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DETERMINING ANTIBODY TITERS

Materials and Reagents
1. The new antibodies to be titered.
2. PBS containing 0.5% sodium azide (use for dilution medium)
3. Falcon #2052 or 2054, 12 x 75mm centrifuge tubes
4. micropipettors and tips
5. QC books: titer log books (rm S635), titer book (QC lab)
6. materials and reagents in staining protocol

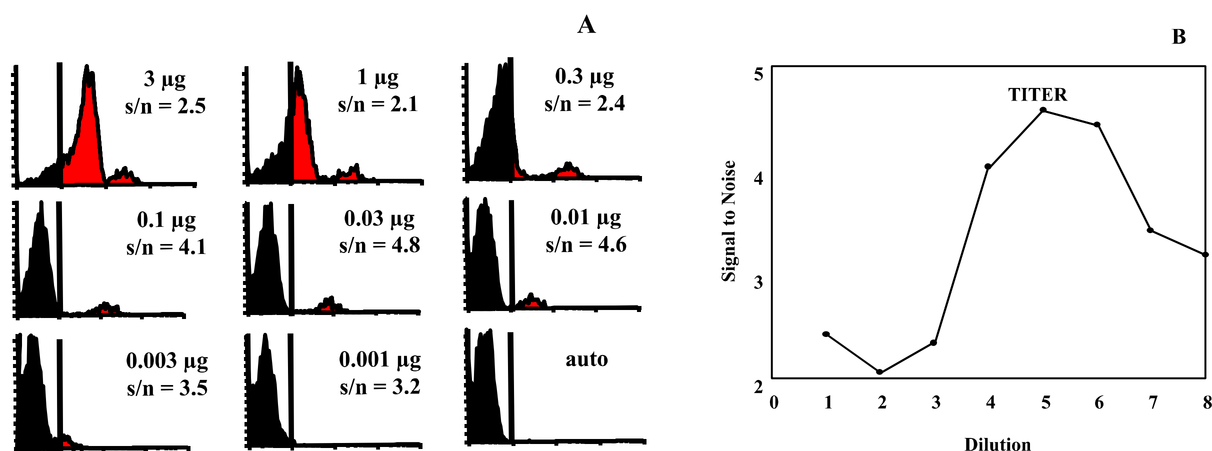
Titering Procedure
When titering the antibodies to be used in four color combinations one must be sure to take into consideration that each antibody will be mixed with three other antibodies and those antibodies will dilute each other. Each antibody should be ordered in bulk at a titer of 5 µl, if possible, and then titered when it is to be used. The same antibody having a new lot number must also be titered. Label each bottle with the date received and the titer to be used. Store in refrigerator in the Quality Control Lab (rm S628).. Dilute using PBS (containing 0.5% sodium azide).

1. Begin by adding 30 µl of PBS (containing 0.5% sodium azide) to four appropriately labeled 12 x 75mm tubes. Then add 10 µl of the stock antibody to be titered to the first tube, mix it and then remove 10 µl and add it to the second tube and continue to the next etc. After mixing the dilution in the last tube discard 10 µl of the total. This way each tube has 30 µl. Then add the 50 µl of washed, blocked PBL or cells to all tubes and continue as per the staining protocol.
2. Record in order, each tube number and contents on a log sheet and give each one a file name.
3. (CD34 must be tested on normal bone marrow and CD10 on granulocytes).
4. These dilutions are equivalent to 0/1, 1/4, 1/16, and 1/64. The first tube contains a one to four dilution (10 + 30 = 40 divided by 10 = 4) of the antibody and is equivalent to approximately (30 µl (antibody volume)/4 =) 7.5 µl of the stock antibody. For simplicity we call this 5 µl. The bulk antibodies have been ordered at a 5 µl titer, so this first tube should be at titer. If it is not, then the company sent the wrong concentration, which is not unusual.
5. When acquiring each sample, use a live gate encompassing the target population, according to the chart of antigens (located in this manual). Collect 5000 events on each sample on the clinical flow cytometer. Transfer the files to the network drive e.g. /E:/sigie/titers/date

Analysis Procedure (using WinList, Verity Software House, Topsham, ME)
1. Open setup.mac for titers F:\verity\titers\setup.mac
2. Next data source, open auto and adjust regions, gated region and appropriate color to be analyzed
3. File open batch files, add batch files to be analyzed
4. Run F:\verity\titers\titer.mac
5. Edit multigraph, label MG with CD#, batch #
6. Print MG
7. For next titers, edit MG – remove all, return to step 2

When titering using 2 mabs (e.g. CD19 and CD23), first compensate in software with the appropriate cmp file. Turn gate on region to be gated (e.g. R4 for PC-CD19). Double click on histogram of desired view to turn on gate.

The optimal antibody titer is the dilution that produces the maximum signal to noise ratio and it is often not the concentration that produces the highest percentage of positive cells as shown in the figure below.



To determine the titer, target cells are stained with various dilutions of antibody and the mean channel fluorescence (MCF) of positive and negative cells determined. The marker is set using unstained cells (auto). The ratio of the MCF of the positive cells to negative cells is calculated. We call this the signal to noise ratio (s/n). Because each antibody, when properly titered, is its own isotype control, there is no need to use a separate one. B. By plotting s/n as a function of the concentration an inverted parabolic shaped curve is produced whose zenith is the titer. This concentration produces the best distinction between positive and negative cells. The left side of the curve represents rapid changes in non-specific binding and very slow changes in specific binding. The right side reflects decreased saturation of epitopes by antibody.

Calculation Procedure:
Calculate the signal to noise ratio in each histogram. The highest ratio is the optimal antibody concentration to use. Record the MCF of this file in the MCF titer log, QC lab. Each time an antibody is titered compare it to the previous batch and record the MCF. If the MCF is < 5 channels different it is acceptable. If less, a new supplier or batch must be found.

If the second tube, when analyzed, has the same MCF or signal to noise ratio as the first tube, then use 5 µl of a 1 to 4 dilution of the stock as the titer. If the third tube is the same, use 5 µl of a 1/16 dilution etc.

If the titer is determined to be 1/16 (the third tube), then make a sample volume of this dilution. For example, take 50 µl and add 750 µl of PBS/azide and label it 1:16 and use 5 µl of it per test. This dilution will be good to keep for several months. Note: If you are only using one antibody in a tube, then you could use 5 µl of a 1:64 dilution, as you are not diluting the antibody with three others.

TESTING A COMBINATION FOR A CLINICAL PROTOCOL

Materials and Reagents

Same as for titering and staining protocols.

This procedure must be performed every time a new panel combination is to be added to the clinical protocols.

1. Determine the titer of all antibodies to be combined according to the SOP for “Determining Antibody Titers”.
2. To four separate tubes, add one test of each of the antibodies and q.s. to 30 µl with PBS. E.g. for a 4 color panel add 25 µl PBS. Note: 30 µl is the total volume amount of each or our four-color panels.
3. To a fifth tube add all the antibodies together and q.s. to 30 µl with PBS. E.g. for a 4 color panel add 10 µl PBS.
4. Stain, using 50 µl of normal healthy donor blood as per staining SOP. (the total volume of each tube will be 30 + 50 = 80 µl)
5. Acquire 20000 events of each sample on flow cytometer.
6. The mean channel fluorescence of each antibody singly acquired must be the within 5 channels of the MCF of the same antibody in the combination of antibodies. If they cannot be so verified the combination cannot be used.
7. Add verification analysis to Panel Log Book.

BATCH PANEL PREPARATION PROCEDURE

Producing a Batch

Once the antibody combination has been validated, batches of the number of tests desired can be created, e.g. 100 test batches. For consistency, to reduce errors and to promote staining efficiency, it is important to create a batch rather than to add the antibodies individually to cells at the time of staining. It is also important that the antibody concentration is three to four times titer so they can be mixed together to give 1x titer because the volume of each antibody dilutes the others.

Materials Needed (QC lab)

Present Panel Log Book
Titered Antibodies
Pipettes, tips
Vials and lids
PBS containing 0.5% sodium azide

Procedure:

1. Rinse out used panel vials and lids with distilled water and air dry. Do not remove labels except for the date made label on the bottom of the vial.

2. Locate the correct panel to be made in the Present Panel Log Book and find the antibodies needed in the antibody refrigerator in the Quality Control Lab (Rm 628). If several vials are available, the one with the dot on top of the lid means opened and should be used first.

3. Write the lot number, titer, amount used for each antibody onto the worksheet. Add each appropriate antibody to the clean, labeled vial. Make as many tests as needed for at least one month, for example add 500 µl for 100 tests or 1000 µl for 200 tests of each antibody. Make dilutions as necessary for the titer of each antibody using PBS/Azide. For 3 color panels, add enough PBS/Azide to make 20 µl per test. For 4 color panels add enough PBS/Azide to make 30 µl per test. Date and sign the logbook worksheet and note the amount to be used per test and the amount of diluent added. Label a small sticker with the day's date and attach it to the bottom of the vial.

4. Place the completed panel vial in the antibody refrigerator in the back up panel sectioned box.

5. Make each panel separately, never do more than one at a time and be sure no one interrupts you. Do not answer the phone and, if necessary, close and lock the door. It is important to be completely undisturbed.

6. After making each panel it must be verified before use in the clinical lab. Follow verification procedure.

BATCH VERIFICATION PROCEDURE

After making a new batch of antibody combinations and before placing in the back up panel section each new panel must be verified to be as good as or better than the previous batch.

Materials Needed (QC lab)
Same as for sample preparation

Procedure:
1. Add panel to be verified to appropriately labeled tubes. Add the same panel now in use to appropriately labeled tubes. Add healthy donor blood or bone marrow as per staining sop. Use bone marrow for verification of all CD34 panels.
2. Prepare Panel Verification Log with appropriate batch numbers
3. Acquire data on the clinical flow cytometer as per protocol.

Analysis Procedure
1. Open verity/panverf/asetup.mac
2. Get next data source- choose file to be verified
3. Open/Run macro e.g. Fxverify (for specific F panel)
4. Open Batch Files: add appropriate files: first one is the one in use, the second the new batch.
5. Open/run Compare.mac
6. Edit multigraph, label with Fx and batch number to be verified.
7. Print multigraph
8. Edit multigraph, remove all items.
9. Start again at step 4

Compare the two panels, if values are low (more than 5 MCF channels) on any mab(s) add more of the appropriate antibody and verify again.