Technical Manual

ProFection® Mammalian Transfection System

INSTRUCTIONS FOR USE OF PRODUCT E1200.

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Part# TM012

ProFection® Mammalian Transfection System

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1. Description

Introduction of DNA into mammalian cells is facilitated by the ProFection® Mammalian Transfection System–Calcium Phosphate. Each system contains sufficient reagents for 40 high-efficiency transfections of cells plated in 100mm tissue culture plates.

Calcium phosphate transfection is the method of choice to produce long-term stable transfectants. This method also works well for transient expression of transfected genes and can be used with most adherent cell lines.

2. Product Components and Storage Conditions

Product	Cat.#
ProFection® Mammalian Transfection System—Calcium Phosphate	E1200

Contains sufficient reagents for 40 transfection reactions. Includes:

- 3ml $CaCl_2$, 2M
- 25ml 2X HEPES-Buffered Saline (2X HBS)
- 20ml Nuclease-Free Water

Storage Conditions: Store the ProFection® Mammalian Transfection System—Calcium Phosphate components at –20°C.

3. General Considerations

The ability to introduce DNA into cultured cells has provided a powerful means to study the function and control of mammalian genes. Calcium phosphate-mediated transfection facilitates DNA binding to cell membranes and entry of DNA into the cell via endocytosis. Calcium phosphate also appears to provide protection against intracellular and serum nucleases (1).

3.A. Calcium Phosphate-Mediated Transfection

Calcium phosphate-mediated transfection involves mixing DNA directly with $CaCl_2$ and a phosphate buffer to form a fine precipitate, which is dispersed over the cultured cells. This method was first reported by Graham and van der Eb (2) and was extended by Wigler *et al.* (3) when they demonstrated that the thymidine kinase gene could be transfected and stably expressed in mouse cells. Long-term stable expression can be achieved by integration of the transfected DNA into the host cell genome (4–6) or by autonomous replication in mini-chromosomal structures (7–9).

The calcium phosphate transfection protocol is routinely used for both transient and stable transfection of a variety of cell types. The protocol is versatile and, although used primarily on adherent cells in culture, also can be modified to transfect cells growing in suspension (10). It also may be the method of choice when independent function is desired for multiple plasmids that are co-transfected (11).

3.B. Factors Affecting the Efficiency of Gene Transfer

Transfection efficiencies can be increased in many cell types by additional treatments after the primary exposure of cells to calcium phosphate-DNA. The most effective and routinely used agents are glycerol (12), dimethyl sulfoxide (DMSO; 13,14), chloroquine (15) and sodium butyrate (16). Since each of these chemicals is toxic to cells, the transfection conditions for individual cell types must be carefully optimized for reagent concentration and exposure time.

Glycerol and DMSO solutions are applied to cells after DNA has been in association with the cells. The glycerol or DMSO solution (usually 10–20%) is applied to cells for a period of 30 seconds to a few minutes, then removed from the cells. The exact mechanism of action is unknown, but these treatments may modify cell membrane structure to enhance uptake of DNA.

Chloroquine, which is generally applied to cells with the DNA, appears to enhance transfection efficiency by inhibiting DNA degradation by lysosomes (15).

Sodium butyrate treatment following transfection has been reported to result in higher levels of transient expression (16). This effect may be mediated by a more "active" chromatin structure of newly transfected genes (9). For any of these additional treatments, the optimal concentration and times must be empirically and systematically determined.

A number of additional factors affect the successful outcome of gene-transfer experiments. For example, certain types of cell lines (such as COS) are intrinsically easier to transfect than others, although the exact reason for these differences is currently unknown. Even clonal variability in the ability to take up DNA has been reported in mouse L cells (17). Cells used for gene transfer must be growing exponentially and in good condition at the time of transfection. Another factor that contributes to transfection success or failure is the purity of transfected DNA. Finally, all chemicals and reagents used for cell culture should be of the highest possible quality.

4. Transfection Preparations

Many excellent references that review tissue culture techniques, such as trypsinizing and plating cells, and preparing DNA for transfection, are available (18–21). For additional information, consult these references and Sections 4.A and 4.B.

4.A. Plating Cells for Transfection

Plate cells the day before transfection. The plating density for any particular cell line will depend on how quickly the cells divide. The cells should be at 30–60% confluency the day of transfection. An optimal plating density produces a nearly confluent dish when the cells are harvested or split into selective medium, which is usually about 48 hours after the transfection. A general guideline is to plate about 8×10^5 cells per 100mm culture plate. Scale down the number of cells proportionately for 35 or 60mm plates.

4.B. Preparation of DNA for Transfection

Supercoiled plasmid DNA is efficiently expressed following calcium phosphate-mediated transfection. The plasmid DNA to be transfected should be free of protein, RNA and chemical contamination. Ethanol-precipitated DNA should be resuspended in a sterile solution such as TE buffer to a final concentration of 0.2–1mg/ml. A good indicator of DNA purity is the ratio of absorbance at 260nm (A_{260}) to 280nm (A_{280}). A DNA solution with an A_{260}/A_{280} ratio of 1.8 or greater is desirable. (For a more detailed description of techniques for plasmid DNA purification, visit our web-based *Protocols and Applications Guide* at: **www.promega.com/paguide/**)

5. Calcium Phosphate Transfection

5.A. Protocol

1. Plate cells the day before transfection as described in Section 4.A.
2. Three hours prior to transfection, remove the medium from cells, and replace it with fresh growth medium.
3. Thaw all system components, and warm them to room temperature. Mix each component thoroughly by swirling the container or vortexing.

4. For each transfection, prepare the DNA and 2X HBS solutions in separate sterile tubes. Add DNA and water to the first tube, mix well, then add the $CaCl_2$ and mix again. Add the specified amount of 2X HBS to the second tube.

	Per 60mm Plate	Per 100mm Plate
Tube 1		
DNA	6–12µg	10–20µg
2M $CaCl_2$	37µl	62µl
sterile, deionized water to a final volume of	300µl	500µl
Tube 2		
2X HBS	300µl	500µl

5. Working in a tissue culture hood, gently vortex the tube containing the 2X HBS solution. The speed should be adjusted such that the tube can be vortexed safely with the cap off and can accommodate the addition of the prepared DNA solution. Continue to vortex while slowly adding the prepared DNA solution in Tube 1 dropwise to the 2X HBS in Tube 2. (Alternatively, using a pipette, bubble air through the 2X HBS while slowly adding the $CaCl_2$-DNA solution.) When DNA addition is complete, the solution should appear slightly opaque due to the formation of a fine calcium phosphate-DNA coprecipitate. Incubate the solution at room temperature for 30 minutes.

   Note: Characteristics of the calcium phosphate-DNA coprecipitate are affected by temperature and methods used to mix the $CaCl_2$-DNA and HBS solutions.

6. Vortex the transfection solution again just prior to adding it to the cells. Add the solution containing the $CaCl_2$-DNA precipitate dropwise and in various locations around the plates. Swirl the plates to distribute the precipitate evenly over the cells. This helps to evenly distribute the precipitate and avoid localized acidification of cells. Return the plates to a 37°C CO_2 incubator.

   Optional: A glycerol or DMSO shock step may be performed 4–16 hours after addition of DNA to the cells (refer to Section 6).

7. When working with sensitive cells, the culture medium should be changed 4–16 hours after transfection. The length of incubation should be optimized for individual cell lines. Primary cells are particularly sensitive and should not be exposed to calcium phosphate for more than 4 hours.

8. In general, cells may be harvested or selective medium applied for up to 48–72 hours after the transfection (see Section 8).

5.B. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptoms	Causes and Comments
No transfection or low transfection efficiency	Poor precipitate: • The $CaCl_2$-DNA and 2X HBS formation solutions should be at room temperature (22–25°C) when they are mixed. Higher or lower temperatures for precipitate formation can lead to decreased transfection efficiency. • Addition of $CaCl_2$-DNA to the 2X HBS solution should be performed dropwise and with continuous mixing. • DNA concentration can affect the size of the precipitate. Low amounts of DNA (less than 1µg) can be supplemented with **sheared** carrier DNA such as salmon or herring sperm DNA. However, there are conflicting reports in the literature as to the efficacy of adding carrier DNA (22). • The precipitate should be added dropwise around the dish to the medium bathing the cells, and the medium should be mixed thoroughly at the end of the addition. This helps to evenly distribute the precipitate and avoid localized acidification of cells. • After adding the calcium phosphate precipitate to the cells, the pH of the medium should be between 7.2 and 7.4. The CO_2 concentration in the incubator should be maintained at an appropriate level (generally 5–10%, depending on the culture medium composition).
	Poor-quality DNA. Purify DNA using methods that result in high-quality DNA. The A_{260}/A_{280} ratio of the DNA should be 1.8 or greater.
	pH was not optimal. The pH of the HBS solution should be 7.1. Adding a large volume of DNA in Tris buffer could change the pH. The DNA should be resuspended in water, 1mM Tris, or if present, in 10mM Tris, and should be fairly concentrated so that a relatively small volume of DNA solution is added to the HBS.
	pH of the 2X HBS may have changed on storage. Check the pH, and adjust it to 7.1 if necessary.

Symptoms	Causes and Comments
Variable transfection efficiencies in replicate experiments	Cells were contaminated with mycoplasma. Test cultures for mycoplasma contamination. Destroy contaminated cultures, and start a new culture from a fresh stock.
	Suboptimal growth of cells. Transfection efficiency may decrease if cells have been passaged for many generations. Start a fresh culture from cell stocks that were frozen at an early passage. Some cells, particularly lymphocytes, will exhibit variability in transfection efficiency if they are left in culture for more than 1–2 weeks.
	Cell confluency. Cells should be at 30–60% confluency the day of transfection. See Section 4.A and references 18–21 for additional information on the preparation of cells for transfection.

6. Glycerol or DMSO Shocks

6.A. Glycerol Shock

High transfection efficiencies can be obtained by leaving the DNA-calcium phosphate solution on the cells until the cells are harvested or selective pressure is applied. HeLa cells, for example, respond well to this treatment. However, transfection of some cell lines, such as CHO cells, is enhanced by a glycerol shock step (see Section 10.B). For this reason, we have included a representative glycerol shock protocol below.

The glycerol shock step may be performed 4–16 hours after transfection. In general, if cells can tolerate the calcium phosphate solution, it is best to leave it on for as long as possible and perform the glycerol shock 16 hours after transfection. Cell lines that are more sensitive to the calcium phosphate solution may respond better to a glycerol shock step performed earlier, such as 4 hours after exposure to the DNA. In either case, **do not** expose the cells to the glycerol shock solution for more than 2 minutes. The optimum time interval before performing the glycerol shock should be determined empirically for each cell line.

Materials to Be Supplied by the User
(Solution compositions are provided in Section 10.A.)

- glycerol shock solution
- wash solution: 1X PBS or 1X HBSS

1. Prepare a fresh glycerol shock solution in 1X HBS, and warm it to 37°C along with the growth medium and wash solution.
2. Wash cells once with 5ml of wash solution per 60mm plate (10ml per 100mm plate).

6.A. Glycerol Shock (continued)

3. Add 2ml of glycerol shock solution per 60mm plate (3ml per 100mm plate).
4. Incubate up to 2 minutes at room temperature.
5. Remove the glycerol shock solution, and wash the cells twice with 5ml of wash solution per 60mm plate (10ml per 100mm plate).
6. Add growth medium, and return the cells to a 37°C incubator.

6.B. DMSO Shock

Certain cell types exhibit enhanced transfection efficiencies after exposure to dimethyl sulfoxide (DMSO). The DMSO step can be added to the calcium phosphate transfection protocol (12,13). However, DMSO, like glycerol, is toxic to cells, and the concentration and exposure times may require careful optimization for each cell type. Most cells **should not** be exposed to DMSO for more than 2.5 minutes. One representative protocol for a DMSO shock is provided below.

Material to Be Supplied by the User
(Solution compositions are provided in Section 10.A.)

- DMSO shock solution

1. Remove medium from cells.
2. Immediately before use, prepare the DMSO shock solution, and warm it to 37°C. You will need 2ml per 60mm plate (3ml per 100mm plate).
3. Add the DMSO shock solution to the cells ,and incubate for 2.5 minutes. Do not return the plates to an incubator during this time.
4. Remove the DMSO shock solution, and add 5ml of growth medium per 60mm plate (10ml per 100mm plate). Return the cells to the 37°C incubator.

7. Monitoring Transfection Efficiencies

Plasmids with "reporter gene" function can be used to monitor transfection efficiencies. An ideal reporter gene product is not endogenous to the cell, can be expressed from a plasmid DNA, and is usually an enzyme that can be conveniently assayed. Such assays are generally performed 2–3 days after transfection. Some reporter genes that are commonly used are described in the following paragraphs.

Many plasmids containing different reporter genes, such as firefly luciferase, *Renilla* luciferase, click beetle luciferase, β-galactosidase and chloramphenicol acetyltransferase, are available from Promega. For example, the pGL4.13[*luc2*/SV40] Vector (Cat.# E6681), which directs synthesis of firefly luciferase, may be used as a transfection control (23). Luciferase activity can be assayed using a luminometer or a scintillation counter with an assay that is rapid and extremely sensitive (24). For a list of available plasmids and reporter assays, see Section 10.C or visit our Web site at: **www.promega.com**

8. Transient versus Stable Transfection

8.A. Transient Transfection

The post-transfection interval for cell harvest should be optimized and depends on the cell type, cell doubling time and 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 on the end-product being assayed.

Isolation of RNA from cultured cells can be accomplished using several products from Promega: the SV Total RNA Isolation System (Cat.# Z3100), PolyATtract® 1000 System (Cat.# Z5400, Z5420) and PureYield™ RNA Midiprep System (Cat.# Z3740, Z3741).

Extracts may be prepared using our Reporter Lysis Buffer (Cat.# E3971). This enables the extracts to be assayed for luciferase, CAT and β-galactosidase activity. If only luciferase activity is to be assayed, our Luciferase Cell Culture Lysis Reagent, 5X (Cat.# E1531) may be used. If extracts are to be prepared with the Dual-Luciferase® Assay Systems, Passive Lysis Buffer, 5X (Cat.# E1941) can be used.

8.B. 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 (25).

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 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 transferred 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 aminoglycoside phosphotransferase can be selected for stable transformation in the presence of the drug G-418 (Cat.# V7981; 26). Similarly, expression of the gene for hygromycin B phosphotransferase from the transfected vector will confer resistance to the drug hygromycin B (27).

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 (28). 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 (29).

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10. Appendix

10.A. Composition of Buffers and Solutions

1X PBS

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

The final pH should be 7.1.

2X HBS (HEPES-buffered saline)

50mM	HEPES (pH 7.1)
280mM	NaCl
1.5mM	Na_2HPO_4

The final pH should be 7.1.

DMSO shock solution

1X	PBS
10%	DMSO (tissue culture grade)

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

glycerol shock solution

1X	HBS
15%	glycerol

TE buffer

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

10.B. Optimal Transfection Protocols for Commonly Used Cell Lines

Table 1. Comparison of Transfection Protocols Recommended for CHO, COS and HeLa Cell Lines.

Cell Line	Protocol
Hamster CHO	CaP for 16 hours, then glycerol shock
Monkey COS	CaP for 24 or 48 hours
Human HeLa	CaP for 24 or 48 hours

Comparisons were made between calcium phosphate (CaP) protocols with and without glycerol shock. Cells were transfected with various reporter plasmids including the pSV-β-Galactosidase Control Vector. Transfection efficiency was monitored by harvesting cells 48 hours post-transfection and assaying for reporter enzyme activity.

10.C. Related Products

Transfection Reagents

Product	Size	Cat.#
Tfx™-50 Reagent	2.1mg	E1811
Tfx™-20 Reagent	4.8mg	E2391
Tfx™ Reagents Transfection Trio	5.4mg	E2400
Transfectam® Reagent for the Transfection of Eukaryotic Cells	1mg	E1231
	0.5mg	E1232
TransFast™ Transfection Reagent	1.2mg	E2431

Antibiotic

Product	Size	Cat.#
Antibiotic G-418 Sulfate	100mg	V7981
	1g	V7982
	5g	V7983
Antibiotic G-418 Sulfate Solution	20ml	V8091

Plasmid DNA Purification System

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495

β-Galactosidase Reporter Vectors and Assay Systems

Product	Size	Cat.#
pSV-β-Galactosidase Control Vector	20µg	E1081
β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer	65 assays	E2000
Beta-Glo® Assay System	10ml	E4720
	100ml	E4740
	10 × 100ml	E4780

CAT Reporter Vectors and Assay Systems

Product	Size	Cat.#
pCAT®3-Control Vector	20µg	E1851
pCAT®3-Promoter Vector	20µg	E1861
pCAT®3-Basic Vector	20µg	E1871
pCAT®3-Enhancer Vector	20µg	E1881
CAT Enzyme Assay System	50 assays	E1000

pGL4 Luciferase Reporter Vectors (Please visit **www.promega.com/vectors/** for a complete listing of reporter vectors.)

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene Promoter	Mammalian Selectable Marker	Cat.#
pGL4.10[*luc2*]	Yes	*luc2*[A]	No	No	No	E6651
pGL4.11[*luc2P*]	Yes	"	hPEST	No	No	E6661
pGL4.12[*luc2CP*]	Yes	"	hCL1-hPEST	No	No	E6671
pGL4.13[*luc2*/SV40]	No	"	No	SV40	No	E6681
pGL4.14[*luc2*/Hygro]	Yes	"	No	No	Hygro	E6691
pGL4.15[*luc2P*/Hygro]	Yes	"	hPEST	No	Hygro	E6701
pGL4.16[*luc2CP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6711
pGL4.17[*luc2*/Neo]	Yes	"	No	No	Neo	E6721
pGL4.18[*luc2P*/Neo]	Yes	"	hPEST	No	Neo	E6731
pGL4.19[*luc2CP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6741
pGL4.20[*luc2*/Puro]	Yes	"	No	No	Puro	E6751
pGL4.21[*luc2P*/Puro]	Yes	"	hPEST	No	Puro	E6761
pGL4.22[*luc2CP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E6771
pGL4.70[*hRluc*]	Yes	*hRluc*[B]	No	No	No	E6881
pGL4.71[*hRlucP*]	Yes	"	hPEST	No	No	E6891
pGL4.72[*hRlucCP*]	Yes	"	hCL1-hPEST	No	No	E6901
pGL4.73[*hRluc*/SV40]	No	"	No	SV40	No	E6911
pGL4.74[*hRluc*/TK]	No	"	No	HSV-TK	No	E6921
pGL4.75[*hRluc*/CMV]	No	"	No	CMV	No	E6931
pGL4.76[*hRluc*/Hygro]	Yes	"	No	No	Hygro	E6941
pGL4.77[*hRlucP*/Hygro]	Yes	"	hPEST	No	Hygro	E6951
pGL4.78[*hRlucCP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6961
pGL4.79[*hRluc*/Neo]	Yes	"	No	No	Neo	E6971
pGL4.80[*hRlucP*/Neo]	Yes	"	hPEST	No	Neo	E6981
pGL4.81[*hRlucCP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6991
pGL4.82[*hRluc*/Puro]	Yes	"	No	No	Puro	E7501
pGL4.83[*hRlucP*/Puro]	Yes	"	hPEST	No	Puro	E7511
pGL4.84[*hRlucCP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E7521

[A]*luc2* = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene.

10.C. Related Products (continued)

Luciferase Assay Reagents

Product	Size	Cat.#
Luciferase Assay System	100 assays	E1500
Luciferase Assay System with Reporter Lysis Buffer	100 assays	E4030
Reporter Lysis Buffer, 5X	30ml	E3971
Luciferase Cell Culture Lysis Reagent, 5X	30ml	E1531
Passive Lysis Buffer, 5X	30ml	E1941
Glo Lysis Buffer, 1X	100ml	E2661
Steady-Glo® Luciferase Assay System	10ml	E2510
	100ml	E2520
	10 × 100ml	E2550
Bright-Glo™ Luciferase Assay System*	10ml	E2610
	100ml	E2620
	10 × 100ml	E2650
Dual-Luciferase® Reporter Assay System	100 assays	E1910
Dual-Luciferase® Reporter Assay System 10-pack	1,000 assays	E1960
Dual-Luciferase® Reporter 1000 Assay System	1,000 assays	E1980
Dual-Glo™ Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
EnduRen™ Live Cell Substrate	0.34mg	E6481
	3.4mg	E6482
	34mg	E6485
ViviRen™ Live Cell Substrate	0.37mg	E6491
	3.7mg	E6492
	37mg	E6495

*For Laboratory Use.