Preparation of DNA for Transfection

The quality of the DNA used for transfection is critical. Purified plasmid DNA should be free from protein, RNA and chemical contamination. DNA may be purified using a plasmid preparation protocol, a CsCl gradient, or column chromatography. One measure of DNA purity is the ratio of absorbance at 260 to 280nm; for transfection the A_{260}:A_{280} ratio should be at or above 1.8. The purified DNA should be ethanol precipitated and resuspended in sterile TE buffer to a final concentration of approximately 1mg/ml. The optimal amount of DNA to use for transfection depends on both the cell type and the reagent used.

Plasmid Preparation Protocol

We have successfully purified transfection quality plasmid DNA using a modified alkaline lysis protocol (29). In the following procedure, membrane lipids are solubilized using SDS. Sodium hydroxide is used to denature and break up a large amount of the chromosomal DNA, which is then precipitated by addition of potassium acetate. Treatment with RNase A and ammonium acetate removes ribonucleic acids (30). Polyethylene glycol (PEG) is used to further purify the plasmid DNA by precipitating it away from other contaminating material (30). Any remaining proteins and oligosaccharides are removed by a high salt phenol extraction; the acid phenol extraction serves to remove residual chromosomal and nicked plasmid DNA.

All reagents used should be molecular biology grade and solutions should be freshly prepared from reliable, nuclease-free stocks.

Materials to Be Supplied by the User

(Solution compositions are provided at the end of this chapter.)

- 25mM Tris-HCl, 50mM EDTA
- 0.1M NaOH, 1% SDS
- 5M potassium acetate
- TE (pH 8.0)
- 5M ammonium acetate
- 5M NaCl
- PEG precipitation solution
- DNase-free RNase
- chloroform:isoamyl alcohol (24:1)
- sterile water
- 2M sodium acetate
- high salt phenol
- acid phenol
- Miracloth (Calbiochem®)
- 2-propanol
- 100% ethanol
- 70% ethanol
- sterile nuclease-free water

1. Harvest the bacterial cells from a 1 liter overnight culture by centrifugation at 6,000 x *g* for 10 minutes. If necessary, the cell pellet may be stored at –20°C or –70°C.
2. Resuspend the pellet in 50ml of 25mM Tris-HCl (pH 8.0), 50mM EDTA.
3. Add 100ml of **freshly prepared** 0.1M NaOH, 1% SDS; mix gently by swirling the container for ~15 seconds. **Do not vortex.** Incubate for 10 minutes on ice.
4. Add 75ml of ice-cold 5M potassium acetate. Mix gently and incubate on ice for 5 minutes. A precipitate will form.
5. Centrifuge at 6,000 x *g* for 15 minutes. Filter the supernatant through Miracloth or through 4 layers of cheesecloth.
6. Add 135ml of 2-propanol, mix and incubate at room temperature for 30 minutes.
7. Centrifuge at 6,000 x *g* for 15 minutes. Decant and discard the supernatant.
8. Resuspend the pellet in 20ml TE (pH 8.0). Add 20ml 5M ammonium acetate. Incubate on ice for 20 minutes.
9. Centrifuge at 12,000 x *g* for 10 minutes. Decant supernatant into a fresh tube.
10. Add 80ml of 100% ethanol to the supernatant. Incubate on ice for 15 minutes. Centrifuge at 12,000 x *g* for 10 minutes.
11. Dissolve pellet in 2ml TE (pH 8.0). Add 20µl of 10mg/ml DNase-free RNase. Incubate for 15 minutes at 37°C.
12. Add 600µl 5M NaCl and 650µl PEG precipitation solution. Mix and incubate on ice for 30 minutes. Centrifuge at 12,000 x *g* for 15 minutes at 4°C. Discard the supernatant. Drain the pellets by inverting the tubes onto paper towels and blot the rim of the tube with a Kimwipes® tissue or a paper towel.
13. Dissolve the pellet in 1ml TE (pH 8.0). Extract the remaining PEG by adding an equal volume of chloroform:isoamyl alcohol (24:1). Mix well by inversion and spin in a microcentrifuge for 5 minutes (or 1,600 x *g* for 10 minutes if using another rotor).
14. Remove the upper aqueous phase to fresh tubes. Add NaCl to a final concentration of 0.5M (a total of 100µl of 5M NaCl). Extract with an equal volume of high salt phenol. Spin for 5 minutes in a microcentrifuge tube. Remove the upper, aqueous phase to a fresh tube.

15. Add two volumes of 100% ethanol. Incubate on ice for 15 minutes. Spin for 10 minutes in a microcentrifuge (20 minutes at 12,000 x *g*). Discard the supernatant and drain the pellets briefly by inverting the tube onto paper towels.
16. Dissolve pellets in a total of 960µl water. Add 15µl of 5M NaCl and 25µl of 2M sodium acetate (pH 4.0). Extract with an equal volume of acid phenol (31). Centrifuge for 5 minutes at room temperature in a microcentrifuge (phenol may crystallize at colder temperatures).
17. Extract any remaining phenol by adding an equal amount of chloroform:isoamyl alcohol (24:1). Invert to mix and centrifuge for 5 minutes in a microcentrifuge. Remove the upper aqueous phase to a fresh tube.
18. Add two volumes of 100% ethanol. Incubate for 20 minutes on ice or store overnight at –20°C. Spin for 10 minutes in a microcentrifuge. Discard the supernatant.
19. Wash the pellet by adding 70% ethanol. Centrifuge for 10 minutes in a microcentrifuge. Carefully remove the supernatant without disturbing the pellet. Dry the pellet briefly under vacuum.
20. Resuspend the DNA in 600µl of sterile, nuclease-free TE. Determine the exact DNA concentration by measuring the absorbance at 260nm. Run an aliquot on a 0.7% agarose gel stained with ethidium bromide to check for the size, purity and integrity of the purified plasmid DNA.

The above protocol is time consuming, but generates high quality DNA that works well in transfections. Alternatively, DNA purified by the alkaline lysis method may be further purified using a cesium chloride (CsCl) gradient.

Cesium Chloride Equilibrium Gradient

The CsCl equilibrium centrifugation method produces transfection quality DNA. Standard protocols for this procedure can be found in references 29 and 30. In this procedure, a high-speed centrifugation step follows a crude DNA isolation protocol such as the alkaline lysis procedure (29). Ethidium bromide (EtBr; a mutagen) is then added to the DNA along with CsCl and the mixture is centrifuged to equilibrium. The DNA "band" is removed, leaving many contaminants behind. A second EtBr and CsCl centrifugation removes additional protein and RNA contaminants. Once the DNA has been isolated, both EtBr and CsCl must be removed. EtBr may be removed using a Dowex AG50 column or by extraction with 1-butanol. The DNA must then be dialyzed or ethanol precipitated and washed thoroughly with 70% ethanol to remove excess CsCl. It is important to remove residual CsCl ions as they can react with charged liposomes or other transfection reagents, making transfection less effective. The purified DNA is resuspended in TE buffer.

Anion Exchange Chromatography

Column chromatography is by far the quickest method of preparing high quality plasmid DNA suitable for transfection. However, care must be taken in choosing the type of column used, as some commercially available columns leave contaminants in the DNA preparation that adversely affect transfection efficiency, sometimes to a dramatic degree.

Preparation of Cells for Transfection

Trypsinization Procedure for Removing Adherent Cells

Trypsinizing cells for purposes of subculturing or cell counting is an important technique that is critical to successful cell culture. The following technique works consistently well when passaging cells.

Materials to Be Supplied by the User

(Solution compositions are provided at the end of this chapter.)

- 1X trypsin-EDTA solution (0.05% trypsin, 0.5mM EDTA)

1. Prepare a sterile trypsin-EDTA solution in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS. The 1X solution can be frozen and thawed for future use, but the activity of the trypsin will decline with each freeze-thaw cycle. The trypsin-EDTA solution may be stored for up to 1 month at 4°C.
2. Remove the media from the tissue culture dish. Add enough PBS or HBS solution to cover the cell monolayer: 2ml for a 150mm flask, 1ml for a 100mm plate. Rock the plates to distribute the solution evenly. Remove and repeat the wash. Remove the final wash. Add enough trypsin solution to cover the cell monolayer.
3. Place the plates in a 37°C incubator until the cells just begin to detach (usually 1-2 minutes).
4. Remove the flask from the incubator. Strike the bottom and sides of the culture vessel sharply with the palm of your hand to help dislodge the remaining adherent cells. View the cells under a microscope to check whether all cells have detached from the growth surface. If necessary, the cells may be returned to the incubator for an additional 1-2 minutes.
5. When all cells have detached, add media containing serum to the cells to inactivate the trypsin. Gently pipet the cells up and down to break up cell clumps. The cells may then be counted using a hemacytometer and/or distributed to fresh plates for subculturing.

Transient Expression vs. Stable Transfection

Transient Expression

Cells are typically harvested 48-72 hours post-transfection for studies designed to analyze transient expression of the transfected genes. The optimal time interval depends upon the cell type, the doubling time of the cells and the specific characteristics of expression for the transferred gene. Analysis of gene products may require isolation of RNA or protein for enzymatic activity assays or immunoassays. The method used for cell harvest will depend upon the end-product being assayed.

Extracts may be prepared using Promega's Reporter Lysis Buffer. This allows extracts to be assayed for luciferase, CAT and β-galactosidase activity. If only luciferase activity is to be assayed, Promega's Cell Culture Lysis Reagent may be used. Passive Lysis Buffer is best for the Dual-Luciferase™ Reporter Assay System. For further information on the preparation and assay of cell extracts, see Chapter 6.

Stable Transfection

The goal of stable, long-term transfection is to isolate and propagate individual clones containing transfected DNA. Therefore it is necessary to distinguish nontransfected cells from those that have taken up the exogenous DNA. This screening can be accomplished by drug selection when an appropriate drug resistance marker is included in the transfected DNA. Alternatively, morphological transformation can be used as a selectable trait in certain cases. For example, bovine papilloma virus vectors produce a morphological change in transfected mouse CI127 cells (32).

Typically, cells are maintained in nonselective medium for 1-2 days post-transfection, then trypsinized and replated in selective medium containing the drug. The use of the selective medium is continued for 2-3 weeks, with frequent changes of medium to eliminate dead cells and debris, until distinct colonies can be visualized. Individual colonies are then trypsinized and subcloned to multiwell plates for further propagation in the presence of selective medium.

Several different drug selection markers are commonly used for long-term transfection studies. For example, cells transfected with recombinant vectors containing the bacterial gene for neomycin phosphotransferase can be selected for stable transformation in the presence of the neomycin analog G418 (8). Similarly, expression of the gene for hygromycin B phosphotransferase from the transfected vector will confer resistance to the drug hygromycin B (33).

An alternative strategy is to use a vector carrying an essential gene that is defective in a given cell line. For example, CHO cells deficient in expression of the dihydrofolate reductase (DHFR) gene survive only in the presence of added nucleosides. However, these cells, when stably transfected with DNA expressing the DHFR gene, will synthesize the required nucleosides (34). An additional advantage of using DHFR as a marker is that gene amplification of DHFR and associated transfected DNA occurs when cells are exposed to increasing doses of methotrexate (35).

Before using a particular drug for selection purposes, it is important to determine the amount of drug necessary to kill the cells you will be using. This may vary greatly between cell types. Design experiments using various concentrations of the drug to determine the amount to use for selection of resistant clones (29).

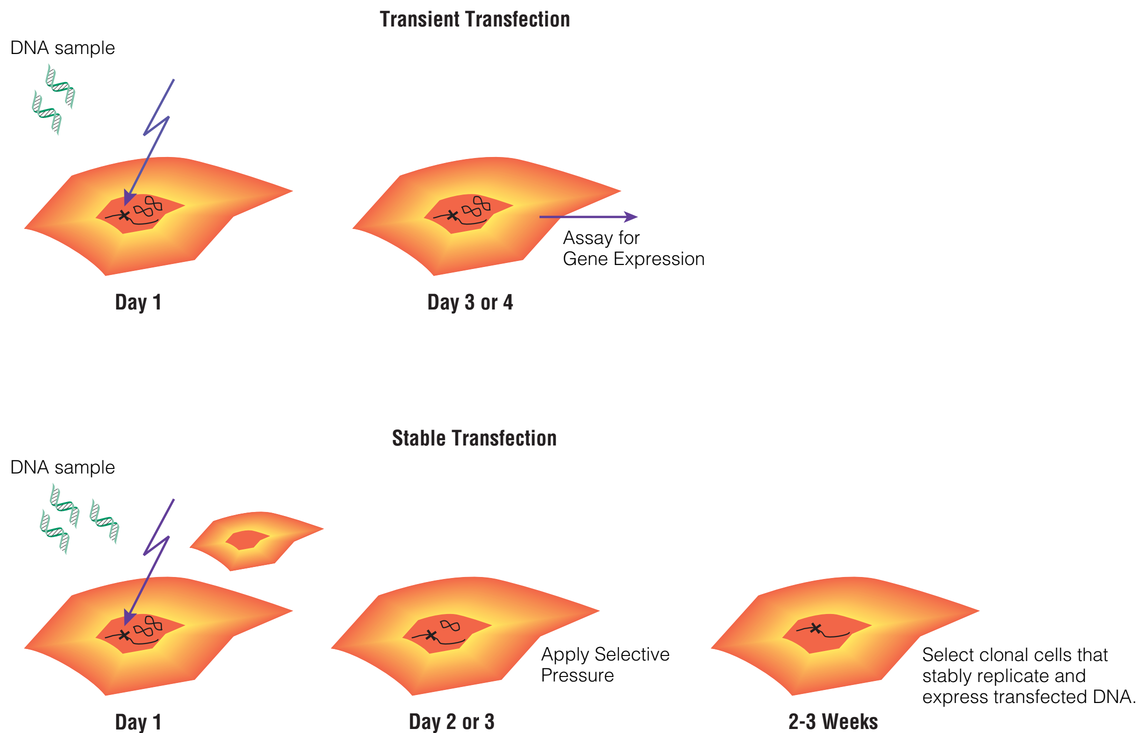

Figure 2.1. Schematic representations of stable and transient transfections.

Composition of Buffers and Solutions

5M ammonium acetate

Dissolve 385g of ammonium acetate in 1 liter distilled water. Filter through a 0.2µm filter. Store at 4°C.

DNase-free RNase A

Prepare a 10mg/ml stock solution of Pancreatic RNase A in 10mM Tris-HCl (pH 7.5), 15mM NaCl. Aliquot to tubes and heat in a boiling water bath for 15 minutes. Cool slowly to room temperature. Store at –20°C.

1X HBSS (Hanks Balanced Salt Solution)

5mM	KCl
0.3mM	KH_2PO_4
138mM	NaCl
4mM	$NaHCO_3$
0.3mM	Na_2HPO_4
5.6mM	D-glucose

The final pH should be 7.1

1X PBS

137mM	NaCl
2.7mM	KCl
4.3mM	Na_2HPO_4
1.47mM	KH_2PO_4

The final pH should be 7.1

PEG Precipitation Solution (30% PEG-8000, 1.5M NaCl)

300g	PEG 8000 (molecular biology grade)
300ml	5M NaCl

Add deionized water to a final volume of 1L. This solution may have to be heated slightly to completely dissolve the PEG. Store at 4°C.

Acid Phenol (phenol saturated with TE + 50mM sodium acetate)

Phenol is caustic; work in a chemical safety hood and wear protective safety equipment. Melt phenol by placing in a 50°C or warmer water bath. Add an equal volume of 50mM sodium acetate (pH 4.0). The pH of the sodium acetate solution is important. Stir with a Teflon-coated magnetic stir bar until the two phases become completely mixed. Stop stirring and allow the phases to separate. Remove and discard the top aqueous phase. Add 50mM sodium acetate (pH 4.0), mix and allow the phases to separate. Remove the aqueous phase. Repeat two more times or until the pH of the aqueous phase after extraction is between 4.0 and 4.2. Add back 1/10 volume of 50mM sodium acetate (pH 4.0) to the phenol. Store protected from light at 4°C.

High Salt Phenol (phenol saturated with TE + 0.5M NaCl)

Phenol is caustic; work in a chemical safety hood and wear protective safety equipment. Melt phenol by placing in a 50°C or warmer water bath. Add an equal volume of TES (TE + 1/10 volume of 5M NaCl). Stir with a Teflon-coated magnetic stir bar until the two phases become completely mixed. Stop stirring and allow the phases to separate. Remove and discard the top aqueous phase. Add TES, mix, and allow the phases to separate; remove the aqueous phase. Repeat two more times. Add back 1/10 volume of TES to the phenol. Store protected from light at 4°C.

5M Potassium Acetate Solution

60ml	5M potassium acetate
11.5ml	glacial acetic acid
28.5ml	deionized water

Store at 4°C. This solution is 3M with respect to potassium and 5M with respect to acetate.

2M Sodium Acetate, pH 4.0

15g	NaOH
115ml	deionized water
115ml	glacial acetic acid

Dissolve the NaOH slowly in 115ml of water. Slowly add the glacial acetic acid. Adjust the final volume to 1L with deionized water. The final pH of the solution should be 4.0. This solution provides a 40X stock for the 50mM sodium acetate solution used to prepare acid phenol.

TE

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

1X Trypsin-EDTA solution

0.05% (w/v)	trypsin
0.53mM	EDTA

Dissolve in a calcium- and magnesium-free salt solution such as 1X PBS or 1X HBSS.

Notes