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Dear Researcher,

Please find enclosed a transfer aggreement that needs to be signed by you and an authorized individual at your Institution. I am sorry about this inconvience; however, my Institution requires that any researcher that would like to use the Tet retroviral vectors sign and return the agreement.

Lastly, if you send me an email message, I will send you the electronic sequence of pBPSTR1.

Good luck with your studies.

Sincerely,

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Transfection of BOSC23 cells with the Tet vector and infection of NIH 3T3 cells

This guide is based on using BOSC23 cells to generate virus. Because you will probably be using other packaging cells and your specific target cells, you will have to change plating densities, etc. For everthing else, you can follow this guide.

References:
W.S. Pear, G.P. Nolan, M.L. Scott, & D. Baltimore. Proc. Natl. Acad. Sci. USA 90 (1993), 8392-6.
W. Paulus, I. Baur, X.O. Breakefield, F. Boyce, & S.A. Reeves. J. Virol. 70 (1996), 62-67.

If you have other questions or feed back, please contact me (S.A. Reeves):
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Please have individuals interested in obtaining the vector contact me (S.A.R.).

All conditions are for 100mm plates, but can be scaled down if needed.

Hints:
When setting up for transfections using the tetracycline regulated retroviral vector, initially you may want to confirm that your gene is expressed and that it can be regulated with tetracycline. Because the pBPSTR-1 vector will express and regulate the expression of genes when transfected into cells using $CaPO_4$ (although not nearly as efficiently as when the gene is transduced with the virus), you may want to examine the transfected BOSC 23 cells (or your specific packaging cells), after collecting the virus-containing supernatants, for expression of your gene using western blotting or immunocytochemistry.

The simplest way to measure tetracycline-regulated expression of your gene is to examine cells infected with virus either in the presense or absense of tetracycline. Initially, all transfections should be done in duplicate where one plate of target cells is infected in the presense of tetracycline and another in the absense of tetracycline. Experiments then can be carried out where tetracycline is removed after a designated period of time.

This Guide is for transient production of virus followed by infection and assaying of target cells; however, you certainly can puromycin select your infected target cells. Just make sure you grow and expand the infected cells in the presence of tetracycline (particularly if your gene is growth suppressive or toxic). We have done this with several target cell types including NIH 3T3, RAT1 and human tumor cell lines. The results are what you would expect in a population of infected cells: we always see excellent tetracycline regulation in the clones picked from a pool of infected cells; however, the induction levels vary with the individual clones. Because the virus is randomly integrating into the host chromosome, this is not surprising. I have included a figure to show these results.

Guide:
2. Plate 4.5 x 10^6 packaging cells/100mm plate (or 2 x 10^6 cells/60mm plate) approximately 18-24 hrs prior to transfection in 10 mls of DME plus 10% FCS. Your transfection efficiency will be higher if the cells appear as single cells rather than clumps. The plates should be about 80% confluent prior to transfection. It is also important to count the cells rather than estimating the split. The above cell number is optimized for the pMFG-lacZ retroviral vector. Other vectors and inserts may slow the growth of the cells and it may be necessary to plate more cells prior to transfection. I try to plate at a density so that the cells are 95-100% confluent 24 hours after transfection.

Note: When transfections are done correctly, transfection efficiencies are about 60% (this is for BOSC23 cells). You may want to use a control LacZ expression vector in your transfections and stain the cells with X-gal to monitor transfection efficiencies. I have provided a pBabe-LacZ (parent vector of pBPSTR) for this

purpose. If you are new at working with retrovirus, you should also use this vector to monitor your infection efficiencies.

3. Just prior to transfection, change media to 5 mls of DME plus 10% FCS plus 25uM chloroquine (the addition of chloroquine appears to increase titers by 2-fold).

4. Transfect by adding 6-10 ug vector DNA to $CaCl_2/H_20$ in a total volume of 500ul. Add 500ul 2X HBS (pH 7.05) while bubbling the solution. Immediately (within 1-2 minutes) add this solution to the cells (Note: Halving the volumes of all of the above reagants will result in a 2-fold decrease in titer). However, it appears IMPORTANT that this solution is added to the cells within 1-2 minutes).

5. At 10 hrs post transfection, aspirate off the media, wash cells twice with PBS, and add 10 mls DME plus 10% FCS. It is important that you do not leave the chloroquine longer that 12 hrs. This will cause a large decrease in titer. The range for chloroquine treatment is 7-11 hrs. (+) Tet

6. 24 hrs prior to harvesting the cells, aspirate off the media, and add 5 mls DME plus 10% FCS **plus 1-2 ug/ml tetracycline** (0.1 ug/ml tetracycline is actually sufficient to respress transcription, but to be safe you can increase the tetracycline concentration without cytotoxicity). Reducing the volume at this point increases the relative titer/ml and for unexplained reasons, the presense of tetracycline during packaging of virus increases regulation in infected cells.

7. The cells should be nearly confluent 24 hrs after transfection. Harvest the cells 48 hrs post-transfection. If the cells are not confluent at this point, you may want to wait 72 hrs. If the cells are not confluent at this time, you should alter plating conditions so that cells are confluent by 48 hrs.

INFECTION OF 3T3 CELLS OR OTHER TARGET CELLS:

8. Plate 5 x 10^5 3T3 cells/100mm plates the night before infection (or 1-2 x 10^5 cells/60mm plate). Remember to make duplicate plates for +/- tetracycline.

9. 3-5 hrs prior to infection, aspirate media from (+) tetracycline 3T3 cell plates, and add media **plus 1-2 ug/ml tetracycline** (preloading cells with tetracycline).

10. Save supernatant from TRANSFECTED PLATES and spin 5 min at 1500 RPM to remove cells (temperature is not important). Alternatively, the supernatants can be filtered through a **0.45 uM** filter. At this point, you can also examine BOSC23 cells for expression of your gene.

10. Add whatever volume of viral supernatant you want to DME plus 10% CS (calf serum) such that the final volume is 3 mls AND CONTAINS POLYBRENE AT A CONCENTRATION OF 4 ug/ml (all other cells, I would recommend using 8 ug/ml polybrene)(1 ml total volume for 60mm plate). Until you know the titer of your packaged virus, you should use as much viral supernatant as possible. You can freeze any remaining viral supernatant at -80 ^oC. Titer drops about 2-fold when thawing the supernatants.

11. Aspirate off media from 3T3 cells and add the 3 mls containing virus and polybrene (add tetracycline here if you are concerned about toxicity due to the gene you are transducing). Leave on cells **overnight**. After this time, aspirate off supernatant and replace with 10 mls DME plus 10% CS. In (+) tetracycline plates, add DME plus 10% CS **plus 1-2 ug/ml tetracycline**.

12. Harvest and process (immunostaining, western blotting, puromycin selection, etc) these cells 24-48 hrs post-infection.

Polybrene can be toxic → run a set of plates for Injection +/— polybrene (↑'s efficiency)

OVERVIEW FOR THE INFECTION OF NIH 3T3 CELLS WITH RETROVIRUS:

Day 1: Plate BOSC cells (4.5×10^6 /100mm plate) in duplicate.
Day 2: Calcium phosphate transfection of BOSC cells (see separate protocol).
Day 3: Put transfected BOSC cells in 5ml media plus tetracycline
Plate 3T3 cells (5×10^5 /100mm plate) for infection (+/- tetracycline).
Day 4: 3-5 hrs before infection add tetracycline (1-2ug/ml) to (+) tet plates.
Infect 3T3 cells with supernatants from transfected BOSC cells (see separate protocol).
Day 5-6:Assay infected 3T3 cells.

Puromycin Selection

The retroviral vector contains the puromcyin resistance gene so you can select your infected target cells. Although this may seem obvious, it is very important to carry out a puromycin kill curve on you target cells. Killing concentrations vary substancially between different cells.

IT IS IMPORTANT TO NOTE THAT IN THIS SPECIFIC VECTOR THE PUROMYCIN RESISTANCE CONFERRED BY THE PAC (PURO) GENE IS NOT AS STRONG AS YOU MAY BE USED TO WITH OTHER EXPRESSION VECTORS THAT CONFER PUROMYCIN RESISTANCE. THEREFORE, WHEN SELECTING YOUR CELLS WITH PUROMYCIN USE THE AMOUNT THAT IS JUST SUFFICIENT TO KILL ALL NONTRANSFECTED OR TRANSDUCED CELLS.

If you are using the BOSC23 cells, forget about selecting these cells. Although these cells are very good at making relatively high transient titers, they select very poorly in our experience.

Note: if you are going to select puromycin resistant clones, make sure you have a reasonable number of resistant clones (100's-1000's)--these numbers should be easy to obtain with the virus. Puromycin does give false positives.

In vivo studies

If you want to use the virus for in situ infection or implant infected cells, you can add tetracycline (or doxycycline) to the animals drinking water (1 mg/ml for tetracycline, less for doxycyline). We have used this concentration of tetracycline in rodents and it is sufficient to regulate gene expression even in the brain.

BETA-GALACTOSIDASE STAINING OF CULTURED CELLS

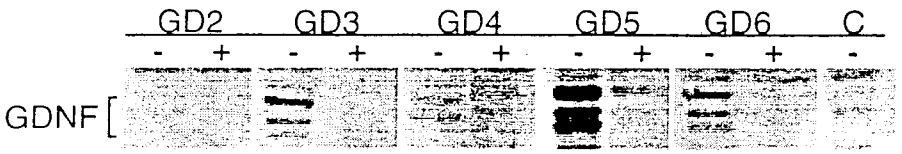
1. Wash cells with 10 mM PBS at RT
2. Fix cells for 5 min at RT in 0.5% Glutaraldehyde/PBS
   4 ml/100mm plate
3. Wash cells 2x with PBS at RT, 5 min each
4. Incubate cultures at 37°C with beta-gal buffer containing:
   1 mg/ml of X-gal (0.1% solution) from a stock of 40 mg/ml prepared with DMSO or 50 mg/ml N,N-dimethlyformide.
   Add X-gal to buffer just prior to use.
5. Staining should be complete from 4 hrs to overnite.

*Buffer can be premade and kept refrigerated in a light protective bottle:

1X beta-gal buffer	100 ml stock
PBS, pH 7.4	88 ml PBS
1-2mM $MgC1_2$	2 ml 100 mM $MgC1_2$
5 mM potassium ferricyanide	5 ml 100 mM K^+ferricyanide
5 mM potassium ferrocyanide	5 ml 100 mM K^+ferrocyanide pH to 7.4
K^+ferricyanide:	M. Wt. 329.26 gm/L = 1M 100mM = 3.9 gm/L or 3.29 gm/100ml
K^+ferrocyanide:	M. Wt. 422.4 gm/L = 1M 100mM = 42.2 gm/L or 4.22 gm/100ml

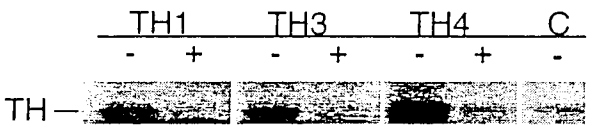
A

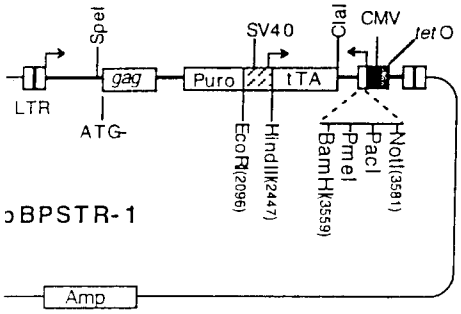
Western blot of clonal Rat1 cells probed with GDNF antibody

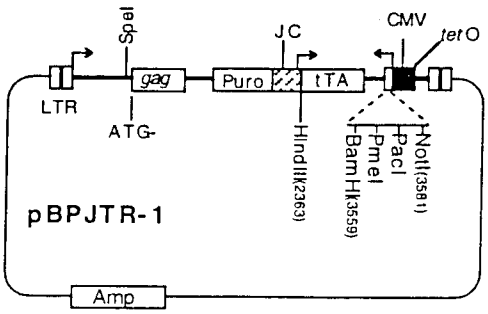


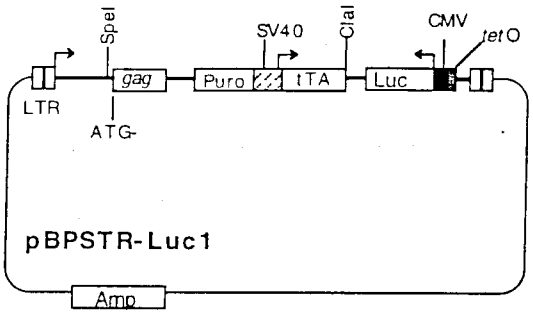
B

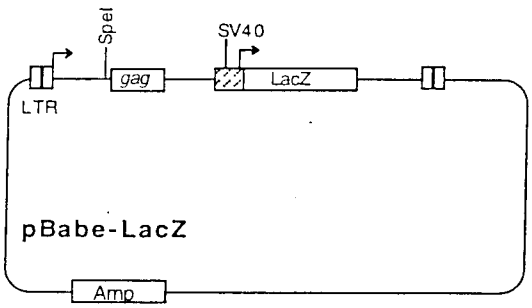
Western blot of clonal Rat1 cells probed with TH antibody











LMP1 B434 inserted into Bam 1 sites