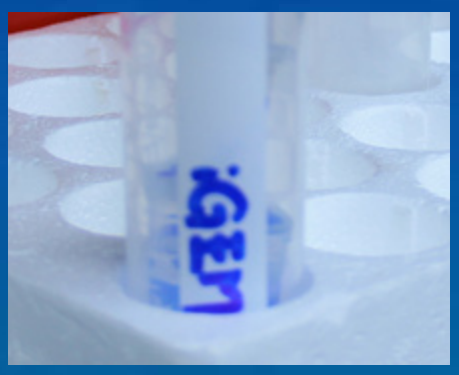
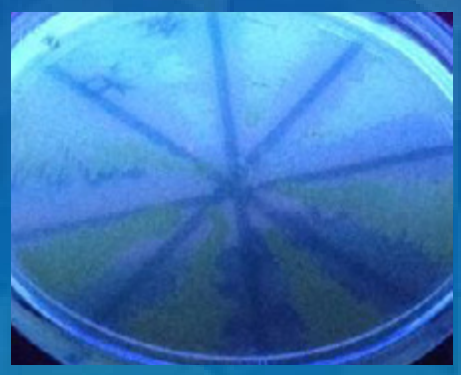
Laboratory Protocols



VIRTUS PARVA

Contents

DNA Extraction

Two methods were pursued for the extraction of DNA from E. Coli

The first method:

1. Incubate the bacteria up to saturation in a Petri dish with LB broth. at 37°C for 24 hr

2. The Bacteria is then passed into an Eppendorf tube, adding each 567 µL of TE buffer and 30 µL proteinase K 20mg/mL. Incubate for an hour and 37°C

3. Add 100 mL NaCl 5M, mix vigorously. Add 80 mL of the CTAB/NaCl solution and leave to dry at 65°C for 10 min in the thermoblock, for further addition of a chloroform/isoamylic alcohol solution and centrigue for 5 minutes

4. Transfer the aquous phase onto a fresher tube and add a phenol/chloroform/isoamylic alcohol volume to later centrigue and resuspend on 100 mL TE buffer

The second method:

1. The bacterian batch is cultivated to saturation in broth and it's centrifuged at 5000 rpm and 4°C for 10 minutes.

2. Add 5 mL TE buffer, centrifuge to the same conditions. Add 5 mL of the ph/chl/isoam solution, and mix vigorously for 3 minutes.

3. Centrifuge at 6000 rpm and 4°C for 20 minutes, dispose of the leftover liquid and let the tubes dry out.

4. 0.5 mL TE buffer are added, as long as two volumes of absolute ethanol, centrifuge at 1300 rpm and add 200 µL to the tubes.

5. At completion of the extraction, run an electrophoresis to prove the presence of DNA.

Plasmid DNA Purification Spin Miniprep Protocol

Using the Axygen Kit

Note: The use of ethanol at 96% induces precipitation of the ADN

1. 1-4 mL of LB culture is collected. Centrifuge at 12,000 rpm at 4°C for 5 minutes to pellet the bacteria. Decant as much of the supernatant as posible. Add, EtOH again, and repeat the process. After assuring that the strand of DNA is inside the Eppendorf. Leave the excess supernatant to dry off, when it almost dries completely off, it's time to begin the procedure.

Note: When using rich broths such as LBG or 2×YT, reduce the culture volume by half. Excessive bacteria will reduce lysis efficiency, resulting in low yield and reduced purity of the plasmid DNA. Do not exceed 2 ml of bacterial culture grown in rich broth.

2. Resuspend the bacterial pellet in 250 µl of Buffer S1 by vortexing. Please be sure that the bacteria are completely resuspended before proceeding.

Note: Be sure that RNAse A has been added into Buffer S1.

3. Add 250 µl of Buffer S2, and mix by gently inverting the tube for half a minute. Do not vortex.

Note: Vigorous shaking or vortexing will cause shearing of the bacterial genomic DNA and result in the contamination of the plasmid DNA.
After use, the buffer S2 bottle should be closed immediately in order to avoid neutralization of NaOH by ambient CO2. Buffer S3 (Step 4, below) must be added within 5 minutes.

4. Add 350 µl of Buffer S3, and mix by gently inverting during half a minute. Centrifuge at 12,000 rpm for 10 minutes to clarify the lysate. Do not vortex.

Note: Vigorous shaking or vortexing will result in contamination with genomic DNA.

5. Place a Miniprep column into an uncapped 2 ml Microfuge tube (provided). Transfer the clarified supernatant from Step 4 into the Miniprep column. Transfer the Miniprep column and 2 ml Microfuge tube to microcentrifuge and spin at 12,000 rpm for 1 minute.

6. Buffer W1 Wash
Washing with Buffer W1 is required only in cases where the plasmid has been propagated in an endA+ bacterial strain. These strains often exhibit high levels of endonuclease activity which will degrade the plasmid DNA.
Proceed to Step 7 if an endA- bacterial strain is used.
Pipette 500 µL of Buffer W1 into each Miniprep column. Centrifuge at 12,000 rpm for 1 minute.

7. Pipette 700 µL of Buffer W2 into each Miniprep column. Centrifuge at 12,000 rpm for 1 minute.

Note: Make sure that the volume of ethanol specified on the bottle label has been added to the Buffer W2 concentrate.

Purification of Genomic DNA from Cultured Animal Cells

Using the Axygen Kit

Cells grown in suspension or a cell suspension freshly-isolated from animal or human tissues:

1A. Collect 1x103-2x106 cells in suspension and transfer to a 2 ml microfuge tube (provided). Centrifuge for 5 minutes at 2,000xg to pellet the cells.

2A. Add 350 µl of PBS to resuspend the cells and then add 150 µl of Buffer C-L. Let the tube stand for 1 minute at room temperature

3. Add 0.8 µL of RNase A and 8 µL Proteinase K. Vortex for 15 seconds and Incubate at 56°C for 15 minutes. Briefly centrifuge to remove drops from inside the lid.

Note: Do not add Proteinase K directly to buffer C-L.

4. Add 350 µL Buffer P-D to the sample and mix by vortexing at top speed for 30 seconds.

5. Centrifuge for 10 minutes at 12,000 rpm at ambient temperature to pellet cellular debris.
Binding, washing and elution on the Miniprep column

6. Place a Miniprep column into a 2 ml microfuge tube (provided). Pipette the clarified supernatant obtained from step 5 into the Miniprep column. Centrifuge for 1 minute at 12,000xg

7. Discard the filtrate from the 2 ml microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube. Pipette 500 µL of Buffer W1 to the Miniprep column and centrifuge for 1 minute at 12,000rpm.

8. Discard the filtrate and place the Miniprep column back into the 2 ml microfuge tube. Add 700 ml of Buffer W2 and centrifuge for 1 minute at 12,000xg. Discard the filtrate from the 2 ml microfuge tube and repeat this wash step with a second 700 µL aliquot of Buffer W2.

Note: Make sure that ethanol has been added into Buffer W2 concentrate.
Two washes with Buffer W2 are used to ensure the complete removal of salt, eliminating potential problems in subsequent enzymatic reactions.

9. Discard filtrate from the 2 ml microfuge tube. Place the Miniprep column back into the 2 ml microfuge tube. Centrifuge for 1 minute at 12,000xg.

10. Transfer the Miniprep column into a clean 1.5 ml microfuge tube (provided). To elute the genomic DNA, add 100-200 µL of Eluent (or deionized water) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge for 1 minute at 12,000xg.

Note: Pre-warming water or Eluent at 65°C will often improve elution efficiency.

DNA Transformation to generate Competent Cells Protocol

Note: Have a plentiful stash of cold $CaCl_2$ prepared

1. Seed E. Coli in 20 mL of LB growth in a sterile beaker
2. Stir at 37°C during 3 hrs
3. Transfer into 2 Falcon tubes
4. Centrifuge for 10 min at 5000 rpm and 4°C. Decant supernatant
5. Resuspend in 10 mL $CaCl_2$, dissolve
6. Centrifuge again with the previous conditions, decant and dispose
7. Repeat steps 5 and 6
8. After centrifuging and disposing, resuspend in 5 mL of $CaCl_2$
9. Leave on ice during half an hour
10. Centrifuge during 10 min at 1500 rpm and 4°C
11. Resuspend 1 mL
12. Take 250 uL and pour them into an Eppendorf tube along with 50 uL pGLO
13. Leave on ice for half an hour
14. Place on a water bath at 42°C for 2 min and another 2 min at room temperature
15. Add 1 mL LB broth
16. Incubate at 37°C in stove for 1 hr
17. LB plate + Ampiciline
18. Incubate for a night in oven

Agarose Gel Electrophoresis

The preparation of the Agarose Gel .5% involves:
40 mL TAE (1x) and .18 g Agarose

1. Place comb at the desired height
2. The process begins as soon as gellification starts to take effect
3. One of the pores will be injected with 30 µL λ marker solution, the rest will receive a 30 µL dose of the samples along with 10 µL of the loading buffer, glicerol 10%
4. The electrochemical chamber is set. Voltage output is 80 V. Running phase will end when the marker signal approaches the far extreme of the gel
5. After the run is complete, the gel is carefully extracted and placed in container with water bromide, and stir gently for 10-15 min. Carefully remove gel after stirring.
6. Place in revealing chamber, and observe on monitor through the UV lens.

UV-Visible Spectroscopy

To perform a slow speed run:

1. Set the wavelenght of the scan from 800-200 nm.
2. The measure mode is Absorbance
3. Place target on both cells
4. Run scan to obtain the baseline, (800-200 nm)
5. Pull out the cell from the nearest side
6. With a micropipette stir the mixture and place samples
7. Hit start. Let the scan run. When the scan has completed an emerging window will appear
8. Save file

To change sample

9. Wash the cell holding the target 2 times (if the target water)
10. If the target is not water, wash first twice with water, following two baths with the target

Electroporation

1. Gently thaw 50 mL of cells, DNA in a tube and 1 mm black capped cuvettes on ice. It is essential that there is no salt present in your DNA preparation.

2. Add 50 µL of cold cells into DNA. Mix well and let sit on ice for 1 min. Keep on ice.

3. Transfer to Cuvette. Transfer the cell/DNA mix into an ice-cold 1-mm electroporation cuvette. Be sure that there are no bubbles or gaps in the cuvette's electrode gap. Keep on ice.

5. For each electroporation, prepare one 17 ´ 100-mm sterile polypropylene culture tube, one 1-mL sterile transfer pipette, and SOC medium. Before each electroporation, draw 1 mL of SOC medium into the transfer pipette. Uncap culture tube and place the transfer tube into the culture tube.

4. Gene Pulser Apparatus Set-Up.
i. Turn on the Gene Pulser apparatus using the power switch. The LED display should illuminate and read "0.00."
ii. Press and SET VOLTS. The LED above the button will illuminate. The LED display is in kilovolts (kV). Use RAISE and LOWER to adjust the voltage to the desired value in the range. Set the voltage at 1.8 kV, or unless indicated otherwise.
iii. Adjust the capacitor by setting the capacitance at 25 mF using the capacitance select knob on the lower right front of the Gene Pulser panel.
iv. Adjust the parallel resistor by setting the multi-position switch to a resistance of 200 W on the Gene Pulser Controller panel.

5. Electroporate.
i. Wipe the cuvette with a Kim Wipe assuring that there is no water or residue on the cuvette.
ii. Insert the cuvette into the white slide (the nodule on the cuvette faces to the rear). Push the slide into the chamber until the cuvette makes firm contact with the chamber electrodes.
iii. To charge the capacitor and deliver a pulse, press and hold both red pulse buttons until a continuous tone sounds. The display will flash "Chg," indicating that the capacitor is being charged to the selected voltage.
iv. Release the pulse buttons once the tone signals that the pulse has been delivered.
You can read the time constant and actual voltage on the LED display by pressing the set voltage and actual voltage on the front panel of the apparatus simultaneously.
v. Remove the cuvette from the chamber and immediately add 1 mL of SOC medium to the cuvette with a pipette. Resuspend the cells gently by pipetting up and down. The cells are very delicate at this stage. Transfer the cell culture to a sterile microcentrifuge tube.
vi. Turn the Gene Pulser apparatus OFF.Rebay Lab Electroporation

6. Incubate for 60 min. Incubate the cells at 37°C on a heat block for 60 min.

7. Plate Cells.
i. Select two LB plates containing the appropriate antibiotic for selection, e.g., ampicillin. In some cases you may also need to add 40 mL of X-Gal stock (20 mg/mL) and 4 mL of IPTG stock (200 mg/mL).
ii. From each transformation, add 50 mL and 100 mL of the cell suspension to the two LB plates.
iii. Cool the glass spreader by touching it to a portion of the agar plate—aside from the cell suspension.
iv. Carefully spread the cells on a turntable, allowing the solution to permeate the medium.
v. Reflame the spreader before setting it down. Permit the plate to set before turning it over.

8. Incubate Overnight. Incubate the plates upside-down overnight at 37°C.