(Transformation of Competent E. coli with Plasmid Vector)

Principle of Transfomation:

Chemical transformation, bacteria are incubated in a solution containing Ca2+ ions, a process that is thought to ① create pores in the bacterial membranes, ②mask the negative charge on DNA, and promote binding of DNA to the membranes. Cells treated in this way are said to be competent.

The Hanahan Method:

E.coli

Plasmid DNA(1μg)

TE(pH 7.6)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | tryptone | Yeast extract | NaCl | Other added substances |
| SOB medium | 20g | 5g | 0.5g | 0.186gKCl, 2M MgCl2 5ml |
| SOC medium | 20g | 5g | 0.5g | 0.186gKCl, 2M MgCl2 5ml 1M Glucose 20ml |

Preparing Seed Stocks of Cells for transformation:

1. Use an inoculating loop to streak E.coli of the desird strain directly from a frozen stock onto the surface of an SOB agar plate.Incubate for 16h at 23℃.
2. Transfer four or five well-isolated colonies into 2mL of SOB in 17×100-mm polypropylene tubes.
3. Incubate the culture for 16h at 23℃ in a shaking incubator.
4. To each culture, add sterile glycerol to a final concentration of 15%,v/v.
5. Aliquot 1-mL quantities into 2.5-mL cryotubes.Insert the tubes into a plastic zipper bag and immerse the bag for 5min in a dry-ice/ethanol bath.
6. Transfer the bag and its contents to a －80℃ freezer.

Preparation of Competent Cells:

1. Inoculate 250mL of SOB medium with a 1-mL vial of seed stock.
2. Incubate the culture at 21℃ in a shaking incubator for ～16h. From time to time,　check the optical density of an aliquot of the culture at 600nm. Do not allow the culture to grow to an OD600>0.26.
3. Transfer the culture to prechilled, flat-bottomed centrifuge bottles and centrifuge for 10min at 4 ℃.
4. Remove as much of the supernatant as possible by pouring, followed by gentle aspiration through a wide-bore pipette.
5. Gently resuspend the cells by adding 20mL of ice-cold CCMB80 buffer and swirling the contents of the centrifuge bottle.When the pellet is reasonably well suspended, add another 60mL of ice-cold CCMB80 buffer and swirl again. If the suspension is still lumpy, disperse the lumps by very gentle up-and down pipetting.Then, incubate the suspension for 20 min in ice.
6. Centrifuge the suspension for 10 min at 4℃. Read the OD at 600nm.
7. Remove the supernatant as before and resuspend the pellet in 10 mL of ice-cold CCMB80.
8. Transfer 200μl of SOB to a plastic tube. Add 50 μl of the E. coli suspension and mix gently, avoiding air bubbles.
9. Calculate the amount of CCMB80 buffer required to adjust the OD of the remainder of the bacterial suspension to between 1.0 and 1.5. Add the required amount of ice-cold CCMB80 to the suspension.
10. Incubate the diluted suspension for 20min on ice.
11. Dispense 50-μl aliquots into prechilled 2-mL screw-top freezing vials. Freeze the susupension as before.
12. Test the competence of the cells as described below.

Concept of colony forming unit

In microbiology, colony-forming unit (CFU) is a rough estimate of the number of viable bacteria or fungal cells in a sample. The visual appearance of a colony in a cell culture requires significant growth - when counting colonies it is uncertain if the colony arose from one cell or 1,000 cells. Results are reported as CFU/mL (colony-forming units per milliliter) for liquids, and CFU/g (colony-forming units per gram) for solids to reflect this uncertainty (rather than cells/mL or cells/g).The plate count is linear for *E. coli* over the range of 30 - 300 CFU on a standard sized petri dish. The CFU/plate is read from a plate in the linear range, and then the CFU/g (or CFU/mL) of the original is deduced mathematically, factoring in the amount plated and its dilution factor.



A solution of bacteria at an unknown concentration is often serially diluted in order to obtain at least one plate with a countable number of bacteria. In this figure, the "x10" plate is suitable for counting.

Calculation example:

Sample: 0.1ml,

Dilution rate: 100-fold dilution

Inoculated dishes: 2dishes (54 colony and 47 colony)

(50+47)/2(colony average number) ×100(dilution rate)×10(1.0 mL/0.1 mL)≒4.9×10^3 cfu/mL

Measuring Competence:

1. Prepare for the vector(plasmid DNA).
2. Transfer E.coli(DH5α: titer 1.6×108CFU/μg pBSKS) from the deep freezer. Place the tubes containing E.coli on ice.
3. Transfer Amp Agarose gels from low-temperature chamber. ※Add 10～15ml of Amp(100mg/ml) into the old gels.
4. Dilute the DNA. The concentration of plasmid DNA in the dilute solution should be ～10-5 μg/μL. Store the DNA solution on ice until needed.

Plasmid DNA 1μl(undiluted solution or ten-fold dilution with DDW of TE)

E.coli 100μl(or 50μl)

1. Place the tubes on ice for 10 min.
2. Incubate the tube 1’, 2’ and 3’ in a water bath at 42℃ for 90sec.
3. Place the amp agarose gel(4℃) upside down in clean bench to avoid dew condensation.
4. Add 900μl of SOC(15ml tube with yellow seal)..
5. Centrifuge them at 15000rpm, for 1 min.
6. Immerse glass rod in alcohol and roast in burner in clean bench.
7. Place dish containing Amp agarose gel on a rotating wheel.
8. Discard 900μl of the supernatant, pipette it up and down using 100μl tip.
9. Spread the inoculum evenly over the surface of Amp agarose gel. Slowly shake the plate back and forth, turning the plate roughly 90°every few seconds. Dry them opening the cover for 5min.
10. Transfer the plates to an incubator and incubate them upside down for 16 hours～overnight at 37℃.
11. Measure the number of colony, calculate the efficiency of transduction.

Culture the competent cell:

Super broth:

Tryptone 32g

Yeast extract 20g

NaCl 5g

10NNaOH 0.5ml

DDW /1L

LB plate:

NaCl 10g

Agar 15g

Polypeptone 10g

Yeast extract 5g

DDW 1L

10NsOH 0.5ml

Small scale amplification: for mini prep

Culture medium:

Super broth(LB medium) 20ml

Amp 20μl(100mg/ml)※The types and concentrations of antibiotics vary depending on what kind of vector is used for transformation.

1. Divide the medium into 6 Eiken tube(3ml) written about the gene name using tape.
2. Number colonies:1〜6 on the bottom of masterplate(LB plate amp＋).
3. Flame the tip of toothpick.
4. Pick up the single colony with the tooth pick. Place the bacteria on the number. (Colony without plasmid; satellite cells can’t grow up in masterplate(LB plate amp＋).
5. Place the bacteria into the medium of the same number.
6. Inoculate 3ml of culture medium.
7. Move to P3 room.
8. Incubate the masterplate upside down at 37℃ overnight
9. Incubate the tubes into the box overlaid with kimwipe at 37℃ with vigourous shaking for over 16h. ※Take care not to over-incubate the liquid media. Harvest bacteria at log phase.
10. Store the masterplate at 4℃.
11. Centrifuge 6 tubes at 3000rpm, 4℃, for 30 min.
12. Remove the supernatant in waste pan. Pour hypochlorous acid into the supernatant and discard it.
13. Stand 6 tubes top down on the KimWipe for 10 min to remove the supernatant completely.
14. Store 6 tubes at -20℃.

Note: Next time frozen bacteria die and elute DNase and RNase. So after thawing it, treat it rapidly.

PureYieldTM Plasmid Miniprep System(Promega):

1. Add 600μl of TE to bacterial culture and transfer the suspension to a 1.5ml microcentrifuge tube.
2. Add 100μl of Cell Lysis Buffer, and mix by inverting the tube 6 times. The solution should change from opaque to clear blue, indicating complete lysis.
3. Add 350μl of cold(4-8℃) Neutralization Solution within 2 minutes after Step2 finished, and mix thoroughly by inverting neutralization to ensure complete neutralization. The sample will turn yellow when neutralization is complete.
4. Centrifuge it at maximum speed (11,000～16,000×g) in a microcentrifuge for 3 min.
5. Transfer the supernatant (～900μl) to a PureYieldTM Minicolumn with a pipette..
6. Place the minicolumn into a PureYieldTMCollection Tube, and centrifuge at maximum speed in a micro centrifuge for 15 sec.
7. Aspirate the flowthrough, and place the minicolumn into the same PureYieldTM Collection Tube.
8. Add 200μl of Endotoxin Removal Wash to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30 sec, aspirate the flowthrough.
9. Add 400μl of Column Wash Solution to the minicolumn. Centrifuge at maximum speed in a microcentrifuge for 30sec. Aspirate the flowthrough and centrifuge at maximum speed for 15sec again.
10. Transfer the minicolumn to a clean 1.5ml microcentrifuge tube, then add 50μl of Elution Buffer directly to the minicolumn matrix.
11. Let stand for 1min at RT.
12. Centrifuge at maximum speed in a microcentrifuge for 15sec to elute the plasmid DNA.
13. Cap the microcentrifuge tube, and store eluted plasmid DNA at－20℃.
14. OD:100～200ng/μl
15. Run a gel with 0.8% agarose gel for 20min.(sample : loading buffer = 1 : 1)

Large scale amplification : for transfection, public

Culture medium:

Super broth(LB medium)50ml(100ml)

Amp 50μl(100μl)(100mg/ml)※The types and concentrations of antibiotics differ according to the type of vector that is used for transformation.

1. Pour the culture medium into 300ml Erlenmeyer flask each 50ml.
2. Roast the tip of tooth pick.
3. Pick up the single colony from and inoculate 50ml of culture medium.
4. Move to P3 room.
5. Incubate the tubes into the box fixed using metal loop at 37℃ with vigourous shaking for over 24h.
6. Store them at 4℃.
7. Centrifuge tubes at 3000rpm, 4℃, for 30 min.
8. Discard the liquid, place the kimwipe on the desk and stand the tube top down on the desk and remove the extra water.
9. Store them at -20℃.

Growth chart(curve) of E.coli:

Lag phase:

Logarithmic growth phase:

Stationary phase(plateau phase): 1×109cells/ml

Death phase:

Purification by PEG:Maxi prep

Alkaline Lysis Solution Ⅰ:

Glucose 50mM

Tris-Cl(pH 8.0) 25mM

EDTA(pH 8.0) 10mM

Autoclave for 15 min and store at 4 ℃.

Alkaline Lysis Solution Ⅲ:

Potassium acetate(5M)　　 60ml

Glacial acetic acid 11.5ml

H2O 28.5ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate. Store the solution at 4℃ and transfer it to an ice bucket just before use.

Principle:

DNA has minus charge and phosphate backbone, and is hydrophilic colloid.

PEG(polyethylene glycol(-CH2-CH2-O-)n-) can aggregate and precipitate DNA to derive hydration water and remove RNA, since RNA has higher affinity with hydration water and is hard to be aggregated.

EtOH and isopropyl alcohol are water-soluble organic solvent, and derive hydration water.

Ability to precipitate nucleic acid: EtOH<isopropyl alcohol

EtOH is easy to be evaporated.

Ammonium acetate is useful to remove protein and precipitate protein.

LiCl can precipitate high-molecular-weight RNA on ice.

Ability to remove protein(intermediate):phenol<phenol/chloroform※wear rubber gloves to protect hands from phenol and chloroform that have toxicity and causticity.

PEG precipitation:

Preparation of SolutionⅡ:

10N NaOH 1ml(0.2N)

10% SDS 5ml(1%)

DDW Mess up

Total 50ml

Lysis by alkali:

1. Add 5ml of SolⅠ(cold room) to frozen pellet not to thaw it, and vortex it and unharden E.coli completely.※DNase elutes when thawed, because E.coli is dead.
2. Add 10ml of SolⅡ, invert the tube upside down quietly to make a hole in cell membrane of E.coli. Do not vortex it because genomic DNA elutes.
3. Add 7.5ml of SolⅢ(cold room), invert the tube upside down quietly and neutralize it. Do not vortex it because genomic DNA elutes.
4. Centrifuge it at 3000 rpm for 15min.
5. Pour 25ml of isopropyl alcohol(2-propanol) into the new 50ml tube and place twofold kimwipe on the tube not to touch isopropyl alchol.
6. Filtrate the supernatant through a kimwipe into isopropyl alcohol and squeeze the kimwipe. ※It can be stored at－20℃.
7. Vortex it.
8. Centrifuge it at 3,000rpm for 10min.
9. Discard the supernatant and stand the tube top down to extract the nucleic acid.
10. Dissolve the pellet in 3ml of TE or DDW, pipette it down.
11. Add 3ml of ice-cold 5M LiCl and place it on ice for 1min until it turns white.
12. Centrifuge it at 3000rpm for 10min to precipitate high-molecular-weight RNA.
13. Transfer the supernatant to new 50ml tube by decantation.
14. Add 5ml of isopropanol and mix well.
15. Centrifuge it at 3000rpm,RT for 15min.
16. Remove the supernatant by decantation.
17. Resuspend a pellet with 1ml of 70%EtOH, vortex it and transfer it to siliconized 1.5ml tube.(×2)
18. Centrifuge it at 15000rpm, RT for 5min.
19. Discard the supernatant using 1000μl tip. Dry up for 1～2 min.
20. Resuspend a pellet with 500μl of TE. Vortex it and incubate it at 55℃to be completely soluble. ※It can be stored at－20℃.
21. Add 5μl of 100mg/ml RNaseA(－20℃).
22. Incubate it at 37℃ for 30min ～ 1h. ※It can be stored at－20.
23. Add 300μl of 2.5M NaCl containing 20%(w/v) PEG and mix well.
24. Centrifuge it at 15000rpm,4 ℃ for 5min.
25. Remove the supernatant rapidly.

Wash:

1. Add 1ml of 70% EtOH and vortex for few sec. Centrifuge it at 15000rpm, 4℃ for 5min. Remove the sup by decantation.(×4 :1min→1min→1min→1min)
2. Add 700μl of TE(55℃) to the pellet and suspend well and incubate it at 55℃ for 3min. In the case of 100ml culture, put together into a whole.
3. Prepare 1 siliconized tubes containing 700μl of Phenol, 4 siliconized tubes containing 500μl of phenol/chloroform, 3 siliconized tubes containg 500μl of chloroform. Mix 1000μl of 100%EtOH with 100μl of 10M ammonium acetate in a siliconized tube.
4. Add the solution into 700μl of phenol(4℃→RT) to denature protein and invert well. Centrifuge it at 15000rpm,RT, for 1min. Check protein and PEG in interphase. Transfer the aqua phase(upper layer) to the eppentube containing phenol(×1).※Avoid aspirating the white layer into the nucleic acid solution.
5. Add the solution into 500μl of phenol/chloroform(1:1, 4℃→RT, avoid light) to the solution to separate protein and invert well. Centrifuge it at 15000rpm, RT for 1 min. Transfer the aqua phase to the eppentube that added phenol chloroform. (×3～4)
6. Transfer the aqua phase to the eppentube that added 500μl of chloroform to remove phenol. Centrifuge it at 15000rpm, RT for 1 min. (×2～3)
7. Transfer upper phase to a fresh eppen tube to 100μl of 10M ammonium acetate and 1000μl of 100%EtOH.(solution : EtOH=1:2.5) ※solution : isopropanol = 1:0.8
8. Centrifuge it at 15000rpm, 4℃ for 15min.
9. Remove the sup.
10. Rinse the pellet with 1ml of 70% EtOH.
11. Centrifuge it at 15000rpm, 5 min for 4 ℃.
12. Remove the sup.
13. Resuspend with 500μl of TE at 55℃ for 10min.
14. Invert it well.
15. Load 1μl of the sample and 1μl of loading buffer run a gel. It’s recommended that yield of supercoiled DNA (cccDNA, covalently closed circular DNA) is more than yield of ocDNA(open circular DNA) and linear DNA.

Purification of plasmid :JET STAR/MAX１, 50-100ml culture

 **Buffer Compositions:**

E1: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/ml RNase A Buffer

 E2: 200 mM NaOH, 1%(w/v) SDS

 E3: 3.1 M potassium acetate (pH 5.5 with acetic acid)

 E4: 100 mM Sodium acetate (pH 5.0 with acetic acid), 600 mM NaCl, 0.15% Triton X-100

 E5: 100 mM Sodium acetate (pH 5.0 with acetic acid), 800 mM NaCl

 E6: 100 mM Sodium acetate (pH 5.0 with acetic acid), 1.500 mM NaCl

1. **Equilibration: The JETSTAR 2.0 Midi columns are delivered with the Lysate Filter Unit (LFU) inserted into the 30ml/column housing**.  Place the JETSTAR 2.0 column into a suitable rack and equilibrate the column with **14 ml of equilibration buffer E4** by pouring the buffer directly into the filter device.  Wait for 30min.

*The equilibration buffer will go through the filtration matrix thereby pre-wetting it. Then the buffer will pass through the JETSTAR resin and equilibrate it. The column will start dripping. While column equilibration is in process, harvest the bacterial cells and prepare the cleared lysate as described below.*  *Shortly after the column has begun dripping, some drops at the outlet may appear turbid. This is normal and due to the interaction of the equilibration buffer with the matrix. The turbid drops will not affect the preparation in any way.*

1. Spin down the bacterial cells from the *E. coli* culture for **3 min** at ≥**12,000 x g**. Remove the supernatant quantitatively with a pipet.
2. Vortex the bacterial cells from the *E. coli* culture.
3. Resuspend the cells in **10ml buffer E1 (reconstituted with 100 μg/ml RNase stored at –４℃)**. Vortex or pipette down it and No cell clumps must be visible.
4. Cell lysis: Lyse the bacterial cells with **10 ml buffer E2.** Mix gently but **thoroughly** (e.g. by multiple inverting) until a **homogeneous mixture** is obtained. **DO NOT VORTEX!!!** Due to the release of genomic DNA the mixture is very viscous at this stage. Incubate at **RT** for **5 min**.
5. Neutralization: Neutralize the lysis mix with **10 ml buffer E3**. Mix gently but thoroughly (e.g. by multiple inverting) until a homogeneous mixture is obtained. DO NOT VORTEX!!! The liquid of the neutralized lysate must be completely non- viscous again. No remainders of the viscous matter obtained after lysis of the cells (step 4) must be left.
6. Centrifuge it at 3000rpm, RT for 20min.
7. Get the supernatant(neutralization mix) with all the precipitated matter through 30ml syringe be packed with sterile cotton.
8. Apply the supernatant in it into the Lysate Filter Unit (LFU) inserted into the previously equilibrated (step 1) JETSTAR / LFU column. Let the lysate run through by gravity flow until the flow stops or dripping becomes very slow (< 1 drop per 10 seconds). Do not force out any remaining liquid!
9. As soon as the JETSTAR column has stopped dripping, take the filtration device out of the column housing and discard it. Wash the JETSTAR column by applying **60 ml of wash buffer E5**. Let the wash buffer flow through the column by gravity flow until all liquid has passed through the resin. Do not force out any remaining buffer!
10. ELUTION: Pour 10.5ml of isopropanol into the new 50ml tube. Remove the waste tray and place a clean, sterile collection 50ml tube under the outlet nozzle of the JETSTAR 2.0 column. Elute the DNA from the column into the clean tube with **15 ml of elution buffer E6**. Precipitate the DNA by mixing the eluate thoroughly with **15ml of isopropanol.**
11. Uncap the tube and wrap it with parafilm.
12. Move to the P3. Spin down the DNA for 30 min at ≥8000rpm(9.000 g) 4°C using ultracentrifugation(Hitachi HimacI
13. After precipitation the DNA is washed with 1ml of 70-80% ethanol.
14. Transfer it into new eppendorf.
15. Centrifuge it at 15000rpm, 4℃ for 5 min. Washing
16. Dry it for 1 min.
17. Dissolve the DNA in 500μl of 10 mM Tris-HCl or TE buffer (pH 8.0 – 8.5) or simply water.
18. Incubate it at 65℃ for 30min〜1hr.