# *TISSUE CELL CULTURE*

## I. GENERAL INFORMATION

### A. Media

Heavily used media are being prepared in the laboratory rather than being purchased from the Medical Center Central Facilities; these currently include DME, RPMI 1640, and Joklik's S-MEM. The powder is purchased from GibcoBRL and is stored at 4°C.

Tissue culture bottles should never be put through the regular glassware washing procedure to avoid the possibility of detergent residue. Instead, used bottles should be rinsed out several times with tap water and stored; then 20 bottles should be washed in automatic washer without detergent and autoclaved prior to media preparation. Equipment required to prepare media are: a 20 L vessel and large stir bar (rinsed in distilled water before use), a Millipore pump and long piece of tubing, a magnetic stir plate, a ring stand with large clamp, and a sterile Millipore Millipak 40 filter. These items except for the filter should be allowed to stand in the hood under the germicidal lamp for 15-30 minutes prior to starting the media preparation. The vessel should be filled with 9.5 liters of double distilled water and the powdered media added slowly with stirring to prevent clumping. When the media is completely dissolved, sodium bicarbonate is added in the amount specified on the media package. When bicarb is dissolved, conc. HCl is added until the medium color looks correct. *For DME, the color should be red-orange; this requires the addition of approximately 2.6 ml of conc. HCl per 10 liters of media*. The color has to be checked in media which has passed through the filter as filtration causes the pH to rise. *For RPMI 1640, the correct pH corresponds to a salmon- orange color. The Joklik's S-MEM does not need to be pH adjusted*. Therefore, filter the pH-adjusted medium into a flask and examine the color. If the pH appears correct, empty the contents back into vessel, adjust the volume to 10 liters with water using the volume marks on the yellow tape, and start filling the bottles. The solution should be stirred at low speed during the entire filtration process.

The filter has a vent to allow air to be expelled when filtration is initiated. When the pump is turned on and media starts to fill the filter, the vent cap should be loose and the filter tilted so that trapped air can be expelled through the vent. When all air is expelled, tighten the cap so that medium will now be forced through the filter. Make sure that any unfiltered medium which may drip down the filter unit during this process is wiped away so that it does not enter the bottles. The inside surface of the bell portion of the filter unit must remain sterile.

The pH of the medium may rise during the filling of the bottles and a few drops of HCl may need to be added, especially toward the end of the process. Remember that the color needs to be checked when the medium in the tubing prior to adjustment has been pumped through the filter.

After all of the bottles are filled, sterile test at least 5 bottles filled at various times throughout the process. Make sure that the last bottle filled is sterile tested. To sterile test, transfer 2.5 ml to a 35 mm dish, using a separate pipet for each bottle. Label the bottles which are tested so that they can be identified if necessary. The dishes should be incubated at 37°C for at least 5 days and then checked for any turbidity.

The tubing should be cleaned by pumping 500 ml of water through it. New filters can be cleaned and autoclaved for one additional use. 500 ml of water should be pumped through and then the liquid above the filter removed by emptying it through the vent or by pumping air through the tubing until the liquid above the filter is just pumped through. Air must not be pumped through

the filter as this will damage it and compromise its effectiveness. The filter, with the vent loosened, can now be wrapped in foil and autoclaved. The vessel should be thoroughly rinsed with water - no detergent should be used.
It is a good idea to retighten the caps on the bottles after they have cooled to 4°C to prevent loss of bicarb and consequent alkalinization.

Media obtained from Central Facilities, although supposedly sterile tested, has frequently been found to be contaminated. Check any bottles for visible cloudiness before using. If critical experiments are being done, or if the cells are precious, sterile test the particular bottle before using.

B. Serum

If possible, serum should be purchased in large amounts, e.g., 20 x 500 ml bottles and stored frozen at -20°C. Reliable companies from which to purchase serum are GibcoBRL and Hazelton (Lexena, KS). Prices vary depending on the supply of cattle, etc. These companies will send out 100 ml samples which you can test while they hold the desired number of bottles of the same lot on reserve. The sample should be tested on all cell lines currently in use, especially if they are finicky in their growth. It is best to plate them at low density in the test serum and compare their growth to that in the serum currently in use in the lab. If any assays are being done which require clonal growth, then the serum should be tested in that assay as well. The ability to support clonal growth is the most stringent test of serum quality.

In general, cell lines will grow more rapidly in fetal bovine serum than in calf serum, and many cell lines show a requirement for FBS. Since FBS is more expensive than calf serum, the latter should be used if the cells are happy in it, e.g. Hela cells do well in calf serum. In general, people tend to grow a particular cell line in medium described for that cell line in the literature; however, that medium is not necessarily optimum. Serum obtained from the companies cited above is always sterile and mycoplasma free. Once the serum is thawed, it is a good idea to aliquot it into 2 or 3 bottles and refreeze all but one aliquot. That way, if someone contaminates one aliquot, you don't lose the whole bottle. However, serum can be stored at 4°C for several months without any loss of activity.

Some media calls for heat inactivated serum. This is serum which has been heated to 56°C for 30 minutes to inactivate complement.

Dialyzed serum is obtained by several techniques. Serum is placed inside dialysis tubing and can be equilibrated against 2-3 changes of 100 x volume of water or isotonic salt over a two-day period. This should be done in the cold. After dialysis, serum has to be resterilized, preferably by pressure rather than by vacuum filtration. If vacuum filtration is being use, the filter will have to be changed frequently.

Stripped serum is treated with charcoal to remove steroids. Each batch of serum has widely varying amounts of steroids. To be certain that stripping has been effective, additional amounts of labeled steroids can be used as a monitor. The label should be added that day before stripping to allow protein binding to occur. Removing steroids from sera is usually accomplished by adding 2% (weight/volume) charcoal (Norit, J.T. Baker Co.) or equivalent to sera and heating to 55°C for 1 hour. In some protocols the use of dextran coated charcoal is substituted for charcoal alone. The Norit is then removed by centrifugation and the serum is sterilized by gentle filtration through a 0.22 μm Millipore filter. Several repeat charcoal treatments may be

necessary. This treatment also removes other low molecular weight compounds from the serum such as free fatty acids which are growth promoters for some cell types.

C. Antibiotics

Ideally antibiotics should be avoided in the medium. However, antibiotics are used by most tissue culture laboratories. Penicillin (50-100 U/ml) and streptomycin (50-100 μg/ml) (GibcoBRL) are routinely added to the media immediately prior to use without apparent deleterious effects. Gentamycin sulfate is more stable at 37°C than pen-strep. Central Facilities supplies it in tubes at a concentration of 50 mg/ml. The tubes can be stored at 4°C. Use 0.5 ml per 500 ml bottle.

D. Sterilizing Solutions

Many filter devices provide an alternative to heat sterilization which denatures proteins. A filter of 0.22 μm removes most bacteria. Protein solutions such as serum or enzymes are best filtered by positive pressure rather than vacuum. Vacuum filtering causes foaming indicative of protein denaturation.

Small volumes can be sterilized by passage through reusable 0.20-0.22 mm filter adapters on syringes or by a variety of disposable commercial tools. Syringe adapters for small volumes are excellent as long as no back pressure is placed on the filter. Vacuum sterilization can be accomplished with set-ups that hold from 150-500 ml. We currently use Uniflo (Schleicher & Schuell) units for syringes and Nalgene 115 ml and 500 ml capacity units for small volumes.

E. Cell Lines

Many certified cell lines are available through the American Type Culture Collection, Rockville, Maryland, and the Human Genetic Repository in Camden, New Jersey. The fee is nominal to acquire the cell lines. Each collection has stipulations on distribution and some hazardous lines require signing a form on the method of handling in the receiving laboratory. Commercial suppliers also handle some primary established cell lines.

Once a cell line has been used in a publication it should be available upon request. Shipping is usually more successful for a line cultured in a closed flask completely full of medium. All collections obtain their material from scientific donations. They will certify your cell line and make it available to other scientists so that you do not have to expend time in growing cells and dealing with shipping requirements.

When a new cell line is obtained, either from ATCC or from another investigator, it should be immediately expanded and frozen in liquid nitrogen in at least 8 vials. When the vials have been depleted to 2 or 3 remaining, a vial should be thawed and immediately expanded again for freezing. It is NOT a good idea to carry cells in culture for longer than a month or two. Many cell lines show a high mutation rate under normal culture conditions, being inherently genetically unstable. This is especially true of many tumor-derived cell lines. Therefore, after a month or two in culture, fresh cultures should be started from the frozen stock.

If you are carrying several lines in culture at once, it is imperative to avoid cross-contamination. If you are using the same bottle of media for several cell lines, never go into that bottle with a pipet which has been used to add media to a plate of cells. The same holds true for

any other solution being used in cell culture, e.g., PBS or trypsin. Fresh pipets should always be used except when adding the solution to empty plates.

F. Viability Stains

These stains are necessary to determine the proportion of live cells in a population following certain manipulations or treatments. A cell that is metabolically alive has a cell membrane that is active and selective in the materials that it will let pass into the cytoplasm. The membrane of a dead cell has lost this selective capacity and many materials readily pass into the cell. Certain dyes qualify for this selective exclusion by a live cell while a dead cell is rapidly stained. The dyes Eosin Y, erthrosine B, and trypan blue are used, the latter being the most common.
The Sigma catalog has a procedure for the use of their trypan blue stain in the tissue culture section. Living cells will appear refactile and colorless, while dead cells will stain blue.

II. EQUIPMENT

A. Hood

Before going into the hood, turn on the blower and germicidal lamp for 15 minutes. Wipe down the working surface with $dH_2O$, then EtOH. Wash all bottles to be put into the hood with 70% EtOH. When finished, wipe down working surface with $dH_2O$, then EtOH. Turn off blower and lamp. It is not necessary to turn on the germicidal lamp afterwards since it is always turned on for 15 minutes prior to hood use. The only exception is that after working with viruses the lamp should be left on.

B. Incubators

The incubators are kept at 37°C and 5% $CO_2$. The atmosphere is kept humidified by placing a pan of $dH_2O$ containing a little Roccal in the bottom. The incubator should be checked periodically for water level and $CO_2$ pressure. Temperature and $CO_2$ levels are regulated by the incubator itself and do not require attention unless their alarms sound. The best way to prevent mold growth in the incubator is to constantly monitor the contents for contaminated cultures. In the event that a contaminated culture is found in the incubator, all of the shelves within the incubator should be removed and washed with Roccal followed by EtOH. The interior of the incubator should be washed in the same manner. If mold growth becomes a persistent problem, the shelves and supports (sides and top) will have to be removed and autoclaved.

C. Waterbaths

The waterbath is maintained at 37°C and filled only with distilled water plus a bit of Roccal. This bath needs to be cleaned frequently to eliminate fungal material.

D. Microscope

The only maintenance which should be required on the scope is bulb replacement and phase

adjustment. The bulb is a 6V, 15W, P 15d, Bt 77Z. As of 2/92, 3 spare bulbs were on hand. To readjust the phase, you need to use the phase telescope in order to align the phase rings. Replace one of the oculars with the telescope. Open the iris almost all the way. Hold the body of the telescope with one hand and slowly pull out the black end piece until the phase rings are in focus. Now tighten or loosen the 2 phase adjustment screws until the two phase rings are exactly superimposed. If adjusting the screws cannot align the rings, it may be necessary to slightly move the entire phase ring assembly, using the black rod.

E. Pipet-aid

The rubber inserts in the pipet handle require periodic replacement, as they become worn from the constant insertion and removal of pipets. This is one of the major causes of pipet leaking. As of 2/92, several spares were on hand. They are available from Becton-Dickinson. These inserts tend to become constantly plugged with cotton, which needs to be removed to eliminate leaking. Occasionally a rubber diaphragm that is part of the pumping mechanism needs to be replaced. These can be purchased at an aquarium store since they are also used in aquarium pumps. Replacement is not a difficult operation. B/D has a technical service dep't. which can be contacted in the event of malfunction of the unit. They can often diagnose the problem over the phone and send an appropriate replacement part.

## III. SUPPLIES

### A. PBS

To prepare 20X PBS:

per liter
4 g KCl
4 g $KH_2PO_4$
23 g $Na_2PO_4$
160 g NaCl
stir to dissolve. pH adjustment is not necessary. Autoclave. Store at room temp. On long standing, material will settle out of solution and warming will be necessary to redissolve. To prepare 1 x PBS containing $Ca^{++}$ and $Mg^{++}$, use sterile 1.0% stocks of $CaCl_2$ and $MgCl_2$ and add gradually with stirring to PBS to give final concentrations of each of .1 g per liter of 1x PBS. This protocol is necessary to prevent formation of calcium phosphate precipitates.

### B. Trypsin

Trypsin comes as a 10x stock from the Cancer Center. Store frozen. Dilute the sterile stock 1:10 with sterile PBS and store at 4°C. The 10x stock should not sit out at room temp. any longer than necessary to minimize autoproteolysis. Take an aliquot and refreeze immediately.

### C. Freezing Media

In general, cells freeze well in 70% media, 20% serum, 10% DMSO. Add DMSO last to this mix. This can be made up fresh each time or made in a larger batch and stored frozen. If a particular line shows poor viability when frozen in this mix, try increasing the % serum.

## IV. MAINTAINING CELLS

### A. Passaging Cells

Cells should be passaged just prior to or soon after they reach confluence. If cells have to be held at confluence for some reason, they should be fed frequently. Transformed cells will pile up into multilayered foci when held at confluence. Often you will get cell sloughing and death if the cells are not passaged soon enough.

Media and trypsin should be prewarmed prior to using it on cells. To trypsinze attached cells, remove media with aspirator. Wash cells with 1 x PBS. Remove wash. Add 2 ml of trypsin for a 75 $cm^2$ flask, 1 ml for a p100 plate or 0.5-0.75 ml for a 25 $cm^2$ flask or p60 plate. Trypsinization time varies with the cell line; usually 4-5 min at 37°C is sufficient. For a new cell line, the optimum time has to be determined experimentally. Quench the trypsin with at least 5 volumes of serum-containing media. Break up the cell clumps by pipetting up and down 8-10 times. Plate the desired number of cells in fresh media. Most cell lines, e.g., HeLa and 293 cells can be split as low as 1:20. There are some cell lines which don't tolerate this high of a dilution. If working with a new cell line, try several dilutions during the first passaging to see how the line behaves.

### B. Freezing Cells

Trypsinize cells (unless working with suspension cells), pellet in clinical centrifuge at low speed (setting of 4 on IEC clinical) for 3 min. Aspirate media, resuspend cells in freezing media by pipetting up and down a few times, transfer 0.5-1.0 ml to freezing vial, and place in freezing tray for at least 6 hours, preferably overnite. Freeze 3-5 x $10^6$ cells per vial. Then transfer vial to cane. If you have a lot of vials in the freezing tray, they can be held on dry ice while you are transferring them to canes. Do not allow them to thaw during this process.

### C. Thawing cells

Have dish with medium ready. Thaw vial rapidly at 37°C. Use a pasteur pipet to transfer thawed cells to plate as soon as contents of vial have thawed. For adherent cells, change media after cells have attached (usually the next day). If the cells are viable, you should see them start to attach within an hour after thawing. If you are thawing suspension cells, transfer thawed cells to 15ml tube containing 10 ml of your media. Pellet cells briefly, aspirate media, resuspend in fresh media (warm) and transfer to dish. Note that for suspension cells, non-viable cells will only be eliminated after several passages. For adherent cell lines, the dead cells can be aspirated away.

### D. Counting cells in hemacytometer

Take up a small aliquot of a well suspended cell suspension in a pasteur pipet, touch the tip of the pipet to the junction between the hemacytometer and the cover glass, and gently eject so that liquid flows under the cover glass and fills the chamber without overflowing out the back. The chamber is divided into 9 large squares, each of which is subdivided into 16 or 25 small squares. Count the number of cells in 1 large square; if there are less than 100, count additional squares and divide the total by the number of large squares counted. Count cells which are touching the top and left hand borders, but not the bottom and right hand borders. Multiplying the # of cells per large square by $1 \times 10^4$ yields cells/ml in the original suspension. Do not count cells which do not look refractile and are probably dead.

E. Mycoplasma

If there is any suspicion that a cell line is contaminated with mycoplasma, e.g., changes in growth rate, morphological changes, changes in metabolism, there are several assays to check this. One way is to stain with Hoechst, which is available from Sigma (bisBenzimide, B-2883).
Plate cells on coverslips in 60 mm dishes. Allow to grow in culture several days such that cells are 50-70% confluent.
To cells + medium add 5 ml MeOH:HAc (3:1)
After 2 min, aspirate
Add add'l 5 ml MeOH:HAc
After 5 min, aspirate
Repeat .
Air dry cover slips by propping up against side of dish.
The following steps should be done with lights out:
Replace dried cover slip in culture dish and stain with Hoechst as follows:

Stain is prepared by dissolving Hoechst in PBS to conc. of 0.2mg /ml and filter sterilizing. This can be stored in amber bottle at 4°C. Cover slip with stain and allow to stand at r.t. for 20 min. Now rinse slip thoroughly in beakers of d. water. Drain stained cover slip well and mount on slide, cell side down, in 90% glycerol in PBS. Store in dark until viewing in fluorescence scope. If cells are harboring mycoplasma, you will see speckles of stained DNA in the cytoplasm (you should obviously see a stained nucleus, which serves as a positive control for the staining) when viewed with 40X objective. Unfortunately, scopes in this department are not equipped with the proper filter, a 53/44 barrier filter with BG-3 exciter filter. I have gone to the Cancer Center downtown where they have a scope with this filter. Supposedly, Central Facilities was going to offer mycoplasma testing. If so, they may be using this method and the Cancer center scope. Other commercial testing for mycoplasma is available.