Calcium Phosphate Transfection Method

Experimental Design Considerations

- The method described here is a modification of some of the original methods but is much simpler (i.e. the drip technique is not required) and is more reliable and consistent.

- While the Calcium Phosphate transfection method is a very efficient means of introducing DNA into cells in many cell systems, it is very inefficient in many others. In our experience, this method works best in cell lines that are 1) highly transformed and 2) adherent (we typically use it for Hela, U2OS, SAOS2, AdAH, NPC-KT and obtain from 20% to 100% transfection efficiency depending on the cell line). The method works well for transient experiments but precautions should be used in the design and interpretation of experiments based on the discussion below. This method also works very well for generating stable cell lines.

- This method is quite sensitive to the amount of input plasmid. Therefore, the total amount of transfected DNA should be 30ug (for a 100mm plate and scaled according to the surface area for other mediums, see table). As mentioned below, we always try to minimize the amount of any plasmid containing a eukaryotic promoter and we make up the difference with a neutral carrier plasmid [pGL3Basic or Bluescript (note: although pGL3Basic doesn't contain a specific eukaryotic promoter sequences, it contains a luciferase gene - therefore, this plasmid should not be used in luciferase reporter experiments)]. For typical transactivation experiments we might use between 50 – 500 ng of an effector plasmid, 500ng of a reporter and ca. 29ug of Bluescript or pGL3Basic. However, if you want to use a functional transdominant negative effector, you might have to use up to 5-10 ug of the respective expression vector and 20-25 ug of carrier DNA.

TABLE of DNA amounts for different surface areas.

Format	Relative Surface Area	Culture Media	Transf. Media	1X HBS	2.5M $CaCl_2$	Total DNA	GFP Plasmid
24 well	0.25X	500ul	280ul	18ul	1.1ul	1.1ug	3.6-9.0ng
12 well	0.5X	1ml	570ul	36ul	2.1ul	2.1ug	7.1-18ng
6 well	1.2X	2ml	1.4ml	86ul	5.1ul	5.1ug	17-43ng
35mm	**1.0X**	2ml	1.15ml	71ul	4.3ul	4.3ug	14-36ng
60mm	2.6X	5ml	3ml	186ul	11ul	11ug	37-93ug
10cm	7X	10ml	8ml	500ul	30ul	30ug	100-250ng
T25	3X	5ml	3.4ml	214ul	13ul	13ug	43-107ng
T75	9X	12ml	10ml	643ul	39ul	39ug	128-321
T150	18X	24ml	20.5ml	1.3ml	77ul	77ug	258-643ng

- There are a couple of important issues that are especially germane to cell cycle and apoptosis experiments [see Rodriguez A, and Flemington EK. Transfection mediated cell cycle signaling. Anal Bio 1999; 272:171-181, for further details]. First, DNA only crosses the nuclear membrane during mitosis. Therefore, expression of the gene of interest [or promoter of interest (for reporter assays)] will not occur until cells pass through mitosis. This results in extreme synchronization of transfected cells at early times following transfection (i.e. at 0-8 hrs following transfection of a GFP expression plasmid, nearly 100% of GFP expressing cells are in G1 phase of the cell cycle). Therefore, one should allow at least 24 hrs., and preferably longer (48hrs), to allow for dissolution of transfection dependent synchronization.

The second cell cycle and apoptosis related issue results from the large amounts of plasmid DNA that enters each sucessfully transfected cell using this method. This results in specific DNA dependent signaling of cell cycle and apoptosis pathways. While this issue can be problematic, meaningful experiments can, in fact, be carried out if steps are taken to minimize these types of signaling events.

We find that the biggest culprits in cell cycle signaling during Calcium Phosphate transfection are plasmids containing eukaryotic promoters (and stronger promoters are usually more problematic). Therefore, we try to minimize the amounts of plasmids that contain a promoter in our transfections and make up the difference with carrier DNA (it may surprise you how little you may need to generate excellent results). If you're using a GFP expression vector to specifically analyze the sucessfully transfected cell population, the use of membrane localized GFP plasmids [e.g. Us9-GFP described by Kalejta et al, Exp. Cell Res. 248; 322 (1999) or GFP-SP described by Kalejta et al, Cytometry 29;286 (1997)] minimizes the amount of expression required to give good results because there is very little loss of GFP during processing of the cells (typically, we use 50ng – 150ng of the plasmids, GFP-SP or Us9-GFP, for a transfection in a 100mm plate). When using effector plasmids, titration experiments should be carried out to determine the lowest amounts which give good functional effects [for example, we obtain optimal transactivation properties with CMV-E2F1 if we use ca. 100ng of input plasmid (for transfection of a 100mm plate).

Protocol (for 100mm plates)

- The day before transfection, split fairly confluent (70-90%) culture between 1:10 and 1:15 (the ratio that we use depends on the cell lines being transfected and we typically split faster growing cell lines at the higher ratios – very slow growing cell lines may be split even less than 1:10).
- The next morning, replace the media on each plate with 8ml of fresh media [usually DMEM (+10%FBS, +pen/strep)]. (note: this seems to help the transfection efficiency at least in part, by helping boost cell cycle progression and therefore increasing entry into mitosis where DNA is taken up into the nucleus).
- Later in the day, cells can be transfected. Set up transfections using 30ug of total DNA and the amounts of effector plasmids based on the discussion above (experimental design considerations).
    - For each transfection, put 0.5 ml of 1X HBS in a sterile tube
    - Add appropriate DNAs to each tube. After adding DNA to all tubes, mix contents of each tube.
    - Add 30ul of 2.5M $CaCl_2$ to each tube (mix each tube immediately after adding 2.5M $CaCl_2$).
    - Let sit for 20 minutes.
    - Add transfection mix to plates in dropwise fashion, mix by rocking back and forth (don't swirl) and put in 5% CO_2 incubator (using a 5% CO_2 incubator is important).
    - Change media the next day with fresh DME (+FBS).
- For transient experiments, cells are typically harvested 48-72hrs later. For generating stable cell lines, 48hrs after transfection, we typically split each plate into a serial dilution of four plates each (i.e. split 4 plates 1:10, split 4 plates 1:20, split 4 plates 1:40). Pick 10-20 colonies from which ever plates give isolated colonies.

If you follow these guidelines, you should be able to carry out some very sucessful experiments!

Solutions

1X HBS	1 liter
HEPES (Acid)	5g
NaCl	8g
Dextrose	1g
KCl	3.7g
$Na_2HPO_4(7H_2O)$	10ml of $Na_2HPO_4(7H_2O)$ *Stock Solution (see below)

- Add HPLC Purified H_2O (Aldrich Cat#32,007-2) up to ca. 900ml
- Adjust pH to exactly 7.1
- Add HPLC Purified H_2O (Aldrich Cat#32,007-2) up to 1L
- Filter through a 0.2u sterile filter

Aliquot into sterile tubes and keep frozen at –20ºC. We then keep one working tube at 4ºC.

*$Na_2HPO_4(7H_2O)$ Stock Solution = 0.94 g of $Na_2HPO_4(7H_2O)$ in 50ml HPLC Purified H_2O (Aldrich Cat#32,007-2).

2.5M $CaCl_2$	100ml
$CaCl_2$	27.75g anhydrous $CaCl_2$
Or	
$CaCl_2$	36.75g $CaCl_2(2H_2O)$

We typically aliquot into sterile 15 ml tubes (10 ml in each) and store at –20ºC (although keeping at –20ºC is probably not necessary – this is primarly to keep it in a fairly germ free environment and to keep it together with the 1X HBS).