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NBP2-29542 Protocol

One Day Before Transfection
1. Seed 1 x 106 293 cells in 6 cm tissue culture plates. This should yield a cell density of about 30% confluence on the day of the experiment.
2. Incubate overnight at 37oC in DMEM supplemented with 10% fetal bovine serum, 1% pen-strep.
TRANSFECTION OF 293 CELLS WITH RETROVIRAL VECTOR

Day 1
1. Add 0.25 ml of transfection buffer (previously tested for optimum transfection efficiency) to the required number of sterile 15 ml polypropylene tubes. Lipofectamine may have certain advantages in reproducibility, but this has not been tested extensively by us for overall virus titers.
2. Dilute 2M CaCl2 to final concentration of 0.25 M in sterile distilled water (30 ul of 2 M CaCl2 and 220 ul of sterile water). Add the following to 1.5 ml sterile Eppendorf tube:
0.25 M CaCl2 - 30 ul
pCL-ECO, pCL-Ampho, or pCL-10A1 - 10 ug
Retroviral vector containing your gene - 10 ug
Mix by vortexing.
3. Add the DNA/CaCl2 mix drop-wise to the transfection buffer tubes (step 1) while lightly vortexing. Incubate at room temperature for 20 min.
4. During this incubation aspirate the transfection media from plates to be transfected and add 2 ml DMEM containing 10% FBS/1% P/S and put back in the incubator until step 3 is done.
5. Add the DNA/CaPO4 mix drop-wise to 293 cells on 6 cm T.C. plates. Place in humidified CO2 incubator for 3-4 h. (Longer times may result in cells coming off the plates).
6. Carefully aspirate medium. Add 2 ml of warm PBS-15% Glycerol -no serum (glycerol shock medium) for 2 min. This step is optional. In some cases it may increase the transfection efficiency by 2-fold.
7. Aspirate glycerol shock medium. Carefully add 4 ml DMEM containing 10% FBS, 1% P/S along the side of the dish. Incubate for 12 h.(See Table 1.)

Day 2

1. Aspirate medium and add fresh medium in the morning and incubate for 24 to 72 hrs.

Day 3 (24 h after addition of fresh medium).

1. Filter sterilize (0.45 mm syringe filters are convenient) the viruscontaining supernatant to remove any cells in suspension. The virus can now be used directly, or stored at -70C until needed.
2. Infect the desired target cells with 1 ml to 4 ml of 293 supernatant in 8 ug/ml Polybrene. The amount of supernatant you use depends on whether you are titering virus or want to infect the maximum number of target cells possible. Do not forget the polybrene. Omission of polybrene will drop your apparent titers 100-1000 fold.

DETERMINING THE VIRAL TITER

Remember that for titering, you must dilute the transfected supernatant at least 50 fold to stay in the linear part of the dilution curve. If you just want the

maximum number of cells infected, then as little as a 2-fold dilution (equal volume mix) with the medium of the intended target cells is usually enough to prevent significant cell cycle inhibition.
1. For tittering, prepare serial dilutions (four 10-fold dilutions) of vector supernatant in order to be sure that you are in the linear part of the titration curve (ie, out of the Poisson region). Infections for accurate titering must be done at effective MOI is 0.1. Target cells must be growing exponentially and only 30-50% confluent for maximum infection efficiencies.
2. Total virus-cell contact time should be a minimum of 12-24 h. This is because cycling cells are continuously entering and exiting the window of infectability. Even though the infective half life of the murine retrovirus particle is just 6-8 h at 37oC the rate of new cells entering the window is greater for the first 24 h, so longer contact times means more infected target cells.
3. Always test your titers on a standard control cell line (we use NIH 3T3) in parallel with infections of other desired target cells. Intrinsic infectability of many target cells can vary widely from 0.01-100% of the titers on NIH 3T3 cells.
Virus titers on NIH 3T3 cells for empty RetroMax vectors are typically 2-3 x 106 CFU/ml for ecotropic virus and 1 x 106 for amphotropic virus, assuming a typical 293 transfection efficiency of 30-50%.

When tittering virus on NIH 3T3 cells, infect 2 x 105 cells on a 6 cm plate (in 4 ml medium), overnight (16 h) with 1, 3, and 10 ml of pCL vector supernatant. You will need larger volumes for lower titer vectors, or cells that are more refractory to infection than NIH 3T3.
If virus stock is limiting: the most efficient use can be made by using 0.5-1 ml volumes to serially infect target cells in 6 cm plates (or 2-3 ml in 10 cm plates), and adding fresh virus every 4-6 h for 3-4 infection cycles. Continuous exposure to virus for about 24 hr is necessary in order to ensure that all cells have cycled through their receptivity window (S-G2) for retroviral infection. Be sure to add polybrene to 8 ug/ml.
4. Check your transfection efficiency by drawing a 1 cm square on the bottom of the plate of transfected 293 cells. Scrape harvest all the cells outside of this square (if desired) for RNA or protein analysis (CAT assays, ONPG-LacZ, Westerns, Northerns, Hirts, etc.) Fix and stain the transfected cells remaining inside the 1 cm square with X-Gal to determine the transfection efficiency (TXE). Typical transfection efficiencies are 30-50% in this subline of 293 cells. The same DNA and reagents will give TXEs of 2-15% on COS cells.

SELECTION FOR STABLE CELL LINES

Day 4 (12-24 h after infection)
1. If using a vector that confers G418 resistance, split the infected target cells at various dilutions (1:20 to 1:200) into 10 cm T.C. plates. A 1:20 dilution is about 105 NIH 3T3 cells. If 0.1% of the cells were infected, you will get about 100 colonies after 8-12 days of selection.
When infecting primary cells:
Accurate titers cannot be obtained when infecting primary fibroblasts, bone marrow or tumor cells because these cell types display density-dependent growth and typically have low plating efficiencies of 0.01%. This means that if 1000-10,000 cells are plated, only 1-100 colonies will actually clone out, even if they are all infected and G418-resistant. Therefore when infecting these cells, do not split them more than they will tolerate and only if they are >80% confluent (this is usually only a 1:2 to 1:4 dilution).
If you are selecting primary cells in G418, you will need to trypsinize and concentrate the cells by replating on sequentially smaller dishes until sufficient G418-resistant cells have grown out that you can begin expanding the infected pool of cells. This process can take 2 weeks. Effective titers for a particular primary cell type and vector will be a constant percentage of the titer observed on NIH 3T3 cells.

If using vectors that do not confer antibiotic resistance (like LacZ or GFP), simply change the medium today. Primary bone marrow cells should always be infected by co-cultivation of autochthonous stromal cells and virus producer cells in the presence of IL-3 (or WEHI-conditioned medium) and GMCSF (a potent stromal cell growth factor). Never select them in G418.
Day 5 (2 days after infection)
1. Begin selection of cells infected with virus vectors conferring antibiotic resistance by adding 100 ml of a 100x stock to a 10 cm dish containing 10 ml of medium.
The correct concentration of G418 (or any antibiotic) varies widely for different cell types. You must determine the concentration empirically. For NIH 3T3 cells this is 400-1000 mg/ml (active) G418. For other cell types, the right concentration is that which results no observable death at Day 2 and about-30-50% on Day 4. Complete G418 selection is usually achieved in 7-10 days.
2. If using a virus vector that does not contain a selectable marker (e.g., pCL-LacZ, MFG-GM-CSF, GFP), or if you would like an rapid assessment of gene expression in the infected target cells (for vectors expressing CAT, Luciferase, GFP, or LacZ), this can be tested today: b-gal staining of fixed cells in situ, (you can calculate the LacZ titer of your virus from this); CAT, Luciferase, or ONPG assays are done from cell lysates.
3. Because of the natural kinetics of retroviral infection, integration, and expression, no selection pressure (antibiotics) or assessment of gene expression should be made until 48 h after infection, i.e., if cells are infected on Day 3, gene expression cannot be accurately tested until Day 5.

Day 9 (4 days after starting selection).

1. Add fresh medium (and antibiotic) to cells under selection.
2. If infected cells were primary fibroblasts or primary tumor cells, you may need to increase the cell density (that has fallen due the death of uninfected cells under selection) by one of two methods, in order to avoid cell death due to densities falling below that tolerated by your particular primary cell type: concentrate the infected cells by trypsinization and plating on a smaller dish, or add uninfected primary cells (of the same type) to bring the density up to 50%, and continue selection. You must let the added (non-G418 resistant) cells attach to the plates for 3-4 h before adding G418 again.
3. Most primary cells will not grow as isolated clones because of densitydependent growth requirements. Attempts to pick clones frequently result in the loss of all infected cells.

Day 14 (10-13 days after infection)

1. Count the antibiotic resistant colonies, and calculate the titer (e.g., Neo titer) in your virus supernatants.
Example: Let us say you count 125 G418-resistant colonies on a 10 cm plate. If you infected (5 x 105) NIH 3T3 cells with 1 ml of virus supernatant, then split out the infected cells 1:20, your calculated titer is 125 x 1000 x 20 =2.5 x 10^6 CFU/ml.
Note: Many cDNAs of interest may have either cytostatic or cytotoxic effects on infected cells, so that stable colony formation under G418 selection does not actually reflect the true number of cells initially infected. Only growing cells make colonies.

V. MAXIMIZING RETROVIRUS TITERS

1. The principal determinant of retrovirus titer is the abundance of packageable RNA, and not the abundance of viral proteins. Viral pro teins are typically made in 20 fold stoichiometric excess. In fact, too much gp85 env can actual lower your titers because of impaired glyco protein processing and assembly.
2. The RetroMax (pCL) system generates the highest abundance of packageable viral RNA of any known transient system by exploiting the

power of the CMV IE enhancer-promoter in E1A-expressing 293 cells.
The natural enhancer of the unmodified MuLV LTR is inhibited by E1Ap300 in 293 cells, so attempts to use non-pCL retroviral vectors in 293 cells will yield 20-50 fold lower titers, even with the same transfection efficiencies.
3. If you are studying cDNAs that do not have cytostatic or cytotoxic phenotypes, it may be possible to generate higher titer virus using traditional retrovirus packaging cells. This process takes 2 months (instead of 2 days for pCL). The highest titers are always obtained from stably infected (not transfected), cloned (not pooled) packaging cell lines. This is because transfected sequences are often inactivated by methylation, and because pro-virus integration position effects can influence gene expression from the same retrovirus vector in different clones of infected cells can vary over a 100 fold range (i.e., integration into heterochromatic regions of the genome gives poor expression, while integration into euchromatic regions gives high expression).
4. In deciding whether to go through the process of selecting and characterizing clones of packaging cells or simply preparing virus by the rapid pCL system, one must consider the intended applications. If you need a rapid test for the stable expression properties of a battery of mutant cDNAs that you have prepared, the pCL system is often adequate, or in the case of cytostatic and cytotoxic cDNAs, it is often the only way to produce usable amounts of virus.
Sometimes producing the virus (with a toxic or static cDNA) in cells from a different species can overcome the titer problems that result from cell growth inhibition.
If on the other hand, you plan to use the virus produced as a reagent that you can go back to many times over the next few years, then you need to pick clones of stable packaging cells.

Scaling Up:

1. Transfect 10 cm plates of 293 cells with 30-40 mg of pCL vector containing your gene of interest in 1 ml of CaCl2-HBS.
2. Replace the medium on Day 2.
3. Harvest and replace the medium every 24 h on Days 3, 4, and 5. This should give you 30 ml of virus supernatant from each transfected plate.
The titers in supernatants harvested on Days 3 and 4 are equivalent.
We suspect that Day 5 will be almost the same.

VI. THE SAFE USE OF MURINE RETROVIRUS VECTORS AND SAFETY PRECAUTIONS

Replication competent retroviruses (RCR) are called helper virus, or simply "Helper".
They require 3 trans- (gag, pol, and env), and 7 major, cis-active control elements (U3, R, U5, PBS, SD, y, and SA) in order to replicate.
The most common retrovirus vectors are based on the Moloney Murine Leukemia Virus (MoMuLV), encoding only the 7 cis elements.
These vectors are defective and can not replicate without picking up 7.1 kb of sequence by homologous recombination with a helper genome (while simultaneously deleting your cDNA). Modern vectors are now "safety modified" by including a stop mutation early in "gag" (or a frame-shift) that pre-vents gag translation and limits the sequence window available for productive recombination with helper genomes.
Packaging cells supply the 9 processed proteins encoded by gag, pol, and env (p15MA, p12 p30CA, p10NC, p14PRO, p85RT, p40IN, gp70SU, and p15ETM) necessary for virion assembly.
Modern packaging cells are safety-modified by dividing the gag-pol genes, and the env gene on two separate plasmids. These two plasmids are serially transfected (not co-transfected) into NIH 3T3 cells. The resulting safety modifications yield the modern split genome packaging cells.
Current evidence suggests that in order to initiate a pathogenic infection in primates with amphotropic murine retroviral vectors, three requirements must be met:
1. The infected host must be immunocompromised.
2. The vector preparation must contain helper virus.

3. Direct body fluid contact, e.g., intravenous inoculation is required for transfer.
However, for safe use of the RetroMax system, the user is strongly advised to follow the following guidelines:
1. According to NIH guidelines all retroviral production and transduction work must be done in a Biosafety Level 2 (BL2) facility.
2. Work in laminar flow, HEPA filtered hoods that receive annual maintenance and recertification.
3. Use sterile technique (flaming is not necessary and not recommended because of convection disturbances to airflow patterns).
4. Aspirate all liquid waste into flasks containing 5-10% (v/v) of a microbiocidal agent.
5. Discard spent plasticware in biohazard bags and autoclave before discarding.
6. Dispose spent glassware in detergent containers for cleaning and autoclaving.
7. Clean all surfaces with 70% ethanol at the end of the work.
8. Switch on the UV light.
Note: Retroviruses are not spread by aerosols.