

PROCEDURE: THREE COLOR FLOW CYTOMETRIC CROSSMATCH

PRINCIPLE:

The detection of circulating anti-HLA antibodies in the serum of potential renal allograft recipients is generally considered to be a contraindication to transplantation. One current method for detecting these antibodies is a complement dependent cytotoxicity (CDC) assay. This assay is adequate for crossmatching in that it detects most antibodies which are responsible for hyperacute graft rejection. However, this methodology does not detect very low concentrations of antibodies which may be the cause of accelerated graft rejection in patients who have high PRAs or in regrant patients. Consequently, patients still suffer from early graft loss possibly due to low levels of anti-HLA antibodies. Consequently, additional screening methods have been developed that utilize flow cytometric methods. These tests include flow screening using latex beads coated with HLA antigen and flow screening with a cell based panel. The flow cytometric crossmatch (FCXM), which is not dependent upon complement fixation, has been shown to be 10 to 100 fold more sensitive than CDC. More importantly, patients with a negative CDC but a positive FCXM have been shown to be at risk for an increased number of rejection episodes and possibly early graft failure. Thus, the FCXM may be important in identifying a subgroup of patients who would be at increased risk for graft failure or for a more complicated post-transplant clinical course.

The FCXM is performed by incubating donor cells with potential recipients serum followed by addition of a fluoresceinated (FITC) goat, anti-human polyclonal immunoglobulin reagent. In addition, a phycoerythrin (PE) labeled monoclonal antibody that detects B cells (CD19) and a peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibody that detects T cells (CD3) are added. This three-color combination allows for the simultaneous detection of alloantibodies reacting with T cells and B cells and eliminates background binding due to natural killer (NK) cells and monocytes. Results are then analyzed by flow cytometry and expressed as positive or negative based on a shift in median channel fluorescence intensity of the test serum with respect to negative control or autologous serum.

SPECIMEN REQUIREMENTS

Lymphocytes or mononuclear cells with viability > 80%, obtained from lymph node, spleen, or peripheral blood are acceptable.

DO NOT USE A CELL PREP THAT HAS BEEN TREATED WITH LYMPHO-KWIKTM or PERCOLL. Both of these products increase the negative background binding which reduces the sensitivity of the assay and may result in a false negative result.

200,000 - 250,000 cells / tube.

Serum sample(s) from potential recipient(s).

NOTE:

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1. All fresh (unfrozen) serum should be flash-frozen, then airfuged, to eliminate a potential false positive crossmatch. (see procedure for "Flash-Freezing" of serum for use in Flow Cytometry).
2. All serum samples should be airfuged at 28psi (100,000g) for 10 minutes prior to use to remove aggregated immunoglobulin and immune complexes.
3. Lympho-Kwik contains Percoll that may produce false negative crossmatch results. If absolutely necessary to use a prep that has been treated with Lympho-Kwik™ or Percoll, incubate the prep for a minimum of 1 hour at 37°C in PRMI w/10%FCS. Wash X3 with flow wash buffer. This will remove the Percoll.

REAGENTS, SUPPLIES AND EQUIPMENT

Negative control (Normal human or Pooled human sera)

Positive control (Pooled positive serum), titered for flow

FITC-conjugated goat, anti-human IgG [F(ab)₂, Fc specific]

(Jackson Labs. Cat.# 109-016-098).

FITC-conjugated goat, anti-human IgM [F(ab)₂, Fc specific]

(Jackson Labs. Cat. #109-016-043).

Anti-human CD3PerCP, peridinin chlorophyll protein (Becton-Dickinson cat#347344)

Anti-Human CD19 PE, phycoerythrin (Becton-Dickinson)

Flow Wash Buffer (PBS, 2% FCS and 0.1% sodium azide- NaN₃)

1% paraformaldehyde in PBS, Ph 7.2 ± .2

Eppendorf Pipet with tips or Pipetman with tips

Beckman Airfuge with microultracentrifuge tubes and protective caps

6 x 50mm glass tubes (kimble)

Beckman GP centrifuge or Fisher Scientific Tabletop centrifuge

Vacuum aspirator

Flow Cytometer capable of 3-color analysis

STAINING PROCEDURE

1. Flash-freeze all fresh (unfrozen) serum, then airfuge all serum samples including the positive and negative controls. (Microultracentrifuge tubes will hold a maximum of 175µl; **DO NOT** over fill as contamination of other tubes may occur - a good rule is to use 150 ul per tube).
2. Pronase treat your cells (see procedure for Pronase Treatment of Cells).
3. Place 200,000 - 250,000 cells in 6 x 50mm glass tubes. An absolute minimum is 100,000. If you have less please consult a supervisor. Note low cell number on flow

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worksheet. Count not only lymphocytes, but monocytes and neutrophils as well, as they all have HLA Class I antigen on their surface. Make sure your tubes indicate the cell sample - i.e. a label, colored tape, etc. **Mix the cell prep well before adding the cells, to ensure consistency from tube to tube** (Maximum tube volume is 450 ul). The number of tubes needed will depend upon the number of serum samples to be tested. The master recording sheet should contain all information regarding each sample, including lot numbers.

Tubes should be labeled and set up as follows:

TUBE #	Primary Antibody/Serum	Secondary Antibody
1	Negative or Autologous	FITC IgG or IgM CD3 PerCP & CD19 PE
2	Pooled positive serum	FITC IgG or IgM CD3 PerCP & C19 PE
3	Patient sample #1	FITC IgG or IgM CD3 PerCP & CD19 PE

** Repeat set-up as in tube #3 for each additional serum. **

4. Centrifuge cells to a pellet in the Fisher-Scientific table top centrifuge for 1 minute at 700 x g
OR the Beckman GP centrifuge for 5 minutes at 700 x g.
5. Aspirate supernatant, **being careful not to aspirate cell pellet**. Residual fluid volume should be <20 µl.
6. Add 25ul of the appropriate serum directly to the pellet in each corresponding tube, being careful not to allow the serum to run down the side of the tube. **Vortex to ensure proper mixing of serum and cells.**
7. Incubate tubes for 30 minutes at 4°C (in refrigerator).
8. Wash cells with 400 µl of **COLD** Wash Buffer. (Add 200 µl, vortex, then add the additional 200 µl).
Centrifuge cells to a pellet in the Fisher-Scientific table top centrifuge for 1 minute at 700 x g
OR the Beckman GP centrifuge for 5 minutes at 700 x g.
9. Aspirate supernatant (as in step #4) and repeat wash as in step #7 two more times for a total of three washes.

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NOTE: Be sure to aspirate positive control **LAST** to decrease the chance of carryover. Rinse tip after aspirating positive control.

10. Add 20 μ l of FITC anti-IgG (or IgM if applicable) to each tube and incubate at 4°C **in the dark**, for 10 minutes. **NOTE:** Check titer and proper dilution of ALL reagents prior to use.
11. After 10 minutes, either add 20 μ l of CD3 PerCP and 20 μ l of CD19-PE **OR** add 40ul of CD3 PerCP/CD19-PE combo to each tube, vortex gently and incubate at 4°C, **in the dark**, for an additional 20 minutes.

NOTE: Although using monoclonal antibodies at the manufacturers recommended concentration is acceptable, some monoclonal antibodies may be titered prior to use. Check results and/or titration procedure for appropriate monoclonal titer, if any, to be used.

12. Wash cells x2 with 400 μ l of **COLD** wash buffer and centrifuge as in step #7.
13. Resuspend cell pellet in 200 μ l **COLD** wash buffer.
14. While vortexing, add 200 μ l of **COLD**, 1% paraformaldehyde to each tube. Cells can then be analyzed or held at 4°C in the dark, for up to 7 days. **The final volume for analysis should always be 400 μ l.** If you must deviate, equal volumes of wash buffer/paraformaldehyde must be used.

PROCEDURAL NOTES

1. Fresh 1% paraformaldehyde should be made every 2 weeks. Check expiration date before using. Unfixed cells should be stored at 4°C, protected from light, and analyzed within 8 hours.
2. **CAUTION: Paraformaldehyde is a Carcinogen!** Use personal protective equipment and take appropriate safety measures.
3. After reconstituting the FITC reagent, airfuge the entire stock and dilute with flow wash buffer to the current working dilution. Aliquot the FITC into 1ml Sarstedt screw cap microtubes and store at -70 up to 3 months. When ready to use just thaw and mix. Place any unused portion in the -4 refrigerator for storage up to 7 days.
4. **BE CAREFUL** not to contaminate sample with pipette tips when aspirating supernatant.
5. CD3 PerCP and CD19 PE may be combined using equal volumes of the appropriate dilution of each reagent. The combination may be stored in the refrigerator for up to a month.

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5. **BE ACCURATE:** Due to the high sensitivity of Flow Cytometry, all cell concentrations, serum dilutions, and volumes must be exact and accurate. Small fluctuations in cell numbers will result in erroneous and inconsistent results. The biggest potential for error lies in performing accurate cell counts. Excess cell numbers can produce false negative results.
6. It is important to remember to keep the reagents COLD and in the DARK!

INTERPRETATION

Subtract the MCF of the negative control from all samples and record the difference under the change in MCF on the provided worksheet.

Below are listed the cutoff values for the interpretation of results when using NHS lot #2104: ***THESE VALUES SHOULD BE CONSIDERED AS GUIDELINES, NOT AS ABSOLUTE "CUT-POINTS."***

T-cells

- ≤ 30 channels = **NEGATIVE**
- ≥ 30 but ≤ 45 channels = **PROBABLE NEGATIVE**
- ≥ 45 channels = **LIKELY POSITIVE**

B-cells

- ≤ 60 channels = **NEGATIVE**
- ≥ 60 but ≤ 120 channels = **PROBABLE NEGATIVE**
- ≥ 120 channels = **LIKELY POSITIVE**

FOR ALL SITUATIONS WHERE VALUES EXCEED THE "CUT-POINTS," RESULTS MUST BE REVIEWED BY A DIRECTOR OR SUPERVISOR BEFORE REPORTING THE RESULT AS POSITIVE. THERE WILL BE SITUATIONS WHERE THE "CUT-POINTS" ARE EXCEEDED BUT THE INTERPRETATION OF THE CROSSMATCH WILL BE NEGATIVE.

Troubleshooting any interpretive problems may begin with evaluation of the MCF for the NHS and PPS controls.

For the current lot of Flow IgG FITC (#42562 at 1:150) the following MCF values fall within the expected ranges:

NOTE: If any of the lot #s listed for the FITC, NHS, PHS or PPS should change then these guidelines will need to be redetermined. (See QC notebook for raw data used in calculation of ranges below).

RANGES FOR PBL

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SAMPLE	T MCF	B MCF
NHS 2104	270-350	275-475
ATL BIO F0192	250-375	250-513
PPS 082100	500-760	600-880

NOTE: On average the difference between the NHS 2104 and ATL F0192 should be within 15 channels for T-cells and 60 channels for B-cells. However, individual outliers may occur. For values that are outside the expected range consult with a supervisor or director before reporting.

For PPS lot # 08212000 the expected value for the change in MCF between the negative Control (NHS 0706) and the positive control (PPS 08212000) is:

Δ MCF ≥ 177 for T-cells.

Δ MCF ≥ 229 for B-cells

If the PPS values are significantly greater than the above values it is not necessarily a cause for concern or indication for repeat of the test. However, if the values are significantly below the above listed ranges, repeating the test may be indicated if the crossmatch is negative.

These values are only to be used as guidelines for interpretation and troubleshooting. If a sample should fall outside of these ranges it does not necessarily invalidate the test but should indicate the need for a closer evaluation of the results and/or review by the director.

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