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

http://www.molecularcloning.com/members/protocol_print.jsp?chpnumber=1&pronumber=24

This protocol reproducibly generates competent cultures of E. coli that yield 1 x 108 to 3 x 108 transformed colonies/mg 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
Buffers and Solutions
DMSO
Inoue transformation buffer (please see Step 1)
Chilled to 0°C before use.
Nucleic Acids

Media
SOB medium for initial growth of culture
SOB agar plates containing 20 mM MgSO4 and the appropriate antibiotic
SOB medium, for growth of culture to be transformed
SOC medium

METHOD
1 Prepare Inoue transformation buffer (chilled to 0°C before use).

a. 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 H2O (Milli-Q, or equivalent). 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 (0.45-µm 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 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. 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) 10 ml 10 mM
   H_2O to 1 liter

d. 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 OD600 of all three cultures. Continue to monitor the OD every 45 minutes.

5 When the OD600 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 2500g (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 2500g (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 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.

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.

RECIPES

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.
Media Containing Agar or Agarose
Prepare liquid media according to the recipes given. Just before autoclaving, add one of the following:
Bacto Agar (for plates)
15 g/liter
Bacto Agar (for top agar)
7 g/liter
agarose (for plates)
15 g/liter
agarose (for top agarose)
7 g/liter

Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar or agarose evenly throughout the solution. Be careful! The fluid may be superheated and may boil over when swirled. Allow the medium to cool to 50-60°C before adding thermolabile substances (e.g., antibiotics). To avoid producing air bubbles, mix the medium by swirling. Plates can then be poured directly from the flask; allow approx. 30-35 ml of medium per 90-mm plate. To remove bubbles from medium in the plate, flame the surface of the medium with a Bunsen burner before the agar or agarose hardens. Set up a color code (e.g., two red stripes for LB-ampicillin plates; one black stripe for LB plates, etc.) and mark the edges of the plates with the appropriate colored markers.

When the medium has hardened completely, invert the plates and store them at 4°C until needed. The plates should be removed from storage 1-2 hours before they are used. If the plates are fresh, they will "sweat" when incubated at 37°C. When this condensation drops on the agar/agarose surface, it allows bacterial colonies or bacteriophage plaques to spread and increases the chances of cross-contamination. This problem can be avoided by wiping off the condensation from the lids of the plates and then incubating the plates for several hours at 37°C in an inverted position before they are used. Alternatively, remove the liquid by shaking the lid with a single, quick motion. To minimize the possibility of

contamination, hold the open plate in an inverted position while removing the liquid from the lid.

NaCl
To prepare a 5 M solution: Dissolve 292 g of NaCl in 800 ml of H2O. Adjust the volume to 1 liter with H2O. Dispense into aliquots and sterilize by autoclaving. Store the NaCl solution at room temperature.

SOB
deionized H2O, to 950 ml
tryptone, 20 g
yeast extract, 5 g
NaCl, 0.5 g
For solid medium, please see Media Containing Agar or Agarose.

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 H2O.) 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 H2O. 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 MgCl2. (This solution is made by dissolving 19 g of MgCl2 in 90 ml of deionized H2O. Adjust the volume of the solution to 100 ml with deionized H2O and sterilize by autoclaving for 20 minutes at 15 psi [1.05 kg/cm2] on liquid cycle.)

SOC
deionized H2O, to 950 ml
tryptone, 20 g
yeast extract, 5 g
NaCl, 0.5 g

For solid medium, please see Media Containing Agar or Agarose.
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 H2O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized H2O and sterilize by passing it through a 0.22-µm filter.)

CAUTIONS

DMSO

DMSO (Dimethylsulfoxide) may be harmful by inhalation or skin absorption. Wear appropriate gloves and safety glasses. Use in a chemical fume hood. DMSO is also combustible. Store in a tightly closed container. Keep away from heat, sparks, and open flame.

REFERENCES

1 Inoue H., Nojima H., and Okayama H. 1990. High efficiency transformation of Escherichia coli with plasmidsGene 96:23-28.