Revised 11/05

ADVL0411 study

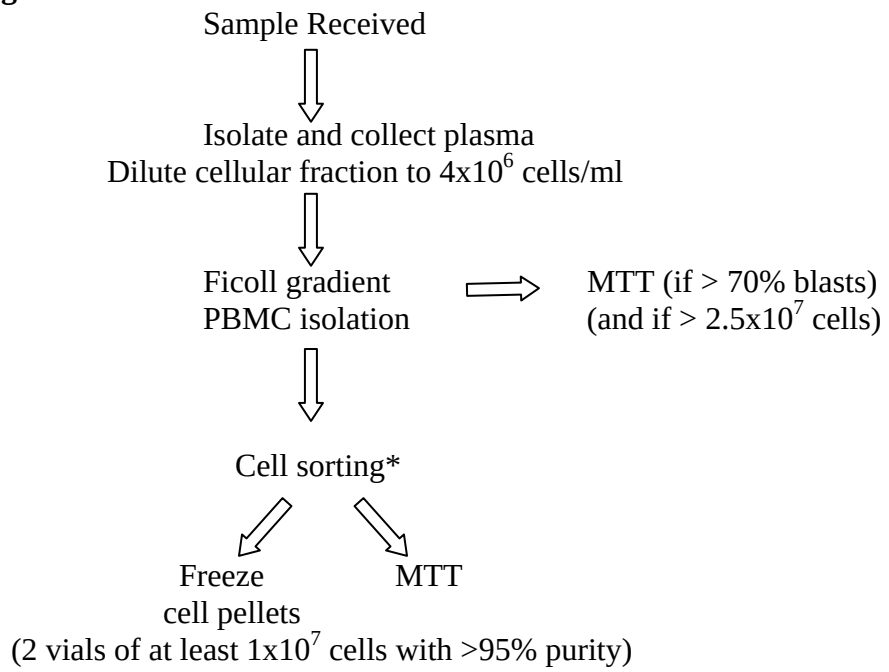
(temozolomide in patients with leukemia)

I. Overview of study:

We are interested in determining the level of MGMT activity and MRS mutations in leukemic samples (plasma and lymphoblasts) before drug treatment. To do this we will **collect serum** and **freeze cell pellets** (for protein and DNA work) and (if cells are available) perform a **cytotoxicity assay** using MTT (Kathy Scorsone will do this).

Since a purified population of lymphoblasts is needed for the MGMT activity assay, the cells will need to be sorted if possible. If the % blasts is <90% in the bone marrow or blood, Chris can sort out the lymphoblasts (and normal lymphocytes if there are enough) to use for cell freezing and (if there are enough) MTT analysis. In this case the timing may be important, so it's important to **note the time elapsed** between acquiring the sample (noted on the sheets received from the site) and the time that cell pellets are frozen.

Overview diagram:



* don't need to sort marrow if >90% blasts. Will need $1x10^7$ cells with >95% blasts for MGMT activity assay; can do from peripheral blood if blast % <90% (and enough cells for $1x10^7$ cells after sort—assume 80% recovery post-ficoll gradient)

II. Sample preparation

A. Contacting folks: We will either receive either a peripheral blood sample (if the WBC count is high) or both peripheral blood and bone marrow. Once we know a marrow is coming, please contact Mike/Chris for cell sorting @4-4252. Samples arrive by FedEx between 10-11 am. Book slots for 11am-1pm for flow sorting if available. In this case it is good to try to sort at least $1x10^7$ cells on the day the samples arrive if possible. However, if there is a sorting slot open the next day, this is also OK, just note the time lag. The samples will be pretreatment only (no day 8 or 18 samples).

- Put the Lymphoprep (if don't plan to sort) and Histopaque (if plan to sort – can get higher yield) at room temperature on the morning of sample arrival.
- When the sample is received
    1. Log into logbook, ask Terzah for sample # (COG#) if not on sheets that come with sample. Record sample type, diagnosis and shipment condition
    2. Log in volume of each sample.
- Take an aliquot of 100ul of marrow in an Eppendorf tube to April Durrett (4-4666, 11^{th} floor) for WBC count. The WBC count tells how to dilute sample for ficoll gradient

Take an aliquot of 200ul marrow in an Eppendorf tube to Mike (4-4252, 10^{th} floor) for blast % (This % helps to decide if the sample needs to be sorted.) and to determine markers. Need to bring immunophenotype report for AML samples). (This % helps to decide if the sample needs to be sorted.)

Step 1: Isolation of Plasma:

- Centrifuge the blood sample at 1000 rpm for 5 min.
- Label Eppendorf tubes with COG#, "plasma" and date.
- Aliquot top layer (plasma) from the centrifuged blood sample into labeled tubes
- Store at @-80^0C. Note location in lab book and logbook.

Step 2: Isolation of PBMC by sucrose gradient:

- Label 50 ml polypropylene tubes. In a 50ml polypropylene tube add 15ml of Lymphoprep solution (for sorted sample use Histopaque 1119 instead of Lymphoprep). If total WBC in the peripheral blood is >4000 cells/ul, dilute to $4X10^6$cells/ml in HBSS medium. Also dilute the bone marrow sample in HBSS medium to a cell count of 4x106 cells/ml (based on WBC count from April)
- Carefully layer diluted blood and marrow samples over an equal volume of ficoll solution. Try not to mix the layers.
- Centrifuge the tubes for 30 min at 1200rpm (300xg) with brake off. After centrifugation the sample will layer:
    - bottom layer: RBC's
    - Next layer: (slightly turbid) lymphoprep or histopaque
    - Third layer: *thin layer of cells (PBMC)*
    - top layer: media/plasma layer.

- Note: The cells are at the *interface* between lymphoprep and serum layer.

- Remove the interface with a pipettman (p1000) into a clean, labeled 50 ml tube. Dilute the PBMC to 10ml with cold 1XPBS and mix gently. Take an aliquot of 90ul and add 10ul of trypan blue (1:10). Count the cells using hemocytometer.
- Add 30ml 1XPBS with 2%FBS (=FACS wash buffer) to cells and spin the cells for 5-7 min @1000rpm. Aspirate supernatant. Resuspend the cells for the assays planned. (Cell sorting and MTT assay)
- If the pellet is red/pink, same 50ul for cytospin analysis (see below)

-----**Step 3: Cell sorting** (don't need to sort if blast %>85% in either marrow or blood.). If marrow is >85% blasts and peripheral blood is less than 85% blasts, sort the blood and freeze the unsorted marrow (as cell pellets) and do the MTT from the unsorted marrow. Freeze sorted cells for the MGMT activity assay (goal: $1x10^7$ cells).

-----**Step 4**. **Freeze cell pellets** $1X10^7$ cells per tube, can freeze up to 10 tubes if lots of cells. Need at least two tubes for each sample type (blood and marrow).

-----**Step 5**. **MTT assay** - If purity is < 70% or if cell # is < $2X10^7$ cells – do not perform MTT analysis. If > 70%, perform on only one type of sample (blood or marrow) whichever you have the most cells (this is usually marrow)

----Step 6. **Cytospin –** If the pellet appears contaminated with red cells, please do cytospin:

--Assemble labeled, coated slide, filter paper and plastic funnel into metal holder.

--Place holder in cytospin apparatus (balance each sample)

--Add 50ul of sample (and 50ul 30% albumin if available)

--spin at 1000 rpm for 2 min.

--Air dry on heat block set on low (fixes cells to slides)

--Put on Terzah's desk for staining in heme lab.

Step 3. Cell Sorting: (done on 10th floor in Flow lab –Mike/Chris@ 4-4252)

- Resuspend cells in 750ul of 1X binding buffer. If more than $4X10^7$ cells, resuspend cells to $4X10^7$ cells/ml in 1X binding buffer.
- Add CD45-PE- 1ul for each $1X10^6$ cells (need to use a minimum of 25 ul of reagent). Vortex briefly and incubate 10min in dark @room temp. If the cells are low CD45 expressers (Mike will let you know), also add CD10-FITC (1 ul/$1x10^6$ cells) if ALL. If AML and low CD45 expressers, ask Mike what counterstain to use (CD33 or CS14).
- If >750ul divide sample into 500ul for each 3ml tube for spinning (for eg: if $8X10^7$ cell, add 2ml 1Xbinding buffer, incubate @ RT, then divide into 4 tubes (500ul each) for centrifugation).
- Wash cells once with 2 ml 1X FACS wash buffer. Take an aliquot and count the cells.

- Resuspend to $4X10^7$ cells/ml in RPMI1640 + 20%FBS + antibiotics in **special polypropylene tubes** for sorting. Place $4X10^7$ cells/ml (1ml) in each tube. Take some extra clear 3ml tubes with 500 ul medium (500ul RPMI1640 + 20%FBS + antibiotics) to FACS lab to place the sorted cells into after sorting.
- After sorting plan the experiments accordingly – (cell count usually on the side of tubes)

  -if $2X10^7$ cells or less –freeze as cell pellets only: go to step 4 (i.e., Freeze pellets for protein lysates) below. **Note time** of freezing.

Step 4. Freeze pellets for protein lysates: (stored at –80^{0}C)

- Freeze all cells remaining after completing MTT (if done)
- Wash cells in 10ml cold 1XPBS
- Resuspend cells to 1X10^{7}cells/ml
- Label the tubes with sample name/no, type of sample (either blood or marrow), cell no and date prepped. For example:
  - Top of tube: COG #____, blood, pretreatment
  - Side of tube: number of cells frozen, date prepped
- Aliquot 1ml into Eppendorf tubes and spin in cold microcentrifuge. (1000 rpm for 7 min) Aspirate the supernatant completely.
- Freeze cell pellets at –80^{0}C and note in the lab notebook and logbook where tubes have been stored.

Step4. MTT assay:

- Resuspend 3.7X10^6 cells to 1X10^7 cells (2X10^7 cells if have extra) @1X10^6 cells/ml in RPMI1640 + 20%FBS + pen-strep & gentamycin.
- Bring cells to Kathy Scorsone (Extension 4-4219) (or Terzah) for plating in MTT assay. NOTE: leave resuspended cells 37^{0}C incubator in tissue culture flask until use. If in 50ml conical tube, loosen 50ml conical cap so cells can breathe

Instructions for Kathy: please modify this section for temozolomide; use concentrations from last temozolomide cell line experiment:

- Dilute +control cells (Jurkat) to $0.75X10^5$/ml
- Plate primary cells using robot (if $1x10^7$ cells) or just three rows by hand if only $4x10^6$ cells (columns 3-11 only)
- On Day 2 add drug temozolomide (__ul stock@__mM + __ml of 1XPBS- 1:4 dilution)

Anu: minimize the volumes here to avoid using up too much drug with each experiment

#	Drug dilutions	Working stock (uM)	Final conc. (uM)
(1)	__ul stock into __ ml 1XPBS	5,000	500
(2)	__ ul of (1) into __ ml 1XPBS		
(3)	__ ul of (2) into __ ml 1XPBS		
(4)	__ ul of (3) into __ ml 1XPBS		
(5)	__ ul of (4) into __ ml 1XPBS		
(6)	__ ul of (5) into __ ml 1XPBS		
(7)	__ ul of (6) into __ ml 1XPBS		
(8)	__ ul of (7) into __ ml 1XPBS		

- Incubate the plates for 48 hrs
- On last day harvest the cells after adding MTT.