Protocol 24

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

AT ITS BEST, THIS METHOD FOR PREPARING COMPETENT *E. coli* from Inoue et al. (1990) can challenge the efficiencies achieved by Hanahan (1983). However, under standard laboratory conditions, efficiencies of 1×10^8 to 3×10^8 transformed colonies/μg of plasmid DNA are more typical. The advantages of the procedure are that it is less finicky, more reproducible, and therefore more predictable than the original Hanahan method.

This protocol differs from other procedures in that the bacterial culture is grown at 18°C rather than the conventional 37°C. Otherwise, the protocol is unremarkable and follows a fairly standard course. Why growing the cells at low temperature should affect the efficiency of transformation is anybody's guess. Perhaps the composition or the physical characteristics of bacterial membranes synthesized at 18°C are more favorable for uptake of DNA, or perhaps the phases of the growth cycle that favor efficient transformation are extended.

Incubating bacterial cultures at 18°C is a challenge. Most laboratories do not have a shaking incubator that can accurately maintain a temperature of 18°C summer and winter. One solution is to place an incubator in a 4°C cold room and use the temperature control to heat the incubator to 18°C. Alternatively, there is almost no loss of efficiency if the cultures are grown at 20–23°C, which is the ambient temperature in many laboratories. Cultures incubated at these temperatures grow slowly with a doubling time of 2.5 to 4 hours. This can lead to frustration, especially late at night when it seems that the culture will never reach the desired OD_{600} of 0.6. The answer to this problem is to set up cultures in the evening and harvest the bacteria early the following morning. The procedure works well with many strains of *E. coli* in common use in molecular cloning, including XL1-Blue, DH1, JM103, JM108/9, DH5α, and HB101.

MATERIALS

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

DMSO <!>

Oxidation products of DMSO, presumably dimethyl sulfone and dimethyl sulfide, are inhibitors of transformation (Hanahan 1985). To avoid problems, purchase DMSO of the highest quality.

Inoue transformation buffer (please see Step 1)

Chilled to 0°C before use.

Nucleic Acids and Oligonucleotides

Plasmid DNA (recombinant plasmid)
Construct using one of the methods described in Protocols 17 through 22 of this chapter.

Media

LB or SOB medium for initial growth of culture
SOB agar plates containing 20 mM $MgSO_4$ and the appropriate antibiotic
Standard SOB contains 10 mM $MgSO_4$.
SOB medium, for growth of culture to be transformed
Prepare three 1-liter flasks of 250 ml each and equilibrate the medium to 18–20°C before inoculation.
SOC medium
Approximately 1 ml of this medium is needed for each transformation reaction.

Centrifuges and Rotors

Sorvall GSA rotor or equivalent

Special Equipment

Liquid nitrogen **<!>**
Polypropylene tubes (17 x 100 mm; Falcon 2059), chilled in ice
Shaking Incubator (18°C)
Water bath preset to 42°C

METHOD

▲ **IMPORTANT** All steps in this protocol should be carried out aseptically.

Preparation of Cells

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

   Organic contaminants in the H_2O used to prepare transformation buffers can reduce the efficiency of transformation of competent bacteria. H_2O obtained directly from a well-serviced Milli-Q filtration system (Millipore) usually gives good results. If problems should arise, treat the deionized H_2O with activated charcoal 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 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.

   b. 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	

   c. 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 LB broth or SOB medium 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.

   The ambient temperature of most laboratories rises during the day and falls during the night. The number of degrees and the timing of the drop from peak to trough varies depending on the time of year, the number of people working in the laboratory at night, and so on. Because of this variability, it is difficult to predict the rate at which cultures will grow on any given night. Using three different inocula increases the chances that one of the cultures will be at the correct density after an overnight incubation.
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.

   The cells are best suspended by swirling rather than pipetting or vortexing.
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.

Freezing of Competent Cells

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.

    Freezing in liquid nitrogen enhances transformation efficiency by ~5-fold.

    For most cloning purposes, 50-µl aliquots of the competent-cell suspension will be more than adequate. However, when large numbers of transformed colonies are required (e.g., when constructing cDNA libraries), larger aliquots may be necessary.
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.

    Glass tubes should not be used since they lower the efficiency of transformation by ~10-fold

Transformation

Include all of the appropriate positive and negative controls (please see the panel on **BACTERIAL TRANSFORMATION** in Protocol 23).

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 supercoiled 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.

    Heat shock is a crucial step. It is very important that the cells be raised to exactly the right temperature at the correct rate. The incubation times and temperatures given here have been worked out using Falcon 2059 tubes. Other types of tubes will not necessarily yield equivalent results.

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.

    To maximize the efficiency of transformation, gently agitate (<225 cycles/minute) the cells during the recovery period.

    If screening by α-complementation, proceed to Protocol 27 for plating.

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.

    When selecting for resistance to tetracycline, the entire transformation mixture may be spread on a single plate (or plated in top agar). In this case, collect the bacteria by centrifuging for 20 seconds at room temperature in a microfuge, and then gently resuspend the cell pellet in 100 µl of SOC medium by tapping the sides of the tube.

    ▲ **IMPORTANT** Sterilize a bent glass rod by dipping it into ethanol and then in the flame of a Bunsen burner. When the rod has cooled to room temperature, spread the transformed cells gently over the surface of the agar plate.

    When selecting for resistance to ampicillin, transformed cells should be plated at low density ($<10^4$ colonies per 90-mm plate), and the plates should not be incubated for more than 20 hours at 37°C. The enzyme β-lactamase is secreted into the medium from ampicillin-resistant transformants and can rapidly inactivate the antibiotic in regions surrounding the colonies. Thus, plating cells at high density or incubating them for long periods of time results in the appearance of ampicillin-sensitive satellite colonies. This problem is ameliorated, but not completely eliminated, by using carbenicillin rather than ampicillin in selective media and increasing the concentration of antibiotic from 60 µg/ml to 100 µg/ml. The number of ampicillin-resistant colonies does not increase in linear proportion to the number of cells applied to the plate, perhaps because of growth-inhibiting substances released from the cells killed by the 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.

    Transformed colonies may be screened for the presence of recombinant plasmids using one of the methods described in Protocols 27, 28, 31, and 32 of this chapter or Protocol 12 in Chapter 8.