Rev. April 27, 2011

Procedure for Enrichment of Circulating Tumour Cells Using RosetteSep® Human CD45 Depletion Cocktail

1. MATERIALS, EQUIPMENT AND FORM

Reagents & Solutions:	Equipment:	Supplies:
0.48 mM EDTA/1x PBS	Swinging bucket benchtop Centrifuge	15mL Polypropylene (PP) Tube.
4% neutral buffered formalin	Microcentrifuge	Low retention large orifice tips
RosetteSep® Human CD45 Depletion Cocktail	Hemocytometer	Sterile Transfer Pipettes
0.1% BSA/0.1% Sodium Azide/1x PBS	Micropipettor	2. 0mL round bottom Eppendorf tubes
1x PBS pH 7.4 +2% Fetal Bovine Serum	Pipet.aid	Sterile Serological Pipette
Complete Growth Medium	Phase Contrast Microscope	1.5mL Eppendorf tube
Trypan Blue		18 gauge needle
Cancer cell line	Fluorescence microscope equipped with recommended filters	5mL syringe
Ficoll Plus density gradient		

2. REAGENTS

<u>0.48 mM EDTA/1x PBS (100mL)</u>
100mL of 1x PBS
0.0199 g of EDTA (Sigma Cat. # E6511)
Store at 4ºC for no more than 2 weeks

<u>0.1% BSA/0.1% Sodium Azide/1x PBS (100mL)</u>
100mL of 1x PBS
0.01g of BSA (Rockland Cat. # BSA-50)
0.01g of Sodium Azide (Sigma Cat. # S2002)
Store at 4 ºC for no more than 2 weeks.

<u>Complete Growth Medium (500mL):</u>
445mL of appropriate base medium for cancer cell line
5mL of penicillin-streptomycin (Sigma Cat. #P4333-100mL)
50mL of HyClone Fetal Bovine Serum (Thermo Scientific Cat. # SH3039603)
Store at 4 ºC for no more than 4 weeks

<u>1x PBS+ 2% FBS (10mL prepared fresh):</u>
9.8mL 1x PBS
200µL HyClone Fetal Bovine Serum (Thermo Scientific Cat. # SH30396.03)
Make fresh by adding 200µL FBS to 9.8mL 1x PBS.

3. PROCEDURES

SAMPLES

- Samples consist of volunteer blood samples spiked with Circulating Tumour Cell (CTC) surrogates
- This procedure provides instructions for enrichment of circulating tumour cells using RosetteSep® Human CD45 Depletion Cocktail. Circulating tumour cell population is enriched from whole blood on the basis of negative selection followed by density gradient separation. The tetrameric antibody complexes (targeted against glycophorin A on the RBCs and hematopoietic/leucocytic markers CD45 and CD66b) contained in RosetteSep® Human CD45 Depletion Cocktail lead to Rosette formation of the haematopoetic cells (including leukocytes) with the RBCs. Density gradient separation further allows the sedimentation of these Rosettes leaving a pure population of CTCs at the Ficoll : plasma interface. Purified CTCs may then be characterized by immunofluorescence for cell surface/cytoplasmic markers and fluorescence *in situ* hybridization for cytogenetic alterations.

QUALITY CONTROL

- Control samples must be run concurrently with patient samples to monitor assay performance
- Ensure that all reagents have reached desired temperatures prior to initiating procedure

PREPARATIONS REQUIRED BEFORE STARTING PROCEDURE

- All reagents and stock solutions should be prepared prior to the start of the procedure
- Label the slides correctly: patient ID, date, lab identification, etc., along with name of person doing the procedure
- Prepare fresh solutions prior to each procedure

PROTOCOL

<u>Harvesting cultured tumour cells</u>

1. Culture cells to no more than 60% confluence in a 75cm^2 cell culture flask (polystyrene, tissue culture treated).
2. Aspirate growth medium from the flask with a 10mL sterile serological pipette and discard.
3. Perform a wash of the flask with 3mL of 0.48 mM EDTA/1x PBS (37ºC) using a 5mL sterile serological pipette.
4. Aspirate 0.48mM EDTA/1x PBS from the flask with a 5mL sterile serological pipette and discard. Repeat steps 3 and 4.
5. Add 3mL of 0.48mM EDTA/1x PBS (37ºC) using a 5mL sterile serological pipette, incubate for 5 minutes at 37°C in a 5 % CO_2 incubator. Give the flask a firm tap against a flat surface to dislodge the cells from the flask.
6. As the cells become detached from the flask, use a 5mL sterile serological pipette to gently re-suspend the cells up and down against the flask's bottom to break the clumps if they occur.
7. Aspirate the 3mL of cell suspension using a 5mL sterile serological pipette and transfer to a new 15mL polypropylene (PP) tube.
8. Add 7mL of complete growth medium (37ºC) using a 10mL sterile serological pipette.
9. With a transfer pipette gently re-suspend the cell suspension up and down.
10. Centrifuge the cells at 300 x *g* for 10 min with the brake speed turned "OFF" at room temperature.

11. Aspirate the supernatant with a 10mL sterile serological pipette and discard.
12. Add 10mL complete growth medium (37°C) to the 15mL PP tube and gently re-suspend the pellet using a transfer pipette. Centrifuge the cells at 300 x *g* for 10 min with the brake speed turned "OFF" at room temperature. Aspirate the supernatant with a 10mL sterile serological pipette and discard.
13. Repeat step 12.
14. Add 3mL of complete growth medium (37°C) using a 5mL sterile serological pipette. Gently re-suspend the cell pellet using a transfer pipette.

Preparation of **stock** cell suspension

- Using 1000µL micropipettor with low retention large orifice tips, dilute 100µL of cell suspension in 900µL of complete growth medium in a new 1.5mL Eppendorf tube. Re-suspend well to mix. This is the stock solution.
- Count cells on a Hemocytometer (Refer to Appendix A for Hemocytometer instructions).
- Based on the results of the cell counting, dilute the cell suspension with PBS +2% FBS to obtain a cell solution with a concentration of $1x10^6$ cells/mL.
- Perform serial dilutions (1:10 each) to obtain a cell suspension with a concentration of $1x10^5$cells/mL:
    - Using a 1000µL micropipettor with low retention large orifice tips dilute 100µL of stock cells ($1x10^6$ cells/mL) in 900µL of room temperature PBS +2% FBS in a new 1.5mL Eppendorf tube. Re-suspend well to mix. This will give you a suspension of $1x10^5$ cells/mL. This is the working cell solution

Verifying the concentration of the working cell suspension

- Using the $1x10^5$ cells/mL working cell suspension, count the cells on a Hemocytometer (Refer to Section 2.3 for Hemocytometer instructions).
- Take the average of the 3 counts and determine the final stock cell concentration.

Spiking the donor blood sample with tumour cells

- Based on the final concentration determined for the stock cell suspension calculated above, determine the volume of cell suspension required to transfer $1x10^5$ cells:

$$X \text{ uL stock solution} = [1000 \text{ cells}] / [\text{final concentration stock solution}]$$

- Using a 200µL micropipettor with low retention large orifice tips, gently take ($1x10^5$ cells) of working cell suspension and transfer to 2mL of blood (in a 15mL conical tube). This gives a final concentration of $1x10^4$ spiked surrogate CTCs in blood.

Enriching the population of surrogate CTCs using RosetteSep® Human CD45 Depletion Cocktail

Bring all the reagents to room temperature in order to ensure efficient antigen-antibody reaction.

- To 2mL of spiked whole blood (in a 15mL conical tube) add 100µL of RosetteSep® Human CD45 Depletion Cocktail. Mix well by inverting the tube gently 5-6 times
- Incubate the tube at room temperature for 20 min
- Dilute the blood-antibody mixture with 2mL PBS + 2% FBS and invert the tube 3-5 times to ensure even mixing
- Layer the diluted blood mixture on 3mL Ficoll-Paque (in a 15mL conical tube).
- Centrifuge the tube at 1200 x *g* for 20 minutes at room temperature with the brake off.
- Carefully collect the circulating tumour cells from the Ficoll: plasma interface (with some Ficoll and plasma to achieve maximum CTC recovery).
- Wash the collected CTCs with 2mL PBS +2% FBS by gentle resuspending. Centrifuge at 1200 x *g* for 5 minutes. Discard supernatant
- Add 1mL PBS +2% FBS to the cell pellet and transfer the CTC suspension to a 1.5mL eppendorf tube
- Repeat the wash step by centrifuging at 200 x *g* in a microcentrifuge for 5 minutes
- Aspirate the supernatant and resuspend the cells in 100µL of 4% neutral buffered formalin
- Follow SOP # 10.10.005 for cell fixation and Immunofluorescence followed by Fluorescence *in situ* hybridization.
- The CTCs can now be used for downstream applications such as IF and/or FISH.

4. APPLICABLE REFERENCES, REGULATIONS & GUIDELINES

- Hemacytometer info: http://www.cascadebio.com/uploads/file/pdf/hemat.pdf
- http://www.stemcell.com/en/Products/All-Products/RosetteSep-Human-CD45-Depletion-Cocktail.aspx
- ATCC: The Global Bioresource Center: http://www.atcc.org
- Refer to the 3rd edition of the Public Health Agency of Canada's Laboratory Biosafety Guidelines,
- Refer to the Queen's University's Biohazards Safety Manual published by the Department of Environmental Health and Safety, when handling biohazardous materials such as blood and tumor samples

APPENDIX A - CELL COUNTING USING LARGE CAPACITY HEMOCYTOMETER:

Stock Solution Cell Concentration

- Place 100µL of Trypan Blue and 100µL of the stock cell suspension into a 1.5mL microcentrifuge tube.
- Gently mix the Trypan Blue and cell suspension, remove 90µL and place onto the hemocytometer. The counting area on the hemocytometer is 50µL.
- Count only viable cells (clear in light blue background).
  Count the entire rectangular area of the hemocytometer.

Calculations for stock solution cell concentration	
Number of Cells Counted	
x by 2 (double count)	
x dilution factor (2)	
x 10	
Cell Concentration (cells/mL)	

Verify Number of Cells Spiked

- Place 100µL of Trypan Blue and 100µL of the $1x10^4$ cell suspension (working cell solution) into a 1.5mL microcentrifuge tube.
- Gently mix the Trypan Blue and cell suspension, remove 90µL and place onto the hemocytometer. The counting area on the hemocytometer is 50ul.
- Count only viable cells (clear in light blue background).
  Count the entire rectangular area of the hemocytometer.

Calculations for 1 x 10^3 cell concentration	
Number of Cells Counted	
x by 2 (double count)	
x dilution factor (2)	
Cell Concentration (cells/mL)	

***Note: This protocol has not yet been tested for CTC recovery efficiency, hence will be modified for the cell numbers once tested.**