

Cationic Lipid Transfection Reagents - Transfectam[®], TransFast[™] and Tfx[™] Reagents for the Transfection of Eukaryotic Cells

Introduction to Promega's Cationic Lipid Reagents

Promega provides three types of cationic lipid-based transfection reagents, Transfectam[®] Reagent, TransFast[™] Reagent and the Tfx[™]-10, Tfx[™]-20 and Tfx[™]-50 Reagents. The cationic lipid component of these reagents associates with negatively charged nucleic acids, resulting in a lipid/nucleic acid complex that has a net neutral or positive charge and therefore allows closer association of the DNA with the negatively charged cell membrane.

Transfectam[®] Reagent for the Transfection of Eukaryotic Cells is a cationic lipid reagent consisting of dioctadecylamidoglycyl spermine (DOGS), a synthetic, cationic lipopolyamine. The spermine group is covalently attached through a peptide bond to the lipid moiety (Figure 3.1). The strong positive charge contributed by the spermine headgroup gives the molecule a high affinity for DNA (10^5 - 10^6 M⁻¹). The positively charged headgroup effectively coats the negatively charged DNA with a cationic lipid layer, allowing it to fuse with the plasma membrane of eukaryotic cells, resulting in internalization of the DNA.

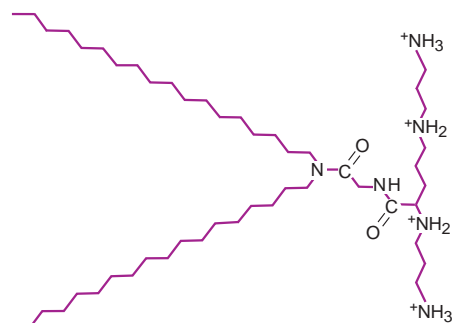


Figure 3.1. Structure of Transfectam[®] Reagent.

Liposome Based Transfection Reagents

The term "liposome" refers to lipid bilayers that form colloidal particles in an aqueous medium (36). Liposome reagents specifically designed for transfection applications incorporate synthetic cationic lipids (16), often formulated together with the neutral lipid DOPE (Figure 1.4), which has been demonstrated to enhance the gene transfer ability of certain synthetic cationic lipids (37,38).

Incubation of cationic lipid-containing liposomes and nucleic acids results in quick association and a compaction of the nucleic acid (39,40), presumably from electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid. Entry of the liposome complex into the cell may occur by the processes of endocytosis, or fusion with the plasma membrane via the lipid moieties of the liposome (41). Once inside the cell, the complexes often become trapped in endosomes and lysosomes. Endosomal disruption is facilitated by DOPE (24), which allows the complexes to escape into the cytoplasm. The cytoplasm is the site of action for RNA or anti-sense oligonucleotides delivered via the liposomes. The nucleus is the target for most DNA delivery and it is not known precisely how the transfected DNA or liposome/DNA complex gains entry to the nucleus.

Promega's **TransFast[™]** and **Tfx[™]** Reagents facilitate liposome-mediated transfer of nucleic acids into eukaryotic cells. The TransFast[™] Reagent is composed of the synthetic cationic lipid, N,N [bis (2-hydroxyethyl)]-N-methyl-N-[2,3 di(tetradecanoyloxy) propyl] ammonium iodide (Figure 3.2a) and the neutral lipid, (DOPE) (Figure 1.4).

The Tfx[™] Reagents contain a mixture of a synthetic, cationic lipid molecule [N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxy-ethyl)-2,3,-dioleoyloxy-1,4-butanediammonium iodide] (Figure 3.2b) and DOPE (Figure 1.4). All of the Tfx[™] Reagents (Tfx[™]-10, Tfx[™]-20, and Tfx[™]-50) contain the same concentration of the cationic lipid component, but contain different molar ratios of the fusogenic lipid, DOPE.

The best transfection reagent and conditions for a particular cell type must be empirically and systematically tested because inherent properties of the cell influence the success of any specific transfection method.

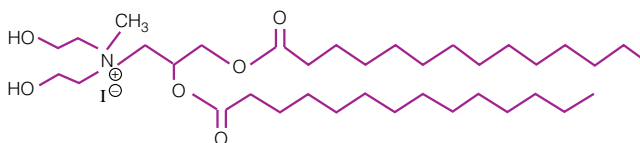


Figure 3.2a. Structure of the synthetic cationic lipid component of the TransFast[™] Reagent.



Figure 3.2b. Structure of the synthetic cationic lipid component of the Tfx[™] Reagents.

Advantages of Using Cationic Lipid Reagents for Transfection

Cationic lipid reagents designed for transfection applications are more versatile than many other traditional methods. The advantages include versatility in the macromolecules delivered, *in vitro* and *in vivo* applications, ability to reproducibly transfect cells that are recalcitrant to other methods, and suitability for transient and stable transfection paradigms. For example, several different types of macromolecules can be delivered to cells using these methods, including RNA and DNA of all sizes ranging from oligonucleotides to plasmids and yeast artificial chromosomes (17-21,42).

TransFast™ Reagent, Transfectam® Reagent and the Tfx™ Reagents offer the advantages that they are easy to optimize and work well for a variety of cell types (43-46). In addition, these reagents are excellent for use with primary cells as they can be used in the presence of serum, can be used for both transient and stable transfections and are of low toxicity.

The Tfx™ and Transfectam® Reagents can also be used for *in vivo* transfection (47-49). It has been shown that Tfx™-50 Reagent is highly active in the presence of amniotic fluid (50), which has implications for its use in intra-amniotic injection and transfection.

Factors Influencing Transfection Efficiency

With any transfection reagent or method, cell health, degree of confluency, passage number, contamination, and DNA quality and quantity are important parameters that can greatly influence transfection efficiency. Plasmid DNA for transfections should be free of protein, RNA and chemical contamination (See Chapter 2). Suspend ethanol-precipitated DNA in sterile water or TE buffer to a final concentration of 0.2-1mg/ml. The optimal amount of DNA to use in the transfection will vary widely depending upon the type of DNA and the target cell line.

It is **essential** to optimize specific transfection conditions to gain optimal transfection efficiencies.

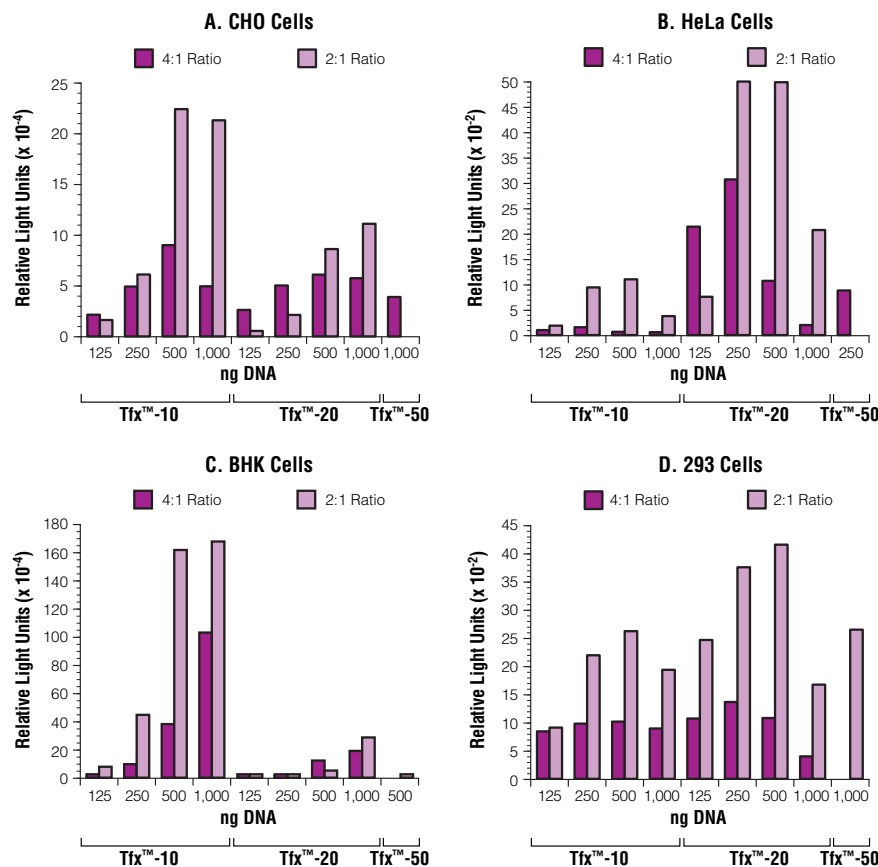


Figure 3.3. Relative levels of gene expression as a function of Tfx™ Reagent, DNA amount and reagent:DNA charge ratio. CHO Cells (Panel A), HeLa cells (Panel B), BHK cells (Panel C) and 293 cells (Panel D) were plated at a density of 50,000 cells/well in 24 well plates. Transfections were performed in the absence of serum using the indicated Tfx™ Reagent and pGL3-Control Vector at reagent:DNA ratios of 2:1 and 4:1. All transfections were overlaid with serum-containing media after one hour, and cells were harvested for luciferase assays after 48 hours. The results represent the mean of 6 replicates and are expressed as relative light units per well of cells. The single Tfx™-50 Reagent conditions reflect the optimal DNA amount and reagent:DNA ratio determined from previous optimization experiments.

The important parameters to optimize in order to maximize transfection efficiencies are the charge ratio of transfection reagent to DNA, the amount of transfected DNA, the length of time the cells are exposed to the transfection reagent and the presence or absence of serum. Figure 3.3 shows an example of optimization experiments for 4 different cell lines using different amounts of pGL3 Control DNA, different reagent:DNA charge ratios and the three different Tfx™ Reagents. Expression of luciferase activity from the transfected DNA is indicated in relative light units on the Y-axis. The graphs show the results of comparisons among the three Tfx™ Reagents: for CHO cells, Tfx™-10 Reagent and 500ng DNA at a 2:1 reagent:DNA charge ratio was most effective; for HeLa cells, Tfx™-20 Reagent and 250ng DNA at a 2:1 charge ratio was most effective; for BHK cells Tfx™-10 Reagent and 1,000ng DNA at a 2:1 ratio was most effective; and for 293 cells Tfx™-20 Reagent and 500ng of DNA at a 2:1 Reagent:DNA ratio was most effective. It should be noted that Figure 3.3 is a comparison of the performance of the three Tfx™ Reagents in these cell lines. For CHO and 293 cells, TransFast™ Reagent has been found to perform better than Tfx™ Reagents.

The transfection efficiency achieved using all of Promega's cationic lipid-based transfection reagents varies depending on the cell type being transfected and the transfection conditions used.

Liposome Based Transfection Protocols - TransFast™ and Tfx™ Reagents

General Considerations

Charge Ratio of Transfection Reagent to DNA

The amount of positive charge contributed by the cationic lipid component of the transfection reagent should equal or exceed the amount of negative charge contributed by the phosphates on the DNA backbone, resulting in a net neutral or positive charge on the multilamellar vesicles associating with the DNA. Charge ratios of 2:1 to 4:1 Tfx™ Reagent:DNA and 1:1 to 2:1 TransFast™ Reagent:DNA have worked well with various cultured cells but ratios outside of this range may be optimal for other cell types or applications. Each of the Tfx™ Reagents contains the same amount of cationic lipid (1mM when the contents of each vial are resuspended in the recommended 400µl volume), but contains varying amounts of the neutral lipid component, DOPE.

DNA

The optimal amount of DNA to use in the transfection will vary depending upon the type of DNA and the target cell line used. For example, HeLa cells are optimally transfected with 0.25µg of pGL3-Control DNA using Tfx™-20 Reagent while NIH/3T3 cells are optimally transfected with TransFast™ Reagent. For adherent cells, we recommend initially testing 0.25, 0.50, 0.75 and 1µg of DNA in a 24 well plate format at a transfection reagent:DNA ratio of 3:1 for each of the Tfx™ Reagents and at transfection reagent:DNA ratios of 2:1 and 1:1 for TransFast™ Reagent. Increasing the amount of DNA does not necessarily result in higher transfection efficiencies.

Time

The optimal transfection time is dependent upon the cell line and DNA used. For the first tests, use a one hour transfection interval. However, in optimization experiments, test transfection times from 30 minutes to 4 hours. Monitor cell morphology during the transfection interval, particularly when the cells are maintained in serum-free medium, as some cell lines lose viability under these conditions. The transfection time with the TransFast™ and Tfx™ Reagents is usually significantly shorter than that required with other cationic lipid compounds, and can be decreased to as little as 30 minutes with certain cell lines (Figure 3.4). In addition to saving time, this shortened transfection time may significantly reduce the risk of cell death during the transfection procedure.

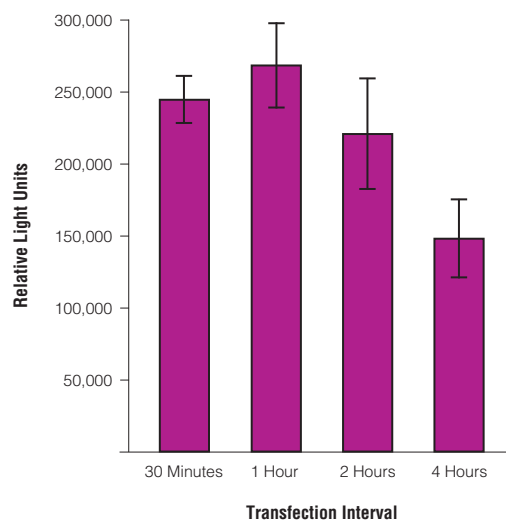


Figure 3.4. Effect of transfection interval on transfection of CHO cells using TransFast™ Reagent. CHO cells were transfected with 250ng of pGL3-Control DNA using TransFast™ Reagent at a 2:1 reagent:DNA charge ratio for various times in the absence of serum. All transfections were performed in 24 well plates and cell lysates were harvested 2 days post-transfection. The results represent the mean of 6 replicates and are expressed as relative light units per well.

Serum

Transfection protocols often require serum-free conditions for optimal performance, as serum can interfere with many commercially available transfection reagents. The TransFast™ and Tfx™ Reagents can be used in transfection protocols in the presence of serum, allowing transfection of cell types or applications that require continuous exposure to serum. Figure 3.5 shows the effect of the presence or absence of serum on transfection of COS-7 cells using TransFast™ Reagent.

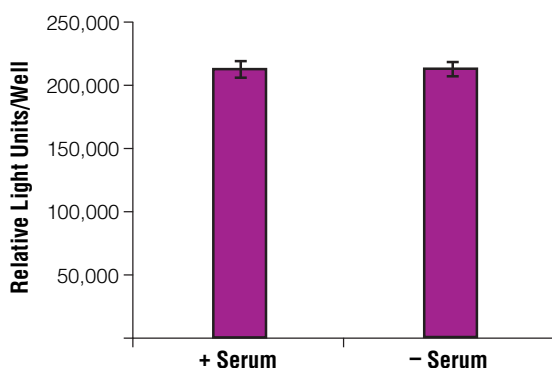


Figure 3.5. Effect of serum on transfection of COS-7 cells using TransFast™ Reagent. Cells were transfected with 500ng of a CMV-promoter driven luciferase reporter plasmid DNA per well, at 1:1 reagent:DNA charge ratios in 10% serum-supplemented or serum-free medium. The transfection interval was one hour. All transfections were performed in 24 well plates and cell lysates were harvested 2 days post-transfection. The results represent the mean of 6 replicates and are expressed as relative light units per well.

Stable Transfection

The TransFast™ and Tfx™ Reagents can be used for the production of stable transfectants. However, we recommend first optimizing the transfection conditions using transient transfection studies.

Lipid Carrier

The TransFast™ and each of the Tfx™ Reagents work optimally for different cell lines. For example, we have determined that BHK cells are optimally transfected with Tfx-10™ Reagent, HeLa cells with Tfx™-20 Reagent, and 293 cells with TransFast™ Reagent (see the table on the inside front cover of this guide). The optimal transfection reagent for each cell line needs to be determined empirically. Table 3.1 gives specific transfection conditions that have worked well for the various Tfx™ Reagents and TransFast™ Reagent in some commonly-used cell lines.

Table 3.1. A Comparison of Transfection Conditions Used for TransFast™, Tfx™-10, Tfx™-20 and Tfx™-50 Reagents with Various Cell Lines.

Cell Line	Cell Type	Reagent	Transfection Solution per Well in a 24 Well Plate	Charge Ratio
293	Attached; Human Epithelial; Ad5-transformed embryonic kidney	TransFast™ Reagent	250ng plasmid DNA 0.75µl TransFast™ Reagent S.F. Media to 200µl	1:1
BHK	Attached; Hamster Fibroblasts; Kidney	Tfx™-10	1.0µg plasmid DNA 3.0µl Tfx™-10 S.F. Media to 200µl	2:1
CHO	Attached; Hamster Epithelial-like; Ovary	TransFast™ Reagent	500ng plasmid DNA 1.5µl TransFast™ Reagent S.F. Media to 200µl	1:1
		TransFast™ Reagent	1.0µg plasmid DNA 3µl TransFast™ Reagent Serum + Media to 200µl	1:1
COS-7	Attached; Monkey Fibroblasts; African Green Monkey Kidney; SV40 Transformed	TransFast™ Reagent	500ng plasmid DNA 1.5µl TransFast™ Reagent Serum + Media to 200µl (or S.F. Media to 200µl)	1:1
CV-1	Attached; Monkey Fibroblasts; African Green Monkey Kidney	TransFast™ Reagent	1.0µg plasmid DNA 3µl TransFast™ Reagent S.F. Media to 200µl	1:1
HeLa	Attached; Human Epithelial; cervical carcinoma	Tfx™-20	250ng plasmid DNA 0.75µl Tfx™-20 S.F. Media to 200µl	2:1
HepG2 ¹	Attached; Human Epithelial; Hepatoblastoma	Tfx™-20	250ng plasmid DNA 1.5µl Tfx™-20 S.F. Media to 200µl	4:1
		Tfx™-50	250ng plasmid DNA 1.1µl Tfx™-50 (or 1.5µl) Serum + Media to 200µl	3:1 (4:1)
Jurkat ²	Suspension; Human T-lymphocytes; T cell leukemia	TransFast™ Reagent	3µg plasmid DNA 9µl TransFast™ Reagent S.F. Media to 1ml per 6 well plate	1:1
K562 ²	Suspension; Human Lymphoblast; Myelogenous Leukemia	TransFast™ Reagent	4µg plasmid DNA 12µl (or 24µl) TransFast™ Reagent S.F. Media to 1ml per 6 well plate	1:1 (2:1)
NIH/3T3	Attached; Mouse Fibroblasts; NIH Swiss Mouse embryo	TransFast™ Reagent	1.0µg plasmid DNA 3.0µl TransFast™ Reagent S.F. Media to 200µl	1:1
PC12	Attached; Rat Adrenal; Pheochromocytoma	Tfx™-20	1.0µg plasmid DNA 2.25µl Tfx™-20 S.F. Media to 200µl	1.5:1
		TransFast™ Reagent	1.0µg plasmid DNA 6µl TransFast™ Reagent S.F. Media to 200µl	2:1
SF9	Insect	Tfx™-20	500ng plasmid DNA 1.5µl Tfx™-20 Reagent S.F. Media to 200µl	2:1
		TransFast™ Reagent	500ng plasmid DNA 3µl TransFast™ Reagent S.F. Media to 200µl	2:1

N.D. = Not Determined

S.F. = Serum-Free

¹TransFast™ Reagent Not Tested.²Procedures are different for suspension cells. See page 23.

Note: Conditions for these cell lines were determined using cells obtained from the American Type Culture Collection (ATCC). All attached cells were tested at low passage number and 80% confluency.

Protocol

A general protocol for use with Promega's liposome-based transfection reagents (TransFast™, Tfx™-10, Tfx™-20 and Tfx™-50 Reagents) is provided below. Figure 3.6 gives a general overview of the steps involved in the procedure. This protocol can be used with serum-supplemented or serum-free medium. For further information on each system, please request the *TransFast™ Transfection Reagent Technical Bulletin #TB260*, or the *Tfx™ Reagents Technical Bulletin #TB216*. These Technical Bulletins are also available on the Internet at www.promega.com.

For a list of references using the Tfx™ Reagents in a variety of cell lines, see Appendix A.

Materials to Be Supplied by the User

- cell culture medium with serum (i.e., complete medium; appropriate for the cell type being transfected)
- serum-free cell culture medium
- 24 well plates or 60mm or 100mm cell culture plates

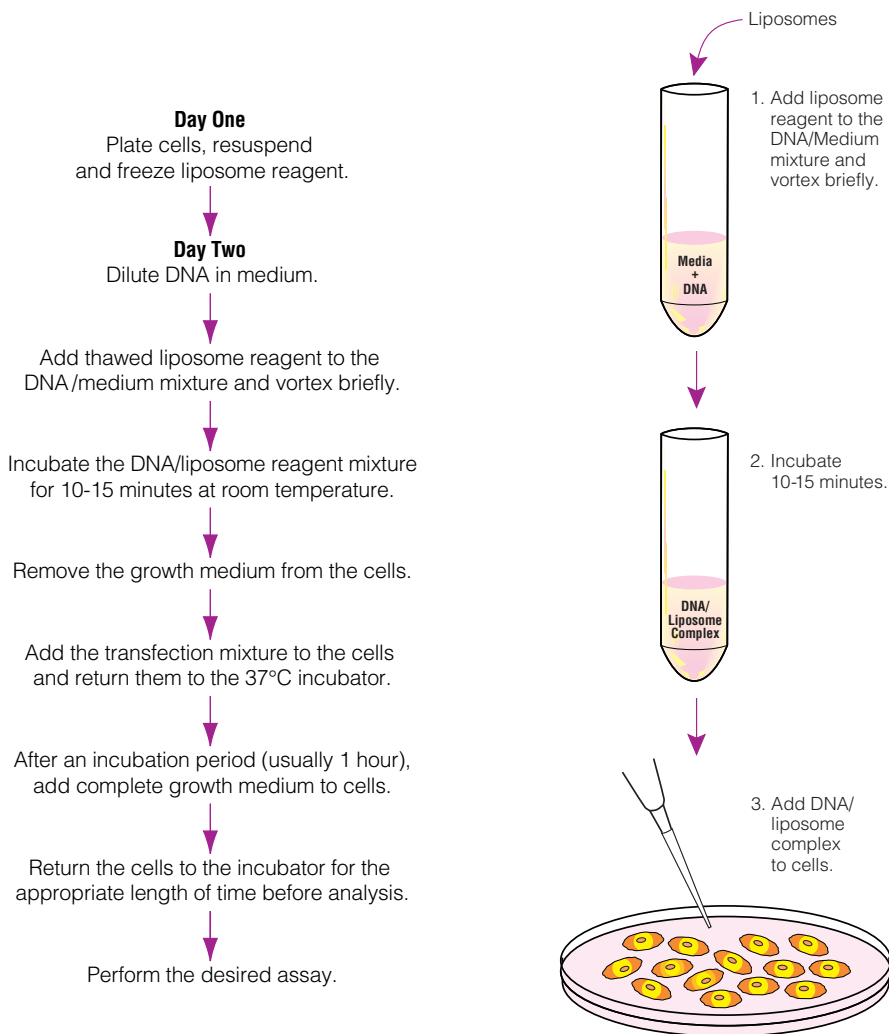


Figure 3.6. Overview of cationic lipid - mediated transfection with adherent cells.

Plating Cells

Cells should be almost confluent by the time they are harvested 48 hours after transfection. The degree of confluency on the day of transfection is a parameter that needs to be optimized for each individual cell line.

As a general guideline, plate cells one day before the transfection experiment so that they will be approximately 50-80% confluent on the day of the transfection. Some cell lines, such as HeLa cells, exhibit higher toxicity effects when transfected at lower cell densities. As a general guideline, plate 5×10^4 cells per well (24 well plate) or 5.5×10^5 cells (60mm culture dish). Change cell numbers proportionately for differently sized plates (see Table 3.2).

Table 3.2. Area of Culture Plates for Cell Growth.

Size of Plate	Growth Area (cm ²) ^a	Relative Area ^b
24 well	1.88	1 X
96 well	0.32	0.2 X
12 well	3.83	2 X
6 well	9.4	5 X
35mm	8.0	4.2 X
60mm	21	11 X
100mm	55	29 X

^aThis information was calculated for Corning™ culture dishes.

^bRelative area is expressed as a factor of the total growth area of the 24 well plate recommended for optimization studies. To determine the proper plating density, multiply 5×10^4 cells by this factor.

Preparation of Liposome Reagent Stock Solution

1. The **day before transfection**, warm the vial of TransFast™ or Tfx™ Reagent to room temperature. Dissolve the contents of the vial in 400μl of Nuclease-Free Water at room temperature (1mM final concentration of the cationic lipid component). After adding the Nuclease-Free Water, vortex the sample vigorously for 10 seconds to dissolve the lipid film. (For Tfx™ Reagents, place the vial in a 65°C water bath for one minute after vortexing. Make sure the level of the water is above the level of the liquid in the vial. Vortex again.) Store the suspended reagent at -20°C overnight. Before each use, thaw and vortex the solution. Store any remaining suspended reagent at -20°C, where it is stable for 8 weeks.

Note: It is necessary to freeze the reagent prior to use.

Note: It is normal for the lipid suspension to appear cloudy and contain particulate matter. A slight, residual “ring” of material may remain in the vial after suspension.

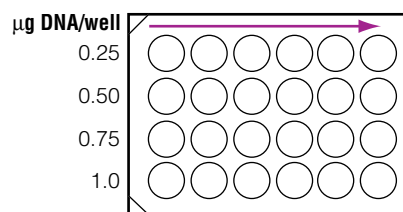
2. Before each use, thaw at room temperature and vortex the solution. If liquid has condensed at the top of the vial or in the vial cap, collect the liquid by placing the reagent vial inside a 50ml centrifuge tube and centrifuging briefly at 300 x g. After use, store the remaining stock in the vial at -20°C.

Optimization of Transfection

Plasmids with reporter gene functions can be used to monitor transfection efficiencies (see Chapter 6). An ideal reporter gene product is one that is unique to the cell, can be expressed from plasmid DNA and can be assayed conveniently. Generally, reporter gene assays are performed 2-3 days after transfection.

We recommend testing various amounts of transfected DNA (0.25, 0.5, 0.75 and 1.0μg per well), using a one-hour exposure time and charge ratios of TransFast™ Reagent:DNA of 1:1 and 2:1, or a 3:1 ratio of Tfx™ Reagent:DNA. This can be done under serum-free conditions with adherent cells in a 24 well plate format. Figure 3.7 outlines a typical optimization matrix.

1:1 Charge Ratio of TransFast™ Reagent:DNA



3:1 Charge Ratio of Tfx™ Reagent:DNA

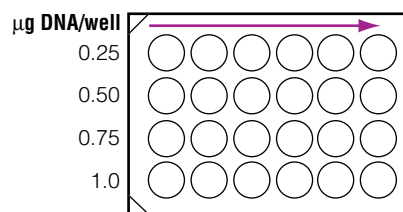


Figure 3.7. Typical optimization matrices for TransFast™ Reagent and Tfx™ Reagent:DNA Ratios.

Table 3.3. Optimization Protocol Using a 1:1 Charge Ratio of TransFast™ Reagent to DNA.

	Amount of DNA Per Well			
	0.25µg	0.5µg	0.75µg	1µg
Medium (to final volume)	1,400µl	1,400µl	1,400µl	1,400µl
DNA	1.8µg	3.5µg	5.3µg	7.0µg
TransFast™ Reagent*	5.3µl	10.5µl	15.8µl	21µl

*Volumes given are for use with TransFast™ Reagent suspended in 400µl/vial and for use with 24 well plates at 200µl/well.

To test 2:1 ratios of TransFast™ Reagent:DNA, simply double the amount of reagent used for each DNA amount.

Table 3.4. Optimization Protocol Using a 3:1 Charge Ratio of Tfx™ Reagent to DNA.

	Amount of DNA Per Well			
	0.25µg	0.5µg	0.75µg	1µg
Medium (to final volume)	1,400µl	1,400µl	1,400µl	1,400µl
DNA	1.8µg	3.5µg	5.3µg	7.0µg
Tfx™ Reagent*	7.9µl	15.8µl	23.6µl	31.5µl

*Volumes given are for use with Tfx™ Reagent suspended in 400µl/vial and for use with 24 well plates at 200µl/well.

- For a 24 well plate, the total volume of the medium, DNA and liposome reagent should be 200µl per well. The volumes in Tables 3.3 and 3.4 were calculated for seven wells, adequate for 6 replicates for each DNA concentration. In a sterile tube, combine the indicated amount of serum-free medium (prewarmed to 37°C) and plasmid DNA and vortex. Add the indicated amount of liposome reagent and vortex immediately.
- Incubate for 10-15 minutes at room temperature. Incubations longer than 30 minutes result in a lowered transfection efficiency.
- Carefully remove the medium from the cells by aspiration.
- Briefly vortex the liposome reagent/DNA mixture. Add the mixture to the cells (200µl per well) and return the plates to the incubator for 1 hour. During the incubation, warm complete medium (cell culture medium containing serum) to 37°C.
- At the end of the 1 hour incubation period, gently overlay the cells with 1ml of complete medium (prewarmed to 37°C). **Do not** remove the transfection medium containing the liposome reagent/DNA mixture. Return the cells to the incubator and continue the incubation for the appropriate length of time before analysis. For many reporter systems (Luciferase, CAT and β-galactosidase) a 48 hour incubation is sufficient.

- Check the transfection efficiency using an assay appropriate for the reporter system.

Transfection Protocol for Adherent Cells

After the transfection parameters have been optimized, use the empirically determined conditions for experimental transfections. If you choose not to optimize the transfection parameters, use the general conditions recommended below. Volumes and amounts are given for transfections performed in 60mm plates (values for 100mm plates are given in parentheses).

- The total volume of medium, DNA and liposome reagent per 60mm dish is 2ml (6ml). To a sterile tube add the appropriate amount of medium, prewarmed to 37°C. Add 2.5-10µg of plasmid DNA to the medium (7.5-30µg) and vortex. We recommend 5µg of DNA per 60mm dish (15µg), a 1:1 reagent:DNA ratio for TransFast™ Reagent and a 3:1 reagent:DNA ratio for Tfx™ Reagents for initial transfection experiments. Add the amount of liposome reagent indicated in Table 3.5 and vortex immediately.

Note: The TransFast™ Reagent and the Tfx™ Reagents are at a final concentration of 1mM cationic lipid per suspension. However, the cationic lipid in the Tfx™ Reagents has two positive charges per molecule while the TransFast™ Reagent has one positive charge per molecule. Therefore, twice the volume of the TransFast™ Reagent is required to provide the same charge ratio to DNA.

Table 3.5. Relationship Between Volume of TransFast™ Reagent and TransFast™ Reagent:DNA Charge Ratio.

Charge Ratio of Liposome Reagent to DNA	Volume of TransFast™ Reagent Per µg of DNA*	Volume of Tfx™ Reagent Per µg of DNA
1:1	3.0µl	1.5µl
2:1	6.0µl	3.0µl
3:1	9.0µl	4.5µl
4:1	12.0µl	6.0µl

*Volumes given are for use with reagents suspended in 400µl/vial.

- Incubate the liposome reagent/DNA mixture for 10-15 minutes at room temperature.
- Remove the medium from the cells.
- Add 2ml (or 6ml) of the liposome reagent/DNA mixture to each plate and return the cells to the incubator. Incubate the plates for 1 hour. During the incubation, warm an appropriate volume of serum-containing medium to 37°C.

- At the end of the incubation period, gently overlay the cells with 4ml (12ml) of the prewarmed medium. Again, do not remove the transfection medium containing the liposome reagent. Return the cells to the incubator and continue incubation for the appropriate length of time before analysis.
- Check the transfection efficiency using an appropriate reporter assay (Figure 3.8). For transient transfection, cells are typically harvested 48 hours after transfection.

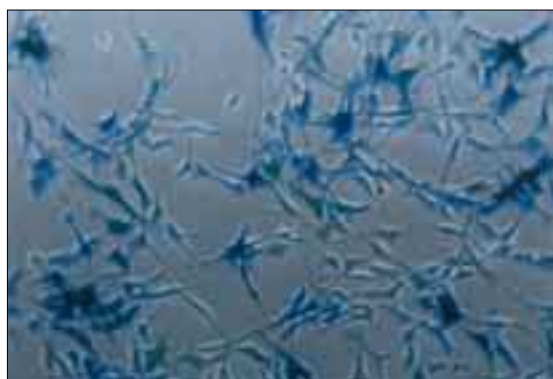


Figure 3.8. Histochemical staining of NIH/3T3 cells for β -galactosidase activity. NIH/3T3 cells were plated in 24 well plates and transfected with 1 μ g DNA containing the β -galactosidase gene under the control of the CMV promoter per well. TransFast™ Transfection Reagent was used at a 1:1 TransFast™ Reagent:DNA charge ratio. Cells were fixed with glutaraldehyde 2 days post-transfection and stained for β -galactosidase activity using standard techniques (See Promega Technical Bulletin #TB097). The cells expressing β -galactosidase are stained blue.

Transfection Protocol for Suspension Cells

Optimization of transfection parameters can be performed with suspension cells using the following general guidelines: For 1×10^6 cells, test 1, 2, 3 and 4 μ g DNA at an initial charge ratio of liposome reagents to DNA of 1:1 for TransFast™ Reagent and 3:1 for the Tfx™ Reagents. Incubate for 1 hour in the absence of serum. Additional optimization studies, to test the effect of serum and other liposome reagent:DNA charge ratios can be performed once the optimal amount of DNA has been determined. We recommend also testing a 2:1 charge ratio for both TransFast™ Reagent and Tfx™ Reagents for optimization.

- Suspend the liposome reagent the day before the transfection and store at -20°C .
- On the day of the transfection, determine the cell density using a hemacytometer and spin down enough cells to complete the transfection

experiments; 1×10^6 cells per transfection is usually sufficient. Spin the cells for 5 minutes at $300 \times g$ in a swinging bucket rotor. Resuspend the cell pellet such that the cells are at a concentration of 2×10^6 cells/ml in serum-free media. Re-count the cells and adjust the volume if necessary.

- Prepare the liposome reagent/DNA mixture. To a sterile tube add the indicated amount of medium (prewarmed to 37°C) and DNA to a total volume of 0.5ml and vortex. Add the indicated amount of liposome reagent and vortex immediately (see Table 3.6).
- Allow the liposome reagent and DNA mixture(s) to incubate for 10-15 minutes at room temperature.

Table 3.6. Optimization Protocols for Suspension Cells.

TransFast™ Reagents (1:1 Charge Ratio)

	Amount of DNA Per Tube			
	1 μ g	2 μ g	3 μ g	4 μ g
Medium (to final volume)	0.5ml	0.5ml	0.5ml	0.5ml
DNA	1 μ g	2 μ g	3 μ g	4 μ g
TransFast™ Reagent*	3 μ l	6 μ l	9 μ l	12 μ l

* Volumes given are for use with TransFast™ Reagent suspended in 400 μ l/vial.

Tfx™ Reagents (3:1 Charge Ratio)

	Amount of DNA Per Tube			
	1 μ g	2 μ g	3 μ g	4 μ g
Medium (to final volume)	0.5ml	0.5ml	0.5ml	0.5ml
DNA	1 μ g	2 μ g	3 μ g	4 μ g
Tfx™ Reagent*	4.5 μ l	9 μ l	13.5 μ l	18 μ l

* Volumes given are for use with Tfx™ Reagent suspended in 400 μ l/vial.

- While the liposome reagent/DNA mixtures are incubating, aliquot 0.5ml of cells (1×10^6 cells) to each well of a 6 well plate.
- Briefly vortex the liposome reagent/DNA mixture and add to the cells (0.5ml/well). Return the cells to the incubator for 1 hour. During the incubation, warm complete medium (containing serum) to 37°C .
- At the end of the incubation period, add 5ml of the prewarmed medium per well. Return the cells to the incubator and continue the incubation for the appropriate length of time before analysis. For many reporter systems (e.g., luciferase, CAT and β -galactosidase), a 48 hour incubation is sufficient.
- Check the transfection efficiency using an assay appropriate for the reporter system.

Stable Transfections

For stable transfections, cells should be transfected with a plasmid containing a gene for drug resistance, such as neomycin phosphotransferase (see Chapter 6 for details of vectors available from Promega). As a negative control, transfect the cells using DNA that does not contain the drug resistance marker.

1. Prior to transfection, determine the killing concentration of the selective drug being used.
2. Forty-eight hours post transfection, trypsinize adherent cells and re-plate at several different dilutions (for example, 1:100, 1:500) in media containing the appropriate selection drug.
3. For the next 14 days, replace the drug-containing media every 3 to 4 days.
4. During the second week monitor the cells for distinct “islands” of surviving cells. Cell death should occur in cultures transfected with the negative control plasmid.
5. Transfer individual clones by standard techniques (e.g., using cloning cylinders) to 96 well plates and continue to maintain cultures in medium containing the appropriate drug.

Neomycin (G418) Selection

G418 blocks protein synthesis in mammalian cells by interfering with ribosomal function. It is an aminoglycoside, similar in structure to neomycin, gentamycin, and kanamycin (8).

Varying concentrations of G418 should be tested as cells differ in their susceptibility to G418. Use 100 to 800 $\mu\text{g/ml}$ of G418 in complete medium. G418 should be prepared in a highly buffered solution (eg. 100 mM HEPES, pH 7.3) so that the addition of drug does not alter the pH of the medium.

Different lots of G418 can have different potencies, causing many investigators to buy a large amount of one lot to standardize selection conditions. G418 concentration should be calculated using the amount of active drug (usually indicated on each lot label) so that variance is controlled.

Cells will divide once or twice in the presence of lethal doses of G418, so the effects of the drug take several days to become apparent. Complete selection can take up to 3 weeks of growth in selective media.

Calculating Stable Transfection Efficiency

The following procedure may be used to determine the percentage of stable transfectants obtained.

Note: The stained cells will not be viable after this procedure.

1. After approximately 14 days of selection in the appropriate drug, monitor the cultures microscopically for the presence of viable cell clones. When distinct “islands” of surviving cells are visible and non-transfected cells have died out, proceed with step 2.
2. Prepare stain containing 2% methylene blue in 50-70% methanol
3. Remove the growth media from the cells by aspiration.
4. Add stain to the cells, sufficient to cover the bottom of the dish.
5. Incubate for 5 minutes.
6. Remove the stain and rinse gently under deionized cold water. Shake off excess moisture.
7. Allow the plates to air dry. The plates can be stored at room temperature.
8. Count the number of colonies and calculate the percent of transfectants based on the cell dilution and original cell number.

For further information on stable transfections see reference 29.

Transfection Protocols - Transfectam® Reagent for the Transfection of Eukaryotic Cells

Table 3.7. List of Cell Lines Transfected Using Transfectam® Reagent.

Origin	Cell Line	Cell Type	References
Established Cell Lines			
Hamster	CHO	Fibroblast	43,52-55
Human	293	Embryonic kidney	56-58
Human	HeLa	Epthelial	43
Human	Hep G2	Hepatocyte	59
Monkey	COS	Fibroblast	53,60-63
Mouse	C2C12	Myoblast	64
Mouse	F9	Teratocarcinoma	43,65
Mouse	LM (tk-)	Fibroblast	43,65,66
Mouse	NIH/3T3	Fibroblast	46
Mouse	AtT20	Hypophysis	43,67
Mouse	S49	Lymphocyte	43
Mouse	Balb/3T3	Fibroblast	68
Mouse	MEL	Erythroleukemia	69
Rat	PC12	Pheochromocytoma	70
Mink	Mv1LU	Lung epithelial	71
Human	A549	Lung carcinoma	72
Human	LoVo/Dx	Colon adenocarcinoma	68
Human		Lymphoblast	73
Human	CCRF-CEM/VLB	Leukemic T-lymphoblast	68
Primary Cells			
Rat		Cerebellum neurons	67
Rat		Striatum neurons	67
Rat		Cortical neurons	67
Rat		Astrocytes	67
Rat		Adipocytes	67
Rat		Anterior pituitary	43
Chicken embryo (<i>in vivo</i>)			74
Chicken		Heart	75
Pig		Melanotrope cells	43,67
Bovine		Chromaffin cells	43,67

For further references using Transfectam® Reagent in a variety of cell lines, see Appendix A.

Plating Cells

Plate cells the day before the transfection experiment according to the guidelines given in the protocol for Tfx™ and TransFast™ Reagents.

Suspension cells can be transfected by the following protocol using the equivalent of 10⁶ suspended cells per assay. The volume of reagents can be scaled up or down proportionately depending on the number of cells used per assay.

Preparation of Transfectam® Reagent Stock Solution

- Resuspend Transfectam® Reagent in 100% ethanol (dehydrated) with vortexing (final concentration is 2mM) and incubate at room temperature for at least 5 minutes. Overnight storage at 4°C ensures complete solubilization and may give improved transfection efficiencies. Store the resuspended Transfectam® Reagent at 4°C, where it is stable for 6 months.
- Note:** For some cells, it may be desirable to minimize the ethanol concentration applied. If so, Transfectam® Reagent may be dissolved with vortexing in as little as 1/10 volume of ethanol (dehydrated), incubated at room temperature for 5 minutes, and then further diluted to working concentration in water.
- Mix the solution before each use. Store the remaining stock at 4°C.

Transfection Protocol for Media Without Serum

We recommend using medium with no added serum for transfection. Some components in serum may degrade the Transfectam® Reagent. The presence of albumin, heparin, trypsin or EDTA in the medium also will decrease the efficiency of transfection. However, if cell viability is low in medium without serum, use the alternative protocol, provided below.

Materials to Be Supplied by the User

- cell culture medium appropriate for the cell type used

The reagent volumes in this protocol are based on use of 60mm culture dishes. Optimization experiments can be performed using adherent cells in 24 well plates. Scale the reagent volumes up or down proportionally if using different sized plates (see Table 3.8).

Table 3.8. Area of Culture Plates for Cell Growth.

Size of Plate	Growth Area (cm ²) ^a	Relative Area ^b
96 well	0.32	0.02 X
24 well	1.88	0.09 X
12 well	3.83	0.18 X
6 well	9.4	0.45 X
35mm	8.0	0.38 X
60mm	21	1.00 X
100mm	55	2.62 X

^aThis information is for Corning™ culture dishes.

^bRelative area is expressed as a factor of the growth area of the 60mm dish. To determine the approximate plating density, multiply 5 x 10⁵ cells by this factor. To determine the reagent volumes needed for plates other than 60mm plates, multiply the volumes by the appropriate "Relative Area" factor.

1. Add 1-5 μ g of plasmid DNA to 500 μ l of serum-free medium in a sterile tube and vortex (Solution A). We recommend 5 μ g per 60mm dish for the initial tests.
2. For each microgram of plasmid DNA used in Solution A, add between 1.5 and 5 μ l of Transfectam[®] Reagent to 500 μ l of serum-free medium in a sterile tube and mix (Solution B). For the initial tests, use 10 μ l of Transfectam[®] Reagent per 60mm dish per 5 μ g plasmid DNA.
3. Immediately mix Solutions A and B and add directly to the cells. The final volume will be 1.0ml for a 60mm plate or per 10⁶ suspended cells.
4. Leave in contact with the cells 30 minutes to overnight. Use 2 hours for the initial tests.
5. At the end of the incubation period, gently overlay the cells with 4ml of complete medium with serum (37°C). It is not necessary to remove the transfection medium containing the Transfectam[®] Reagent/DNA mixture. Return the cells to the incubator and continue the incubation for the appropriate length of time before analysis. For many reporter assays 48 hours after addition of DNA is sufficient.
6. Check the transfection efficiency using the appropriate reporter assay.

Transfection Protocol for Medium With Serum

Materials to Be Supplied By the User

- 0.15M NaCl (sterile)
- complete medium with serum

The reagent volumes in this protocol are based on use of 60mm culture dishes. Scale the reagent volumes up or down proportionately if using different size plates (see Table 3.8).

1. Add 1-5 μ g of plasmid DNA to 50 μ l of 150mM NaCl solution in a sterile tube and vortex (Solution A). We recommend 5 μ g per 60mm dish for the initial tests.
2. For each microgram of plasmid DNA used in Solution A, add between 1.5 and 5 μ l of Transfectam[®] Reagent to 50 μ l of 150mM NaCl solution in a sterile tube and mix (Solution B). For the initial tests, use 10 μ l of Transfectam[®] Reagent per 60mm dish, for 5 μ g plasmid DNA.
3. Immediately mix solutions A and B, wait 10 minutes, then add to the cells.

4. Leave in contact with the cells 30 minutes to overnight. Use 2 hours for the initial tests.
5. At the end of the incubation period, gently overlay the cells with 4ml of the complete medium (37°C). It is not necessary to remove the transfection medium containing the Transfectam[®] Reagent/DNA mixture. Return the cells to the incubator and continue the incubation for the appropriate length of time before analysis. For many reporter assays 48 hours after addition of the DNA is sufficient.
6. Check the transfection efficiency using the appropriate reporter assay.

Optimization of Transfection Efficiency

Follow these recommendations to obtain the best results possible:

- Optimize the volume/weight ratio of Transfectam[®] Reagent/DNA in the range of 1.5-5 μ l/ μ g DNA. Ten microliters of Transfectam[®] Reagent stock to 5 μ g DNA is a good initial test for a 60mm plate.
- Optimize the amount of DNA used in the range of 1-10 μ g DNA. It may not be necessary to increase the quantity of DNA significantly to obtain optimal results. In fact, if the first transfection results are satisfactory, a reduced DNA quantity can be tested (while keeping the optimal Transfectam[®] Reagent/DNA ratio constant).
- The transfection time depends on the specific DNA and cell system used and should be optimized between 30 minutes and overnight. It may be necessary to monitor cell viability if using serum-free medium for a prolonged period because some cells do not thrive under this condition. Usually, the transfection time using the Transfectam[®] Reagent is significantly shorter than that with standard techniques, reducing the risk of cell death significantly during transfection.
- Calibrate the system using a test plasmid with reporter gene function (see Chapter 6).

Tip

Studies by Boukhnikachvili et al. suggest that the efficiency of transfection using DOGS (Transfectam[®] Reagent) is related to the structure of the lipid/DNA complex formed, and increasing both pH and ionic strength (76) can increase formation of such complexes.

