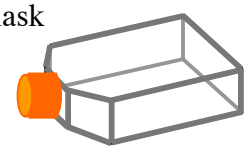
MAMMALIAN CELL CULTURE PROTOCOL

Some useful info:

1. # cells to inoculate and volume of medium
    - 96-well plate: 3000 cells/well; 100 μl
    - 24-well plate: 20,000 cells/well; 700 μl
    - 6-well plate: 100,000 cells/well; 2 ml
    - 15–cm plate: 1.77x10^6 cells/plate; 20 ml
2. Prepare final growth medium with FBS and glutamine for use fresh or within a week and avoid repeated heating at 37°C which accelerates the degradation of glutamine and growth factors in FBS. The same precaution should also apply to the trypsin solution used for cell detachment.
3. All cell culture media or wash should be treated with dilute bleach before discard into the sink.
4. All lab wares and gloves brought into the biosafety hood should be sterilized to minimize contamination. The outer surface of lab wares can be sterilized with 70% ethanol spray.

Primary Culture

1. Thaw quickly (1-2 min) the frozen stock culture (5 x 10^6 cells/ml) in warm water
2. To the 1 ml stock culture, add slowly 9 ml of [1]culture medium+10% fetal bovine serum (FBS) + 100 IU/ml pencillin-100 μg/ml streptomycin (1 ml stock per 100 ml medium) to 25 cm^2 flask
   [1] A549 lung cancer cell uses RPMI 1640 medium (+2 mM Gln & 0.2% glucose); MCF-7 breast cancer cell uses αMEM (modified Eagle medium) + 1 mM Na pyruvate (0.1 ml of 1M stock per 100 ml medium) + 0.1 μg/ml bovine insulin + 1 mM non-essential amino acids (1 ml stock, CellGro, Inc per 100 ml medium)
3. Centrifuge at 1,200xg for 5 min
4. Resuspend cell pellet in 1 ml of stock culture and add to 25 cm^2 (T-25, 25 cm^2) culture flask



5. Add additional 4 ml culture medium and incubate at 37°C, 5% CO^2
6. Change medium every two days for a total of 3-4 changes; check cells under phase-contrast optical microscope

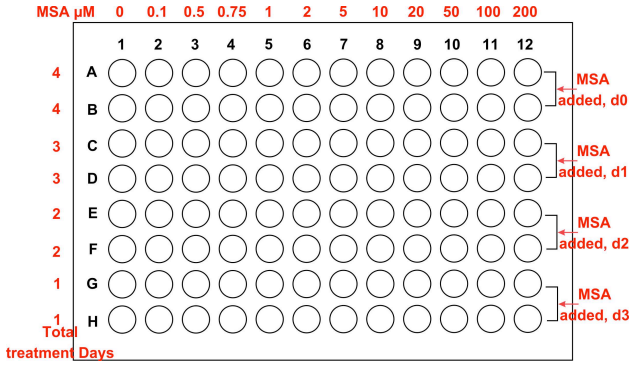
Secondary Culture

7. When cells become 70% confluent, remove medium, wash cells once with 11-2 mls 1X PBS (phosphate buffer saline) minus Ca and Mg
8. Add trypsin solution ([2]0.25% trypsin, Co??) to flask and incubate at 37°C, 5% CO^2 for 5 min
   [2] Use 0.05% trypsin for primary cells and 0.25% for A549 cancer cells; use 0.7 ml to cover T-25 flask bottom and 1 ml for T-75 (75 cm^2) flask

9. Bang on desk to loosen cells from bottom and check under microscope.
10. Add same volume of culture medium to inactivate trypsin
11. Transfer the solution to 15 ml centrifuge tube and spin at 1200 rpm for 7 min or 10-12 min for 50 ml centrifuge tube @ 4 degrees C.
12. Remove medium and add 1 ml medium to the cell pellet and resuspend cells using 1ml pipetter[3]
    [3] Aspirate slowly but expel quickly during resuspension
13. Pipette 10 μl of cell suspension onto the Hemacytometer counting chamber with cover slip[4]
    [4] Wet the edges of the chamber with a small amount of water using gloved finger tip to help seal the cover slip
14. Count 5 x 5 squares in the center of the chamber. The cell no. in the suspension should be the cell counts x 10^4
15. Add sufficient volume of the stock suspension to 10 ml medium in T-75 (75 cm^2) flask to make the cell density $10^4/cm^2$.
16. Incubate at 37°C, 5% CO^2 until cells reach 70% confluent

Treatment within 96 well plate for cell proliferation assay

1. Trypsinize the attached cells as in steps 9-13.
2. Count cell number as in steps 14-15.
3. Make a dilution of the cell suspension in medium to seed 3,000 to 5000 cells per well in a volume of 100 μl and add to each cell of a plate (A total of 11 ml @ 30-50,000 cell/ml is sufficient for one plate).
4. Incubate at 37°C, 5% CO_2 for 24 hours. Check plate for cell adhesion.
5. Synchronize cells by replacing media with 0.5% BSA in RPMI medium with antibiotics and no FBS.
6. Incubate at 37°C, 5% CO^2 for 24 hours.
7. Replace media with standard RPMI medium augmented with treatment substance (e.g. methylseleninic acid or MSA) according the the example scheme below.



8. Incubate at 37°C, 5% CO^2 for up to a total of 96 hours and replace treatment media daily.
9. Cell proliferation assay (e.g. MTT test) is performed at the end of the treatment. Replace all wells with 100 μl fresh control medium; add 15 μl MTT reagent and incubate at 37°C/5% CO_2 for 1-4 hr, depending on cell density (for A549 and PC14PE6, 1 hr is adequate); add 100 μl solubilization reagent and incubate at 37°C/5% CO_2 for 1-2 hr;

shake plate in the microplate reader for at least 1 min to dissolve residual dye at well bottom before reading at 570 nm

10. Well contents should be discarded in waste bottle for organic waste pick up.

Cell cryopreservation

17. Trypsinize the attached cells as in step 10-12
18. Prepare cell suspension as in step 13-15
19. Count cell no as in step 16-17 to determine the cell density in the stock solution
20. Dilute the stock solution with medium to 3-5 x 10^6/ml density
21. Transfer 0.8 ml of 3-5 x 10^6/ml cell stock into 2 ml cryovials and add 0.1 ml FBS* and 0.1 ml DMSO
22. Mix the vial gently by inverting the vial several times
23. Store at –80°C for 24 h or longer, after which the vials can be transferred to liquid N_2 storage tank

* May get better cell recovery using final 50% FBS for cryopreservation

Selenium Treatment

24. Trypsinize the attached cells as in step 10-12
25. Prepare cell suspension as in step 13-15
26. Count cell no as in step 16-17 to determine the cell density in the stock solution
27. Plate 10^4 cells/cm^2 into T-75 flask containing 10 ml culture medium and incubate at 37°C, 5% CO_2 for 20-24 hr
28. Add appropriate amount of Se stock solution[5]

    [5] Prepare sodium selenite, selenomethionine (Se-Met), and methylseleninic acid (MSA) stock from standards stored at –80°C and filter the stock solution through 0.22 μm sterile syringe filter (with aqueous compatibility, e.g. polysulfone or nitrocellulose)
29. Incubate at 37°C, 5% CO_2 for 24-96 hr and check for cell morphology using phase contrast inverted optical microscope (Olympus)

Immunocytostaining

30. Use cells grown to 70% confluent
31. Trypsinize the attached cells as in step 10-12
32. Prepare cell suspension as in step 13-15
33. Count cell no as in step 16 to determine the cell density in the stock solution
34. Plate cells at 10^4/cm^2 (total 10^5 cells/well) onto a 6-well microplate (11 cm^2 per well) containing a 18 mm cover slip in each well [6]
    [6] Sterilize cover slip by washing in 100% ethanol, irradiated under UV for 15 min, flamed quicky to dry, or followed by autoclaving at 121°C/15 psi for 20 min in a beaker
35. Add cells on the cover slip before adding 2 ml medum to the side of each well and incubate at 37°C, 5% CO_2 for 22-24 hr
36. Add appropriate amount of Se to each well and incubate at 37°C, 5% CO_2 for 48 hr
37. Remove medium, wash cells briefly with 1X PBS miuns Ca and Mg to remove FBS
38. Fix cells with 1 ml 3.7% paraformaldehyde for 15 min at 37°C, 5% CO_2 or RT to maintain cell shape during fixation [7]
    [7] Dissolve 3.7 g paraformaldehyde in 100 ml PBS minus Ca and Mg (Ca promotes cadherin function, Mg promotes cadherin and integrin functions), adjust pH to 7.5, store at 4°C, and warm to room temperature before use.
39. Remove paraformaldehyde solution and wash cells 3 times with 2ml each 1X PBS minus Ca and Mg [8]
    [8] Pipette the wash solution up and down for at least 10 time for each wash
40. Permeabilize the fixed cells in 0.2% Triton X-100 in PBS for 3 min at room temperature
41. Remove Triton X-100 and wash cells 3 times as in step 41
42. Prepare a 10 cm petridish containg an upsidedown 3.5 cm small petridish surrounded by a piece of wetted kimwipe, covered with an aluminum foiled lid, and left at room temperature to create a humid chamber
43. Place a piece of parafilm on top of the 3.5 cm petridish in the humid chamber and add 20 μl blocking solution (1% bovine serum albumin or BSA + 10% normal serum IgG (e.g. Santa Cruz Biotech in 1x PBS-Ca-Mg) [9]
    [9] IgG should be from the same source as the primary antibody; Store the blocking solution at 4°C for up to one month
44. Place the cover slip (with cell side facing down) onto the block solution on the parafilm, eliminate bubbles, and incubate at room temperature for 15 min
45. Place the cover slip onto 20 μl primary antibody solution [10] in another humid chamber as in step 45, and incubate at 37°C for 1 hr or 4°C overnight (the latter may give better result)
    [10] Dilute Western antibody in 1% BSA/1X PBS-Ca-Mg with dilution factors ranging from 20-100X which should be tested; for antibody designated for immunostaining, use 1:400 dilution or as vendor's recommendation; rabbit anti-c-Myc (Epitomics): 1:250
46. Transfer the cover slip to a 6-well microplate with the cell side facing up and wash extensively [11] (min. 3 times 5 min each) with 2-3 ml each 1X PBS-Ca-Mg to remove the antibody
    [11] Pipette up and down rigorously using a disposable transfer pipette during wash
47. Repeat step 45 using fluorescent-labeled secondary antibody[12] for 1 hr at 37°C

[12] Use1:500 dilution from stock (2 mg/ml) in 1X PBS/1% BSA; if 2nd antibody is of goat origin, include goat IgG in the blocking solution in Step 45, if it is of rabbit origin, include rabbit IgG in the blocking solution

48. Transfer cover slip to a 6-well microplate with the cell side facing up and wash extensively (min. 10 times 3 min each) with 2-3 ml each 1X PBS under reduced light e.g. in the biosafety hood without light [12]

[11] Pipette up and down rigorously using a disposable transfer pipette during wash

49. Stained with DAPI at 0.5 μg/ml in PBS for 20 min at RT & wash 3 times with 1X PBS
50. Clean a slide with kimwipe, place 20 μl 1X PBS onto the slide, and place the cover slip (with cell side facing down) onto the PBS droplet without bubbles.
51. Soak up excess PBS with kimwipe around the cover slide
52. Seal the cover slip edge with nail polish and let dry
53. Wipe clean the surface of cover slip (opposite to the cell side) with H2O in tissue paper
54. Store the slide (covered with wetted kimpwipe) in a 10 cm petridish covered with aluminum foil at 4°C [13]

[13] The stain may last 1-2 months under this condition

For H2-DCFDA staining:

1. Wash cells in PBS and then incubate cells on cover slip in 20 μl 10 μM H2-DCFDA (Molecular Probes) in PBS at room temperature or 37°C/5% CO_2 for 20 min for dye uptake

For 6-well plates, use 1 ml H2-DCFDA

2. Incubate cover slip in 20 μl medium or change to 3 ml fresh medium in 6-well plate and incubate 37°C/5% CO_2 for 30 min to allow dye to hydrolyze.
3. Wash cover slip in PBS and mount it (cell side faced down) onto slide preloaded with 20 μl PBS *

For microplate quantification, add treatment (e.g. 0.5 ppm Na2SeO3) & incubate at 37°C, at appropriate time points, trypsinize cells, wash in PBS twice, resuspend in PBS (e.g. 170-200 µl), count cells and measure the rest of cell suspension at Ex 495 nm, Em 527 nm, filter cutoff 515 nm in an M5 microplate reader w/ 10 sec shaking

4. Seal slide edge with finger nail polish
5. Wipe clean the surface of cover slip with H_2O in tissue paper or Q-tip
    - Do not fix cells

For DAPI staining

1. Fix and permeabilize cells as described above
2. Stained with DAPI at 0.5 μg/ml in PBS for 20 min at RT
3. Wash cover slip extensively in PBS

For Hoechst 33342 and MitoTracker Red CMXRos and phalloidin-Alexa Fluor 488 staining

1. Incubate cover slip in Hoechst (10 μg/ml PBS) at 37°C for 20-30 min
2. Followed by incubation in MitoTracker Red (25-100 nM) for 30 min-1 hr at 37°C
3. Fix in 3.7% paraformaldehyde in PBS for 15 min at RT or 37°C
4. Wash extensively (at least 6 x 5 min) in PBS with shaking to remove free dye
5. Permeabilize cells in 0.1-0.2% Triton X-100 in PBS for 3-5 min
6. Block cover slip in (10% normal goat serum, optional)+1% BSA in PBS for 20 min at RT

7. Incubate in Alexa Fluor 488-conjugated phalloidin (5 μl 0.2U/μl in 100 μl 1% BSA in PBS) for 1 hr at 37°C or RT

Phalloidin stock solution: 600 U/ml methanol; working stock solution: 200U/ml methanol; use 20 µl @ 10U/ml in 1% BSA

8. Wash 3x in 2 ml PBS before mounting on the slide
9. Mount cover slip (cell side faced down) onto slide preloaded with 20 μl Anti-fade solution (Invitrogen) and allow it to dry overnight in the dark
10. Seal slide edge with finger nail polish
11. Wipe clean the surface of cover slip with H_2O in tissue paper or Q-tip

Slide transport method from Gary Cherr: I am certainly happy to take a look if you send me the slides. I assume they are mounted in some stable manner. If not, you can ship cold with a glycerol (90% glycerol, 10% PBS) mounting medium with a coverslip on top. We usually use nail polish to make sure the coverslip does not slide off. I know there are more permanent mounting media available from Mol. Probes.

Immunofluorescence Cell Staining protocol from Santa Cruz Biotech

- Prepare slides as described above for immunoperoxidase staining, omitting the final step involving treatment of cells with H2O2.
- Use suction to remove reagents after each step, but avoid drying of specimens between steps. Use sufficient reagent to cover the specimen (approximately 100–500 μl per slide is adequate).
- Incubate specimens with 10% normal blocking serum (Normal Sera for Immunohistochemistry) in PBS (Buffers and General Solutions) for 20 minutes to suppress non-specific binding of IgG. Blocking serum ideally should be derived from the same species in which the secondary antibody is raised. Wash with PBS.
- Incubate with primary antibody for 60 minutes. Optimal antibody concentration should be determined by titration; recommended range is 0.5–5.0 μg/ml in PBS with 1.5% normal blocking serum. Wash with three changes of PBS for 5 minutes each.
- Incubate for 45 minutes with either biotin-conjugated or fluorochrome-conjugated secondary antibody (Secondary Antibodies for Immunohistochemistry) diluted to 1–5 μg/ml in PBS with 1.5%–3% normal blocking serum. Optimal antibody concentration should be determined by titration. Wash with three changes of PBS. If fluorochrome-conjugated secondary antibody is used, incubate in a dark chamber and omit the next step.
- Incubate with streptavidin-fluorescein for 15 minutes in a dark chamber. Optimal streptavidin conjugate concentration for a given application should be determined by titration; recommended range is 10–20 μg/ml in PBS. Wash extensively with PBS.
- Mount coverslip with aqueous mounting medium or 90% glycerol in PBS.
- Examine using a fluorescence microscope with appropriate filters. Store slides in a dark location at room temperature (UltraCruz™ Mounting Medium: sc-24941) or at 4° C (glycerol/PBS mount).

Preservation of cells for microscopy

Hi Jagan,
The best way to preserve the slides is fixed the cells in -20C cold methanol:acetone (1:1) for 3 min. at RT..or 15 min in 3.7% paraformaldehyde. Wash in PBS 3 times and put to the fridge . They can stay there couple weeks.
If you have any question please let me know.
Laura