Clontech® Laboratories, Inc.

CalPhos™ Mammalian Transfection Kit User Manual

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I. Introduction

The ability to introduce exogenous DNA into cultured cells is a powerful tool for molecular and cell biologists. Of the many methods to introduce DNA into mammalian cell cultures, the calcium phosphate method is one of the most widely used because it is inexpensive, simple, and suitable for a range of different cell types (Ausubel *et al*,1994; Graham & van der Eb, 1973). The CalPhos Mammalian Transfection Kit provides high-quality, pretested reagents suitable for both transient and stable transfections. The kit includes all the reagents necessary to perform 100 transfections in 10-cm plates, or 700 transfections in wells of a 6-well plate.

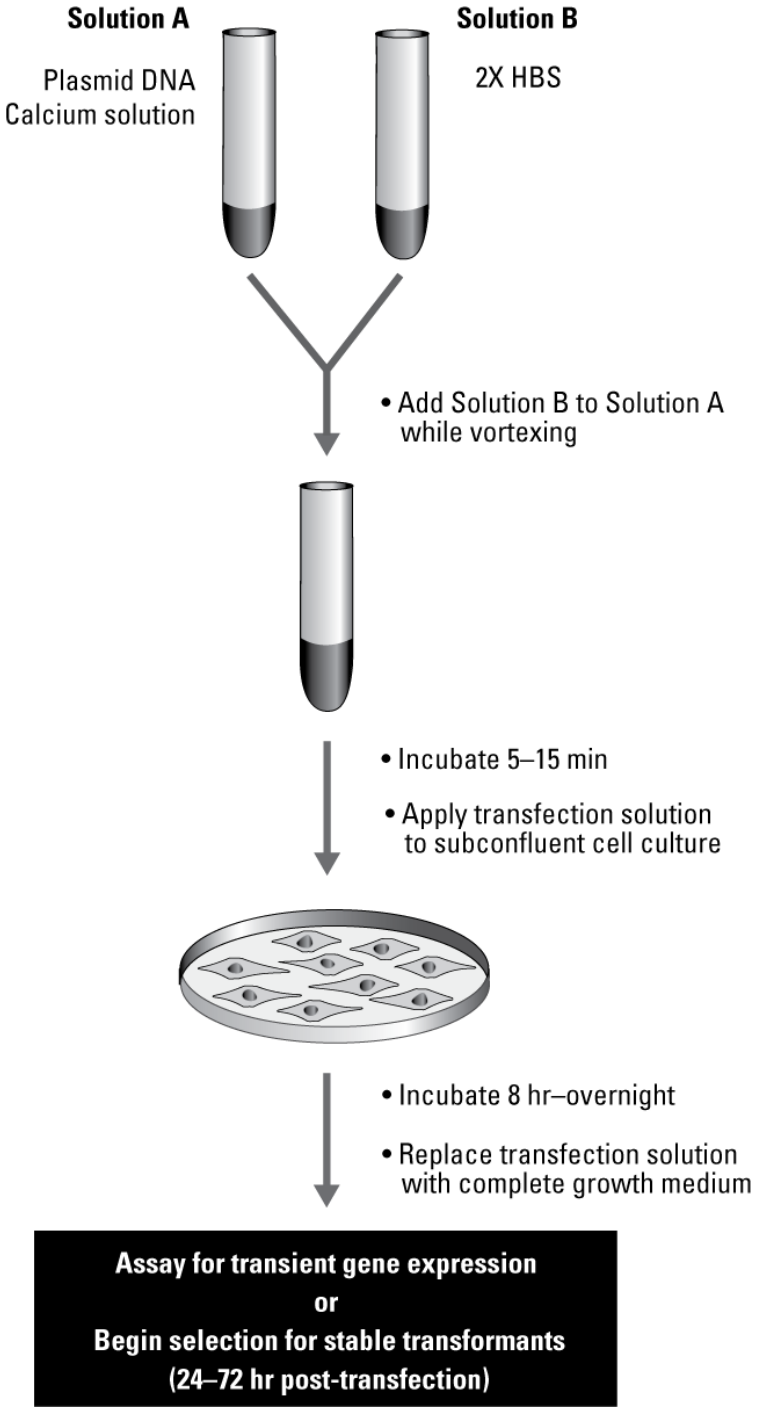

Figure 1. Flow chart for using the CalPhos Mammalian Transfection Kit.

II. List of Components

Store HBS at –20°C; store all other components at 4°C after thawing. The following reagents are sufficient for 100 transfections in 10-cm plates or 700 transfections (6-well plate format).

- 9 ml 2 M Calcium Solution
- 2 x 35 ml 2X HEPES-Buffered Saline (HBS)
  We recommend dispensing this buffer into small aliquots, to be stored at –20°C. Avoid multiple freeze-thaw cycles. When an aliquot is in use, store it at 4°C for up to one week.
- 2 x 35 ml Sterile H_2O

III. Additional Materials Required

- Cell culture plates or flasks
- Tubes (12 x 75-mm sterile tubes)
- Cell culture medium (appropriate growth medium for mammalian cells in culture)
- Fetal bovine serum, newborn calf serum, or equivalent (to supplement the growth medium)
- Phosphate buffered saline (PBS; pH 7.4)

	Final Conc.	To prepare 2 L of solution
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O. Adjust to pH 7.4 with 0.1 N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature.

- 1X Trypsin/EDTA (Life Technologies Cat. No. 25300-054)
- Plasmid DNA
  The DNA should be of high quality, e.g., double CsCl-banded or column-purified DNA. Clontech offers many NucleoBond Plasmid Purification Kits and cartridges which yield “transfection-grade” plasmids. See www.clontech.com for more information.

IV. CalPhos Mammalian Transfection Protocol

The following protocol is designed for use with adherent cultures growing in a well of a 6-well plate. If you are using plates, wells, or flasks of a different size, adjust the volume of the transfection solution in accordance with your culture volume. See Appendix B for culture plate conversions.

All steps of the following protocol should be performed in a sterile tissue culture hood.

1. Plate the cells the day before the transfection experiment. The cells should be 50–80% confluent the day of transfection. Generally, we plate 2–4 x 10^5 cells/well of a 6-well plate.
2. For each transfection, prepare Solution A and Solution B in separate sterile tubes.
   Solution A: add components in the following order:

____ µl	(1–3 µg) Plasmid DNA
____ µl	Sterile H_2O
12.4 µl	2 M Calcium Solution
100 µl	Total Volume

   Solution B: 100 µl 2X HBS

   NOTE: To reduce variability when transfecting multiple plates with the same plasmid DNA, prepare master solutions of Solutions A and B sufficient for all plates.

3. Carefully and slowly vortex Solution A while adding Solution B dropwise.
4. Incubate the transfection solution at room temperature for 5–15 min.
5. Gently vortex transfection solution and then add solution dropwise to culture plate medium. (Add 200 µl of transfection solution per well of a 6-well plate.)
6. Gently move plates back and forth to distribute transfection solution evenly. (Do not rotate plates as this will concentrate transfection precipitate in the center of the well or plate.)
7. Incubate plates at 37°C for 8 hr–overnight in a CO_2 incubator.
8. Remove calcium phosphate-containing medium and wash cells with medium or 1X PBS.
9. Feed plate with 2 ml fresh complete growth medium and incubate at 37°C until needed for assay.
10. Assay for transient gene expression or start selection for stable transformants 24–72 hr post-transfection.

V. Troubleshooting Guide

Problem	Possible Explanation	Solution
Low transfection efficiency	Poor precipitate formation	Addition of the 2X HBS (Solution B) to the calcium/DNA (Solution A) should be performed dropwise and with continuous mixing. Adding Solution B too quickly or with too little mixing can result in a poor precipitate.
	Poor quality DNA	The A_{260}/A_{280} ratio of the plasmid DNA should be ≥1.7.
	pH not optimal	The pH of the HBS should be between 7.05 and 7.12. However, during prolonged storage, the pH of the solution may change; therefore, use the transfection kit within 12 months of purchase.
Variable transfection efficiency in experiments	**Note:** There will always be some variability in transfection efficiencies. We recommend performing transfections in triplicate to minimize the variability.	
	Variable cell density	Keep cell density constant after optimizing transfection procedures. Generally we use cultures that are 50–80% confluent at the time of transfection.
	Suboptimal cell growth	Keep cells healthy in culture. Cells should be in mid-log phase growth when plated for transfection. Transfection efficiencies may decrease for cell lines that have been passaged for too many generations.
Toxicity	Cells dead or dying within 24 hr after addition of CaPO4 to cells.	Optimize cell density (see Appendix A): Higher cell density can help maintain viability.
		Optimize exposure time of cells to the transfection reagent (see Appendix A): Some cells are more sensitive to $CaPO_4$.
		The transfected gene product may be toxic.

VI. References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1994) In Current Protocols in Molecular Biology (Greene Publishing Associates and John Wiley & Sons, Inc., NY) Vol. 1, Ch. 9.

Freshney, R. I. (2000) Culture of Animal Cells, Fourth Edition (Wiley-Liss, NY).

Appendix A: Optimization of Transfection

The efficiency of a mammalian cell transfection is primarily dependent on the host cell line used. Optimization of the transfection parameters for each cell type is crucial to obtaining consistently successful transfections. Therefore, for each cell type you plan to use, perform preliminary experiments to determine the optimal: 1) cell density; 2) amount and purity of DNA; and 3) transfection incubation time.

For the preliminary experiments, the host cell line can be transfected with a reporter expression vector, such as pZsGreen1-N1 (Cat. No. 632448) or pMetLuc Control Vector (Cat. No. 631729). The success of the transfection can then be estimated by assaying for green fluorescence or secreted luciferase. Once the transfection parameters have been optimized, they should be kept consistent from one experiment to the next to obtain reproducible results.

The following is a general guideline for optimizing the transfection parameters. To optimize transfection parameters, it is best to perform a series of small-scale transfections. This can be done conveniently in 12-well or 6-well plates.

To optimize cell density: keeping all other parameters constant, plate host cells in individual wells of a 6-well plate at varying densities (e.g., 5×10^4, 1×10^5, 2×10^5, 4×10^5, 8×10^5). 24–72 hours post-transfection, assay for reporter gene (ZsGreen or Met luc) activity. Record results. Repeat the experiment once or twice to account for day-to-day variation. Choose the density with the highest reporter gene activity.

The other parameters can be optimized in much the same way. Hold all other variables constant while varying the parameter you are testing. Transfection incubations should be maximal at 2–16 hours using the CalPhos Mammalian Transfection Kit. After transfections have been optimized, scale-up or scale-down as necessary for the size of culture plate you are using (see Appendix B for a table of conversions).

Appendix B: Culture Plate Conversion

Table 1. Culture Plate Conversions.

	Solution A			Solution B	
Plate/ Flask	**Plasmid DNA**	**Calcium Solution**	**Sterile H_2O**	**2X HBS**	**Culture Volume**
96-well	30–100 ng	0.5 µl	to 4 µl total	4 µl	200 µl
24-well	200–600 ng	3.1 µl	to 25 µl total	25 µl	500 µl
12-well	0.4–1.2 µg	6.2 µl	to 50 µl total	50 µl	1 ml
6-well	1–3 µg	12.4 µl	to 100 µl total	100 µl	2 ml
35 mm	1–3 µg	12.4 µl	to 100 µl total	100 µl	2 ml
60 mm	2.5–7.5 µg	32 µl	to 260 µl total	260 µl	5 ml
10 cm	6–18 µg	87 µl	to 700 µl total	700 µl	10 ml
T25 flask	3–9 µg	37 µl	to 300 µl total	300 µl	5 ml
T75 flask	8–24 µg	112 µl	to 900 µl total	900 µl	12 ml

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