**FLOW CYTOMETRY SAMPLE STAINING**

The purpose of this procedure is to create samples for flow cytometry enumeration. This method if used with sterile materials and under sterile technique conditions can be also used for cell sorting. For this procedure use biohazard safety precautions at all times.

**PREPARING THE SAMPLES**

1. Obtain sample to be tested and proceed to do a cell count on the sample. Refer to the proper procedures for sampling and cell counting.
2. Calculate the amount of sample required per stain. See step 8 if the amount of sample per tube is greater than ½ ml. Most samples require 1 million cells/tube. Multiple stains can be used in a single tube. The maximum stains (antibodies) per tube for this lab is 4. If a viability stain is used, then the maximum number of stains (antibodies) per tube is 3.
3. The viability stain used in this lab is 7AAD. It is a mutagen and is to be handled very carefully. The spectrum that this stain encompasses crosses between the Per-CP and APC color spectrum therefore it cannot be used in conjunction with either of these stains.
4. Appropriately label a sufficient number of tubes (5 ml snap cap tubes) required for the stain panel that is needed.
5. This label should include the sample control number and rack location of the tube in the panel.
6. If this is a new stain or it is being run separately from our conventional panels then label the tube with the antibody type. By convention the labeling is as follows, FITC is first, PE is second, Per-CP is third, and APC is fourth. If 7AAD is to be put in the stain tube, then it will be listed last after FITC and PE.
7. The appropriately labeled tubes should be placed in a rack in the specific order they will be run on the flow cytometer. Remove all caps from the tubes.
8. To each tube add approximately 2-3 ml of Hemolytic buffer (cap and return this buffer to the refrigerator when done). It is best if this buffer is at room temperature. Make sure not to have the bottle of this buffer exposed to the ambient room air for extended periods of time. If that occurs the pH of this buffer will become deleterious to the cells. If the amount of the blood sample needed is greater than ½ ml of blood it is best to use a 15 ml tube to hemolyze the red cells of the sample. Warming the sample in a 37 degree water bath will assist in the hemolysis process. This tube can be then washed beginning on step 12 of this procedure.
9. If you are going to immediately stain the blood sample, then the caps on the tubes can remain off. If these tubes are being prepped for a later stain procedure, then it is best to put the caps back on the tubes.
10. Pipet the appropriate amount of sample into the tubes containing the Hemolytic buffer. Refer to the **Tube Sampling** procedure for how to correctly obtain a sample from a tube of blood.
11. When the sample has been pipeted into all the stain tubes containing Hemolytic buffer, then set a timer for 3 minutes.
12. On completion of the 3-minute incubation add ½ ml of flow wash to each of the tubes.
13. Centrifuge the tube(s) in the tabletop Sorvall centrifuge for 3 minutes at room temperature at 1600 RPM with brake.
14. On completion of centrifugation remove the tubes from the centrifuge checking each tube to see if the cells have pelleted. Dump the supernatant off each tube with a gentle motion and “flick” of the wrist. This will allow about 100 ul of supernatant to remain in the tube. This “flick” method is important later in the staining procedure.
15. When all tubes have had the supernatant drained, then carefully rake the tubes against the tube rack. This action will break up the cell pellet.
16. Add 3-4 ml of flow wash to the tubes and repeat the centrifugation process as in step 13.
17. Check and drain the tubes as in step 14.
18. Rake the tubes as in step 15. If the flow wash is not to be used in the immediate future the it is recommended to return it to the refrigerator.
19. The tubes are now ready for the addition of stain.

**STAINING THE PREPARED SAMPLES**

1. Prepared samples can be held for 1-2 hours prior to staining if more emergent procedures arise.
2. Determine what stains are required for the samples to be stained. Remember antibody stains are light sensitive and should be kept cold and in the dark. Bottles should be kept closed when not in use. Exposing the stains to fluorescent light will degrade the stains over time.
3. Set your pipet(s) to the correct amount of stain to add to each tube. Usual stains require 10 ul stain/tube/1 million cells in 100 ul of liquid. Some other stains require other amounts, refer to that particular stain for more information. For ease of reading this procedure we will only use 10 ul for the amount to pipet.
4. Check to make sure all stain tubes are labeled and in their correct panel order in the tube rack. Have the tubes places in a single file line to the extreme right of the rack.
5. Pipet 10 ul of the stain antibody into the first tube. Pipet the antibodies one at a time. Only have one antibody bottle open at a time. This will avoid any possibility of cross contamination.
6. Do your pipet work methodically and do not allow any interruptions in your work at this time.
7. When the first sample is done. Move the tube to the extreme left of the rack.
8. Pipet the antibody stain into the second tube. Repeat step 7 with this tube.
9. Pipet all the remaining tubes, moving the tubes from right to left.
10. When completed, rake all the tubes and place in a dark refrigerator (4 degrees C).
11. Set a timer for 25 minutes.
12. On completion of the incubation add 1 ml of flow wash to each stain tube.
13. If there is a tube that will have a viability stain (7AAD) then 15 ul of 7AAD can be added to it after the flow wash has been added.
14. The 7AAD stain will need to incubate for 5 minutes in the dark.
15. On completion of the incubation the stain tubes can be centrifuged in the tabletop Sorvall centrifuge for 3 minutes at room temperature at 1600 RPM with brake.
16. After the centrifugation process check to see that the cells have pelleted and dump the supernatant from each tube.
17. Add 300 ul of flow wash to each tube. The stain tubes are now ready to be run on the flow cytometer. These samples can be held for 2-3 hours in the dark in a refrigerator if there are more emergent procedures that need to be done. 7AAD will remain active and functional for that amount of time.

**MATERIALS NEEDED FOR THIS PROCEDURE**

* Hemolytic buffer
* Flow wash
* Staining antibodies
* Pipets
* Pipet tips
* Tube rack
* Sorvall tabletop centrifuge
* Waste container
* Timer
* Calculator