Standard Operating Procedure V:
Bronchoalveolar Lavage Isolation and Processing

NOTE: The following procedure is to be performed wearing laboratory coat, gloves, eye protection, and mask. Perform all open tube procedures in a biological safety cabinet (BSC) in a level 2 laboratory. If Level 3 pathogens are suspected to be in the sample, do not proceed.

The bronchoalveolar lavage (BAL) fluid must be fresh and processed immediately after collection.

1. Label all the tubes and slides needed for this procedure BEFORE the start of the protocol.

2. ICU Doc's will collect BALF in sterile traps (tri-trap assembly). Tubes should be documented for volume of normal saline infused, anatomical area lavaged (i.e. right middle lobe, and volume aspirated back into trap). **MEASURE AND RECORD TOTAL FLUID RETURN FOR BRONCHOPIST**

3. ICU Nurse will take fluid required for diagnostic purposes and the remainder can be used for research. Record the volume of fluid taken by the nurse.

4. Place the remaining trap(s) on ice immediately.

5. After the procedure, transport the specimens back to the lab immediately for processing.

6. Pool the traps of BALF and record the volume obtained.

7. Filter the lavage sample through a 40µm nylon filter into a sterile 50ml falcon tube.

8. Record the volume obtained after filtering (some fluid loss is expected).

9. Transfer 2ml of filtered lavage sample to a clean and identified 15ml Falcon tube.

10. Centrifuge the remaining fluid at 1200rpm (235 x g) for 15 minutes with brakes at *4°C*.

11. In the mean time, determine the number of cells present in the sample:
    a. Mix 10µl of lavage fluid (from the 0.5 ml saved above) with 80µl PBS + 10µl Turks.
    b. Load 10µl of this mix into each chamber of a hemocytometer. Count four squares in each chamber. Maintain two counts, one viable, one non-viable.
    c. Calculate and record cell concentration for every chamber, as follows:
        i. **(**Total cells counted**/**4**) x** 10 **x** 4000 **=** number of cells per ml
    d. Do this for each chamber and average the numbers.

    e. Calculate and record **total** cell number by multiplying "cells per ml" by the volume of BAL after filtering (***less 2ml fraction removed***).
    f. Cell viability will be recorded as % of viable cells: **(**viable cells**/**total cells**) x** 100

12. Pour the centrifuged lavage supernatant into a 50ml Falcon tube and place on ice.

13. Put 1.5 ml of supernatant into each of the ten cryovials and store the remaining supernatant in 10ml aliquots in the 15ml identified conical Falcon tubes.

14. Place all aliquoted supernatant into the -70°C freezer.

15. Set up the slides with the cytofunnels in the cytospin.

16. Compute the volume needed for 50,000 cells per slide.

    a. **(**$5x10^4$cells/slide**) / (**Y x 10^4cells/ml**) x** 1000µl/ml **=** X µl/slide
    b. X = µl to give 50 000 cells/slide.
    c. Y = cell concentration from Step 6.

17. Multiply X by 12.

18. Remove this volume from the 2ml BAL aliquot.

19. Bring total volume to 6ml with PBS in a separate tube. Mix well.

20. Load 500µl into each cytofunel.

21. Centrifuge the slides in the cytospin at 600 rpm (Cytospin2 by Shandon) for 10 minutes

22. Discard funnels and allow the slides to air dry at least two hours.

23. Stain two slides with Hemacolor stains as follows:
    a. 30 seconds in fixative #1
    b. 15 seconds in eosin Y (red) #2
    c. 15 seconds in thiazine (blue) #3
    d. Rinse in distilled water

All remaining slides are fixed. At least two slides should be fixed in *each* of the following ways: Methanol, acetone or formalin. Indicate on the slide with #2 pencil how slide was fixed.