OSUCCC LTB Laboratories Procedure Procedure to thaw cryopreserved mononuclear cells from bone marrow, peripheral blood or products of leukapheresis[1]			Effective: 10/1/1997
Written by: Donna Bucci, Laboratory Supervisor		Reviewed by: Donna Bucci	
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Version 1.0:		10/01/1997	5
		9/30/1998	5
		9/30/1999	5
		9/30/2000	5
		9/30/2001	5
		9/30/2002	5
Revised Version 1.1: Angela Lang BS	1.0	4/15/2003	6
		9/30/2003	6
		9/30/2004	6
		9/30/2005	6
		9/30/2006	6
		9/30/2007	6
		9/30/2008	6
		9/30/2009	6
		8/13/2010	6
		7/19/2011	6
		9/26/2012	6
Version 1.2: Wacharaphon Vongchucherd BS	1.1	9/25/2013	7
Version 1.3: Donna Bucci BS	1.2	10/04/2014	7
		12/29/2015	7

1.0 PRINCIPLE

Every attempt is made to prepare cryopreserved viable cells that may be retrieved with the highest level of viable sample of recovery. By using carefully standardized techniques for cell preparation we are able to obtain an average of cell viability at the time of freezing of ≥ 80%. The following protocol has been used in the LTB and has been demonstrated to provide consistently high recovery of viables. The key is not to rush or try to thaw too many aliquots at one time. Cryopreserved cells are fragile and require gentle handling. Cryopreserved cells are thawed quickly and plated directly into complete growth medium. If cells are particularly sensitive to cryopreservative (DMSO or glycerol), they are centrifuged to remove cryopreservative and then plated into complete growth medium.

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as

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Trypan blue, Eosin, or Propidium Iodide, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye. A viable cell will have a clear cytoplasm whereas a nonviable cell will have a blue cytoplasm. [1]

Hemacytometers were developed for counting blood cells, but can also be used to count other cell types. A hemacytometer has two chambers and each chamber has a microscopic grid etched on the glass surface. The chambers are overlaid with a glass coverslip that rests on pillars exactly 0.1 mm above the chamber floor. Thus, the volume of fluid above each square of the grid is known with precision.

2.0 SPECIMEN

Cryopreserved viable cells from peripheral blood, bone marrow aspirate, single-cell suspension of tissue sample or products of leukapheresis.

3.0 MATERIALS AND REAGENTS

Sterile 15 or 50cc conical tube
Sterile pipets (5ml, 10ml and 25ml)
Sterile Dulbecco's PBS (Invitrogen # 19140-144)
Sterile Fetal Bovine Serum (Invitrogen # 16140-071)
RPMI-1640 Phenol Red free (Invitrogen # 11835-030)
Sterile 4x4 gauze
Sterile pipet tips (200µl and 1000 µl)
Micropipetters (20-200µl and 1000 µl)
Neubauer Brightline Hemacytometer
Trypan Blue stain (0.4% - Invitrogen # 15250-061)
Wexcide solution

4.0 EQUIPMENT

Biosafety cabinet
Benchtop centrifuge with swinging bucket rotors to hold 15 and 50cc conical tubes

5.0 QUALITY CONTROL AND SAFETY

It is recommended that specimen collection be carried out in accordance with NCCLS document M29T2. No known test sample can offer complete assurance that human blood samples will not transmit infection. Therefore, all derivatives are potentially infectious. Always spray alcohol on the caps before opening solutions. The alcohol can be dried off using gauze. If you have been out of the hood for a while and are wearing the same pair of gloves, use a new pair of gloves.

6.0 PROCEDURE

6.0.1 Prepare a volume of phenol red free RPMI + 10% FBS and warm it to 37°C in a water bath for 15-20 minutes. Phenol red has been reported to stimulate certain cell types within a given population; therefore phenol-red free medium is preferred. Prepare 10ml of completed media for each sample to be thawed.

6.0.2 After the media has been warmed, prepare sterile 15cc conicals tubes, 1 for each sample to be thawed. Add 10 ml of the pre-warmed media to each conical tube.

6.0.3 Retrieve samples from LN2 storage and keep them on dry ice until the media is ready to use. Don't attempt to thaw more than a couple of vials at a time. The DMSO containing cryopreservative should be diluted as soon as the sample is thawed.

6.0.4 Do NOT allow thawed samples to sit at room temperature in undiluted freezing medium.

6.0.5 Once the media tubes are ready, thaw the cryovials at 37°C in a water bath, by gently agitating the vials in the water.

6.0.6 When there's just a small bit of ice left, working in a biosafety hood, add 1ml of pre-warmed media to one of the cryovials, pipetting no more than 2 times to transfer the entire volume into the prepared 15cc conical.

6.0.7 Do not vortex thawed cells in the 15cc conical but gently pipet as few times as possible to transfer the all of the cell aliquot from the cryovial. The total volume in the tube is now approximately 10ml. Repeat with the second cryovial.

6.0.8 Spin the resuspended cells at 1100rpm for 10 minutes.

6.0.9 Gently resuspend the cell pellet in 2 or 5 ml of media, depending upon the size of the pellet.

6.0.10 Perform a manual (hemacytometer) cell and viability count using 100ul of sample and 100ul of trypan blue.

6.0.11 Dilute per the user's protocol for assay or culture.

6.1 SAMPLE EVALUATION – TRYPAN BLUE EXCLUSION STAIN WITH HEMACYTOMETER COUNT

6.1.1 It is important not to overload the chamber, as doing so will give an inaccurate count. The same is true if the cover slip is moved after the sample is loaded (Figure 1).

6.1.2 The sample is allowed to settle for 2 or 3 minutes so that the cells stop drifting around the chamber and most will be in the same plane of focus (Figure 2). It is important not to allow the sample to settle too long or it will dry out, concentrating the cells over the grid. To avoid drying, the hemacytometer can be placed on straws within a Petri dish containing a moistened filter paper.

6.1.3 The full grid on a hemacytometer contains nine squares, each of which is 1 mm square.

6.1.4 The central counting area of the hemacytometer (as it will be called here) contains 25 large squares and each large square has 16 smaller squares.

6.1.5 When counting, count only those cells on the lines of two sides of the large square to avoid counting cells twice.

6.1.6 All 25 large squares can be counted (Figure 3), or a counting pattern using fewer squares can be used like the ones below. It is important to distribute the counting areas in a non-biased manner since cells can be more concentrated on one side of the chamber.

6.1.7 If you count over only 5 of the 25 large squares, then multiply that value by 5 to obtain the number of cells per central counting area (Figure 5).

6.1.8 Each of the nine squares on the grid, including the central counting area of 25 large squares, has an area of 1 square mm, and the cover glass rests 0.1 mm above the floor of the chamber. Thus, the volume over the central counting area is 0.1 mm^3 or 0.1 microliter. You can thus multiply the average number of cells over each central counting area by 10,000 to obtain the number of cells per ml *of diluted sample*.

6.1.9 In other words, to calculate the number of cells per ml of original sample:

6.1.10 Calculate the mean number of cells counted for each chamber (i.e. for each of the central counting areas of each chamber).

6.1.11 Multiply the mean obtained in (1) by 10,000 to obtain the number of cells per ml of diluted sample.

6.1.12 Multiply the count obtained in (2) by the dilution factor.

Example: Assume that you dilute the original sample by adding 0.1 ml of cell suspension to 9.9 ml of diluent (1:100 dilution factor). You then count the number of cells in 5 of the 25 large squares within the central counting area of two chambers, obtaining counts of 132 and 128 cells.

1. The mean number of cells per chamber is thus 130 x 5 or 650 cells per counting area (650 cells per 0.1 microliter).
2. Multiply the 650 cells per counting area by 10,000 to obtain the number of cells per ml of diluted sample (answer = 6,500,000)

Multiply 6,500,000 cells per ml of diluted sample by 100 (the dilution factor) to obtain 650,000,000 per ml of original sample.

Figure 1. Neubauer Hemacytometer

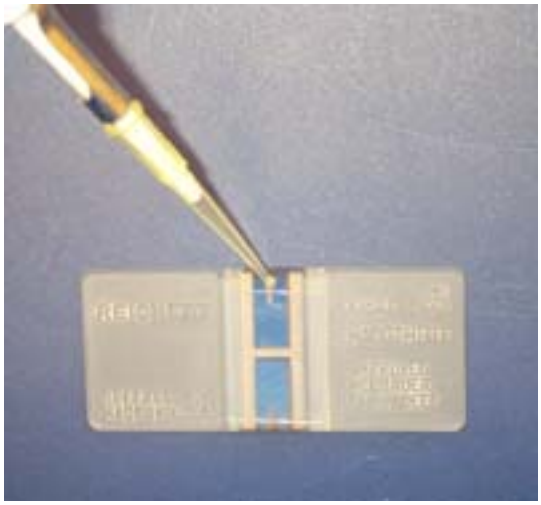
Figure 2.Adding sample to hemacytometer

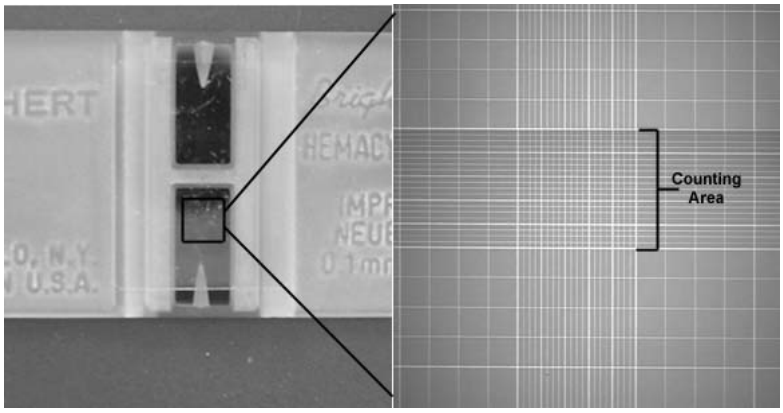

Figure 3. Counting area

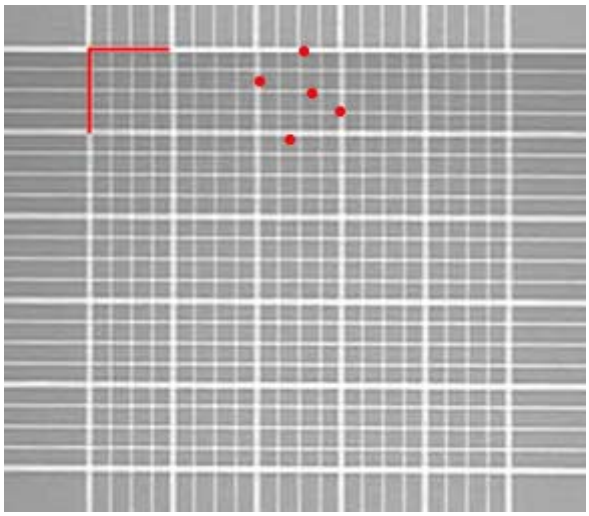
Figure 4.Close up of counting area

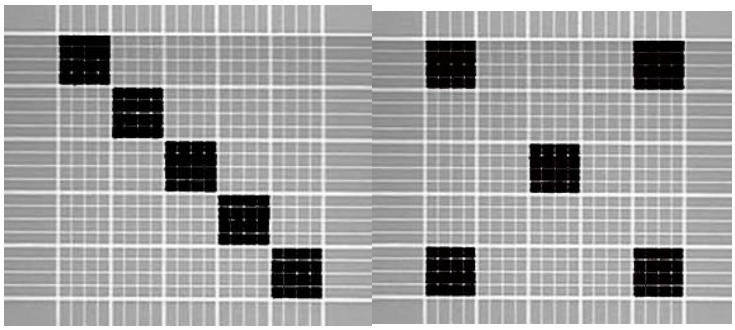
Figure 5.Detail showing possible counting schema

7.0 LIMITATIONS OF THE PROCEDURE

Dye exclusion is a simple and rapid technique measuring cell viability but it is subject to the problem that viability is being determined indirectly from cell membrane integrity. Thus, it is possible that a cell's viability may have been compromised (as measured by capacity to grow or function) even though its membrane integrity is (at least transiently) maintained. Conversely, cell membrane integrity may be abnormal yet the cell may be able to repair itself and become fully viable. Another potential problem is that because dye uptake is assessed subjectively, small amounts of dye uptake indicative of cell injury may go unnoticed. In this regard, dye exclusion performed with a fluorescent dye using a fluorescence microscope routinely

results in the scoring of more nonviable cells with dye uptake than tests performed with Trypan blue using a transmission microscope.

A more sophisticated method of measuring cell viability is to determine the cell's light scatter characteristics or propidium uptake.[2] However, this technique is far more time consuming and is necessary only when precise measurements on the number of dead cells in a cell mixture must be obtained. Trypan blue exclusion, as described in the above protocol, can be performed in 5 to 10 minutes.

8.0 REFERENCES

1. Strober, W. Commonly user immunological techniques. Current Protocols in Immunology, Appendix 3. 2007.).
2. Shapiro, H.M. 1988. Practical Flow Cytometry, 2nd ed., p. 129. John Wiley & Sons, New York.