SOP 2
Solid Tumor Processing for Cell Culture and Xenografts

Receiving the Sample:

Samples will generally arrive in prepared L-15 transport medium with 100 mg/ml gentamicin and 5% FBS. They should be transported or shipped in ambient conditions and should be processed immediately upon arrival. Shipping should be in Styrofoam containers to insulate from heat or cold during shipping.

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. Add a "T" to the patient number to reference a second tumor has arrived (3^{rd} - T2, 4^{th} - T3, 5^{th} - T4 etc. for any additional tumors)
        ii. Notate original patient identifier given by the institution (only if different than our system)
    d. Sample type – specifically referencing the anatomic site the tumor was removed from
    e. Pre-Operative/Suspected diagnosis (if none, notate unknown DX)
    f. Institution the sample came from
    g. Initials of who processed the sample
    h. Notate 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) Aseptically cleanse the bio-safety cabinet with 70% ethanol and sterile wipes for sample processing

Processing and Culturing:

Materials Needed

- (2) 10-blade scalpels
- (3-6) 25 cm^2 cell culture flasks
- (3) 35 x 10mm tissue culture dish
- 15 mL centrifuge tubes
- Gentamicin antibiotic (cell culture grade)
- 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, 4 mM L-Glutamine, 4X ITS (20 µg/mL insulin, 20 µg/mL transferrin, 20 ng/mL selenous acid)
    - Additional Neurobasal-A medium formulation (*for neural tumors only*): Neurobasal-A plus the following supplements (to a final concentration): 0.5mM L-Glutamine, 1X B-27 serum-free supplement and 1X N-2 supplement
        - Additionally you will need rhFGF (fibroblast growth factor, basic, human, recombinant) and rhEGF (epidermal growth factor, human, recombinant)
- 3 Incubators corresponding to 2% O_2, 5% O_2, and 20% O_2. All at 37^O C and 5% CO_2.
- (2) 2 mL pre-weight inter-threaded cryovials
- (2) 2 mL outer-threaded cryovials
- Cold Cryopreservation media

- 1 tube of pure Fetal Bovine Serum (FBS)
- 1 tube of 15% DMSO in RPMI-1640 medium

- Isopropanol-jacketed cryogenic freezing tub
- Frozen matrigel (for mouse injection)
- 1 mL sterile syringe (for mouse injection)
- 18 1-1/2 G needle (for mouse injection)
- No additive RPMI -1640 (for mouse injection)
- Ice (for mouse injection)
- Liquid nitrogen

Tumor Processing

1) Remove tumor vial from the transport container/bag and cleanse the outside with 70% ethanol and place into a clean bio-safety cabinet
    a. For non-solid tumors, centrifuge tumor vial prior to entering sterile hood at 500 x g (1600rpm) for 6 minutes to concentrate tumor
2) Once the tumor is visible in the hood, estimate the tumor size and determine its needs with the following conditions** (*note that they are in order of priority*):
    a. ~10 mg/flask (prioritize 5% O_2 then 20% O_2, 5% O_2 in neuralbasal (neural tumors only), and 2% O_2 last)
    b. 1-2 x ~50mg for dry snap freezing in LN_2 within pre-weighed inner-threaded vials
    c. ~50-100mg for mouse sub-cutaneous injection (~25-50 mg/mouse)
    d. 1-2 x ~50 mg for cryopreservation in outer-threaded vials with cryopreservation media
3) 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 (tumor)
    g. Any extra notes
        i. Medium other than IMDM needs to be notated
        ii. Antibiotics other than gentamicin 50 μg/ml
4) 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 15 μL of gentamicin antibiotic to the 15 mL of medium, final concentration of gentamicin = 50 μg/ml
        iii. For all tumors originating from the colon, mouth, stomach etc. increase antibiotic concentration to 4X this amount to better prevent contamination
        iv. Antibiotics should be added directly to the small volumes of medium used the same day.
        v. 15mL is used when all three oxygen conditions are possible, make less medium if not all oxygen conditions are possible (5 mL/flask)
    b. **Neurobasa**l:
        i. Aliquot 15mL of the warm Neurobasal media into a new 15 mL centrifuge.
        ii. Add 3 μL each of rhFGF anf rhEGF human growth factors (1:5000 concentration) to the 15 mL of medium.
        iii. Additionally add 15 μL of gentamicin antibiotic to the 15 mL of medium, final concentration of gentamicin = 50 μg/ml
        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)
5) Using a 5 mL or 10 mL serological pipette remove the tumor piece(s) and place into the 35 x 10 mm tissue culture dish – discard transport medium, tube and pipette
6) Using the scalpels, place desired amount for snap frozen tumor sections directly into the pre-weighed inner-threaded vials without mincing the pieces
    a. Weigh the vials after adding the tumor and calculate the tumor weight. Record on the label along with the patient identifier, diagnosis, sample type (including site of draw), snap frozen, todays date, and your initials.
    b. Snap freeze these vials quickly with liquid nitrogen vapor and inventory immediately into a -

80^{O} C freezer

7) Next set aside the desired amount to be injected into mice and to be cryopreserved separately into the other two tissue culture dishes. Leaving behind all of the tumor to go into culture.
    a. Please note that neural (neuroblastoma and brain tumors) will be placed in both types of medium (IMDM and Neurobasal) and will need to be separated into two separate dishes at this step
8) Finely mince all sectioned tumors using the two scalpels.
    a. Move quickly, but effectively to ensure the tumor pieces are cut very small and the tumor remains viable and covered with medium through the whole process.
9) Once all of the tumor is cut, begin with placing tumor into culture, setting aside those dishes for mouse injection and cryopreservation.
    a. Using a 5 mL or 10 mL serological pipette, add 1-2 mL of the prepared medium to the dish to gather all tumor and cells. Return the medium and tumor pieces to the 15 mL of prepared medium. Repeat until the dish is free of all cells and tumor pieces.
    b. Discard tissue culture dish.
    c. Thoroughly mix all medium and tumor pieces together and then add 5 mL of mixed medium + tumor pieces to each labeled flask.
        i. If there is only enough tumor for one or two culture conditions, only prepare the correct volume of medium required (5 mL/flask)
    d. Store the flasks into their appropriate incubator corresponding to the oxygen condition labeled on the flask
10) Clean area with 70% ethanol

Sub-Cutaneous Mouse Injection

1) Mouse injection materials needed:
    a. Ice
    b. Matrigel (held on the ice)
    c. 1 mL syringe
    d. 18 1-1/2 G needle
    e. RPMI -1640 (no additive)
    f. Tissue culture dish containing minced tumor pieces
    g. For primary injections use NSG mice for all types of samples
2) For each mouse being injected (~25-50 mg/mouse), add 100 µL of RPMI-1640 (with no additives) followed by 100 µL of partially thawed matrigel directly into the tissue culture dish
    a. DO NOT fully thaw matrigel, once it reaches room temperature it will solidify and will need to be discarded
    b. All extra unused matrigel should be discarded and not re-frozen
3) Mix with the tip of the micro-pipette tip
4) Suck up ALL tumor and injection media from the dish with the 1mL syringe– minimize air bubbles.
5) Wipe off the syringe with an alcohol pad before adding the needle
6) Carefully push the 18 1-1/2 G needle onto the syringe
7) Place on ice quickly to keep matrigel from solidifying
8) Label the syringe or ice bucket with:
    a. Patient identifier, diagnosis, sample type (including site of draw), todays date and your initials. Finally add the type of injection (Sub-Cutaneous), total syringe volume and how many mice are to be injected
9) 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-1640 medium
    d. Isopropanol tub
    e. Tissue culture dish containing minced tumor pieces
2) For every vial cryopreserved (~50 mg/vial), add 0.8mL (800 µL) of FBS and 0.8mL (800 µL) of 15% DMSO in RPMI-1640 medium directly to the tissue culture dish
3) Mix with the serological pipette tip.
4) Add all cryopreservation media and tumor into the 2 mL outer-threaded vials.
5) Label vials with:

a. Patient identifier, diagnosis, sample type (including site of draw), viable tumor, todays date and your initials. Finally add an estimated total weight of the minced tumor.

6) 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 tank
7) Clean area with 70% ethanol

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

Maintaining Tumor 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) Medium needs to be changed every 5-7 days to maintain fresh antibiotic and medium nutrient concentrations (especially glutamine).
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 7-10 days
    d. Continue this procedure until cells have reached 1 150 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 the sample or part of it was contaminated.
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 cryoprserve 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 EBV+ (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 provide a safety reserve vial as early as possible and 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), medium 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 100th 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.

Continuous Cell Lines

Cell lines that meet the above criteria and can be sub-cultured for 3 passages, grow well enough for subsequent passages, and have at least 2 sets of cells cryopreserved are considered continuous cell lines.

Continuous cell lines that can be recovered from cryopreservation, and can be expanded to generate at least 25 vials of cryopreserved vials are considered distributable continuous cell lines.

*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 these are only estimates and ALL tumor volumes are subject to change based on the initial tumor size.