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Protocol for culturing of Osteosarcoma cell line: NCTC clone 2472 (s.c connective tissue, areolar, adipose 82 day old C3H/HeN mouse; morphology fibroblasts)[1]

Culture flasks: 75cm^2 Corning treated tissue culture flasks (Corning 430641, 0.2 micros Vent Cap)
Growth characteristics: adherent
Split ratio: a subcultivation ratio of 1:2 or 1:5 is recommended.
Recommended fluid renewal 3 times per week; pH 7.3

I. Thawing Frozen Sample

A. Preparation of culture medium, NCTC-135 medium (Sigma N3262):
   1. Add powdered medium in 900 ml of sterile ddH_2O at RT while stirring. Do not heat.
   2. Add 2.2 g sodium bicarbonate of 29.3ml of sodium bicarbonate solution (7.5%w/v) per liter.
   3. Adjust pH to 0.1-0.3 units below 7.3 since it may rise during filtration. (initial pH ≅ 7.6)
   4. Add 100 ml of normal horse serum.
   5. Antibiotics not recommended, but if needed: 50-100 units per ml or 50-100 ug per ml of penicillin G
   6. Filter using 0.22-micron membrane.

B. Warm culture medium to 37^{o}C.
C. Hold and gently agitate ampoule in 37^{o}C water bath. Keep O-ring and cap out of water. Thaw for 2 minutes.
D. Remove ampoule as soon as contents are thawed. Spray ampoule with 70% EtOH and place in culture hood for all manipulations.
E. Nick the neck of the ampoule with a sterile file and snap top off with sterile paper towel.
F. Transfer contents to culture vessel and immediately add 10-15ml of warmed medium.
G. Replace cap and incubate for 24 hours.

II. Cell Maintenance

A. Replace medium with fresh medium 3 times per week.

B. Splitting adherent cells using cell scraper
   1. Aspirate medium from the cell monolayer.
   2. Add 12 ml of fresh medium, and scrape flask with a cell scraper. Check under the inverted scope for suspended cells.
   3. Add an additional 12 ml of medium, and pipette vigorously to break cell clumps. Check under the inverted scope for a single cell suspension or pipette until so.
   4. Aspirate 12 ml to place in a new flask.
   5. Remove 1 ml to a microfuge tube for cell counts.
   6. Calculate proper dilution to achieve recommended seeding concentration.
      a. *Ex: If you have 10^6 cells/ml and the desired seeding concentration is 10^5, you want a 1:10 dilution. Thus, add 1 ml of cell suspension to 9 ml of culture medium.*
   7. Rotate flask gently to be sure cells are dispersed evenly.
   8. Place cells in incubator, making sure cap is loose.

C. Splitting adherent cells using 0.25% trypsin-EDTA
   1. Aspirate medium from the cell monolayer.
   2. Add 4 ml of Ca^{2+}, Mg^{2+}-free PBS with EDTA for 30 sec and aspirate from cell monolayer.
   3. Add 4 ml of 0.25% trypsin-EDTA and incubate at RT until cells detach (1-5 min).
   4. Add 8 ml of fresh medium and break cell clumps by vigorously mixing solution with a pipet.
   5. Transfer cells to a centrifuge tube and centrifuge at 1000 rpm for 2.5 min.
   6. Remove supernatant, replace with fresh medium, and resuspend cells.
   7. Transfer cells to desirable number of flasks.

D. Microscopic count of viable cells
   1. Remove 200ul of cells and medium to a microfuge tube. Use 100ul if sample volume is low.
   2. Mix 50ul of the cells and 50ul of the trypan blue solution in a microfuge tube. Allow 5 min for trypan blue to stain.
   3. Place coverslip evenly in the middle of the hemocytometer.
   4. Remove 20ul from the cell/trypan blue mix. Add 20ul to each side of the coverslip by allowing a drop held at the end of the tip to be taken under the slide by capillary action.
   5. Place the hemocytometer on the stage of the inverted microscope. Count viable and dead cells in any one of the 1-mm-square areas, 25 boxes worth, at high magnification. Ideally, you should have between 30 and 300 cells/mm. If you have more, make a 1:5 or 1:10 dilution.
   6. Count cells in a total of three different 1-mm squares, and divide by three to get an average.
   7. Calculate the number of cells per ml.
      a. *Ex: You have counts of 113, 99, and 118, with an average of 110 cells in 0.1 mm^3.*
      1,100 cells/mm^3 x 1,000 mm^3/ml = 1.1 x 10^6 cells/ml
      We diluted 1:2 with trypan blue: 2 x 1.1 x 10^6 cells/ml = 2.2 x 10^6 cells/ml in original culture

[1] The above cell line (ATCC cat. # CCL-11) will be supplied as a frozen inoculum of 1.7 X 10^6 cells. The freeze medium contains culture medium, DMSO 5%. The cells have a viability of 79-84%.