DYSSEE

Inoue Chemically Competent Cells.

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Protocols

Inoue competent cells

Abstract

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 unknown. 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 h. To avoid reaching desired OD late at night, 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, DH5a, and HB101.

Material and reagents

1. Strains of *E. coli*: XL1-Blue, DH1, JM103, JM108/9, DH5a, and HB101.
2. 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. (Merck KGaA/EMD Millipore, catalog number: ES-002-10F)
3. PIPES (Alfa Aesar, catalog number: 3p B21835-22)
4. Deionized H_2O
5. Yeast Extract
6. Tryptone
7. KCl

8. NaCl
9. NaOH
10. $MgCl_2$
11. $MgSO_4$
12. Antibiotic
13. $MnCl_2 \cdot 4\ H_2O$
14. $CaCl_2 \cdot 2\ H_2O$
15. Glucose
16. Liquid nitrogen
17. Ethanol
18. Sugar
19. Inoue transformation buffer (see Recipes)
20. SOB medium (see Recipes)
21. SOC medium (see Recipes)
22. Luria-Bertani (LB) medium (see Recipes)
23. 0.5 M piperazine-1,2-bis(2-ethanesulfonic acid) (PIPES) (pH 6.7) (see Recipes)

Equipment

1. Centrifuges and Rotors (American Laboratory Trading)
2. Milli-Q filtration system (EMD Millipore)
3. Polypropylene 2059 tubes (17 x 100 mm) (BD Biosciences, Falcon®), chilled in ice
4. Shaking Incubator (18 °C)
5. Water bath (42 °C)
6. Nalgene filter
7. Disposable prerinsed Nalgene filter (0.45-mm pore size)
8. 250-ml flask
9. Sorvall GSA rotor
10. Vacuum aspirator
11. Bent glass rod
12. Bunsen burner

13. 0.22 µm filter

Procedure

A. 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 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). 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. 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.
c. Sterilize Inoue transformation buffer by filtration through a prerinsed 0.45-mm 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 h 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 h 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-L 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 min.

5. When the OD_{600} of one of the cultures reaches 0.55, transfer the culture vessel to an ice-water bath for 10 min. Discard the two other cultures.
6. 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.
7. Harvest the cells by centrifugation at 2,500 *x g* (3,900 rpm in a Sorvall GSA rotor) for 10 min at 4 °C.
8. Pour off the medium and store the open centrifuge bottle on a stack of paper towels for 2 min. Use a vacuum aspirator to remove any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck.
9. 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.
10. Harvest the cells by centrifugation at 2,500 *x g* (3,900 rpm in a Sorvall GSA rotor) for 10 min at 4 °C.
11. Pour off the medium and store the open centrifuge tube on a stack of paper towels for 2 min.
12. Use a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge tube or trapped in its neck.

B. Freezing of competent cells

1. Resuspend the cells gently in 20 ml of ice-cold Inoue transformation buffer.
2. Add 1.5 ml of DMSO. Mix the bacterial suspension by swirling and then store it in ice for 10 min.
3. Working quickly, dispense aliquots of the suspensions into chilled, sterile microfuge tubes.
4. 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 ml 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.

5. 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 min.
6. 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.

C. Transformation

1. Include all of the appropriate positive and negative controls.
2. Add the transforming DNA (up to 25 ng per 50 ml 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 min.
3. Transfer the tubes to a rack placed in a preheated 42 °C circulating water bath. Store the tubes in the rack for exactly 90 sec. 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.
4. Rapidly transfer the tubes to an ice bath. Allow the cells to cool for 1-2 min.
5. Add 800 ml 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 min 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.

6. Transfer the appropriate volume (up to 200 ml 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 sec at room temperature (RT) in a microfuge, and then gently resuspend the cell pellet in 100 ml 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 RT, 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 h at 37 °C. The enzyme b-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 mg/ml to 100 mg/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.
7. Store the plates at RT until the liquid has been absorbed.
8. Invert the plates and incubate them at 37 °C. Transformed colonies should appear in 12-16 h.

Recipes

1. LB medium

   Per Liter: To 950 ml of deionized H_2O, add

   Tryptone 10 g

Yeast Extract 5 g

NaCl 10 g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 ml). Adjust the volume of the solution to 1 L with deionized H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

2. SOB medium

a. Per Liter: To 950 ml of deionized H_2O , add

Tryptone 20 g

Yeast Extract 5 g

NaCl 0.5 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 (~0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 min 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 min at 15 psi (1.05 kg/cm^2) on liquid cycle).

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

c. Standard SOB contains 10 mM $MgSO_4$.

d. SOB medium, for growth of culture to be transformed.

e. Prepare three 1-liter flasks of 250 ml each and equilibrate the medium to 18-20 °C before inoculation.

3. Inoue transformation buffer

Reagent	Amount/L	Final concentration
$MnCl_2 \cdot 4\ H_2O$	10.88 g	55 mM
$CaCl_2 \cdot 2\ H_2O$	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 L	

4. Chilled to 0 °C before use.
5. SOC medium

   Approximately 1 ml of this medium is needed for each transformation reaction. SOC medium is identical to SOB medium except 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 sterilized by passing it through a 0.22 μm filter).
6. 0.5 M PIPES (pH 6.7)

   Dissolving 15.1 g PIPES in 80 ml of pure H_2O.

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