

SOP 1 Primary Patient Blood and Bone Marrow

Receiving the Sample:

Samples will arrive in either heparin treated or non-treated tubes. They should be transported or shipped in ambient conditions and should be processed immediately upon arrival.

Log in sample

- 1) Access the correct Excel® file (correlating to the institution the sample was submitted from)
- 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 was drawn/surgery date
 - b. Date and time that sample processing began
 - c. Patient identifier* (year patient consented-institution-patient number)
 - i. For blood samples add a "B" to the patient number to denote the blood sample (for subsequent draws: 2nd = B2, 3rd = B3, etc.)
 - ii. For bone marrow samples add an "A" to the patient number to denote the bone marrow samples (for subsequent draws: 2nd = A2, 3rd = A3, etc.)
 1. If two samples arrive together one drawn from the left and one drawn from the right, denote A1 for the left side and A2 for the right. In this case, no stand alone "A" will be used.
 - iii. 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. Notate whether or not the sample is TCRB qualified (will be referenced within the patient paperwork)
 - i. Note the approximate temperature of the sample when the container was first open and whether or not any leakage of the sample outside the container occurred.
- 3) File paperwork and shipping label (if sample was shipped) away to be scanned and organized by the institution and year
- 4) Aseptically clean the bio-safety cabinet with 70% ethanol and sterile wipes for sample processing

Processing and Culturing:

Materials Needed

- (3-6) 12.5 cm² cell culture flasks (use these small flasks to keep ratio of medium to cells low initially, expand to 25 cm² as necessary once growth and media acidification indicate the need)
- 15 mL centrifuge tubes
- Warm (37°C) PBS (without magnesium or calcium)
- Lymphocyte Separation Medium (Ficoll)
- RBC lysis buffer (recipe at the end of protocol)
- Gentamicin antibiotic (cell culture grade)
- Warm (37°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% O₂, 5% O₂, and 20% O₂. All at 37°C and 5%CO₂.
- (2) 2mL inter-threaded vials
- (2) 2mL outer-threaded vials
- Cold Cryopreservation medium
 - 1 tube of pure Fetal Bovine Serum (FBS)
 - 1 tube of 15% DMSO in RPMI-1640 medium
- Isopropanol-jacketed controlled rate freezing tub
- Frozen matrigel (for mouse injection)
- (2) 28g ½” insulin syringe (for mouse injection)
- RPMI-1640 without additives (for mouse injection)
- Ice (for mouse injection)

Blood and Bone Marrow Processing

- 1) For each vial of blood or bone marrow, label a new 15 mL centrifuge tube with the sample ID
- 2) For each vial of blood or bone marrow, aliquot 10 mL of warm PBS into a new 15 mL centrifuge tube
- 3) Using a 5 mL serological pipette, pipette each individual sample into their newly labeled 15mL centrifuge tube
 - a. Wash out every original sample vial with the 10 mL of PBS with the same pipette used to transfer the blood or bone marrow sample as to not lose any of the sample left behind in the old tube or pipette. Set aside the 15 mL tube with PBS and residual patient sample for later use. Discard original sample vial and pipette.
- 4) Centrifuge the original sample (not PBS) at 1900 x g (3150 rpm) for 6 minutes
 - a. Two layers will form after centrifugation: top layer = plasma and bottom = mononuclear cells, neutrophils, and RBCs
 - b. Save the plasma in 1.8mL inner-threaded cryopreservation vials.
 - c. Label plasma vials with: sample ID, diagnosis, sample type (blood or bone marrow), plasma, date, technician initials, and volume
 - d. Inventory and freeze at -80°C
- 5) For each vial of blood or bone marrow label a new 15 mL centrifuge tube with the sample ID and - layer 3 mL of lymphocyte separation medium to the bottom of the tube
- 6) Re-suspend the cell pellet obtained after collecting the plasma and dilute with the 10mL PBS that was used to wash the sample tube in step 3a and slowly layer diluted sample onto the 3 mL lymphocyte separation medium layer by tilting tube and pipetting along the side of the tube ****do not mix****
 - a. For multiple tube samples, repeat process for each subsequent tube
 - b. For large volume samples, process in multiple 15 ml tubes as this provides the best recovery of mononuclear cells at the interface of the lymphocyte separation medium and medium after centrifugation
- 7) Centrifuge at 20° C, 400 x g, for 30 minutes
- 8) After the 30 minute spin is complete, three layers will form:
 - a. Top Layer = Waste (discarded using a pipette)
 - b. Middle Layer = Buffy coat (containing mononuclear cells) needs to be transferred into a newly labeled 15 mL centrifuge tube for counting
 - c. Bottom layer = RBCs, save and aliquot into 1.8 mL inner-threaded cryopreservation vials
 - i) Label RBC vials with: sample ID, diagnosis, sample type (blood or bone marrow), RBCs, date, technician initials, and volume
 - ii) Inventory and freeze at -80°C
 - iii) Record how many were frozen and the corresponding volumes within the Excel® spreadsheet
- 9) Access the newly transferred buffy coat layer.
 - a. If the buffy coat (mononuclear cell layer) is clear and free of excess RBCs, add PBS to the buffy coat, bringing the total volume to 10.5 mL and proceed to step 10
 - b. If the buffy coat contains visible RBCs, wash cells with RBC lysis buffer, repeating until RBCs are no longer readily visible within the centrifuge tube.
 - i) To wash the cells add 10 mL of the RBC lysis buffer
 - ii) Mix well with the serological pipette
 - iii) Let sit for 2-3 minutes
 - iv) Centrifuge at 1600 rpm (500 x g) for 6 minutes

- v) Poor off waste and repeat 1-3 times, only until pellet is no longer red with RBCs.
- vi) Re-suspend in 10.5 mL of PBS and proceed to step 10
- 10) Get a Vi-Cell™XR counting vial and add 500 µL the sample
- 11) 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
- 12) Multiply total viable cells by the volume the cells were re-suspended in and record the cell viability
 - a. For blood samples obtained as match to solid tumor samples (i.e. the sample is from a patient that has a non-hematological cancer and is *not* collected to try and obtain metastatic cells from the blood), prepare to cryopreserve all of the mononuclear cells obtained from the blood sample and proceed to that step
 - b. If the sample is possibly a hematological malignancy OR collected to attempt culture of metastatic tumor cells, then access your cell counts by the following criteria** (organized in order of priority):
 - i) ~1-3e⁶ cells/flask for culturing
 - o Prioritize 5% O₂ then 20% O₂, and 2% O₂ last
 - o If the sample will be cultured in both medium conditions and not all six conditions are possible, prioritize as follows: 1st = 5% O₂ IMDM, 2nd = 5% O₂ neurobasal, 3rd = 20% O₂ IMDM, etc.
 - ii) 1-2e⁶ cells/mouse for tail vein injection
 - o For cell counts greater than 40e⁶, prepare sub-cutaneous injections as well (~10-30e⁶ cells/mouse)
 - iii) All extra cells are to be cryopreserved in increments no greater than 5e⁶ cells/vial
- 13) Once the cell destinations are determined (either for culture, mouse injection etc.), calculate necessary volumes to be centrifuged and centrifuge at 500 x g (1600 rpm) for 6 minutes
 - a. Please note that any neural tumors (neuroblastoma and brain tumors) will be placed in both types of medium (IMDM and Neurobasal) and will need to be separated into two separate centrifuge tubes at this time
- 14) 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. Medium other than IMDM needs to be notated
 - ii. Antibiotics other than gentamicin at a concentration of 100 µg/ml
- 2) Prepare medium (5 mL/flask):
 - a. **IMDM medium:**
 - i. Aliquot 15 mL of the warm IMDM media into a new 15 mL centrifuge tube.
 - ii. Add 15 µL of gentamicin antibiotic to the 15mL of medium (final concentration of gentamicin = 50 µg/ml)
 - iii. Antibiotics should not be added directly to the stock medium bottle but added instead to the small volumes of medium used for the cultures.
 - 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)
 - b. **Neurobasal medium:**
 - i. Aliquot 15 mL of the warm Neurobasal media into a new 15 mL centrifuge.
 - ii. Add 3 µL each of rhFGF and rhEGF human growth factors (1:5000 concentration) to the 15 mL of media.
 - iii. Additionally add 15 µL of gentamicin antibiotic to the 15mL of medium (final concentration of gentamicin = 50 µg/ml). 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 13 above). Pour off supernatant (waste) and re-suspend in the appropriate media and aliquot 5mL/flask of medium + cells 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

Tail-Vein and Sub-Cutaneous Mouse Injections

- 1) Mouse injection materials needed:
 - a. Ice
 - b. Matrigel (held on the ice)
 - c. (2) 28g ½" insulin syringe
 - d. RPMI-1640 (no additive)
 - e. (2) microcentrifuge tubes
 - f. Centrifuge tube containing cells to be injected
 - g. For primary injections use NSG mice for all types of samples
- 2) Tail-Vein Injection
 - a. For each mouse being injected, add 200 µL of RPMI-1640 (with no additives) –transfer to a 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 medium 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), today's date and your initials. Finally add the type of injection (t.v. = tail-vein, total syringe volume and how many mice are to be injected)
- 3) Sub-Cutaneous Injection
 - a. For each mouse being injected, add 100 µL of RPMI-1640 (with no additives) followed by 100 µL of partially thawed matrigel directly into the centrifuge tube (transfer to a microcentrifuge tube for ease of access with the syringe later on)
 - i. DO NOT fully thaw matrigel, once it reaches room temperature it will solidify and will need to be discarded, so keep matrigel cold until injection into mice
 - ii. Mix with micropipette
 - iii. All extra matrigel should be tossed and not re-frozen
 - b. Remove orange insulin cap, set aside and suck up ALL cells and injection medium from the microcentrifuge tube with the 28 g 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 quickly to keep matrigel from solidifying
 - e. Label the syringe or ice bucket with:
 - i. Patient identifier, diagnosis, sample type (including site of draw), today's date and your initials. Finally add the type of injection (s.c. = sub-cutaneous), total syringe volume and how many mice are to be injected
- 4) Clean area with 70% ethanol

Cryopreservation

- 1) Cryopreservation materials:
 - a. Outer-threaded vials
 - b. 1 tube of pure FBS
 - c. 1 tube of 15% DMSO in RPMI-1640 medium
 - d. Isopropanol tub
 - e. Centrifuge tube containing cells for freezing
- 2) The minimum cell number to cryopreserve is 0.5 million/tube, optimal is 1 million/tube, and maximum is 5 million/tube. Adjust the number of cells per tube as needed, with ideally a minimum of 3 vials being cryprservd per patient sample.
- 3) For every vial cryopreserved, add 0.8mL (800 μ L) of FBS and 0.8 mL (800 μ L) of 15% DMSO in RPMI-1640 medium directly to the 15mL centrifuge tube (from step 13 above). Final concentrations of cryopreservation reagents are: 50% FBS and 7.5% DMSO
- 5) Mix with the serological pipette tip.
- 6) Add 1.6 mL of the viable mononuclear cells suspended in the cryopreservation medium + FBS to each 2 mL outer-threaded vial 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.
- 7) Place vials into the isopropanol control rate freezing tub and into a -80^oC freezer
 - a. Samples are to be inventoried and transferred 12-24 hours later into a liquid nitrogen vapor freezer
- 8) Clean area with 70% ethanol

Notate 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 media 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 the glutamine and growth factors from additive and FBS)
- 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 (acidification, i.e. yellow medium) > than every 7-10 days
 - d. Continue this procedure until cells have reached at least one 150 cm² flask
- 4) Discard the cells immediately when microbial contamination is present.
 - a. Notate within the spreadsheet corresponding to that patient that contamination is present and cells were discarded.
- 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.

Designating a Culture as an Established Cell Line:

- 1) Primary samples may be considered established as a cell line when the following conditions are met:
 - a. The expanded flask (typically in 1-3 150 cm²) is confluent with at least 80-90% cancerous tumor cells
 - b. Short tandem repeat (STR) verified to match the original patient material and is unique in the COGcell/TXCCR STR database.
 - c. Mycoplasma contamination-free
 - d. EBV results known
 - i. EBV+ cell lines are still established, but the cell line is designated EBV+ (often are lymphoblastoid lines) 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 information
 - a. Name will consist of: Study ID (COG or TX) – cancer diagnosis code – cell line number, oxygen condition (20% O₂ = no indicator, 5%O₂ = h, and 2% O₂ = h2), medium type (IMDM = no indicator, Neurobasal = nb)
 - i. Ex. TX-BR-100h2. This is a TXCCR breast cancer cell line grown in 2% O₂ 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% O₂ in Neurobasal media.
 - iii. Ex3. TX-V-112h. This is a TXCCR, lymphoblastoid (EBV+) cell line grown in 5%O₂.
- 3) For all cell lines established in IMDM the formulation used for growth of cells 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 and documentation of growth without contamination and mycoplasma-free must occur.

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.

RBC Lysis Buffer Recipe

- Materials
 - 1) 1 mM Ammonium Bicarbonate (NH₄HCO₃) – 0.079g/Liter
 - 2) 114 mM Ammonium Chloride (NH₄Cl) – 6.1g/Liter
 - 3) 1 Liter deionized water (H₂O)
 - 4) 0.22µm filter
- Dissolve the solid chemicals in 500 mL of deionized water
- Once dissolved, combine with the remaining deionized water
- Filter 1 Liter solution using 0.22 µm 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 returned to the clinical pathology laboratory or 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.