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Purpose: The purpose of this document is to set forth guidelines for the culturing, splitting and freezing and thawing of the attachment Hela cell lines.

Culturing Conditions:

Grow the cells in high glucose DMEM supplemented with 10% FBS and antibiotics. (Typically penicillin and streptomycin). Use 8.0ml of media in a T25 flask, and 20.0ml media in a T75 flask. Maintain the cells in a 37°C, 5% CO_2 incubator checking the cells daily by looking at them on an inverted microscope.

Splitting:

1) Grow the cells to confluence.
2) Aspirate the media.
3) Wash the cells 3X with sterile PBS. (The volume of PBS should be approximately the same as the volume of media used for culturing the cells).
4) Add 1.0ml of 1X trypsin-EDTA, then rock the flask 4-5 times to coat the cell monolayer. Using 1.0ml of trypsin-EDTA will simplify calculating split ratios in step 8.
5) Wait about 2 minutes, then check the cells using an inverted microscope. You should see most of the cells floating loose in the trypsin-EDTA.
6) Make sure the cap is on the flask tightly, then firmly rap the flask against the palm of your hand two or three times to dislodge any cells that were still attached.
7) Again, check the cells on the inverted microscope. By now, all the cells should be loose and floating freely. If not, wait another minute, then rap the flask again. It is very important not to leave the cells in trypsin-EDTA for extended periods of time.
8) Resuspend the cells in growth media containing serum as the serum inactivates the trypsin. The amount of media used is determined by the split ratio. For example, the recommended split for Hela cells is 1:6, so we would add 5.0ml of media to the 1.0ml of trypsin-EDTA. L929cells are also split at 1:6, but J774 cells can be split at 1:8. Remember, the split ratios are *recommendations.* You may have to adjust the ratio depending on the density and condition of the cells. This is one case where experience is absolutely necessary.
9) Pipette the cell suspension across the back of the flask to dislodge any cells that may still be attached. Pipette the cell suspension into the lower corner of the flask 2-3 times to break up any cell clumps. If you're going to use the old flask for a second passage, remove all but 1.0ml of cell suspension. (You can use a 15ml conical tube for the waste). Add 7.0ml of fresh media to the flask if it's a T25, and 19.0ml of media to a T75. If going into a new flask, add 1.0ml of cell suspension to the new flask which should be waiting already labeled and containing fresh media. (As above, 7.0ml in a T25 and 19.0ml in a T75).
10) Rock the flask to distribute the cells, then return the flask to the CO_2 incubator. Remember to loosen the flask cap slightly to help with gas exchange.

Freezing:

Follow the steps above for splitting, but reduce the amount of trypsin-EDTA used. In a T25, use 0.3ml and in a T75, use 0.5ml. After the cells have detached, add growth media containing 10% DMSO. (Add 3.0ml of media to a T25, and 6.0ml to a T75). At confluence, a T25 will have about $2.8X10^6$ total cells while a T75 will have about $8.4X10^6$ total cells. I like to start a new T25 flask with about $1.0X10^6$ cells, so I'll dilute the cells to give me about that number in each cryovial. Immediately pipette 1.0ml aliquots of cell suspension into cryovials suitable for use in liquid nitrogen. Place the cryovials on ice until you're done, then place them into a controlled rate freezing chamber in an -80°C freezer overnight. Finally, transfer the cryovials to liquid nitrogen for long term storage. It is advisable to pull a cryovial out of liquid nitrogen to "proof" the cells to make sure that they're viable and uncontaminated.

Thawing:

If you froze the cells as outlined above each cryovial will contain about $1.0X10^6$ cells. This is the recommended seeding density for a T25, so start with a new T25 containing 5.0ml of DMEM supplemented with 10% FBS and antibiotics. Remove a cryovial from LN_2 and immediately place it into a small container of warm sterile water, (A small beaker works well). Do not "float" the vial, but use enough water cover the frozen cell pellet inside the cryovial. As soon as the cells have thawed, pipette them into the waiting T25 (which already contains 5.0ml of growth media). Rock the flask to distribute the cells, the place the flask into a tissue culture incubator. Wait about an hour, then remove the flask from the incubator and look at the cells inside with an inverted microscope. Most of the cells should be attached, but there will probably be a few "floaters". These may settle down, or they may be cells that didn't survive being frozen. If most of the cells are still floating, you may wish to return the flask to the incubator. Experience has shown me that an hour is usually enough time for the cells to reattach and any that are still floating after two hours will never reattach. In any case, it is necessary to remove the freeze media since the DMSO may be toxic to the cells, so aspirate the freeze media and replace it with 8.0ml of fresh growth media. Return the flask to the incubator. Be sure to check the cells again in about 24 hours. The cells should all be attached and growing. Remember that the shock of freezing will probably introduce a bit of a lag in the growth cycle, so adjust your time frame for splitting. After the cells have come to confluence once, you should be able to resume your normal schedule for passing the cells.