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Laboratory procedures Involving Human Diploid Fibroblasts Cultures

6.1. Human Diploid Fibroblasts – Obtaining Sterile Skin Sample

16.1.1. Obtain sterile skin biopsy after wiping with isopropyl alcohol pad (2X) using a sterile #20 scalpel and sterile forceps contained in the Tissue Culture Kit.

6.1.1.2 Emphasize the need for a sterile skin sample. It is not feasiblé to initiate a fibroblast culture if the skin sample is not sterile.

6.1.1.3. Necessary supplies: Sterile packets of 70% isopropynyl wipes, 50 ml tube with sterile MEM medium supplemented with gentamycin 10% fetal bovine serum, sterile forceps with teeth, and a sterile #20 scalpel blade with handle.

6.1.1.4. Wipe the intended sampling area 3 times with separate 70% isopropanol wipes.

6.1.1.5. Use sterile forceps with gripping teeth to hold tissue.

6.1.1.6. Use a sterile #20 scalpel to cut a sample 5-5 mm in diameter and about 3 mm deep.

6.1.1.7 The usual biopsy side is the lower abdomen close to the incision site.

6.1.1.8. Place skin biopsy in tissue culture media tube labeled with the UMB case number.

6.1.1.9. Tube with tissue media and sample can be stored in a CO_2 incubator, with top of tube loosened, for up to 48 hours before initiation of culture.

6.2. Initiation of Human Diploid Fibroblasts

6.2.1. Necessary supplies: Vertical Class II laminar flow hood, tissue culture medium consisting of MEM supplemented with gentamycin and 10% fetal bovine serum, water bath, a range of sterile plastic pipets, sterile glass Pasteur pipets, electric pipeting aid, 60 mm diameter sterile plastic tissue culture plates, sterile #20 scalpel blades with handles, spray bottle of 70% ethanol, paper towels, disposal

container for plastic waste and sharps.

6.2.2. Work is performed in a Vertical Class II laminar flow hood.

6.2.3 Label 4-5 P60 dishes with case number.

6.2.4 Use a 10 ml pipet under a slight vacuum of the pipeting aid to transfer tissue to first P60.

6.2.5 Use 2 #20 scalpels in a cross scissor fashion to cut the biopsy into 4-5 pieces.

6.2.6 Using the tip of the scalpel, transfer one piece into each P60.

6.2.7 Select one P60 and using the same scalpels cut tissue into segments less than 1 mm cubes.

6.2.8. Place one scalpel on metal container holding the glass Pasteur pipets so that the tip remains sterile is out of the work area.

6.2.9. Holding the open P60 in one hand, use the scalpel close to the tip to force the tissue into a cut in the plastic caused by the pressure on the sample and slide the blade forward.

6.2.10. Use the same scalpel to make additional passes over the tissue to ensure that part of it is imbedded.

6.2.11. Repeat for the remaining pieces (5-10) in that petri dish.

6.2.12. Place the petri without the top towards the back of the hood to allow the sample to adhere to the plastic due to minimal drying.

6.2.13. Repeat with the next P60.

6.2.14. When the second P60 is completed, replace the top on the first dish.

6.2.15. Continue for all P60s. Leave the top off the last dish while media is added to the initial dishes.

6.2.16. Add 7ml of medium warmed to 37^{o}C to each dish. Use a separate 10 ml pipet. Touch the pipet to the bottom of the dish away from an embedded sample and slowly add the medium. Do not squirt it from above the surface of the dish as it will dislodge embedded samples.

6.2.17. Place in CO_2 incubator at 37^{O}C and 5% CO_2 undisturbed for 5-7 days.

6.3. Refeeding Human Diploid Fibroblast Cultures

6.3.1 Required supplies: as listed above.

6.3.2. The medium is suctioned off using a sterile 9 inch glass Pasteur pipet connect to a vacuum flask and a vacuum line.

6.3.3. Fresh medium, 7 ml per P60 and 12 ml per P100, is added using a 25 ml pipet such that 2-3 dishes can be refed using one pipet. Use a new pipet to reefed additional dishes.

6.3.4. Once a pipet has been used to feed a culture dish, it is not to enter the media bottle again.

6.3.5. For P60 dishes containing biopsy material, place the tip on the surface and add media slowly. For cultures without biopsy material, add the medium slowly from about 3-4 cm above the dish.

6.3.6. Refeed twice a week until the plates are confluent.

6.4. Subculturing Human Diploid Fibroblast Cultures

6.4.1. Rinse dishes with sterile PBS, 1ml/P60 or 2 ml/P100.

6.4.2. Add 0.25% trypsin, 0.7 ml/P60 or 1ml/P60.

6.4.3. Return dishes to CO2 incubator for 5-6 minutes until the cells are floating.

6.4.4. Sometimes it is necessary to employ a sharp tap of the dish against the inside surface of the hood. The path of motion should not be greater than 3 inches.

6.4.5. Add about 4.3 ml medium per P60 and 7 ml per P60. Gently triturate to remove remaining cells from surface and distribute contents to four new dishes. The plates are labeled with the UMB #, date and passage. Dilution of plating area following trypsinization is always 4 fold.

6.4.6. The dish with the biopsy is considered Passage 0. Following each trypsinization the passage increases by one.

6.4.7. Add additional medium to bring the volume to either 7 or 12 ml. Place the dish on the surface of the hood and gently swirl the contents and then make a cross. This will distribute the cells equally on the surface of the plate. Return plates to incubator.

6.5. Freezing Human Diploid Fibroblasts

6.5.1. Trypsinize the cells and stop the action of the trypsin as described above.

6.5.2. Transfer the contents of all dishes of the same UMB # in a 50ml centrifuge tube.

6.5.3. Centrifuge at 600 RPM for 6 minutes.

6.5.4. Suction off the medium, carefully! Add 1.5 ml for cells from one P60 of medium containing 6% sterile glycerol.

6.5.5. Using a sterile cotton plugged Pasteur pipet, transfer 1 ml of cell suspension into sterile 1.0 ml vials (Nunc #366656, Thermo Scientific)).

6.5.6. Add 7 ml of medium to the 50 ml tube, tritrate and transfer to a new P60 dish for sterility check.

6.5.7. Cut 5 cm sections of Nunc CryoFlex tubing (Thermo Scientific)

6.5.8. Insert a colored disk, one color per UMB#, and place in plastic Nunc tubing.

6.5.9. Using a Master-Mite heat gun with a circular air deflector on its tip, melt the plastic ends so that the vial is completely encased in the plastic tubing. Use forceps to hold the vial during the heating process. Cut off excess material from the vials.

6.5.6. Place the vials into a slow freezing unit Cool Cell (Biocision) and place the unit in a -80^{o}C freezer overnight.

6.6. Storing Cells in Liquid Nitrogen and Recording Location

6.6.1. Vials are transferred directly to a Liquid Nitrogen tank.

6.6.2. The cells are stored in the vapor phase of the tank.

6.6.3. Record the location, number of vials and the passage number.

6.7. Thawing Cells from Liquid Nitrogen

6.7.1. Find cell location in database.

6.7.2. Fill a 250 ml PLASTIC beaker half full with water at 37^{o}C.

6.7.3. Use a plastic face mask! If liquid nitrogen leaked into the vial, the vial can explode when placed in water.

6.7.4. Swirl the vial until the media and cells are thawed. If the vial has a plastic sleeve protecting it, carefully cut it with a scalpel and remove immediately before thawing..

6.7.5. Dry the vial with a Kimwipe. Place vial in laminar flow hood.

6.7.6. Wipe the top with a sterile 70% isopropanol wipe and open. If it is a glass vial, hold the top with another 70% isopropanol wipe and snap off the top.

6.7.7. Add the contents of the vial to a P60 dish using a cotton plugged heat sterilized glass Pasteur pipet and swirl to distribute cells in one P100 dish with 12 ml medium.

6. 8. Shipping of fibroblasts

6.8.1. Ship either as frozen ampule or as a growing culture.

6.8.2. If shipping as a frozen ampule, place it in 50 ml centrifuge tube. Cushion ampule with paper. Keep at -80^{o}C until shipping in dry ice.

6.8.3. If the cells are to be shipped at room temperature, thaw the vial as above but contents to three 25 square centimeter tissue culture flasks (T25) with 5 ml media each. The T25 have to have a solid cap.

6.8.4. When the cells are confluent, add media to fill the flask, leaving about 0.5ml of air space.

6.8.5. Wrap the top with Parafilm. Place the T25s in sealable plastic vials that contain liquid absorbing material.

6.8.6. Ship the samples at room temperature with instructions to suction off most of the medium, leaving approximately 7 ml behind. Place in incubator for 2 days before subculturing the cells.