SOP 3
Processing of Ascites and Pleural Fluid for Cell Culture and Xenografts

Receiving the Sample:

Samples should be transported or shipped in ambient conditions, sealed with Parafilm® and should be processed immediately upon arrival.

Log in sample

1) Access the correct Excel® file (correlating to the institution the sample was drawn)
2) Enter all of the following information into the spreadsheet (information will be found either directly on the tube label or within the patient paperwork provided by the institution):
    a. Date and time sample were drawn/surgery date
    b. Date and time that sample processing began
    c. Patient identifier* (year patient consented-institution-patient number)
        i. For all fluid samples, add an "F" to the patient number (for multiple fluid samples per patient denote F2, 3rd = F3, 4th = F4 etc.)
        ii. Notate original patient identifier given by the institution (only if different than our system)
    d. Sample type – specifically referencing the site the sample was drawn (if known)
    e. Pre-Operative/Suspected diagnosis (if none, notate unknown DX)
    f. Institution the sample came from
    g. Initials of who processed the sample
    h. Total volume initially received of fluid
    i. Note whether or not the sample is TCRB qualified (will be referenced within the patient paperwork)
3) File paperwork and shipping label (if sample was shipped) away to be scanned and organized by the institution and year
4) Sterilize the bio-safety cabinet with 70% ethanol and sterile wipes for sample processing

Processing and Culturing:

Materials Needed

- (3) 25 cm^2 cell culture flasks
- 15mL centrifuge tubes
- 50mL centrifuge tubes
- Warm (37^O C) PBS (without magnesium or calcium)
- RBC lysis buffer (recipe at the end of protocol)
- Gentamicin antibiotic
- Thinprep® tube
- Warm (37^O C) medium
    - Medium formulation: Iscove's Modified Dulbecco's Medium (IMDM) plus the following supplements (to a final concentration): 20% Fetal Bovine Serum, 4mM L-Glutamine, 4X ITS (20 µg/mL insulin, 20 µg/mL transferrin, 20 ng/mL selenous acid)
- 3 Incubators corresponding to 2% O_2, 5% O_2, and 20% O_2. All at 37^O C and 5% CO_2.
- (10) 2mL inter-threaded vials
- 2mL outer-threaded vials
- Cold Cryopreservation media
    - 1 tube of pure Fetal Bovine Serum (FBS)
    - 1 tube of 15% DMSO in RPMI media
- Isopropanol freezing tub
- 28g ½" insulin syringe (for mouse injection)
- No additive RPMI-1640 (for mouse injection)

- Ice (for mouse injection)

Fluid Processing

1) Depending on the initial volume of fluid received, begin to aliquot the fluid sample into 50 mL centrifuge tubes (the number of 50 mL centrifuge tubes will always change to match the initial volume received) and centrifuge at 500 x g (1600 rpm) for 6 minutes.
    a. If less than 50 mL of fluid is initially received centrifuge the whole sample, saving only 10 vials at 1.8 mL each of supernatant, re-suspend in 10.5 mL of PBS and proceed to step 5
2) After centrifugation, save all the following volumes of supernatant (in order of priority):
    a. 10 vials at 1.8 mL
    b. 1 centrifuge tube at 15 mL
    c. 1 centrifuge tube at 50 mL
    d. Label all vials and tubes with: sample ID, diagnosis, sample type (Fluid), date, initials, and "supernatant"
    e. Inventory, freeze and store at -80° C
3) Once all supernatant vials are saved continue repeating step 1, except now pour off waste, discard and continue mixing cell pellets into one 50mL tube.
    a. This process may take several rounds of centrifugation to combine the sample depending on the initial volume of the fluid
    b. For very large volumes, only combine into 3-4 50 mL centrifuge tubes instead of just one.
4) Once the sample is combined into one (or more) 50 mL centrifuge tube(s), wash the pellet(s) with RBC lysis buffer
        i) To wash the cells, add 30-40 mL of the RBC lysis buffer
        ii) Mix well with the serological pipette
        iii) Let sit for 2-3 minutes
        iv) Centrifuge at 500 x g (1600 rpm) for 6 minutes
        v) Poor off waste and repeat 1-3 times, only until pellet is no longer red with RBCs.
        vi) Once the pellet is clean and free of visible RBCs, re-suspend in 10.5 mL of PBS and proceed to step 5
5) Get a Vi-Cell™XR counting vial and add 500 µL the sample
6) Count the cells using the Vi-Cell™XR
    a. Click: Log in sample
    b. Enter: Sample ID, Cell Type (Blood/BM primary), Dilution Factor (1)
    c. Click: Ok and start queue
7) Multiply total viable cells by the total volume (10mL) and record the cell viability
    a. Access your cell counts by the following criteria** (organized in order of priority):
        i) ~1-5e^6 cells/flask for culturing
        o Prioritize 5% O_2 then 20% O_2, and 2% O_2 last
    b. Add 0.5e^6 directly to a thinprep® tube – label with sample information for histology processing
    c. ~10-30e^6 cells/mouse for Intra-Peritoneal injection
    d. All extra cells are to be cryopreserved in increments 5e^6 cells/vial
8) Once the cell destinations are determined (either for culture, mouse injection etc.), calculate necessary volumes to be centrifuged, separate into newly labeled centrifuge tubes and centrifuge at 500 x g (1600 rpm) for 6 minutes. Pour off all waste.
9) Clean area with 70% ethanol

Culturing Cells

1) Label all necessary flasks with:
    a. Oxygen condition
    b. Your initials
    c. Today's date
    d. Sample ID or patient identifier
    e. Diagnosis
    f. Sample type
    g. Any extra notes
        i. Antibiotic concentrations greater than 1:1000

2) Prepare medium (5 mL/flask):
    a. IMDM:
        i. Aliquot 15 mL of the warm IMDM medium into a new 15 mL centrifuge tube.
        ii. Add 1 5µL of gentamicin antibiotic to the 15 mL of media (1:1000 concentration)
        iii. Antibiotics should be added directly to the medium that will be used that day, i.e. to small volumes of medium only
        iv. 15 mL is used when all three oxygen conditions are possible, make less medium if not all oxygen conditions are possible (5 mL/flask)
3) Once the centrifuge is complete (step 8 above). Pour off supernatant (waste) and re-suspend in the appropriate media and aliquot 5mL/flask of medium +c ells into each labeled flask.
4) Store the flasks into their appropriate incubator corresponding to the oxygen condition labeled on the flask
5) Clean area with 70% ethanol

Intra-Peritoneal and Sub-Cutaneous Mouse Injections

1) Mouse injection materials needed:
    a. Ice
    b. 28g ½" insulin syringe
    c. RPMI-1640 (no additive)
    d. Microcentrifuge tube
    e. Centrifuge tube containing cells to be injected
    f. For primary injections use NSG mice for all types of samples
2) Intra-Peritoneal Injection
    a. For each mouse being injected, add 20 0µL of RPMI-1650 (with no additives) to the centrifuge tube (from step 8 above) – transfer to a sterile microcentrifuge tube for ease of access with the syringe later on
        i. Mix with micropipette
    b. Remove orange insulin cap, set aside and suck up ALL cells and injection media from the microcentrifuge tube with the 28g ½" insulin syringe– minimize air bubbles.
    c. Carefully replace the cap and wipe off the syringe with an alcohol pad to sterilize
    d. Place on ice
    e. Label the syringe or ice bucket with:
        i. Patient identifier, diagnosis, sample type (including site of draw), todays date and your initials. Finally add the type of injection (IP), total syringe volume and how many mice are to be injected
3) Clean area with 70% ethanol

Cryopreservation

1) Cryopreservation materials:
    a. Outer-threaded cryovials
    b. 1 tube of pure FBS
    c. 1 tube of 15% DMSO in RPMI-1650 medium
    d. Isopropanol tub
    e. Centrifuge tube containing cells for freezing
2) For every vial cryopreserved, add 0.8 mL (800 µL) of FBS and 0.8 mL (800 µL) of 15% DMSO in RPMI-1650 medium directly to the 15 mL centrifuge tube with the cell pellet (from step 8 above)
3) Mix with the serological pipette tip.
4) Add 1.6 mL of cryopreservation media and viable cells to each 2 mL outer-threaded cryovial being frozen
    a. Label vials with: Patient identifier, diagnosis, sample type (including site of draw), patient viable cells, cell count, viable, todays date and your initials.
5) Place vials into the isopropanol tub and into a -80^{O} C freezer
    a. Samples are to be inventoried 12-24 hours later into a liquid nitrogen vapor tank
6) Clean area with 70% ethanol

Note all culture conditions, number of mouse injections, number of cryopreserved vials and any processing notes within the Excel® spreadsheet. Save and close.

Maintaining Cultures:

1) Flasks need to be routinely checked via a microscope every 5-7 days.
    a. Incubators need to be monitored daily to verify that no flask is visibly contaminated or if the medium, is acidifying and needs replenishing earlier than 5-7 days.
    b. Watch for cell proliferation, contamination, and growth of non-cancerous cells via the microscope.
2) Media needs to be changed every 5-7 days to maintain fresh antibiotic and media nutrient concentrations.
3) Expand to a larger flask when one or more of the following conditions are met:
    a. The cells need more room to grow (appear to be greater than 70% confluent)
    b. The tumor pieces are too concentrated and need more room to allow for cell growth
    c. The medium pH is changing faster than every 7-10days
    d. Continue this procedure until cells have reached one 150 cm^2 flask
4) Discard the cells immediately when contamination is present.
    a. Note within the spreadsheet corresponding to that patient that contamination is present and cells were discarded and whether all were contaminated or only part of a given sample.
5) Freeze cells and discontinue culturing if no growth or live cells are seen after 3-4 months of culturing OR if non-cancerous cells have overtaken the flask and NO VISIBLE cancerous cells are seen after at least 4-5 weeks of culturing.
    a. Never discard cells, unless contamination is present. Always cryopreserve for future research.

Establishing a Cell Line:

1) Primary samples may be established into a cell line when the following conditions are met:
    a. The expanded flask (typically in 1-3 150 cm^2) is confluent with at least 80-90% cancerous tumor cells
    b. Short tandem repeat (STR) verified to match the original patient material
    c. Mycoplasma bacterium free
    d. EBV results known
        i. EBV+ cell lines are still established, but the cell line is now a known as an EBV+ line (likely a B lymphoblastoid line) and will be treated as such
    e. A minimum of one vial is cryopreserved at an expansion passage of zero
        i. To preserve cells before the cell line expansion begins
2) A new name will be given to denote cancer diagnosis information
    a. Name will consist of: Study ID (COG or TX) – cancer diagnosis – cell line number, oxygen condition (20% O_2 = no indicator, 5% O_2 = h, and 2% O_2 = h2), media type (IMDM = no indicator, Neurobasal = nb)
        i. Ex. TX-BR-100h2. This is a TXCCR breast cancer cell line grown in 2% O_2 and it is the 100^{th} cell line established via TXCCR.
        ii. Ex2. COG-N-450nb. This is a COG, neuroblastoma cell line grown in 20% O_2 in Neurobasal media.
        iii. Ex3. TX-V-112h. This is a TXCCR, lymphoblastoid (EBV+) cell line grown in 5% O_2.
3) For all cell lines established in IMDM the formulation will switch from **4X** ITS (20 µg/mL insulin, 20 µg/mL transferrin, 20 ng/mL selenous acid) to **1X** ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid)
4) Upon establishment, any and all antibiotics must be removed from culture conditions.

RBC Lysis Buffer Recipe

- Materials
    1) 1m M Ammonium Bicarbonate (NH_4HCO_3) – 0.079 g/Liter
    2) 114 mM Ammonium Chloride (NH_4Cl) – 6.1 g/Liter
    3) 1 Liter deionized water (H_2O)
    4) 0.22µm filter
- Dissolve the solid chemical in 500mL of deionized water
- Once dissolved, combine with the remaining deionized water
- Filter 1 Liter solution using 0.22µm filter

*All patients will be given a patient identifier upon completing the consent paperwork. ANY sample received without consent will still be processed. For this circumstance a temporary patient identifier will be generated until the new one is assigned. If no consent is received, the patient refuses or cannot consent, ANY and ALL patient samples will be immediately destroyed.

**Keep in mind that all cell counts are different and each sample needs to be individually accessed for culture, mouse injection and cryopreservation differently.