Alpha-Complementation Fusion Assay

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Principle: Takes advantage of the ability of the lacZ gene to be separately expressed as two different fragments, producing two different peptides. These two peptides (the alpha and omega) subsequently assemble into a functional tetrameric β-galactosidase enzyme in the absence of new protein synthesis. The alpha fragment, which consists of the first 85 amino acids of β-gal, is expressed in one population of cells along with a viral envelope glycoprotein. The omega fragment, which consists of the remainder of β-gal is expressed in a separate target cell population along with the appropriate receptor(s) for the envelope. The two types of cells are mixed and allowed to fuse under various conditions, including different temperatures or pH, for different lengths of time, or in the absence or presence of one of a variety of fusion inhibitors. At the end of the incubation, the cells are lysed and the □-gal activity in each lysate is quantified, and used as a measure of the degree to which fusion of the cells took place.

Reagents:

GalactoStar Kit: Applied Biosystems, catalog #T1014, $387 for 1800 samples.
Preparation Racks for PCR Thin-Walled Tubes: Fisher, catalog #05-541-55, $214.20 for 20 racks.
Lipofectamine 2000: Invitrogen, catalog #11668-019, $334 for 1.5 mL.
200 µL 8 Thin Wall Tube with Hinged Caps: Phenix Research Products, catalog #MPC-628, $89.90 for a box of 125 strips.
96 tube trays, from various suppliers. Doc Frugal makes a good one.
Black 96-well plates: Costar, Part #3916.

Protocol

Day 1: Seed cells for transfection.

For cells that will express alpha and envelope, seed 2.5 x 10^6 293T cells per 10-cm tissue culture dish. Make enough plates for the experiment plus a few extras that often come in handy in case your envelope is a little toxic.

For target cells that will express omega and CD4 and coreceptor, *either:*
A. seed stable omega/CD4/coreceptor target cell line so that will have enough cells on day 4,
or:
B. seed target cell line of choice for transfection on Day 2 with omega and appropriate receptors.

Day 2: Transfect. I use lipofectamine 2000 (Invitrogen). Follow manufacturer's instructions, except reduce lipofectamine volume to 25 µL per 10-cm plate.

For alpha/envelope cells, transfect 7 µg each of the following three plasmids per 10-cm dish:
pSCTZ-alpha-N85
pRSV-Rev
envelope expression vector of choice
Note: if a certain envelope is toxic to cells, use less. For example, I only use 1 µg of pcDNA-VSV-G envelope per plate.

For omega/CD4/coreceptor cells, if don't have the appropriate stable omega cell line, transfect appropriate plasmids into target cells. For example,
10 µg pSCTZ-omega
5 µg pcDNA-CD4
200 ng pcDNA-CCR5
Note: very little CCR5 expression is needed, and using more makes the IC_{50} of any inhibitors go up.
Note: if your transfections don't add up to 20 µg and you want to make up the difference with an empty vector, beware of cryptic alpha expression. Many plasmids contain alpha sequence for the purposes of blue/white colony screening, and this often isn't indicated on the vector map. Good plasmids to use include:
pcDNA 3.1 (Invitrogen) (Don't use older versions like pcDNAI/amp)
pSV7d
pEGFP-N1 (Clonetech)

Day 3: Check cells. I often trypsinize cells on this day and transfer them to 15 cm dishes such that they will be nearly confluent on Day 4. I conserve transfection reagents by seeding the cells fairly densely for transfection, so often by day 3 they are confluent and need larger plates.

Day 4: Fusion Assay.

1. Remove media from plates and wash with 37° PBS.

2. Add warm PBS/5mM EDTA. Return to incubator for about 15 min. Can tap plates or pipet cells up and down to disperse clumps and encourage release from surface of plate.

3. When cells are released from plate and dispersed into a single-cell suspension, neutralize EDTA with an equal volume of complete medium.

4. Mix. Take an aliquot to a hemocytometer and count cells.

5. Spin 1250 rpm (314 x g) 5 min and aspirate medium.

6. Resuspend each cell pellet in fresh medium to a concentration of 5 x 10^6 cells/mL.

7. Aliquot 50 µL of one cell type into 96-well PCR plate or thin-walled PCR strip tubes. Start with whichever cell type is the target of any inhibitor to be added to the reaction. For example, if using a coreceptor inhibitor, start with the omega/CD4/coreceptor cell.

8. Add inhibitor, if using. (Often dissolved in DMSO; the reaction tolerates addition of a 2 µL volume of inhibitor in DMSO).

9. Incubate a few min if desired to allow to mix.

10. Add 50 µL of second cell type. In above example, this would now be the alpha/envelope cell. This gives a total of 5 x 10^5 cells per sample.

11. Cap tubes and invert to mix thoroughly.

12. Briefly spin to lightly pellet cells. I allow our tabletop Eppendorf centrifuge to accelerate to 1000 rpm (about 5-7 seconds) and then stop.

13. Incubate at desired temperature, usually 37°, for desired length of time. Float tubes in plastic PCR tube preparation racks in water baths, or else incubate in PCR machines. For time courses, collect samples at 30 min, 1 h, 2 h, and 5 h. For inhibition assays, incubate 4 h.

14. Spin samples 4000 rpm (3220 x g) for 1 min.

15. Aspirate medium and add 100 µL lysis buffer (comes with GalactoStar kit) to cell pellets. Pipet up and down in lysis buffer using multichannel pipettor, then incubate 5 min at rm temp.

16. Freeze lysates at –20°C until ready to assay.

17. Thaw lysates. At same time, bring GalactoStar buffer and substrate to room temperature.

18. Spin lysates 4000 rpm (3220 x g) for 5 minutes to pellet cellular debris.

19. Transfer 10 µL supernatant into a black 96-well plate.

20. Dilute GalactoStar substrate into its buffer at a 1:50 dilution. Add 100 µL of diluted substrate to each well.

21. Seal plate with a clear plate sealer.

22. Incubate at rm temp, in dark, for 60-90 min.

23. Read relative light units (cps). Use the same program as for luciferase expression.

Notes:

Steps 5, 12, 14, and 18: All spins are done in an Eppendorf tabletop centrifuge, model #5810-R with inserts for spinning plates.

Step 6: We have tried reducing the number of cells used per sample and then using more of the lysate in the end, but the data seems to be noisier. We're still working out the bugs.

Step 16: It would probably be better to freeze at –80°C for longer-term storage.