Protocol 1

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation

PLASMID DNA MAY BE ISOLATED FROM SMALL-SCALE (1–2 ml) bacterial cultures by treatment with alkali and SDS. The resulting DNA preparation may be screened by electrophoresis or restriction endonuclease digestion. With further purification by treatment with PEG, the preparations may be used as templates in DNA sequencing reactions (please see the information panel on **PURIFICATION OF PLASMA DNA BY PEG PRECIPITATION**).

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.

Alkaline lysis solution I

Alkaline lysis solution II

Solution II should be freshly prepared and used at room temperature.

Alkaline lysis solution III

Antibiotic for plasmid selection

Ethanol

Phenol:chloroform (1:1, v/v) **<!>**

Optional, please see Step 8.

STE

Optional, please see Step 3.

TE (pH 8.0) containing 20 µg/ml RNase A

Media

LB, YT, or Terrific Broth

METHOD

Preparation of Cells

1. Inoculate 2 ml of rich medium (LB, YT, or Terrific Broth) containing the appropriate antibiotic with a single colony of transformed bacteria. Incubate the culture overnight at 37°C with vigorous shaking.

To ensure that the culture is adequately aerated:

- The volume of the culture tube should be at least four times greater than the volume of the bacterial culture.
- The tube should be loosely capped.
- The culture should be incubated with vigorous agitation.

2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at maximum speed for 30 seconds at 4°C in a microfuge. Store the unused portion of the original culture at 4°C.

3. When contrifugation is complete, remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

   This step can be conveniently accomplished with a disposable pipette tip or Pasteur pipette attached to a vacuum line and a side arm flask (please see Figure 1-7). Use a gentle vacuum and touch the tip to the surface of the liquid. Keep the tip as far away from the bacterial pellet as possible as the fluid is withdrawn from the tube. This minimizes the risk that the pellet will be sucked into the side arm flask. Alternatively, remove the supernatant using a pipettor or Pasteur pipette and bulb. Use the pipette tip to vacuum the walls of the tube to remove any adherent droplets of fluid.

   The penalty for failing to remove all traces of medium from the bacterial pellet is a preparation of plasmid DNA that is resistant to cleavage by restriction enzymes. This is because cell-wall components in the medium inhibit the action of many restriction enzymes. This problem can be avoided by resuspending the bacterial pellet in ice-cold STE (0.25x volume of the original bacterial culture) and centrifuging again.

Lysis of Cells

4. Resuspend the bacterial pellet in 100 µl of ice-cold Alkaline lysis solution I by vigorous vortexing.

   Make sure that the bacterial pellet is completely dispersed in Alkaline lysis solution I. Vortexing two microfuge tubes simultaneously with their bases touching increases the rate and efficiency with which the bacterial pellets are resuspended.

   The original protocol (Birnboim and Doly 1979) called for the use of lysozyme at this point to assist in dissolution of the bacterial cell walls. This step can be safely omitted when dealing with bacterial cultures of less than 10 ml in volume.

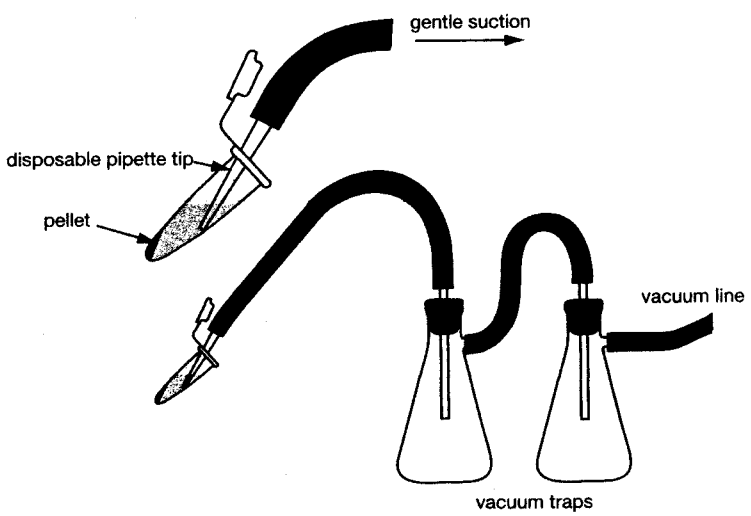

FIGURE 1-7 Aspiration of Supernatants

Hold the open microfuge tube at an angle, with the pellet on the upper side. Use a disposable pipette tip attached to a vacuum line to withdraw fluid from the tube. Insert the tip just beneath the meniscus on the lower side of the tube. Move the tip toward the base of the tube as the fluid is withdrawn. Use gentle suction to avoid drawing the pellet into the pipette tip. Keep the end of the tip away from the pellet. Finally, vacuum the walls of the tube to remove any adherent drops of fluid.

Blocking Agents Used for Western Blotting. The best and least expensive blocking reagent is nonfat dried milk (Johnson et al. 1984). It is easy to use and is compatible with all of the common immunological detection systems. The only time nonfat dried milk should not be used is when western blots are probed for proteins that may be present in milk.

One of the following recipes may be used to prepare blocking buffer. A blocking solution for western blots is phosphate-buffered saline containing 5% (w/v) nonfat dried milk, 0.01% Antifoam, and 0.02% sodium azide.

Blocking Buffer (TNT Buffer Containing a Blocking Agent)

10 mM Tris-Cl (pH 8.0)
150 mM NaCl
0.05% (v/v) Tween-20
blocking agent (1% [w/v] gelatin, 3% [w/v] bovine serum albumin, or 5% [w/v] nonfat dried milk)

Opinion about which of these blocking agents is best varies from laboratory to laboratory. We recommend carrying out preliminary experiments to determine which of them works best. Blocking buffer can be stored at 4°C and reused several times. Sodium azide <!> should be added to a final concentration of 0.05% (w/v) to inhibit the growth of microorganisms.

Extraction/Lysis Buffers and Solutions

Alkaline Lysis Solution I (Plasmid Preparation)

50 mM glucose
25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Prepare Solution I from standard stocks in batches of ~100 ml, autoclave for 15 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle, and store at 4°C.

Alkaline Lysis Solution II (Plasmid Preparation)

0.2 N NaOH (freshly diluted from a 10 N stock) <!>
1% (w/v) SDS

Prepare Solution II fresh and use at room temperature.

Alkaline Lysis Solution III (Plasmid Preparation)

5 M potassium acetate	60.0 ml
glacial acetic acid <!>	11.5 ml
H_2O	28.5 ml

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

STET

10 mM Tris-Cl (pH 8.0)
0.1 M NaCl
1 mM EDTA (pH 8.0)
5% (v/v) Triton X-100

Make sure that the pH of STET is 8.0 after all ingredients are added. There is no need to sterilize STET before use.

5. Add 200 µl of freshly prepared Alkaline lysis solution II to each bacterial suspension. Close the tube tightly, and mix the contents by inverting the tube rapidly five times. *Do not vortex!* Store the tube on ice.

   Make sure that the entire surface of the tube comes in contact with Alkaline lysis solution II.

6. Add 150 µl of ice-cold Alkaline lysis solution III. Close the tube and disperse Alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube on ice for 3–5 minutes.

7. Centrifuge the bacterial lysate at maximum speed for 5 minutes at 4°C in a microfuge. Transfer the supernatant to a fresh tube.

8. (*Optional*) Add an equal volume of phenol:chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at maximum speed for 2 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.

   Some investigators find the extraction with phenol:chloroform to be unnecessary. However, the elimination of this step sometimes results in DNA that is resistant to cleavage by restriction enzymes.

   The purpose of extracting with chloroform is to remove residual phenol from the aqueous phase. Phenol is slightly soluble in H_2O, but it can be displaced into the organic phase by chloroform. Years ago, it was common practice in some laboratories to detect residual phenol in DNA preparations by smell. This practice is no longer recommended.

Recovery of Plasmid DNA

9. Precipitate nucleic acids from the supernatant by adding 2 volumes of ethanol at room temperature. Mix the solution by vortexing and then allow the mixture to stand for 2 minutes at room temperature.

10. Collect the precipitated nucleic acids by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.

    It is best to get into the habit of always arranging the microfuge tubes in the same way in the microfuge rotor, i.e., in order, with their plastic hinges always pointing outward. The precipitate will collect on the inside surface furthest from the center of rotation. Knowing where to look makes it easier to find visible precipitates and to dissolve "invisible" precipitates efficiently. Labeling both the sides and tops of tubes provides clear identification of each tube, even if the ink smudges.

11. Remove the supernatant by gentle aspiration as described in Step 3 above. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Use a Kimwipe or disposable pipette tip to remove any drops of fluid adhering to the walls of the tube.

12. Add 1 ml of 70% ethanol to the pellet and invert the closed tube several times. Recover the DNA by centrifugation at maximum speed for 2 minutes at 4°C in a microfuge.

13. Again remove all of the supernatant by gentle aspiration as described in Step 3.

    Take care with this step, as the pellet sometimes does not adhere tightly to the tube.

14. Remove any beads of ethanol that form on the sides of the tube. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube (5–10 minutes).

    If the pellet of DNA is dried in a desiccator or under vacuum, it becomes difficult to dissolve under some circumstances and may denature (Svaren et al. 1987). Drying the pellet for 10–15 minutes at room temperature is usually sufficient for the ethanol to evaporate without the DNA becoming dehydrated.

15. Dissolve the nucleic acids in 50 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). Vortex the solution gently for a few seconds. Store the DNA solution at –20°C.

    For recommendations on troubleshooting, please see Table 1-5 in Protocol 3.