Protocol

DNA Transfection Mediated by Cationic Lipid Reagents

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Liposomal transfection reagents vary in their ability to transfect cell lines efficiently. Some are generalists, whereas others are best used with specific cell types. The nonliposomal FuGENE 6 and the cationic liposomal Lipofectamine 2000 are examples of reagents that can successfully transfect most adherent and suspension cell types (including several primary and hard-to-transfect cell types) with negligible toxicity and a minimal number of manipulations. Importantly, both reagents can be used to transfect cells in the presence of serum, minimizing the number of manipulations during the transfection procedure. We also provide an alternative protocol that uses the cationic lipid reagents Lipofectin and Transfectam.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Cell culture growth medium (complete, serum-free, and [optional] selective)
Exponentially growing cultures of mammalian cells
FuGENE 6 (Promega Corporation)
Giemsa stain (10%)

The Giemsa stain should be freshly prepared in phosphate-buffered saline or H_2O and filtered through Whatman No. 1 filter paper before use.

Lipofectamine 2000 (Life Technologies)
Lipofection reagents for use in the Alternative Protocol only
  Lipofectin (Life Technologies)
  Transfectam (Promega)
Methanol, ice-cold
NaCl (300 mM)

This is used as a diluent for DOGS in the Alternative Protocol only.

Plasmid DNA

If performing lipofection for the first time or if using an unfamiliar cell line, obtain an expression plasmid encoding Escherichia coli *β-galactosidase or green fluorescent protein. These can be purchased from Addgene (a nonprofit plasmid repository) or several commercial manufacturers (e.g., pCMV-SPORT-β-gal, Life Technologies, or pEGFP-F, Clontech) (see Figs. 1, 2).*

From the Molecular Cloning collection, edited by Michael R. Green and Joseph Sambrook.

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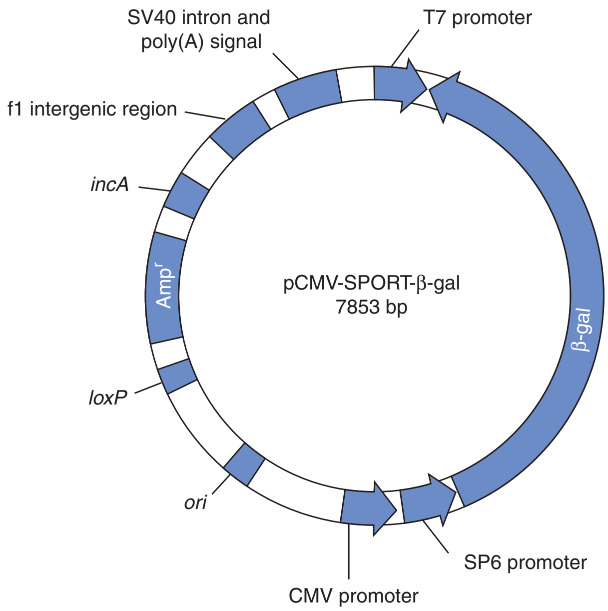

FIGURE 1. pCMV-SPORT-β-gal. pCMV-SPORT-β-gal is a reporter vector that may be used to monitor transfection efficiency. It carries the *E. coli* gene encoding β-galactosidase preceded by the CMV (cytomegalovirus) immediate early promoter, which drives high levels of transcription in mammalian cells. The SV40 polyadenylation signal downstream from the β-galactosidase sequence directs proper processing of the 3′ end of the mRNA in eukaryotic cells. (Reproduced, with permission, from Life Technologies Corporation.)

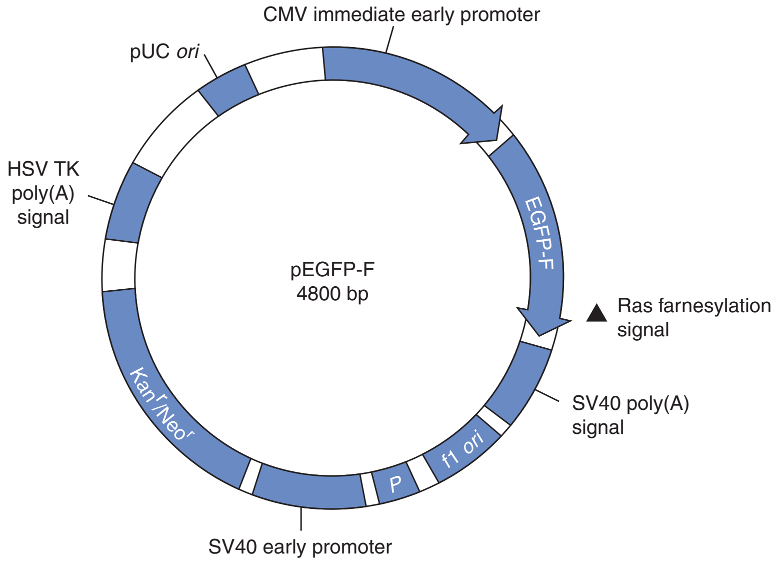

FIGURE 2. pEGFP-F. pEGFP-F is a reporter vector that may be used both to monitor transfection efficiency and as a cotransfection marker. The vector encodes a modified form of the green fluorescent protein, a farnesylated enhanced GFP (EGFP-F) that remains bound to the plasma membrane in both living and in fixed cells. The EGFP-F coding sequence is preceded by the CMV (cytomegalovirus) promoter, which drives high levels of transcription, and is followed by the SV40 polyadenylation signal to direct proper processing of the 3′ end of the mRNA in eukaryotic cells. The plasmid carries (i) sequences that allow replication in prokaryotic (pUC ori) as well as eukaryotic (SV40 ori) cells, and (ii) markers that facilitate selection for the plasmid in prokaryotic (kanamycin) cells as well as eukaryotic (neomycin) cells. The presence of EGFP-F can be detected by fluorescence microscopy. (Adapted, with permission, from Clontech.)

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TABLE 1. Dimensions of dishes used for cell culture

Size of plate	Growth area (cm^2)	Relative area[a]	Recommended volume
96 wells	0.32	0.04×	200 µL
24 wells	1.88	0.25×	500 µL
12 wells	3.83	0.5×	1.0 mL
Six wells	9.4	1.2×	2.0 mL
35 mm	8.0	1.0×	2.0 mL
60 mm	21	2.6×	5.0 mL
10 cm	55	7.0×	10.0 mL
Flask	25	3.0×	5.0 mL
Flask	75	9.0×	12.0 mL

[a]Relative area is expressed as a factor of the growth area of a 35-mm culture plate.

Purify closed-circular plasmid DNAs using a transfection-grade plasmid DNA preparation kit that preferably removes bacterial endotoxins during the purification procedure (e.g., EndoFree Plasmid Maxi Kit from QIAGEN). Alternatively, DNA may be purified by column chromatography or ethidium bromide–CsCl gradient centrifugation. Dissolve the DNAs in sterile Tris–EDTA buffer or sterile H_2O at 0.2–2 µg/µL. Determine DNA purity using a 260 nm/280 nm ratio; the ratio should be 1.8.

Linear DNA, that is, DNA linearized with restriction enzymes, is sometimes preferred, particularly if the aim is to generate stable transfectants (see Introduction: ***Selective Agents for Stable Transfection*** *[Kumar et al. 2018a]).*

Sodium citrate (20 mM, pH 5.5), containing 150 mM NaCl

This reagent is used as a diluent for the plasmid DNA in the Alternative Protocol only if DOGS is the lipofection reagent (Kichler et al. 1998).

Equipment

Microcentrifuge tubes, polypropylene (sterile)

Test tubes, polypropylene or polystyrene

These are used in the Alternative Protocol only; see Step 15.

Tissue culture dishes (60 mm)

These are used in the Alternative Protocol only.

Tissue culture multiwell plates (six well) or culture dishes (35 mm)

This protocol is designed for cells grown in six-well tissue culture plates or 35-mm culture dishes. If multiwell plates, flasks, or dishes of a different diameter are used, scale the cell density and plating volume according to Table 1. The corresponding starting volume of the transfection reagent and the amount of DNA can be scaled according to the manufacturer's instructions.

METHOD

The main protocol below has been modified from its original form after obtaining permission from Roche Applied Science and Life Technologies, manufacturers of FuGENE 6 and Lipofectamine 2000.

Growing Cells for Transfection

1. Twenty-four hours before lipofection, harvest exponentially growing mammalian cells by trypsinization, and replate them in six-well or 35-mm dishes at a density appropriate for the desired transfection reagent (see the table below). Add 2 mL of growth medium, and incubate the cultures for 20–24 h at 37°C in a humidified incubator with an atmosphere of 5%–7% CO_2.

   If the cells are grown for <12 h before transfection, they may not be well anchored to the substratum and are likely to detach during exposure to lipid.

   For suspension cells, use freshly passaged cells at the appropriate concentration as indicated in the following table (use 2 mL in a 35-mm culture dish or six-well plate).

Transfection reagent	Adherent cell density (cells per well)	Suspension cell concentration (cells/mL)	Confluency at lipofection
FuGENE 6	1×10^5 to 3×10^5	5×10^4 to 1×10^6	50%–80%
Lipofectamine 2000	2×10^5 to 8×10^5	2×10^5 to 3×10^6	90%–95%
DOTMA	1×10^5 to 3×10^5	5×10^4 to 1×10^6	75%
DOGS	1×10^5 to 3×10^5	5×10^4 to 1×10^6	75%

Preparation of FuGENE 6–DNA Complexes

For initial optimization, use reagent (in microliters) to DNA (in micrograms) ratios of 3:1, 3:2, and 6:1. These ratios provide good transfection efficiencies for commonly used adherent and suspension cells.

2. Bring the FuGENE 6 reagent to room temperature and mix before use by vortexing for 1 sec or by inverting the bottle. Dilute the reagent into serum-free medium (without antibiotics or fungicides). Label three small sterile tubes: "3:1," "3:2," and "6:1." Pipette 97 µL of serum-free medium into the first two tubes and 94 µL into the last tube. Pipette the FuGENE 6 directly into the medium without touching the walls of the plastic tube: 3 µL of FuGENE 6 into each of the first two tubes, and 6 µL into the tube labeled "6:1." Vortex for 1 sec or flick the tube to mix. Incubate for 5 min at room temperature.

   The reagent–DNA complex must be prepared in serum-free medium, even if the cells are transfected in the presence of serum. The order and manner of addition of components to form the transfection complex is critical. Serum-free medium must be pipetted first. Undiluted FuGENE 6 should not come into contact with any plastic surfaces (such as the walls of the tube that contains the serum-free medium) other than pipette tips.

3. Add 50 µL of DNA to the diluted transfection reagent from Step 2. Add 1 µg of plasmid DNA into each of the tubes labeled 3:1 and 6:1, and 2 µg of DNA (also in a total of 50 µL) into the tube labeled 3:2.
4. Tap the tubes or vortex for 1 sec to mix the contents and incubate for 15–45 min at room temperature.
5. Proceed to Step 10.

Preparation of Lipofectamine 2000–DNA Complexes

For initial optimization, prepare complexes using a Lipofectamine 2000 (in microliters) to DNA (in micrograms) ratio of 2:1–3:1.

6. Mix Lipofectamine 2000 gently, then dilute 10 µL in 250 µL of serum-free medium. Incubate for 5 min at room temperature.

   Proceed to Step 8 within 25 min.

7. Dilute 4 µg of DNA into 250 µL of serum-free medium. Mix gently.
8. Combine the diluted Lipofectamine 2000 with the diluted DNA (total volume = 500 µL). Mix gently and incubate for 20 min at room temperature (the solution may appear cloudy).

   Complexes are stable for 6 h at room temperature.

9. Proceed to Step 10.

Transfection of Cells

10. Add the complexes dropwise to cells in the existing growth medium in the six-well plate or 35-mm dish (from Step 1). Swirl the plate or dish to ensure distribution over the entire surface. Return the cells to a 37°C humidified incubator with an atmosphere of 5%–7% CO_2.

    There is no need to remove and replace with fresh growth medium. However, the growth medium may be changed after 4–6 h if Lipofectamine 2000 causes any kind of toxicity.

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11. Analyze cell viability at 6–24 h after transfection by a Trypan Blue exclusion test, or quantify cell viability using cytotoxicity tests incorporating alamarBlue, lactate dehydrogenase, or MTT (see Protocol: **Analysis of Cell Viability by the alamarBlue Assay** [Kumar et al. 2018b], Protocol: **Analysis of Cell Viability by the Lactate Dehydrogenase Assay** [Kumar et al. 2018c], or Protocol: **Analysis of Cell Viability by the MTT Assay** [Kumar et al. 2018d], respectively).

    See Troubleshooting.

12. If the objective is stable transfection of the cells, proceed to Step 13. To assay for transient transfection, examine the cells 24–96 h after lipofection using one of the following assays:

    i. If a plasmid DNA expressing *E. coli* β-galactosidase was used, follow the steps outlined in Protocol: **Assay for β-Galactosidase in Extracts of Mammalian Cells** (Uchil et al. 2017) to measure enzyme activity in cell lysates. Alternatively, perform a histochemical staining assay as detailed in Protocol: **Histochemical Staining of Cell Monolayers for β-Galactosidase** (Kumar et al. 2019a).

    ii. If a fluorescence protein expression vector was used, examine the cells with a microscope under appropriate illumination conditions. (Green fluorescent protein expression can be observed at 450–490 nm.) Alternatively, a small portion of the cells can be analyzed by flow cytometry to obtain an estimate of transfection efficiency and viability.

    iii. For other gene products, newly synthesized protein may be analyzed by radioimmunoassay, by immunoblotting, by immunoprecipitation following in vivo metabolic labeling, or by assays of enzymatic activity in cell extracts.

    To minimize the effects of dish-to-dish variation in transfection efficiency, it is best to (1) transfect several dishes with each construct, (2) trypsinize the cells after 24 h of incubation, (3) pool the cells, and (4) replate them on several dishes.

    See Troubleshooting.

13. Isolate stable transfectants.

    i. Incubate the cells for 48–72 h in complete medium to allow time for expression of the transferred gene(s).

    ii. Trypsinize the cells, and replate them in the appropriate selective medium.

    iii. Change the medium every 2–4 d for 2–3 wk to remove the debris of dead cells and to allow colonies of resistant cells to grow.

    iv. Clone individual colonies, and propagate them for assay (for Methods, see Jakoby and Pastan 1979 or Spector et al. 1998).

    v. Obtain a permanent record of the numbers of colonies by fixing the remaining cells with ice-cold methanol for 15 min followed by staining with 10% Giemsa for 15 min at room temperature before rinsing in tap water.

Alternative Protocol: Transfection Using DOTMA and DOGS

The following protocol details the use of DOTMA (Lipofectin) and DOGS (Transfectam) for transfecting cells in 60-mm tissue culture dishes and varies from the protocol outlined above mainly in the fact that transfections need to be performed in the absence of serum (see Box 1).

14. Prepare cells in 60-mm dishes as described in Step 1.

15. For each 60-mm dish of cultured cells to be transfected, dilute 1–10 µg of plasmid DNA into 100 µL of sterile deionized H_2O (if using Lipofectin) or 20 mM sodium citrate containing 150 mM NaCl (pH 5.5) (if using Transfectam) in a polystyrene or polypropylene test tube. In a separate tube, dilute 2–50 µL of the lipid solution to a final volume of 100 µL with sterile deionized H_2O or 300 mM NaCl. Incubate the tubes for 10 min at room temperature.

    When transfecting with Lipofectin, use polystyrene test tubes; do not use polypropylene tubes because the cationic lipid DOTMA binds nonspecifically to polypropylene.

BOX 1. DOTMA AND DOGS

Although it is now commonplace to use commercially available transfection reagents composed of a proprietary blend of lipids, it is also possible to prepare a homebrew of lipid-based transfection reagents; two are presented here.

- Lipofectin (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride; DOTMA) (Fig. 3). This monocationic lipid mixed with a helper lipid is usually purchased at a concentration of 1 mg/mL. DOTMA can also be synthesized with the help of an organic chemist (Felgner et al. 1987). If synthesized in-house, dissolve 10 mg each of dried DOTMA and the helper lipid dioleoyl phosphatidylethanolamine (DOPE; purchased from Sigma-Aldrich) in 2 mL of sterile deionized H_2O in a polystyrene tube (do not use polypropylene tubes). Sonicate the turbid solution to form liposomes before diluting to a final concentration of 1 mg/mL. Store the solution at 4°C.
- Transfectam (spermine-5-carboxy-glycinedioctadecyl-amide; DOGS) (Fig. 3). Prepare a stock solution of the cationic lipid DOGS as follows: Dissolve 1 mg of polyamine in 40 µL of 96% (v/v) ethanol for 5 min at room temperature with frequent solute vortexing. Add 360 µL of sterile H_2O, and store the solution at 4°C. Vortex the solution just before use. Polyamines, such as DOGS, do not require the use of polystyrene tubes; polypropylene tubes (i.e., standard microcentrifuge tubes) can be safely used with these reagents.

Although in-house preparation of these two lipid reagents may prove to be cost effective (Loeffler and Behr 1993), the disadvantages include higher cell toxicity, the ability to transfect only a restricted number of cell types, and the need for a large number of manipulations during transfection, because efficiencies are severely compromised in the presence of serum.

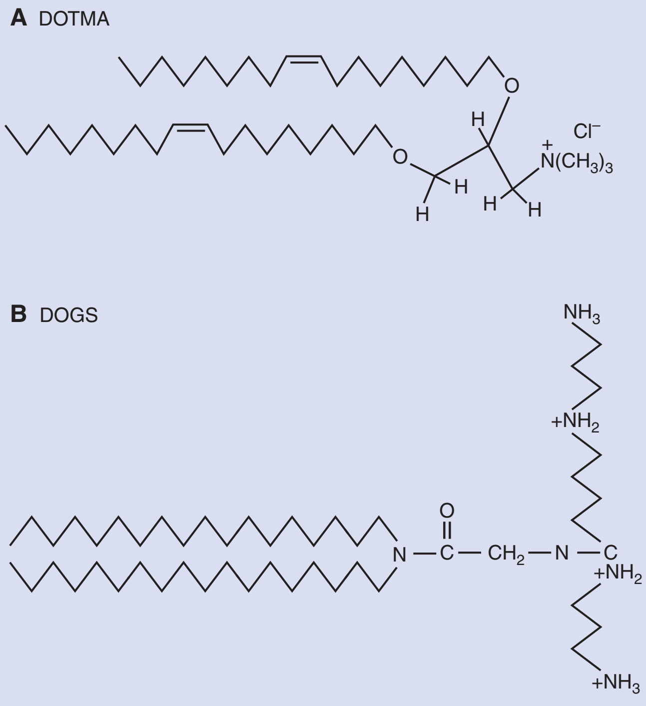

FIGURE 3. Structures of lipids used in lipofection.

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16. Add the lipid solution to the DNA, and mix the solution by pipetting up and down several times. Incubate the mixture for 10 min at room temperature.
17. While the DNA–lipid solution is incubating, wash the cells to be transfected three times with serum-free medium. After the third rinse, add 0.5 mL of serum-free medium to each 60-mm dish, and return the washed cells to a 37°C humidified incubator with an atmosphere of 5%–7% CO_2.

    The cells are rinsed free of serum before the addition of the lipid–DNA liposomes. In some cases, serum is a very effective inhibitor of the transfection process (Felgner and Holm 1989). Similarly, extracellular matrix components such as sulfated proteoglycans can also inhibit lipofection, presumably by binding the DNA–lipid complexes and preventing their interaction with the plasma membranes of the recipient cells.
18. Add 900 µL of serum-free medium to each tube. Mix the solution by pipetting up and down several times. Incubate for an additional 10 min at room temperature.
19. Transfer each tube of DNA–lipid–medium solution to a 60-mm dish of cells. Incubate the cells for 1–24 h at 37°C in a humidified incubator with an atmosphere of 5%–7% CO_2.
20. After the cells have been exposed to the DNA for the appropriate time, wash them three times with serum-free medium. Feed the cells with complete medium and return them to the incubator. Analyze for viability and transfection efficiency and/or proceed to make stable transfectants as detailed in Steps 11–13.

TROUBLESHOOTING

Problem (Step 11): Cells sicken.
Solution: The antibiotic used for selection is the cause. Consider the following:

- Wait to add the selection antibiotic until 24–48 h after the transfection procedure.
- Use a lower (or a range of) concentration of the selection antibiotic.

Problem (Step 11): Cells sicken.
Solution: The expressed protein is cytotoxic or is produced at levels that are too high. Consider the following:

- Analyze cytotoxicity by preparing experimental controls: untransfected cells, cells exposed to DNA alone without a transfection reagent, and cells exposed to transfection reagent alone. Compare the transfected cells with the experimental construct to the wells containing these experimental controls.
- Consider repeating the experiment with a secreted reporter gene assay such as secreted alkaline phosphatase or human growth hormone. Secreting cells should show little or no evidence of cytotoxicity.

Problem (Step 11): Cells sicken.
Solution: The cultures are contaminated with mycoplasma. Consider the following:

- Use a commercial mycoplasma detection kit to determine whether the culture is contaminated.
- Treat the cells with an antibiotic like BM-Cyclin (Roche Applied Sciences) to eliminate the mycoplasma, or start over with fresh clean cultures.

Problem (Step 11): Cells sicken.
Solution: If none of the solutions above are effective, then consider the following:

- DNA containing higher endotoxin levels may cause cytotoxicity to sensitive cell lines such as Huh-7 and primary cells. Use an endotoxin removal kit or restart the experiment using a kit that incorporates this step for generating the plasmid.
- In rare cases, the transfection reagent may be toxic to the cell line used. Try repeating transfection in the presence of fetal bovine serum, under reduced exposure time to the transfection reagent, at a higher cell density, and with varying ratios of lipid:DNA.

Problem (Step 12): Transfection efficiency is low.
Solution: The nucleic acids may be of poor quality or are insufficient in quantity. Consider the following:

- Use only high-quality plasmid preparations and at the recommended concentration.
- Perform a control transfection experiment with a commercially available transfection-grade plasmid preparation containing a marker gene like GFP.
- DNA containing higher endotoxin levels may cause cytotoxicity to sensitive cell lines like Huh-7 and primary cells. Use an endotoxin removal kit, or start over with a kit that incorporates this step for generating the plasmid.

Problem (Step 12): Transfection efficiency is low.
Solution: The transfection reagents were not stored as recommended. Consider the following:

- Store the reagent in the original container from the manufacturer and do not aliquot it.
- Do not freeze lipid transfection reagents.

Problem (Step 12): Transfection efficiency is low.
Solution: The lipid-to-DNA ratio is suboptimal. It may be necessary to empirically determine the ratio of the lipid transfection reagent to the DNA used for transfection. This can be performed in a multiwell plate and then scaled up accordingly.

Problem (Step 12): Transfection efficiency is low.
Solution: If none of the solutions above are effective, then consider the following:

- Some lipofection reagents, particularly FuGENE 6, are highly sensitive to contact with plastic. Make sure to pipette the reagent directly into the medium.
- In some cases, serum and other additives in the medium can inhibit complex formation. Prepare complexes in medium that does not contain additives (e.g., serum, antibiotics, growth enhancers).

DISCUSSION

Because a large number of variables affect the efficiency of lipofection, we suggest that the conditions outlined in the protocol be used as a starting point for systematic optimization of the system (for further details, see Introduction: **Lipofection** [Kumar et al. 2019b]). Once a positive signal has been obtained with a plasmid carrying a standard reporter gene, each of the parameters discussed in that introduction may be changed systematically to obtain the maximal ratio of signal to background and to minimize variability between replicate assays. From these results, optimal protocols can be developed to assay the expression of the genes of interest.

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