Look What They've Done To My Shoes!

SCU_China Project 2016

Protocol

General Protocol

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Protocol of Plasmid Miniprep

E.Z.N.A.™ Plasmid Mini Kit I (OMEGA)

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1-5 ml LB media containing the appropriate selective antibiotic. Incubate for 12-16 hr at 37℃ with vigorous shaking (about 300rpm). Use a 10-20 ml culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of E.coli be used for routine plasmid isolation. Examples of such strains include DH5α and JM109.

2. Pellet 1.5-5.0 ml bacteria by centrifugation at 10,000 x g for 1 min at room temperature. Decant or aspirate medium and discard.

3. Resuspend the bacterial pellet by adding 250 μl of Solution I/RNase A and vortexing (or pipetting up and down). Complete resuspension (no visible cell clumps) of cell pellet is vital for obtaining good yields.

4. Add 250 μl of Solution II and gently mix by inverting and rotating the tube several times to obtain a clear lysate. A 2 minutes incubation may be necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 min. (Store Solution II tightly capped when not in use to avoid acidification from CO2 in the air.)

5. Add 350 μl of Solution III and mix immediately by inverting the tube several times until a flocculent white precipitate forms. It is vital that the solution is mixed thoroughly, and immediately after the addition of Solution III to avoid localized precipitation.

6. Centrifuge at ≥ 13,000 x g for 10 minutes at room temperature. A compact white pellet will form. Promptly proceed to the next step.

7. Add the cleared supernatant by CAREFULLY aspirating it into a clean HiBind Miniprep Column I assembled in a provided 2 ml collection tube. Ensure that the pellet is not disturbed and that no cellular debris has been carried over into the column. Centrifuge for 1 min at 10,000 x g at room temperature to completely pass lysate through the HiBind Miniprep Column I

8. Discard flow-through liquid and re-use the 2 ml collection tube. Add 500 µl of Buffer HB to wash the HiBind Mniprep Column I. Centrifuge for 1 min at 10,000 x g at room temperature to completely pass solution through the HiBind Miniprep Column I. This step ensures that residual protein contaminations are remved, thus ensuring high quality DNA that will be suitable for downstream applications.

9. Discard flow-through liquid and re-use the 2 ml collection tube. Add 700 µl of DNA Wash Buffer diluted with absolute ethanol to wash the HIBind Miniprep Colun I. Centrifuge for 1 min at 10,000 x g at room temperature to completely pass solution through the HiBind Miniprep Column I and discard flow-through liquid.

Note: DNA Wash Buffer concentrate must be diluted with absolute ethanol before use. See label for directions. If refrigerated, DNA Wash Buffer must be brought to room temperature before use.

10. Optional Step: Repeat wash step with another 700 µl of DNA Wash Buffer diluted with absolute ethanol.

11. Centrifuge the empty column for 2 min at ≥ 13,000 x g to dry the column matrix. Do not skip this step, it is critical for good yields.

12. Place the column into a clean 1.5 ml micro-centrifuge tube. Add 30 µl to 50 µl (Depending on desired concentration of final

product) of Elution Buffer(10mM tris-HCl, PH 8.5) or sterile deionized water directly onto the column matrix and let it sit at roon temperature for 1-2 minutes. Centrifuge for 1 min at ≥13,000 x g to elute DNA. An optional second elution will yield any residual DNA, through at a lower concentration.

Protocol of Gel Extraction

E.Z.N.A.™ Gel Extraction Kit (OMEGA)

1. Perform agarose gel/ethidium bromide electrophoresis to fractionate DNA fragments. Any type or grade of agarose may be used. It is strongly recommended however, that fresh TAE buffer, or TBE buffer be used as running buffer, Do not reuse running buffer as its pH will increase and reduce yields.

2. When adequate separation of bands has occurred, CAREFULLY excise the DNA fragment of interest using a wide, clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

3. Determine the appropriate volume of the gel slice by weighing it in a clean 1.5 ml microcentrifuge tube. Assuming a density of 1g/ml of gel, the colume of gel is derives as follows: a gel slice of mass 0.3g will have a volume of 0.3ml. Add an equal volume of Binding Buffer (XP2). Incubate the mixture at 50 ℃ -55 ℃ for 7min or until the gel has completely melted. Mix by shaking or vortexing the tube in increments of 2-3 minutes.

IMPORTANT: Monitor the pH of the Gel/Binding Buffer mixture after the gel has completely dissolved. DNA yields will significantly decrease when pH>8.0. If the color of the mixture becomes orange or red, add 5 μl of 5M sodium acetate, pH 5.2 to bring the pH down. After this adjustment, the color of the Gel/Binding Buffer mixture should be light yellow.

4. Place a HiBind DNA column in a provided 2 ml collection tube.

5. Apply 700 µl of the DNA/agarose solution to the HiBind DNA column, and centrifuge at 10,000 x g for 1 min at room temperature.

6. Discard liquid and place the HiBind DNA column back into the same collection tube. For volumes greater than 700 µl, load the column and centrifuge successively, 700 µl at a time. Each HiBind DNA column has a total capacity of 25 µg DNA. If the expected yield is larger, divide the sample into the appropriate number of columns.

7. Add 300 µl of Binding Buffer (XP2) into the HiBind DNA column, Centrifuge at 10,000 x g for 1 min at room temperature to wash the column. Discard the flow-through and re-use the collection tube.

8. Wash the HiBind DNA column by adding 700 µl of SPW Wash Buffer diluted with absolute ethanol. Centrifuge at 10,000 x g for 1 min at room temperature.

Note: SPW Wash Buffer Concentrate must be diluted with absolute ethanol before use, See label for directions. If refrigerated, SPW Wash Buffer must be brought to room temperature before use.

9. Optional: repeat step 8 with another 700 µl of SPW Wash Buffer diluted with absolute ethanol.

NOTE: Perform the second wash step for any salt sensitive downstream applications.

10. Discard liquid and centrifuge the empty HiBind DNA column for 2 min at maximal speed to dry the column matrix. Do not skip this step, it is critical for the removal of ethanol fro the HiBind DNA column.

11. Place a HiBind DNA column into a clean 1.5 ml microcentrifuge tube. Add 15-30 µl (depending on desired concentration of final product) of Elution Buffer (10 mM tris-HCl, pH 8.5) directly on to the column matrix and incubate at room temperature for 1 minute. Centrifuge for 1 min at maximal speed to elute DNA. This represents

approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

Protocol of PCR Product Purification

E.Z.N.A.™ Cycle-Pure Kit (OMEGA)

1. Perform agarose gel/ethidium bromide electrophoresis to analyze PCR product.

2. Determine the volume of the PCR reaction, transfer the sample into a clean 1.5ml microcentrifuge tube, and add 4-5 volumes of Buffer CP. For PCR products <200 bp add 6 volumes of Buffer CP.

3. Vortex thoroughly to mix. Briefly spin the tube to collect any drops from the inside of the lid.

4. Place a HiBind DNA column in a provided 2 ml collection tube.

5. Apply the sample from Step 3 to the HiBind DNA column, and centrifuge at 10,000 x g for 1 min at room temperature.

6. Discard liquid and place the HiBind DNA column back into the same collection tube.

Note: The HiBind DNA column has a maximal capacity of 700μl. For volumes greater than 700μl, repeat Step 5-6 until all the remaining of the sample pass through the HiBind DNA column. Each HiBind DNA column has a total capacity of about 15ng DNA.

7. Wash the HiBind DNA column by adding 700μl of DNA Wash Buffer diluted with absolute ethanol. Centrifuge at 10,000 x g for 1 min at room temperature.

IMPORTANT: DNA Wash Buffer Concentrate must be diluted with absolute ethanol before use.

8. Discard liquid and repeat step 7 by adding 700 µl of DNA Wash Buffer diluted with absolute ethanol.

9. Discard liquid and centrifuge the empty HiBind DNA column for 2 min at maxi speed (≥ 13,000 x g) to dry the column matrix. This is critical for good yields.

10. Place a HiBind DNA column into a clean 1.5 ml microcentrifuge tube. Add 15-30 µl (depending on desired concentration of final product) **of Elution Buffer** (10 mM tris-HCl, pH 8.5) **directly on to the column matrix and incubate at room temperature for 1-2 minute. Centrifuge for 1 min at ≥ 13,000 x g to elute DNA.** This represents approximately 80%-90% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.

Protocol of PCR

Taq DNA Polymerase (Biomed™)

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and template DNA. Taq DNA polymerase should be the last component added. Prepare sufficient master mix for the number of reactions plus one extra to allow for pipetting error.

1. Gently vortex and briefly centrifuge all solutions after thawing.

2. Place a thin-walled PCR tube on ice and add the following components for each 50 µl reaction:

Water, nuclease-free	variable

10X Buffer with $MgCl_2$	5 μl
dNTP Mix, 2.5mM each	4 μl
Forward primer	10 μM
Reverse primer	10 μM
Template DNA	< 0.5 μg
Taq DNA Polymerase (5U/μl)	0.5-1 μl
Total volume	50 μl

3. Gently vortex the samples and spin down.

4. If using a thermal cycler that does not use a heated lid, overlay the reaction mixture with 25 μl of mineral oil.

5. Perform PCR using the following thermal cycling conditions:

Step	Temperature, ℃	Time	Number of cycles
Initial denaturation	94	1-3 min	1
Denaturation	94	30s	30
Annealing	Tm-5	30s	
Extension	72	1 min/1-2kb	
Final extension	72	5-10 min	1

To prepare several parallel reactions and to minimize the possibility of pipetting errors, prepare a PCR master mix by mixing water, buffer, dNTPs, primers and template DNA. Pfu DNA polymerase should be the last component added. Prepare sufficient master mix for the number of reactions plus one extra to allow for pipetting error.

1. Gently vortex and briefly centrifuge all solutions after thawing.

2. Place a thin-walled PCR tube on ice and add the following components for each 50 µl reaction:

Water, nuclease-free	variable
10X Pfu Buffer with $MgSO_4$	5 µl
dNTP Mix, 2mM each	5 µl (0.2 mM of each)
Forward primer	0.1-1 µM
Reverse primer	0.1-1 µM
Template DNA	50 pg-1 µg
Pfu DNA Polymerase	1.25-2.5u
Total volume	50 µl

3. Gently vortex the samples and spin down.

4. If using a thermal cycler that does not use a heated lid, overlay the reaction mixture with 25 µl of mineral oil.

5. Perform PCR using the following thermal cycling conditions:

Step	Temperature, ℃	Time	Number of cycles

Initial denaturation	95	1-3 min	1
Denaturation	95	30s	25-35
Annealing	Tm-5	30s	
Extension	72	2 min/kb	
Final extension	72	5-15 min	1

Protocol of Enzymatic Digestion (EcoR I, Xba I, Spe I, Pst I) Takara™

General Reaction Mixture:

Restriction enzyme	1 μl
10 x Buffer	2 μl
DNA	≤ 1 μg
Steriled Water	Up to 20 μl

Reaction temperature: 37℃

Note: 2 types of 10 x Buffer are used. 10 x M Buffer is used for Xba I and Spe I, 10 x H Buffer is used for EcoR I and Pst I.

Reaction Time: Generally, 5min reaction under 37℃ is enough to digest target DNA completely. If it doesn't work well for special substrate DNA, the reaction time can be extended to 1h.

Protocol of Ligation T4 DNA Ligase

Thermo™ #EL0014

1. Prepare the following reaction mixture:

Linear Vector DNA	20-100 ng
Insert DNA	1:1 to 5:1 molar ratio over vector
10X T4 Ligase Buffer	2 µl
T4 DNA Ligase	1 Weiss U
Water, nuclease-free	To 20 µl
Total volume	20 µl

2. Incubate 10 min at 22°C.

3. Use up to 5 µL of the mixture for transformation of 50 µL of chemically competent cells or 1-2 µL per 50 µL of electrocompetent cells.