competent cells onto agar SOB medium containing 20 mM $MgSO_4$ and the appropriate antibiotic.

21. Store the plates at room temperature until the liquid has been absorbed.

22. Invert the plates and incubate them at 37°C. Transformed colonies should appear in 12-16 hours.

REFERENCES

1. Inoue, H., Nojima, H., and Okayama, H. 1990. High efficiency transformation of Escherichia coli with plasmids. *Gene* **96:** 23–28.[Medline]