Notes

ALL SOLUTIONS AND EQUIPMENT THAT COME IN CONTACT WITH THE CELLS MUST BE STERILE.

BEFORE YOU START, PREHEAT: GROWTH MEDIA AT 37°C (WATER BATH).

Flask and plate volumes of media used:

75cm^2 - 10ml

175cm^2 - 25ml

500cm^2 - 90ml

Seeding adherent cell cultures from stocks

*Prepare culture flasks containing medium that the cells will be plated into before thawing the cells. E.g. add 9ml to a 75cm^2 flask, then add the 1ml resuspended stock.

1. Get the cell stock from liquid N_2 tank.
2. Thaw cell stock (in hands or 37°C water bath).
3. Spin the cells down at 1000g for 5min (Sorvall RT 6000B, ~2000rpm)
4. Remove supernatant from the vial
5. Resuspend the cell pellet in 1ml of growth medium
6. Add cells to a 75cm^2 culture flask containing 9ml of fresh growth media.
7. Gently rock the flask back and forth and in a rotational motion several times (~25 total oscillations is sufficient).

Example work flow for preparing large quantities of adherent cells

1. Seed cells as above
2. When cells at ≳70-95% confluent transfer the culture to a 175cm^2 flask - see **Splitting cells**, below
    1. typically ~2-3 days to reach confluence
    2. throughout this example, ~2-4 days should be sufficient to reach ~70-95% confluence at each step depending on the number of plates seeded from the resuspended cells.
    3. resuspend trypsinized cells in 10ml medium and transfer to a 175cm^2 flask with 15ml medium in it - as in **Making frozen stocks from adherent cell cultures,** below
    4. a second 175cm^2 flask may be prepared from the same 75cm^2 flask (split in half) for making stocks if needed.
        1. In this case, resuspend in 20ml and split to two 175cm^2 flasks as in 2.
        2. follow **Making frozen stocks from adherent cell cultures.**
3. From one 175cm^2 flask, split into 2-4x 175cm^2 flasks
    1. 20-40ml used to resuspend cells and transfer in 10ml volumes to 175cm^2 flasks with 15ml in them
    2. use 2 dishes if growing to 8x 500cm^2 dishes, or 4 if growing to 16x 500cm^2 plates.
4. From e.g. 2x 175cm^2 flasks split into 8x 500cm^2 plates
    1. Followed by **Tetracycline induction and Harvesting Cells**, below
    2. Once cells are at 70-90% confluence, remove the medium and replaced with 90ml fresh medium (DMEM, std. glutamine, FBS, P/S) with 5ng/ml doxycycline (a 5ug/ml stock made in DMEM from the 5mg/ml stock in 50% EtOH). Return plates to the incubator overnight (up to 24hrs, typically 16hrs).

Tetracycline / Doxycycline induction

1. For each cell line, grow cells in desired number of culture flasks (depending of different concentrations of antibiotic used for the induction)
    1. for Westerns 75cm^2 dishes may be used
    2. single, direct affinity capture, 175cm^2 dishes may be used
    3. for cryo-milling large amounts of material, 500cm^2 dishes (or **Suspension**, page 3) should be used
2. When culture reaches >70% confluence, change medium and add Tet with concentrations of your choice (including a 0 control)
3. Incubate O.N. before harvesting (4pm-10am)

Splitting cells

*Prepare new culture flasks containing medium that the released cells will be diluted into, either before beginning the dissociation, or while cells are incubating.

1. Remove and discard the spent culture media from the culture flas
2. Wash cells using 10ml of PBS. Add the solution against the side, so as not to disturb the cells. Gently rock the vessel back and forth several times.
3. Remove and discard the PBS.
4. Add 1ml of dissociation reagent (0.5% Trypsin w/ EDTA); use enough to cover the cell layer. Add the solution against the side, so as not to disturb the cells, and gently rock the flask to complete coverage of the cell layer.
5. Remove the dissociation reagent and place the culture flask back to incubator for approximately 5 min or till detached. You may also tap the flask to expedite the cell detachment.
6. Add 10ml of media and pipette the suspension up and down against the flask wall to disrupt cell clumps (10 times). Dilute cell suspension to seeding dilution; pipette appropriate volume of the cells into new flask containing fresh media.
7. Recommended dilutions for cell culture maintenance (e.g. maintaining 75cm^2 dishes): 1:5 - 1:10

Making frozen stocks from adherent cell cultures

1. Plate out 10^7 cells in a big bottle (175cm^2). A simple way to do this is to grow a 75cm^2 culture flask to 90%+ confluency, then trypsinize (see above, **Splitting Cells**) and transfer the cells in 10ml medium to a 175cm^2 with 15ml medium in it.
2. 2 days later (or whenever the cells reach 90-95% confluence):
    1. Aspirate the media
    2. Wash briefly in 10ml PBS. Aspirate
    3. Add 3-4ml trypsin and 'wash' briefly. Aspirate
    4. Put the flask (without media) into the 37°C – incubator for 5 min until the cells detach.
    5. Resuspend the cells in 10ml serum with 10% v:v DMSO (9ml serum, 1ml DMSO)
    6. Prepare 5 aliquots in 1.8ml cryo-tubes
    7. Put the cryo tubes in the cryo-container with isopropanol (or just wrap it in a paper towels/styrofoam box)
    8. Freeze at -80°C o/n
    9. After 1-7 days Transfer the tubes to the -140°C freezer (or N_2 tank)
    10. OR use a freezing container - we have this one: http://www.thermoscientific.com/en/product/mr-frosty-freezing-container.html

Harvesting cells

The simplest way for westerns and checks using 75cm^2 flasks

1. Seed cells on a dish/flask that allows easy access for scraping.
    1. Note these product for this purpose from MidSci :
        1. http://shop.midsci.com/scategory/M50/981 (flexible scrapers)
        2. http://shop.midsci.com/scategory/M50/985 (easy to scrape cells)
        3. http://shop.midsci.com/scategory/M50/987 (peel off resealable)
2. Remove media
3. Wash with 10ml PBS
4. Remove PBS
5. Add 500uL lysis buffer (e.g. 20mM HEPES pH7.4, 100mM NaCl, 0.5%v/v Triton X-100)
6. Scrape the cells off and pipette them up and down using a p1000
7. Sonicate the cells - 3 x 10 sec on setting 3 - cooling on ice in between (Misonix XL2020 Sonicator)
8. Spin them down (top speed, 10min, 4*C)
9. Do a Bradford assay if extracts need to be normalized for protein concentration
10. Load ~5uL of the extract on the gel (typically 5 - 10ug total protein per lane).

OR - More consistent for the same

1. After washing cells, add (e.g. 500ul PBS to a 75cm^2 flask) and scrape off cells
2. Transfer to a tube and spin down at 1000g, 5min - remove supernatant
3. Add lysis solution (e.g. 20mM HEPES pH7.4, 100mM NaCl, 0.5%v/v Triton X-100) and pipette up and down vigorously with a p1000
    1. note: this explores only what is soluble in this buffer -- for whole cell extraction use 1%+ w/v SDS and e.g. 0.5M Salt
4. Sonicate the cells - 3 x 10 sec on setting 3 - cooling on ice in between (Misonix XL2020 Sonicator)
5. Spin them down (top speed, 10min, 4*C)
6. Do a Bradford assay if extracts need to be normalized for protein concentration
    1. if SDS is present do a BCA assay
7. Load the extract on the gel (typically not to exceed 25ug of protein per lane)

When you grow the cells on the 500cm^2 dishes

1. Pour off the medium
2. Place the culture dish on ice in a large rectangular bucket
3. Add 20ml of 1x PBS and scrape off the cells using large Fisher scrapers (model number) and transfer the cells to a 50ml tube using the same 25ml pipette used to add the PBS
4. Add 10ml of 1x PBS to the plate (use a new 25ml pipette) and wash the remaining cells from the dish, and transfer using the same pipette to the 50ml tube.
5. Fill the 50ml tubes ~equally - 16 plates results in 10 x 50ml tubes
6. Spin 5min at ~1k RCF, 4*C (~2k RPM in Beckman Allegra 6R w/ GH 3.8 rotor or Sorvall T 6000D)
7. Resuspended each set of 5 tubes in 50ml 1x PBS (use a 25ml pipette set to low speed)
8. Spin 5min at 1k RCF, 4*C
9. Resuspended each pellet in 10ml 1x PBS (use a 25ml pipette set to low speed)
10. Transfer to a 20ml syringe with the opening capped, placed in a 50ml tube
11. Spin 5min at 1k RCF, 4*C
12. Drawn off the supernatant with the vaccum system until just the top layer of cells begin to be sucked up
13. Insert plunger and drip the cells directly into a 50ml tube filled with liquid N2
14. A larger container can be used, but materials should be transferred to capped 50ml tubes
15. Replace caps on tubes loosely - hold overnight at -80C, next day tighten caps fully-
16. Mill the cells

We are spinning down in a Sorvall H1000B rotor at 2000 RPM which = 834 RCF