

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 μ l, 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 labeling 4, 12x75mm tubes as follows: X, 1/4, 1/16, 1/64, where X is the manufactures recommended concentration. Typically this is 5ul. If it is not adjust the amount of PBS and MAB added to tube 1 appropriately.**
2. Add 33.3 μ l of PBS (containing 0.5% sodium azide) to the first tube. Then add 30 μ l of the PBS to the remaining 3 tubes. To the first tube, add the manufactures recommended concentration multiplied by 1.33. For example, if the recommended concentration is 5ul/test then add 6.7ul ($5 \times 1.33 = 6.7$), of the stock MAB. Mix and then remove 10 μ l and add it to the second tube and continue to the next etc. After mixing the dilution in the last tube discard 10 μ l of the total. This way each tube has 30 μ l. Then add the 50 μ l of washed, blocked PBL or cells to all tubes and continue as per the staining protocol.
3. Record in order, each tube number and contents on a log sheet and give each one a file name.
Note: (CD34 must be tested on bone marrow , CD10 on granulocytes and CD138 on MDA-123 cells).
4. The bulk antibodies have been ordered at a 5 μ l titer, so this first tube should be at titer. If it is not, then the company sent the wrong concentration, which is not unusual.
If this is not a bulk antibody, then start the titer at the at the recommended concentration.
5. Stain, lyse, wash and fix as per SOP on staining procedure.
6. Follow acquisition SOP.

Manual Acquisition Procedure

1. Load tubes in carousel put into FACSCalibur. High Speed Mix
2. Macintosh hard drive/AA SJS setup
3. Menu/Acquire/ connect to cytometer
4. Menu/Acquire/Counters (make large – right corner click)
5. Menu/Cytometer/Instrument settings – BD files, instrument settings/select Fworksettings
6. Insert data zip or use hard drive to save data
7. Menu/Acquire/Parameter Description, id = titers, pt id = day's date, file = e.g. 001, folder = data drive or hard drive – select correct folder or create one with current date

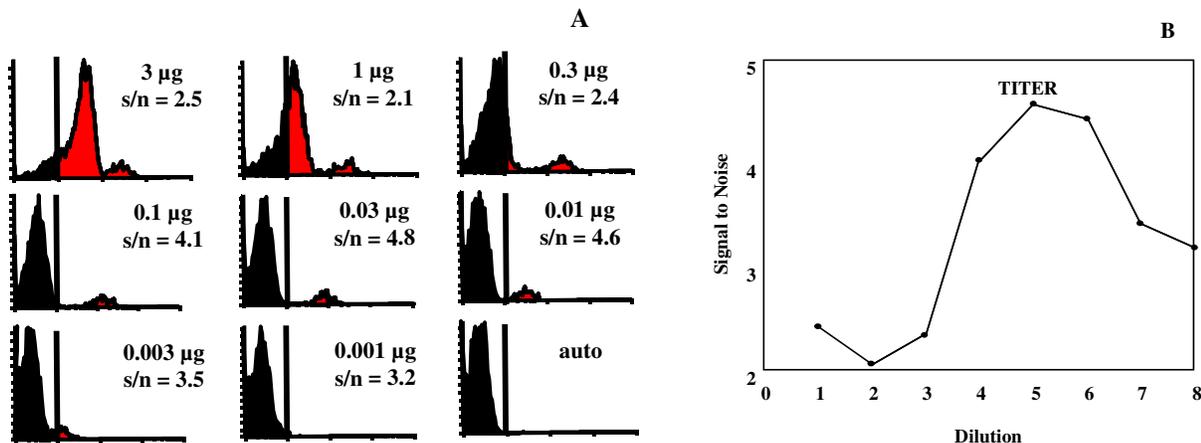
8. Menu/Acquire/Acquisition and Storage: 5000 events. When acquiring each set to be titered, use a live gate encompassing the target population, according to the chart of antigens (located in this manual).
9. Click on setup to make sure all events are on scale. Then uncheck.
10. Menu/Acquire/Acquisition and Storage: select correct gate, click ok
11. Acquire data
12. If using the hard drive to store files, 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 D:\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 D:\verity\titers\titer.mac
5. Edit multigraph, label MG with CD#, back up batch #
6. Print MG
7. For next titers, edit MG – remove all, return to step 2
8. File printout in titer books under appropriate CD number. File log worksheet in Titer log book. (in QC lab rm S635)

When titrating 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 μ l of a 1 to 4 dilution of the stock as the titer. If the third tube is the same, use 5 μ l 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 μ l and add 750 μ l of PBS/azide and label it 1:16 and use 5 μ l 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 μ l of a 1:64 dilution, as you are not diluting the antibody with three others.

rev. 6/18/97 sjs
rev. 08/0698 sjs
rev. 02/02/01 sjs
rev. 08/22/02 sjs
rev 04/30/03 sjs
rev 08/14/03 aec/pkw
rev 12-9-03 aec

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 μ l with PBS. E.g. for a 4 color panel add 25 μ l PBS. Note: 30 μ l is the total volume amount of each of our four-color panels.
3. To a fifth tube add all the antibodies together and q.s. to 30 μ l with PBS. E.g. for a 4 color panel add 10 μ l PBS.
4. Stain, using 50 μ l of normal healthy donor blood as per staining SOP. (the total volume of each tube will be $30 + 50 = 80 \mu$ l)

Acquisition:

1. Load tubes in carousel and put into FACSCalibur. High Speed Mix
2. Macintosh hard drive/AA SJS setup
3. Menu/Acquire/Connect to cytometer
4. Menu/Acquire/Counters (make large – right corner click)
5. Menu/Cytometer/Instrument settings – BD files, instrument settings/select Fworksettings
6. Insert data zip or use hard drive to save data
7. Menu/Acquire/Parameter Description, id = combo verf, pt id = day’s date, file = e.g. 001, folder = data drive or hard drive – select correct folder or create one with current date
8. Menu/Acquire/Acquisition and storage: 20000 events with no gate
9. Click on setup to make sure all events are on scale
10. Acquire data

Analysis:

1. 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.
2. Add verification analysis to Panel Log Book.

rev 7/14/97 sjs

rev. 9/21/98 sjs

rev. 02/02/01 sjs

rev. 04/30/03 sjs

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 μ l for 100 tests or 1000 μ l 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 μ l per test. For 4 color panels add enough PBS/Azide to make 30 μ l 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. For each panel set up the 3rd color antibody as a separate tube. Add cells as in step 1.
3. Prepare Panel Verification Log with appropriate batch numbers

Manual Acquisition for Panel Verifications

1. Load tubes in carousel (back up tubes 1 thru x and in use tubes 11 thru x) and put into FACSCalibur. High Speed Mix
2. Macintosh hard drive/AA SJS setup
3. Menu/Acquire/ connect to cytometer
4. Menu/Acquire/Counters (make large – right corner click)
5. Menu/Cytometer/Instrument settings – BD files, instrument settings/select Fworksettings
6. Insert data zip or use hard drive to save data
7. Menu/Acquire/Parameter Description, id = panverf, pt id = day's date, file = 001 for first set of back up files, folder = data drive or hard drive – select correct folder or create one with current date
8. Menu/Acquire/Acquisition and storage: 20000 events with no gate
9. Click on setup to make sure all events are on scale
10. Acquire data on back up panels
11. Menu/Acquire/Parameter Description, change file = 101
12. Acquire data on panels in use

Analysis Procedure

1. Verify weekly each newly made batch by comparing it with the batch currently in use.
2. Prepare Panel Verification Log with appropriate batch numbers
3. Open WinList
4. Open D/verity/panverf/asetup.mac
5. Get next data source- choose backup file to be verified (xxx.001 etc)
6. Open/Run macro e.g. Fpanelxx (for specific F panel) This brings up regions and axis labels.
7. Adjust R5, if necessary. If R5 is adjusted, save the regions for panel x and remove multigraph items and begin again at step 5.
8. Open the correct compensation matrix for the panel to be verified. (tcfxx.cmp)
9. Open the single 3rd color file for this panel.
10. Check and adjust as needed.
11. Save in the newcomp file. Find appropriate panels folder. Save as the date the panel was set up (ie 121003.cmp).
12. Now go to macro and run .

13. Get next data source- choose currently in use file (xxx.101 etc)
14. Go to macro and run
15. Edit multigraph, add batch # being verified. Adjust the columns to **5 and rows to 3.**
16. Print multigraph
17. Edit multigraph, remove items
18. Start again at step 5

Compare results, if low (more than 5 MCF channels) on any mab(s), adjust by adding more of the same lot number. Verify panel again.

All CD34 panels have mnc gate, but since the verifications are performed on normal donors the R4 CD34 regions are non-evaluable.

Panel	Gate
F1	Mnc
F2	Lg
F3	Mnc
F4	Lg
F5	Mnc
F7/59	All cells
F8	Lg
F9*	Mnc
F19/56	Lg
F20/57	Lg
F21	Lg
F22	Lg
F23	Lg
F24	Lg
F25	Lg
F26	Lg
F27	Lg
F28	Lg
F30	Lg
F31	Mnc
F32	Lg
F33	Lg
F34	Lg
F36	Lg
F37	Mnc
F38	Mnc

F39	Mnc
F41	Mnc
F42	Mnc
F43	Mnc
F44	Include all monocytes and some granulocytes (lymphocytes not necessary)
F51	Mnc
F52	Lg
F53	Lg
F58	Lg
F60	Mnc

* on F9 compensation problem shows up in R4

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

9/20/96 sjs
 rev 4/5/00 sjs
 rev 08/22/02 sjs
 rev 04/30/03 sjs
 rev 8/18/03 aec

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