ViraPack Transfection Kit

Instruction Manual

Catalog #200488

Revision C.0

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ViraPack Transfection Kit

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MATERIALS PROVIDED

Materials provided	Composition	Quantity
Solution 1	2.5 M $CaCl_2$	5 ml
Solution 2	2× BBS[a] (pH 6.95)	25 ml
Solution 3	Modified bovine serum (MBS)	40 ml
Control plasmid	pCMV β-gal	40 µg

[a] BBS, *N*,*N*-bis(2-*hydroxyethyl*)-2-aminoethanesulfonic acid and buffered saline.

ADDITIONAL MATERIALS REQUIRED

Phosphate-buffered saline (PBS) (see *Preparation of Media and Reagents*)
Potassium ferricyanide
Potassium ferrocyanide
Formaldehyde and gluteraldehyde
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)
5-ml BD Falcon polystyrene round bottom tubes (BD Biosciences catalog #352054)

STORAGE CONDITIONS

All Components: –20°C

NOTICE TO PURCHASER

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY.

Revision C.0

INTRODUCTION

When making recombinant virus, one of the critical steps affecting the final titer of the virus is the transfection efficiency into the packaging cell line, most often the HEK293 cell line. The ViraPack Transfection Kit* is based on a modified $CaPO_4$ method of DNA transfection that is exceptionally efficient in HEK293 cells.

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 $CaPO_4$ enables transfection by enhancing the adsorption of DNA to cell membranes, thus facilitating the DNA uptake by mammalian cells. The $CaPO_4$ precipitate also limits the digestion of DNA by DNase associated with mammalian cells.

All the necessary reagents and protocols for efficient, rapid and simplified transfection of viral plasmids into the HEK293 packaging cell line are included. The kit has been optimized for 50 transfections in 60-mm tissue culture dishes using a modified $CaPO_4$ protocol. Solutions 1 and 2 are used in the $CaPO_4$ precipitation protocol. Solution 3 is a specially modified bovine serum (MBS) solution that is added to the culture medium instead of fetal calf serum (FCS) during the incubation of the cells with the $CaPO_4$-DNA precipitate.

* U.S. Patent No. 5,589,368.

TRANSFECTION PROTOCOL

Note *Please read the entire transfection protocol before beginning. See the final page of this instruction manual for a condensed, quick-reference protocol that can be detached and used when detailed instructions are no longer required.*

This protocol outlines the procedure necessary for transient transfection using 60-mm tissue culture dishes. If using 100-mm tissue culture dishes, double the quantities and the volumes of the solutions included in the protocol.

1. Inoculate 60-mm tissue culture dishes with 1–5 × 10^5 exponentially growing cells per dish, 24 hours before the transfection.

2. Grow the cells overnight in 5 ml of the appropriate culture medium.

Important *Roswell Park Memorial Institute (RPMI) media cannot be used for $CaPO_4$ transfection. The excess positive charge in this media will cause formation of a dense precipitate that is toxic to the cells.*

Preparing the DNA Suspension for Transfection

The optimal amount of DNA must be determined for each plasmid and cell line separately. The recommended amount is between 1 and 10 µg of DNA per dish. Start with 5 µg of the pCMV β-gal control plasmid DNA per dish when preparing the DNA for the control transfection.

The amounts indicated in the following steps are for duplicate 60-mm tissue culture dishes (500 µl each).

1. For each duplicate transfection, dilute the optimal amount of DNA (2–20 µg) with sterile water to a final volume of 450 µl in a 5-ml BD Falcon polystyrene round bottom tube.

   Note *Polystyrene tubes yield superior results compared to tubes composed of polyethylene or polypropylene.*

2. Add 50 µl of solution 1 and 500 µl of solution 2. Mix the contents of the tube by gently tapping the sides.

3. Incubate the DNA suspension at room temperature for 10–20 minutes. During this incubation period, prepare the cells as indicated in the following section (see *Preparing the Cells for Transfection*).

Preparing the Cells for Transfection

1. Remove the standard culture medium from the tissue culture dishes by aspiration.
2. Wash the cells twice with phosphate-buffered saline (PBS).
3. Add 5 ml of culture medium containing 4–7% solution 3 (MBS).

   Note *The optimal concentration of MBS should be determined by titration. A good starting point for most cell lines is 6%.*

   The cells are now ready to receive the DNA.

Applying the DNA Suspension

1. ***Gently*** resuspend any precipitate in the DNA suspension by pipetting the suspension up and down with a pipettor set at 500 µl. The DNA suspension should appear clear to opaque.
2. Slowly add 500 µl of the DNA suspension to each tissue culture dish ***dropwise in a circular motion*** to distribute the DNA suspension evenly onto each tissue culture dish**.**
3. Swirl each tissue culture dish ***once***.

Incubating the Transfection Reactions

1. Incubate the dishes for 3 hours at 37°C under 5% CO_2.

   Note *Incubation at 37°C under 5% CO_2 works well; however, for optimal transfection efficiency, the 3-hour incubation should be performed at 35°C under 3% CO_2.*

2. After 3 hours, check the precipitate, which will vary in consistency from slightly perceptible to noticeably granular.
3. Remove the culture medium by aspiration and wash the tissue culture dishes three times with PBS, which should be added gently to avoid dislodging the cells. Check for residual precipitate by microscopic inspection. Add additional wash steps as required to ensure complete removal of the precipitate.
4. Add the appropriate volume of culture medium and incubate the dishes at 37°C under 5% CO_2. For the pCMV β-gal control plasmid, add 5 ml of medium per dish and incubate the cell cultures overnight–24 hours at 37°C under 5% CO_2. For the viral DNA transfection reactions, follow the manufacturer's recommendations for virus production and titering.
5. For the pCMV β-gal control plasmid, perform an assay to detect the β-galactosidase gene product in order to monitor the transfection efficiency. See either the *β-Galactosidase Activity Assay* section or the *β-Galactosidase Histochemical Staining Assay* section.

β-GALACTOSIDASE ACTIVITY ASSAY

Harvesting the Transfected Cells

1. Gently wash the transfected cells twice with PBS.
2. Add 1.5 ml of PBS per 60-mm tissue culture dish.
3. Gently remove the cells from each tissue culture dish by scraping with a PTFE-coated scraper. Pipet the cells from each dish into a separate 1.5-ml microcentrifuge tube.
4. Pellet the cells by spinning in a microcentrifuge at 12,000 × *g* for 5 minutes. Remove the PBS by aspiration.
5. Resuspend the cell pellet in 200 µl of lysis buffer.[§]
6. Incubate the tubes for 5 minutes at room temperature.
7. Spin the tubes in a microcentrifuge at 12,000 × *g* for 5 minutes.
8. Transfer the supernatant to a fresh microcentrifuge tube on ice.
9. Assay 100 µl of cell extract for β-galactosidase activity using the protocol described in the following section.

Performing the β-Galactosidase Assay

Notes *We recommend performing the β-galactosidase assay using the same reagents supplied in the β-Galactosidase Assay Kit.*

Add fresh β-mercaptoethanol to buffer A[§] prior to performing the β-galactosidase assay.

1. Add 100 µl of the cell extract to 900 µl of buffer A.
2. Incubate for 5 minutes at 37°C.
3. Add 200 µl of *o*-nitrophenyl-β-D-galactopyranoside (ONGP) substrate solution[§] and vortex.
4. Incubate the mixture at 37°C. Note the time, and monitor the reaction until a bright yellow color develops.

   Note *Incubation times for color development will vary.*

[§] See *Preparation of Media and Reagents*

5. Terminate the color reaction by adding 500 µl of stop solution[§] and record the incubation time from step 4 above to use in calculating the enzyme activity.

6. Read the optical density at 420 nm (OD_{420}). (The optimum OD_{420} is between 0.6 and 0.9.)

β-GALACTOSIDASE HISTOCHEMICAL STAINING ASSAY

Caution ***To avoid inhaling vapors, stain the cells in a fume hood.***

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

The quantities of the reagents given in this protocol are optimized for a 60-mm tissue culture dish.

1. Aspirate the medium from the cells.

2. Add 4 ml of freshly prepared fixing solution[§] to the tissue culture dish and incubate the dish for 10 minutes at room temperature.

3. Remove the fixing solution from the dish and gently wash the cells twice with 5 ml of PBS.

4. Add 2 ml of freshly prepared histochemical staining solution[§] to the tissue culture dish.

5. 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.*

6. Remove the staining solution and wash the cells two or three times with 5 ml of PBS.

7. Add 2 ml of 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.*

8. 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.

[§] See *Preparation of Media and Reagents*

GENERAL TISSUE CULTURE TECHNIQUES: TRANSFECTION OF MAMMALIAN CELLS

Culture Inoculation

To ensure the greatest transfection efficiency, minimize trauma to the cells during incubation. For adherent cells, minimize the length of trypsinization. Remove all serum from the culture by washing with PBS, washing the culture with a medium without serum or washing the culture very quickly with a trypsin solution (serum inhibits the action of trypsin). Warm a minimal volume of trypsin to 37°C before applying to the culture. Minimal volumes for specific culture vessels are indicated in the following table:

Size and type of culture vessel	Minimum volumes
60-mm tissue culture dish	0.5 ml
100-mm tissue culture dish	1 ml
150-mm tissue culture dish	2 ml
75-cm^2 tissue culture flask	1.5 ml

Proceed with trypsinization until the cell pseudopods just begin to recede toward the nucleus, a process that takes from ~30 seconds to 2 minutes, depending on the cell line. Free the cells from the dish surface by tapping the dish firmly and then immediately dilute to the desired density using medium with serum.

The best transfection efficiencies are obtained when transfected cells are grown exponentially. To allow the cells to grow as rapidly as possible, avoid contact inhibition, which occurs when the cells actually touch. Therefore, it is best to inoculate the cultures at a density that will cover no more than 15–30% of the culture vessel surface at the time of transfection.

The optimal cell density should be determined by transfecting cells plated at concentrations between 5×10^4 and 5×10^5 per 60-mm tissue culture dish.

TROUBLESHOOTING

Observation	Suggestion
The precipitate is not visible or is too fine after adding the DNA and incubating	Too much DNA was added. Check the OD_{260} of the DNA and adjust the volume used or the concentration appropriately. Transfect with different amounts of DNA to determine the optimal concentration.
The precipitate is too dense, with aggregated clumps	Too little DNA was added. Check the OD_{260} of the DNA and adjust the volume used or the concentration appropriately. Transfect with different amounts of DNA to determine the optimal concentration.
Unable to remove the precipitate from the plate	Ensure that the PBS does not contain Ca^{2+} or Mg^{2+}. It is important that the PBS wash solution contain only the cations contributed by the components listed in the recipe.

PREPARATION OF MEDIA AND REAGENTS

PBS

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4
Adjust the pH to 7.4 with HCl

Lysis Buffer

0.25 M Tris-HCl (pH 7.8)
0.5% NP40

Buffer A

100 mM sodium phosphate (pH 7.5)
10 mM KCl
1 mM $MgSO_4$

Note *Add fresh β-mercaptoethanol to buffer A prior to the assay*

50 mM β-mercaptoethanol

X-Gal Stock Solution

40 mg/ml in DMSO

ONGP Substrate Solution

4 mg/ml in 100 mM sodium phosphate (pH 7.5)

Stop Solution

1 M Na_2CO_3

Fixing Solution (100 ml)

Note *Always use formaldehyde in a fume hood*

5.41 ml of 37% formaldehyde (2% final concentration)
2.00 ml of 10% gluteraldehyde (0.2% final concentration)
10.00 ml of 10× PBS (1× final concentration)
Add sterile water to a final volume of 100 ml

Histochemical Reaction Mixture (250 ml)[2]

0.411 g of potassium ferricyanide [$K_3Fe(Cn)_6$] (5 mM final concentration)
0.53 g of potassium ferrocyanide [$K_4Fe(Cn)_6$] (5 mM final concentration)
5 ml of 100 mM $MgCl_2$ (2 mM final concentration)
25 ml of 10× PBS (1× final concentration)
Add X-gal just before use [final concentration of 1 mg/ml from the X-gal stock solution (see above)]
Add sterile water to a final volume of 250 ml

REFERENCES

1. Chen, C. and Okayama, H. (1987) *Mol Cell Biol* 7(8):2745-52.
2. Sanes, J. R., Rubenstein, J. L. and Nicolas, J. F. (1986) *Embo J* 5(12):3133-42.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.

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QUICK-REFERENCE PROTOCOL

- Inoculate 60-mm tissue culture dishes with 1×10^5–5×10^5 exponentially growing cells/dish and grow the cells overnight in 5 ml of appropriate medium
- Dilute the optimal amount of DNA for duplicate transfections (2–20 µg) with sterile water to a final volume of 450 µl
- Add 50 µl of solution 1 and 500 µl of solution 2
- Incubate the DNA suspension at room temperature for 10–20 minutes
- Remove the culture medium from the cells by aspiration
- Wash the cells twice with PBS
- Add 5 ml of culture medium containing 4–7% solution 3. (The cells are now ready to receive the DNA)
- Gently resuspend any precipitate in the DNA suspension with a pipettor
- Slowly add 500 µl (containing 1–10 µg DNA) of the DNA suspension to each tissue culture dish ***dropwise in a circular motion***
- Swirl the tissue culture dish ***once***
- Incubate for 3 hours at 37°C under 5% CO_2
- Remove the culture medium by aspiration and gently wash the tissue culture dishes three times with PBS
- For the viral DNA transfection reactions, proceed to viral production and titering protocols appropriate to the specific virus
- For the pCMV β-gal control, add 5 ml of standard culture medium and incubate the dishes at 37°C under 5% CO_2 overnight–24 hours
- Harvest the cells and perform a β-galactosidase activity assay or fix the cells and perform a β-galactosidase histochemical assay