Mammalian Transfection Kit

INSTRUCTION MANUAL

Catalog #200285 and #200385

Revision C.0

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200285-12

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Mammalian Transfection Kit

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Mammalian Transfection Kit

MATERIALS PROVIDED

Materials provided	Catalog #200285	Catalog #200385
Modified $CaPO_4$ Method		
Solution #1 (2.5M $CaCl_2$)	1.5 ml	1.5 ml
Solution #2 [2 × BBS (pH 6.95) consisting of 50 mM N, N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid and buffered saline, 280 mM NaCl and 1.5 mM Na_2HPO_4]	15 ml	15 ml
DEAE-Dextran Method		
Solution #3 (2 mg/ml DEAE–dextran in 0.9% NaCl)	5 ml	—
Control Plasmid Vectors		
pWLneo [G418 (neomycin) selection]; 1μg/μl	40 μg	40 μg
pCMV β-gal; 1μg/μl	15 μg	—

STORAGE CONDITIONS

All components: –20°

Revision C.0

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INTRODUCTION

The $CaPO_4$ method of DNA transfection was initially developed as a technique for measuring the infectivity of isolated viral DNA.[1] The precipitate formed by $CaPO_4$ facilitates transfection by enhancing the adsorption of DNA to cell membranes and also acts to limit the digestion of DNA by DNase associated with mammalian cells.

This technique has been adapted to introduce DNA from a variety of sources into mammalian cells. Initial attempts to produce stably transfected clones relied on the conversion of mutant cell lines, deficient in a selectable trait (e.g., thymidine kinase [TK]), to a wild-type phenotype.[2, 3] Later, DNA sequences not associated with vital functions were stably introduced into mammalian cells by either physically linking them to or co-transfecting them with the TK gene.[4, 5] The utility of the $CaPO_4$ precipitation method was greatly enhanced by the development of the neomycin antibiotic selection technique.[6] This technique allows for the selection of transfected cells by the acquisition of resistance to the neomycin analog G418.

Transfection has been used for the isolation of functional genes,[7-14] as well as the analysis of transcriptional regulatory elements and translational and RNA processing signals.[15-20] Most traditional techniques produce a low frequency of transfection ranging from 1 in 10^6 to 1 in 10^4 cells.[21-26] The technique on which the Stratagene Mammalian Transfection Kit is based can be used to stably transfect cells at a frequency of greater than 1 in 100 cells.[27]

Our mammalian transfection kit contains the necessary reagents and protocols for efficient, rapid and simplified DNA transfection of mammalian cells. The kit has been optimized for thirty transfections in 100-mm tissue culture dishes using a modified $CaPO_4$ protocol for the production of stable transfectants, and thirty transfections using a DEAE-dextran protocol for transient transfections.

Solutions #1 and #2 are used in the modified $CaPO_4$ precipitation protocol. This method is most frequently used to produce stable transfectants. The modified $CaPO_4$ protocol may also be useful in performing transient transfection assays in cases where it may not be appropriate to use the DEAE-dextran method.

Solution #3 is used in the DEAE-dextran protocol. This method is simple, quick and efficient, and is generally used for transient transfections.[28]

$CaPO_4$ Transfection Protocol

The following protocol is optimized for 100-mm culture dishes and all volumes are for single transfection.

1. Seed appropriate number of cells (~10^6 cells per 100-mm dish) 24 hours prior to transfection to achieve 60–80% confluency. These cells should be grown overnight in 10 ml of the appropriate medium to a confluency of approximately 10–20%. The RPMI series of media cannot be used for $CaPO_4$ transfection. The excess positive charge in this media will cause a dense precipitate to form.

Preparation of the DNA Precipitate Solution

Note *The optimal amount of DNA will vary depending on the cell type being transfected. For the control plasmid pWLneo, use 10 μg/dish, (10–30 μg of circular DNA/dish is recommended in most cases).*

2. Dilute desired amount of DNA with distilled, deionized water to 450 µl. Add 50 µl of Solution #1.

3. Add 500 µl of Solution #2 to the above and mix gently. Allow mixture to incubate 10–20 minutes at room temperature.

Application of the DNA to the Cell Culture

4. Gently mix the solution to ensure adequate suspension. Add the 1-ml DNA suspension to the 100-mm culture dish dropwise and swirl plate gently to distribute evenly.

5. Incubate cells for 12–24 hours. Depending upon cell type, optimal incubation time may vary. A fine precipitate will be seen after incubation.

Note *If spare incubator space is available, transfection efficiency can be improved 2–3 times by using lower CO_2 concentrations (2–4% CO_2).[1] Return to normal CO_2 concentrations after precipitation removal in the following step.*

6. After 12–24 hours incubation, remove medium and rinse culture twice using PBS (without Ca or Mg), or medium without serum. Apply fresh, complete medium (serum-containing), and allow the cells to incubate 24 hours under a CO_2 concentration optimal for the cell line.

7. Split cells at desired ratios (at least 1:10) and incubate an additional 24 hours before applying selection for stable transfectants.

ASSAY TO DETERMINE STABLE TRANSFECTION EFFICIENCY

The Stratagene mammalian transfection kit has been optimized for the transfection protocol described above. We have found that it is beneficial to wait approximately 36–48 hours after first applying the DNA before splitting the cells and waiting an additional 24 hours before applying selection.

To determine the percentage of surviving cells that have been transfected stably with DNA, the cells should be counted, using a hemocytometer, during the cell split described in step 7 above. Approximately 1000 trypsinized cells should be plated per 100-mm tissue culture plate. Use six plates for the assay. 24 hours later, three of these plates should be placed under selective conditions (e.g., G418 for the neomycin gene—see *G418 Selection of Transfectant Clones*) and the medium on the other three plates should be changed, but selection should not be applied. Plates should be selected once every 4–7 days with the desired concentration of G418 in media with the appropriate supplements.

Approximately 4–7 days later, colonies can be counted on the nonselective plates. This number will reflect the actual number of viable cells (times the plating efficiency of the cell line) seeded onto the 6 dishes. To facilitate colony counting, stain cells with methylene blue in 50% methanol after rinsing the plates twice with PBS.

From 7–14 days after applying the selection, colonies can be counted on the selective plates. This number will reflect the cells that were stably transfected (again, × the plating efficiency for the cell line). Once again, staining facilitates counting.

The transfection efficiency ratio consists of the number of colonies appearing under selection divided by the number of colonies appearing without selection. The efficiency will vary greatly with the cell line and the selectable marker chosen. Using neomycin/G418 and NIH3T3 fibroblasts, we typically obtain 5–15% transfection efficiencies.

DEAE–DEXTRAN TRANSFECTION PROTOCOL

The following protocol has been optimized for 100-mm culture dishes and all volumes are for a single transfection.

DEAE–Dextran Transfection

1. Exponentially growing cells should be seeded at a density which will yield ~100% confluency within 72 hours. Transfection should be performed between 6–24 hours after seeding.

2. Mix DNA (1–2 μg/100-mm dish) with phosphate buffered saline (1× PBS) to a final volume of 170 μl.

3. Dilute 85 μl of Solution #3 with 85 μl of 1× PBS.

4. Combine the PBS–DNA mixture with the Solution #3–PBS mixture.

5. Remove media from cultures and rinse twice with PBS.

6. Add the DNA mixture (from step 4) dropwise to the center of the culture and swirl gently to distribute the solution evenly. Incubate for 15 minutes at room temperature (~25°C) without CO_2.

7. Remove the solution and **gently** rinse the culture with PBS (cells will be poorly attached at this point).

8. Add 10 ml of fresh, complete media to the culture and allow to grow under optimal culture conditions.

G418 Selection Of Transfectant Clones

This section provides general guidelines for establishing conditions for G418 selection. Specific conditions for using G418 must be determined by the individual researcher.

Culture Preparation

Cultures which will be subjected to antibiotic selection should be seeded at low densities. Adherent cells should cover no more (and preferably less) than 50% of the growth surface at the time selection is applied. If the cultures are seeded at too great a density, the cells may cover the entire surface of the dish. If this is the case, when the cells begin to die, instead of becoming detached from the dish individually, they may slough off in large, connected groups. Under these conditions, healthy, resistant cells might be removed along with non-viable cells. As a result, the efficiency of the selection process might be significantly reduced.

When nonadherent cells are being studied, high-cell densities also should be avoided. High-cell densities require that the growth medium be changed frequently and this in turn may result in crucial soluble cell factors being removed. Since many cell lines seem to require the presence of other cells of the same type to grow properly, frequent medium changes may reduce their overall viability and reduce the efficiency of the system.

Selection

When using the G418 antibiotic selection method, it is important to remember that not all mammalian cell lines are equally sensitive to G418. The minimal lethal concentration can range from 100 μg/ml to 1 mg/ml. Therefore, the concentration to be used for selection must be determined for **each** cell line **before** the experiment can begin.

Since many cell lines have already been subjected to this type of selection, it may be useful to consult the available literature. If no information about the sensitivity of a particular cell line is available, a simple way to determine its sensitivity is to grow cultures in a multiwell plate with a range of G418 concentrations between the individual wells. The optimal concentration is the lowest one that kills all of the cells within 10–14 days. (Rapidly dividing cells may be killed more readily since the antibiotic appears to act mainly on dividing cells.)

In some cases, it may be possible to reduce the concentration of G418 after the initial selection and still maintain the selectable marker gene. For example, NIH 3T3 cells are generally selected in 400 μg/ml G418 and the presence of the *neo*^{r} gene can be maintained in 250 μg/ml.

β-Galactosidase Assay

The β-Galactosidase assay provides an easy and rapid method of determining transfection efficiency by indicating β-galactosidase activity in individual intact cells, following transfection of the pCMV β-gal control plasmid. The transfected cells can be stained in tissue culture dishes without having to remove or manipulate the cells.[29]

β-Galactosidase is an enzyme that catalyzes the hydrolysis of β-galactosides, including lactose. The β-galactosidase gene functions well as a reporter gene in transfection experiments for two major reasons: its protein product is extremely stable and resistant to proteolytic degradation, and most importantly, the enzyme activity is assayed easily.

The cells are incubated with a glutaraldehyde–formaldehyde fixing solution and then with a staining solution that contains X-Gal. In transfected cells, β-galactosidase cleaves 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal) to produce a blue stain. The transfection efficiency is determined by counting stained and unstained cells under a microscope and calculating the percentage of stained cells in the total population.

Protocol

The following materials will be required for the β-galactosidase assay:

PBS (10×)
Fixing solution (10×)
Staining buffer
X-Gal solution (100×)

Caution ***Wear gloves, goggles, and a lab coat when handling the fixing solution and the staining buffer. Prepare the solutions and stain the cells in a fume hood.*** *The glutaraldehyde and formaldehyde in the fixing solution and the potassium ferricyanide and potassium ferrocyanide in the staining buffer are harmful if inhaled, swallowed, or absorbed through the skin.*

Notes *Perform the staining protocol within 24–72 hours after transfection.*

The quantities of the solutions and reagents given in this protocol are optimized for a 100-mm tissue culture dish. See Table I for solution volumes for dishes of other dimensions.

Use only freshly prepared 1× fixing solution and 1× staining solution. Prepare just enough of each solution to complete the fixation and staining. Aldehyde-based fixatives oxidize with time, and X-Gal is unstable in the staining buffer and precipitates during long-term storage.

TABLE I

Volumes of Fixing Solution and Staining Solution Recommended for Tissue Culture Plasticware

Plasticware	Well diameter	1× Fixing solution volume	1× Staining solution volume
60-mm dish	60 mm	4.0 ml/dish	2.0 ml/dish
100-mm dish	100 mm	8.0 ml/dish	4.0 ml/dish
150-mm dish	150 mm	18.0 ml/dish	9.0 ml/dish
96-well plate	6.4 mm	0.1 ml/well	0.05 ml/well
48-well plate	10 mm	0.25 ml/well	0.125 ml/well
24-well plate	15 mm	0.5 ml/well	0.25 ml/well
12-well plate	22 mm	1.0 ml/well	0.5 ml/well
6-well plate	35 mm	2.0 ml/well	1.0 ml/well

1. Prepare the fixing solution, staining solution, and PBS buffer (see *Preparation of Reagents*). Alternatively, use the prepared solutions with the *In Situ β-Galactosidase Staining Kit* (Stratagene catalog #200384).

2. Aspirate the medium from the cells.

3. Add 8 ml of freshly prepared 1× fixing solution to the tissue culture dish and incubate the dish for 10 minutes at room temperature.

4. Remove the fixing solution from the dish and gently wash the cells twice with 8 ml of 1× PBS.

5. Add 4 ml of freshly prepared 1× staining solution to the tissue culture dish.

6. Incubate the cells between 15 minutes and overnight at 37°C in a humidified incubator.

   Note *The optimal time of incubation depends on the cell type and the transfection efficiency. Observe the intensity of the blue stain in the cells under a microscope and adjust the incubation time accordingly.*

7. Remove the staining solution and wash the cells two or three times with 8 ml of 1× PBS.

8. Add 4 ml of 1× PBS to the tissue culture dish.

   Note *For long-term storage, cover the dish with a glycerol-based mounting medium and store at 4°C.*

Results

Analyze the dish under a light microscope to determine the transfection efficiency. Count the stained and unstained cells in randomly selected fields. The transfection efficiency is the percentage of stained cells in the total population.

Cell Culture Techniques

This section covers those culture techniques which may be especially troublesome in the isolation of clones from transfected cultures.

Culture Inoculation

To ensure the greatest possible efficiency of transfection, it is important to minimize the trauma suffered by the cells during inoculation. For adherent cells, the length of time of trypsinization must be minimized. Wash the cells with PBS, or medium without serum, or very quickly with a trypsin solution to remove all serum from the culture (serum inhibits the action of trypsin). Warm a minimal volume of trypsin to 37°C before it is applied (minimal volume for a 60-mm dish is 0.5 ml; 100-mm dish is 1 ml; 150-mm dish is 2 ml; 75-cm^2 flask is 1.5 ml).

Trypsinization should proceed until the pseudopods of the cells just begin to recede toward the nucleus (in 30 seconds to 2 minutes, depending upon the cell line). The cells should be freed from the dish surface by tapping the dish firmly, and the cells should be diluted immediately to the desired density using medium with serum.

The best transfection efficiencies are obtained when the cells being transfected are grown exponentially. In order for the cells to grow as rapidly as possible, avoid contact inhibition which occurs when cells actually (not nearly) touch. Therefore, it is best to inoculate the cultures to be used for transfection at a density resulting in no more than 15–30% of the dish surface being covered at the time of transfection.

It appears that maximum efficiency is also obtained when cultures are inoculated at minimal densities following transfection. NIH 3T3 cells yield their greatest efficiencies when plated at a density of 10^3 cells/dish.

Clone Isolation

After the selection process progresses to the point where colonies are visible, it is important to isolate the colonies in a manner that obtains the maximum number of cells (increasing the likelihood the clone will survive), and to minimize the chances of contaminating one colony with cells from another. To help achieve both these goals, we recommend using cloning rings when isolating colonies. The rings may be made of any autoclavable material, and have an internal diameter of 5–10 mm. Sterilize them along with a pair of medium forceps and some form of adhesive (vacuum grease works well).

Before isolating the colonies, prepare 24-well microtiter dishes to receive the new clones. The volume used for the new clones should be kept to a minimum, since many types of cells appear to require the support of other cells of the same type in order to grow in culture. This support may involve some kind of soluble growth factor which may become ineffective if diluted too much. In cases where cell growth is initially very sparse (<5% of the dish surface), it is often helpful to add growth factors at higher concentrations than usual.

Note *For example, if a cell type grows well in media supplemented with 10% fetal calf serum under normal culture conditions, it may require 20% fetal calf serum to grow well when the cells are seeded at a very low density. It may also be useful to remove media from a more densely populated culture and, after sterile filtering, use it to supplement the growth medium of the sparsely populated cultures.*

To isolate single colonies using cloning rings, the culture containing the colonies should be washed twice in PBS, then all fluid should be removed from the plate. The rings should be handled only with sterile forceps.

1. Dip the rings in the sterile adhesive and lightly dab them against a dry, sterile surface to spread the adhesive evenly around the outer edges.
2. Apply the rings around the colonies to be harvested (work quickly to prevent drying out).
3. Add 4–5 drops of trypsin to each ring with a sterile, cotton-plugged Pasteur pipet.
4. Wait ~30 seconds per colony, then break up the colony by pipetting the trypsin up and down 2–3 times.
5. Transfer the trypsinized cells to a 24-well plate.
6. Remove some of the media from the 24-well plate, rinse the inside of the ring to remove any residual cells and transfer to the dish.

7. After ~2 hours, examine the plate with a microscope to determine if the cells have attached. If they have attached, replace the media in the 24-well plate with fresh, complete media in order to remove any remaining trypsin.

During the early stages of new clone growth, it is best to change the media as infrequently as possible (about once a week for very sparsely populated cultures), which helps maintain any soluble factors. It is best to add exogenous growth factors to the media, instead of replacing the old media with media containing fresh growth factors.

Once the small cultures have grown to confluency, they may be passed to larger culture vessels (usually 6-well microtiter plates first) and treated the same way as other cells of the same type (the original selection should be maintained throughout the life of the new clone).

TROUBLESHOOTING

Observation	Suggestion
Precipitate is not visible or is too fine after adding DNA and incubating	Too much DNA has been added. Check OD_{260} of DNA. Adjust appropriately. Transfect with different amounts of DNA to determine optimal concentration.
Precipitate is too dense, with aggregated clumps	Too little DNA has been added. See above.
Unable to remove precipitate from the plate	PBS washes contained Ca^{++} or $Mg.^{++}$ Wash without cations.

PREPARATION OF REAGENTS

10× Fixing Solution

20% (v/v) formaldehyde
2% (v/v) glutaraldehyde
PBS

Prepare 1× fixing solution by diluting one part of 10× fixing solution with 9 parts of 1× PBS.

10× PBS

1370 mM sodium chloride
26 mM potassium chloride
100 mM disodium phosphate
18 mM potassium phosphate
pH adjusted to 7.4 with HCl

Prepare 1× PBS by diluting one part of 10× PBS with 9 parts of dH_2O.

1× Staining Solution

Prepare 1× staining solution by diluting one part of 100× X-Gal solution with 99 parts of staining buffer.

Staining Buffer

5 mM potassium ferricyanide
5 mM potassium ferrocyanide
2 mM magnesium chloride
10% dimethyl sulfoxide (DMSO)
buffer stabilizer
PBS

100× X-Gal Solution

100 mg/ml 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal)
dimethyl sulfoxide (DMSO)

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STRATAGENE

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Mammalian Transfection Kit

MODIFIED $CaPO_4$ QUICK-REFERENCE PROTOCOL

- Inoculate dishes and grow cells overnight
- Dilute DNA with 450 μl sterile water
- Add 50 μl Solution #1 and 500 μl Solution #2
- Incubate for 10–20 minutes at room temperature
- Gently resuspend DNA with pipettor
- Add the DNA suspension to each dish and swirl once
- Incubate 12–24 hours at 37°C under 5% CO_2
- Aspirate off medium and wash plates 2 times with PBS
- Add 5 ml of culture medium and incubate overnight
- Split cells at desired ratio (If there is noticeable cell death after splitting, cells may be washed with PBS and resuspended in 5 ml of fresh media)
- Incubate an additional 24 hours
- Apply antibiotic selection
- Assay stable transfection efficiency