DMEM / 10% FCS

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Transfection of BOSC 23 cells and infection of 3T3s

6 x 10^7 cells.

Source: Warren S. Pear (10/06/93) v2.1
Reference: W.S. Pear, G.P. Nolan, M.L. Scott, & D. Baltimore. Proc. Natl. Acad. Sci. USA 90 (1993), 8392-6.

All conditions are for 60 mm plates. I have not tried to scale up.

1. **(IMPORTANT: See Note 1, this is how I am currently carrying the cells).** If carrying the cells in gpt selection, see below:
The cells are carried in gpt selective media See recipe on the accompanying page. It is best to pass the cells at 1:3-1:4 to prevent cell clumping which occurs when the cells are passed at low density or when they are allowed to become overconfluent. I pass the cells by rinsing once with PBS and then trypsinizing for about 30 seconds. Note: Plating the cells may be the most important step in obtaining high titers. It is extremely important that the cells are not clumped and are at the correct density. Unlike most adherent cell lines, the BOSC 23 cells do not form nice monolayers. Instead they tend to clump before confluence (at which time the media will become acidic). To overcome the clumping, I usually split the cells 1:1 one or two days before I split them for transfection. This may need to be repeated if the cells do not spread well. After the 1:1 splits, it is best to pass the cells 1:2 for 1 or 2 passages, and then at 1:3 or 1:4. The cells grow much slower than 293 cells, and the 1:3 split should take about 3-4 days to reach confluence. Transfections are better if you plate the cells for transfection before the plate becomes confluent.

2. Plate 2 x 10^6 cells/plate approx 18-24 hrs prior to transfection in 4 mls of 10% FCS without selection. Your transfection efficiency will be higher if the cells appear as single cells rather than clumps (see #1 on how to prevent this). Note: 2 x 10^6 cells/plate is not a typo. The dish 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 MFG-lacZ. Other 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 at 24 hours after transfection.**

3. Just prior to transfection, change media to 4 mls of 10 FCS containing 25 uM chloroquine. (chloroquine addition increases titers by 2-fold in my hands).

4. Transfect by adding 6-10 ug DNA to $CaCl_2/H2O$ in a volume of 500 ul. Add 500 ul 2X HBS (pH 7.05) by bubbling. 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. I have added up to 20 μg of DNA without toxicity; however, it is very important that the cells are nearly confluent prior to transfection. If the cells are not very confluent, there will be significant cell death) Recipes for HBS and $CaCl_2$ are on the attached pages. **(It is probably fine to add the reagents without bubbling in the order suggested above. It appears IMPORTANT that this solution is added to the cells within 1-2 minutes.)**

5. At 10 hours, suck off the media and replace with 4 mls 10% FCS without selection. **It is important that you do not leave the chloroquine longer than 12 hours. This will cause a large decrease in titer.** The range for chloroquine treatment is 7-11 hours.

Note: 24 hours prior to harvest, you may want to change the volume of media to 2.5-3 mls in order to increase the relative titer/ml.

6. The cells should be nearly confluent by 24 hrs after transfection. I harvest the cells at 48 hours post-transfection. If the cells are not confluent at this point, you may want to wait until 72 hrs. If the cells are not confluent at this time, you should play with the conditions so that they are confluent by 48 hours.

To infect:

8. Plate 5 x 10^5 3T3s the night prior to infection on a 100 mm plate (1-2 x 10^5 cells for a 60 mm dish).

9. Suck off supe from TRANSFECTED PLATES and spin 5 min at 1500 to remove cells (temp is unimportant). (Alternatively, the supes can be filtered through a 45 μM filter.

10. Add whatever volume of viral supe you want to DME containing 10% CS such that the final volume is 3 mls **AND CONTAINS POLYBRENE AT A CONCENTRATION OF 4 μg/ml** (1 ml total volume is fine for 60 mm dish). You can freeze the rest of the viral supe at -80. I do not experience more than a 2-fold loss of titer when thawing the supes (see Note #2).

11. Suck off media from 3T3s and pour on the 3 mls containing virus and polybrene. Leave on cells from 3-5 hours (although the cells can survive even longer without loss of titer). After this time, suck off supe and replace with 10 mls DME with 10% CS.

12. Harvest (stain, neo select, etc) these cells at 48 hours.

FREEZING THE CELLS:

1. We freeze the cells in a solution containing 90% FCS, 10% DMSO.

2. When thawing the cells, grow them initially in DME with 10% FCS. Once the cells are ready to split, place them in gpt selection media.

NOTES:

1. As you will quickly find out, it is not trivial to prepare the gpt selection media. My initial impression was that the BOSC 23 cells must be maintained in gpt selection media (see PNAS paper). In this media, the cell line has been able to consistently produce retroviral stocks with titers in excess of 2 x 10^6 for at least 30 passages.

Because I hate preparing the gpt selection media and the cells grow slowly and clump in this media, I have altered the way I carry the cells.

At present, I carry the BOSC 23 cells in the following way. I have multiple frozen vials. Each month, I unfreeze a vial which I maintain in DME with 10% FCS (and Pen/Strep, glutamine, and Fungizome). I obtain lacZ titers of at least 2 x 106/ml for at least 6 weeks. I am currently checking the long term effects of carrying this cell line without selection. The cells are much happier in the DME with 10% FCS and tend to grow faster and clump less. **Prior to splitting the cells for transfection, it is important that the cells are not overgrown. As mentioned above, the key to successful transfection and high titers is to have a large number of unclumped cells evenly spread on the dish.**

2. With regards to freeze/thawing retroviral stocks: I freeze the stocks immediately at -70. On the first thaw, I find that the titer is similar to fresh virus. After the first thaw, however, the titers drop MARKEDLY. As a result, I aliquot the stocks and throw them away after the first thaw.

3. We use MFG-lacZ as our standard for transfection and infection. This plasmid is a gift from Richard Mulligan's lab (Whitehead Institute, MIT). You should obtain a retroviral titer of at least 1-2 x 10^6 with this vector. You may have to play with the initial BOSC 23 densities to optimize the titer for your particular construct. Judging from the experience of people in this lab, it will take a little time to get used to working with the BOSC 23 cells. As mentioned above, cell densities are critical and it may take some practice to get his right. I suggest that you initially concentrate on the transfections and stain the cells for lacZ 48 hours after transfection. Once you are able to transfect at least 30% of the cells, everything else should work fine. For troubleshooting purposes, I suggest that working out the conditions using MFG-lacZ before trying your own constructs.

4. We prepare our plasmids using either double spun CsCl gradients or Quiagen columns. The 2 methods give roughly similar results. Once the DNA is resuspended in TE, I do not do anything special to it prior to transfection.

5. The cells which are sent are in DME with 10% fetal calf serum, pen/strep, and glutamine. I suggest growing these cells in this medium until they reach confluence and then freezing several vials. If you plan to grow them for several passages, you may wish to reselect in gpt selection medium.

6. The Bosc 23 cells are resistent to: G418, gpt, and hygromycin.

I would appreciate any comment on these protocols, especially regarding additions or deletions which I should make for future versions. My fax # is 212-327-8959. Good Luck.

Reagents*

Reagent	Final []	grams dry	
1) 2x HBS			
HEPES, pH 7.05	50mM	5.0	5.96
KCl	10 mM	0.37	.37g
dextrose	12 mM	1.0	1.0 + .18g.
NaCl	280 mM	8.0	
Na_2HPO_4 (FW 141.96)	1.5 mM	0.1065 g	.2cl g
		500 ml total, pH to 7.05	
2) $CaCl_2$			
$CaCl_2$	2 M	29.4 g/ 100 ml	

*stock solutions may be prepared and frozen at -20 C. I am not sure why, but the HBS goes bad after 6 months-1 year, even when stored in the freezer.

V_{total} (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5
2xHBS (ml)	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0	2.25
$CaCl_2$ (µl)	31	62	93	124	155	186	217	248	279
H_2O + DNA (µl)	219	438	657	876	1095	1314	1533	1752	1971

Hepes MW: $\frac{x}{\frac{238.3}{.5l}} = 50mM$

$NaPO_4$: $\frac{x}{\frac{268.07}{.5l}} = 1.5mM$

KCl: MW: $\frac{x}{\frac{74.55}{.5l}} = 10mM$

dextrose: $\frac{x}{\frac{180.16}{.5l}} = 12mM$

NaCl: $\frac{x}{\frac{5.8\ M}{.5l}} = 280mM$