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RECOMMENDED METHODS

FOR

ANTI-HUMAN GLOBULIN EVALUATION

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OFFICE OF BIOLOGICS RESEARCH AND REVIEW (OBRR)

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OFFICE OF BIOLOGICS RESEARCH AND REVIEW'S (OBRR)

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I. CONTROLS FOR SEROLOGIC PROCEDURES

(A) Red blood cells coated with complement or immunoglobulin shall be tested each day of use with the following positive and negative controls to assure reactivity.

Test Cells	Positive Controls	Negative Controls
C3b	Anti-C3b or Anti-C3b,-C3d	Anti-C3d;Anti-C4b,-C4d;Anti-IgG
C3d	Anti-C3d	Anti-C3b;Anti-C4b,-C4d;Anti-IgG
C4b	Anti-C4b	Anti-C4d;Anti-C3b,-C3d;Anti-IgG
C4d	Anti-C4d or Anti-C4b,-C4d	Anti-C4b;Anti-C3b,-C3d;Anti-IgG
IgA	Anti-IgA	Anti-C3b,-C3d;Anti-C4b,-C4d;Anti-IgG (Heavy chain specific)
IgG	Anti-IgG	Anti-C3b,-C3d;Anti-C4b,-C4d

When specific control antisera are unavailable, approval to use alternate procedures may be obtained from the Director, OBRR.

For the purpose of these methods, the OBRR has defined the antibody which reacts only with C3b coated cells as anti-C3b. It is recognized that the antibody produced in response to immunization is usually directed against the antigenic determinant A which is located on the C3c subunit; some workers have called this antibody "anti-C3c".

It is accepted that cells coated with large amounts of C3b usually react with potent anti-C3d reagents.

Cells coated with IgA myeloma proteins may also be coated with IgG. Therefore, to avoid misleading test results, the anti-IgG component of anti-human globulin may be neutralized by adding an equal volume of immune serum globulin solution diluted 1:1000.

(B) Red blood cells coated with immunoglobulin or complement by one of the following methods may be frozen and thawed for use in potency and specificity testing. Frozen cells shall be used on the day of thawing and appropriate controls shall be used to demonstrate the desired reactivity and specificity of the thawed red blood cells. The method of freezing, storing, and thawing red blood cells, including a description of the cryoprotective medium, shall be described in detail and shall be approved by the Director, OBRR, as a license amendment before use in control testing of antisera.

(C) Throughout these methods the diluent for cell suspensions shall be isotonic saline containing 1 percent (v/v) bovine serum albumin unless otherwise specified, and the concentration of cells shall be 2 percent. Red blood cells may be washed with isotonic saline. The 2 percent cell suspensions shall be prepared from cells collected within seven days or prepared from cells frozen within seven days of collection. Uncoated cells shall give negative direct antiglobulin tests. Cells shall be collected in approved anticoagulants: anticoagulant citrate dextrose solution (ACD), citrate phosphate dextrose solution (CPD) and citrate phosphate dextrose adenine solution (CPDA-1).

(D) The diluent for serum dilutions shall be one percent bovine serum albumin in isotonic saline. Twofold, serial dilutions for potency titrations shall be made in 10X75 mm or 12X75 mm tubes. A separate clean pipette or plastic tip shall be used for each dilution to avoid carryover.

(E) Unless otherwise specified, all tests are centrifuged at approximately 1000 rcf for 20 seconds. Cell buttons shall be gently dislodged from the tube and observed macroscopically. The reactions shall be graded and recorded as follows:

4+	cell button remains in one clump.
3+	cell button dislodges into several clumps.
2+	cell button dislodges into many small clumps of equal size
1+	cell button dislodges into finely granular but definitely small clumps
D	cell button dislodges into fine granules but not definite small clumps. Results shall be recorded as doubtful. For the purpose of potency testing, doubtful reactions are deemed to be negative.
Negative	cell button dislodges with no visible clumps.

II. POTENCY TEST METHODS

Reference standards shall be provided by the OBRR to evaluate final potency testing for anti-IgG and anti-C3d. These reference antisera shall be run in parallel with the test reagent.

(A) Test procedure for determination of anti-IgG using anti-D.

(1) Selection of anti-D serum for coating test cells

The undiluted anti-D shall not directly agglutinate a 2 percent suspension of group O, cDe or CcDe red blood cells. If undiluted single donor antisera are not available, you may request OBRR approval for the use of pooled or single donor antisera which are diluted.

The anti-D serum used for cell coating shall have a 1+ titration endpoint no less than 16 and no greater than 64 by the following test procedure. Potency testing of the anti-D serum need not be performed each day of test provided that the same lot of serum and the same red cell donor source are used to determine the anti-IgG potency of the antiglobulin reagent.

(i) Beginning with undiluted anti-D serum, prepare separate twofold serial dilutions (1:2, 1:4 etc.) of the serum.

(ii) To 0.1 mL of each anti-D serum dilution add 0.1 mL of a 2 percent suspension of cDe or CcDe red cells.

(iii) Mix and incubate at 37° C for 30 minutes.

(iv) Wash 4 times with large volumes of isotonic saline.

(v) To each cell button add 0.1 mL of a released lot of licensed anti-human globulin containing anti-IgG. Mix.

(vi) Centrifuge and record reactions.

(2) Coating red blood cells with anti-D

(i) Beginning with the anti-D dilution meeting the requirements for cell coating, prepare serial twofold dilutions of this serum. Include at least 2 dilutions of anti-D immediately following the endpoint observed when selecting the serum in II.(1)

(ii) Add to the dilutions of anti-D an equal volume of the 2 percent red blood cell suspension used in selecting the anti-D serum and mix thoroughly.

(iii) Incubate at 37° C for 30 minutes.

(iv) After incubation, wash the coated red cells 4 times with large excesses of isotonic saline and resuspend to the original 2 percent red blood cell concentration.

(3) Anti-human Globulin dilutions

Beginning with undiluted serum, prepare separate twofold dilutions (undiluted through 1:8) of the test serum and the Anti-IgG reference serum.

(4) The Test.

(i) For both the test serum and the Anti-IgG reference serum, prepare at least 4 sets of test tubes, each containing no fewer than 7 tubes per set. Each set shall represent a dilution of the anti-human globulin serum as prepared. To each tube of the first set add 0.1 mL of undiluted anti-human globulin; to each tube of the second set add 0.1 mL of anti-human globulin diluted 1:2, etc.

(ii) To the first tube of each set add 0.1 mL of red blood cells coated with the selected anti-D dilution at its highest concentration. To the second tube of each set, add 0.1 mL of red blood cells coated with a 1:2 dilution of the anti-D. Continue the serial addition of red blood cells coated with decreasing amounts of anti-D until all sets are completed. Mix.

(iii) Centrifuge and record reactions observed.

(B) Test procedure for determination of anti-IgG using anti-Fy .

(1) Selection of anti-Fy for coating test cells

Antibodies from pools of donors that have been deliberately hyperimmunized to produce reagent quality antiserum may not be suitable because they may not demonstrate the variations in reactivity that are sometimes seen with weaker antibodies observed in patient samples. However, the OBRR will consider approval of pooled or single donor antisera which are diluted if undiluted single donor antisera are not readily available.

The anti-Fy^a used for coating cells shall have a 1+ titration endpoint of no less than 8 and no greater than 32 by the following procedure. Potency testing of the anti-Fy^a serum need not be performed each day of test provided that the same lot of serum and the same red blood cell donor source are used to determine the anti-IgG potency of the antiglobulin reagent.

A 2 percent suspension of group O, Fy(a+b+), direct antiglobulin test negative, red blood cells shall be prepared.

(i) To 0.5 mL of anti-Fy^a add 0.07 mL of 0.11 M (4.45 percent) dipotassium ethylenediamine-tetracetic acid (K_2EDTA). Incubate the serum-EDTA mixture at room temperature (20° C to 30° C) for 15 to 20 minutes.

(ii) Beginning with undiluted anti-Fy^a serum, prepare separate twofold dilutions (1:2, 1:4, etc.) of the serum.

(iii) To 0.1 mL of each anti-Fy^a serum dilution add 0.1 mL of the 2 percent suspension of Fy(a+b+) red cells.

(iv) Mix and incubate at 37° C for 30 minutes.

(v) Wash 4 times with large volumes of isotonic saline.

(vi) To each cell button add 0.1 mL of a released lot of licensed anti-human globulin containing anti-IgG. Mix.

(vii) Centrifuge and record reactions observed.

(2) Coating red cells with anti-Fy

(i) To 3 mL of anti-Fy^a, add 0.38 mL of 0.11 M (4.45%) K_2EDTA. Incubate the serum-EDTA mixture at room temperature (20° C to 30° C) 15 to 20 minutes.

(ii) Prepare twofold serial dilutions (undiluted 1:2, 1:4, etc.) of the selected anti-Fy^a, including at least 2 dilutions immediately following the end point observed when selecting the anti-Fy^a serum.

(iii) Add to the dilutions of anti-Fy^a an equal volume of the red blood cell suspension used in selecting the anti-Fy^a serum.

(iv) Incubate at 37° C for 30 minutes.

(v) After incubation, wash the coated red blood cells 4 times with large excesses of isotonic saline and resuspend to the original 2 percent red blood cell concentration.

(3) Anti-Human Globulin Dilution

Beginning with undiluted serum, prepare separate twofold serial dilutions (undiluted through 1:8) of the test anti-human globulin and the Anti-IgG reference serum.

(4) The Test.

(i) For both the test serum and the Anti-IgG reference serum, prepare at least 4 sets of test tubes, each containing no fewer than 6 tubes per set. Each set shall represent a dilution of the anti-human globulin. To each tube of the first set, add 0.1 mL of undiluted anti-human globulin; to each tube of the second set, add 0.1 mL of anti-human globulin diluted 1:2, etc.

(ii) To the first tube of each set, add 0.1 mL of red blood cells coated with the selected anti-Fy^a at the highest concentration. To the second tube of each set, add 0.1 mL of red blood cells coated with a 1:2 dilution of anti-Fy^a. Continue the serial addition of red blood cells coated with decreasing amounts of Anti-Fy^a until all sets are completed. Mix.

(iii) Centrifuge and record reactions observed.

(C) Test procedure for determination of anti-C3b.[1,2]

(1) Coating red cells with C3b

At least two normal whole blood samples shall be treated as follows:

(i) In a 500 mL volumetric flask containing approximately 250 mL of H_2O, dissolve:
46.2g sucrose
345 mg $NaH_2PO_4 \cdot H_2O$
789 mg $Na_2EDTA \cdot 2H_2O$
q.s. to 500 mL with distilled water

(ii) In a second 500 mL volumetric flask containing approximately 250 mL of H_2O dissolve:
46.2g sucrose
355 mg Na_2HPO_4
789 mg $Na_2EDTA \cdot 2H_2O$
q.s. to 500 mL with distilled water

(iii) Adjust the pH of the solution prepared in (i) to pH 5.1 by the addition of solution (ii).

(iv) Place 19.8 mL of the sensitizing diluent prepared in (iii) in a 0° C ice bath and stir gently with magnetic stirring bar.

(v) Remove 2 mL of fresnly drawn whole blood from ACD, CPD or CPDA-1 tube. Wash 3 times with isotonic saline. To the washed packed cells, add 1 mL of the same donor's plasma diluted 1:50 in isotonic saline. Add 1 mL of this blood mixture to the chilled diluent.

(vi) Immediately add 0.1 mL of 0.4M $MgCl_2$ to the above mixture.

(vii) Incubate 30 minutes at 0° C with constant, gentle, stirring.

(viii) Wash cells 4 times with large excesses of isotonic saline and remove supernatant from the packed cells.

(ix) Resuspend cells to a 2 percent suspension.

(2) Dilution of Anti-human Globulin.

Beginning with undiluted serum, serial twofold dilutions (1:2, 1:4, etc.) of the test anti-human globulin shall be prepared to a dilution of at least 1:8.

(3) The Test

(i) To 0.1 mL of each test serum dilution, add 0.1 mL of the prepared C3b coated test cell suspension.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(D) Test procedure for determination of anti-C3d.[3]

(1) Preparation of C3d coated cells

(i) Prepare C3b coated packed cells as previously outlined in C.

(ii) To 9 mL of 0.1 M Na_2HPO_4 add 1 mL of 0.1 M $NaH_2PO_4 \cdot H_2O$ (resulting pH 7.7).

(iii) To 9 mL of mixture prepared in (ii) add 1 mL of freshly prepared 1 percent trypsin in 0.05 N HCl.

(iv) Add 0.4 mL of packed C3b coated cells to 1.6 mL of 0.1 percent trypsin prepared in (ii) and (iii).

(v) As a negative control, add 0.4 mL of packed red cells to 1.6 mL of 0.1 percent trypsin. These cells shall be prepared from the same blood sample used to prepare the C3d test cells.

(vi) Incubate both tubes at 37° C for 30 $\pm$ 3 minutes.

(vii) Immediately wash cells 4 times with large excesses of isotonic saline and resuspend to a 2 percent concentration.

(2) Dilution of Anti-Human Globulin and Reference Serum.

Beginning with undiluted serum, serial twofold dilutions of the test serum and the Anti-C3d reference serum shall be prepared to a dilution of 1:8.

(3) The Test.

(i) To 0.1 ml of each test serum dilution, add 0.1 mL of the C3d coated test cell suspension.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(4) Negative control test.

(i) To 0.1 mL of the undiluted anti-human globulin add 0.1 mL of the negative control cell suspension prepared with the C3d coated cell.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(L) Test procedure for determination of anti-C4b.[4]

(1) Preparation C4b coated test cells.

At least two normal blood samples shall be treated as follows:

(i) Add 10 mL of 10 percent sucrose in distilled water to a tube containing 12 to 15 mg K_3EDTA and agitate until dissolved.

(ii) To the above solution add 1 mL of fresh whole blood collected the same day in an approved anticoagulant. Mix.

(iii) Incubate at 37° C for 15 minutes.

(iv) Wash cells 4 times with isotonic saline.

(v) Resuspend cells to a 2 percent concentration.

(2) Dilution of Anti-Human Globulin.

(i) Beginning with undiluted serum, serial twofold dilutions of the test shall be prepared to a dilution of 1:8.

(3) The Test.

(i) To 0.1 mL of each anti-human globulin dilution, add 0.1 mL of the C4b coated test cell suspension.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(F) Test procedure for determination of anti-C4d.[4]

(1) Preparation of C4d coated test cells.

(i) Prepare C4b coated packed cells as previously outlined.

(ii) To 9 mL of 0.1 M Na_2HPO_4 add 1 mL of 0.1 M NaH_2PO_4 H_2O (resulting pH 7.7).

(iii) To 9 mL of mixture prepared in (ii) add 1 mL of freshly prepared 1 percent trypsin in 0.05 N HCl.

(iv) Add 0.3 mL of packed C4b coated cells to 1.2 mL of 0.1 percent trypsin prepared in (ii) and (iii).

(v) As a negative control, add 1.2 mL of 0.1 percent trypsin to 0.3 mL of packed cells prepared from the same blood sample used in (i). Mix.

(vi) Incubate at 37° C for 10 minutes.

(vii) Immediately wash cells 4 times with large excesses of isotonic saline and resuspend to a 2 percent concentration.

(2) Dilution of Anti-Human Globulin.

(i) Beginning with undiluted serum, serial twofold dilutions of the test serum shall be prepared to a dilution of at least 1:8.

(3) The Test.

(i) To 0.1 mL of each test serum dilution, add 0.1 mL of the C4d coated cell suspension.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(4) Negative control test.

(i) To 0.1 mL of the undiluted anti-human globulin add 0.1 mL of the negative control cell suspension prepared in F(1)(v) above.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record reactions observed.

(g) Test procedure for IgA determination[5]

(1) Preparation of IgA coated cells.

Reagents required:

1 percent Chromic Chloride Stock Solution (store in dark bottle at 4° C). Dilute 1:20 in unbuffered isotonic saline for use.

Purified IgA myeloma protein (0.1 mg/mL in unbuffered isotonic saline).

Group O red blood cells from at least two donors, washed four times with unbuffered saline.

(i) To a test tube add 0.1 mL of washed packed red blood cells.

(ii) Add 0.1 mL of diluted IgA protein. Mix.

(iii) Add 0.1 mL of diluted $CrCl_3$ and mix immediately.

(iv) Mix continually for four minutes at room temperature.

(v) Wash the cells four times with large excesses of unbuffered isotonic saline and discard supernatant from the packed cells.

(vi) Resuspend cells to 2 percent concentration.

(2) The Test.

(i) To 0.1 mL of each test serum add 0.1 mL of the IgA coated cells.

(ii) Mix thoroughly and incubate at 20° C to 30° C for 5 minutes.

(iii) Centrifuge and record macroscopic reactions observed.

(3) Negative Control Test.

(i) To 0.1 mL of the undiluted Anti-IgA add 0.1 mL of a 2% suspension of the untreated cells used in paragraph (g)(1).

(ii) Incubate for 5 minutes at 20° C to 30° C and centrifuge.

(iii) Centrifuge and record reaction.

(H) Potency Test Requirements

The following requirements shall apply to all antiglobulin specificities claimed to be present:

(1) Potency requirements for the anti-IgG component. Each dilution of the antisera tested in parallel shall give reactions equal to or greater than the correspondingly diluted reference serum when tested against cells coated with the smallest amounts of IgG detectable by the reference serum. The test serum shall not prozone-ie-no dilution of the test serum shall give significantly stronger reactions than those observed with the undiluted test serum.

(2) The undiluted anti-human globulin shall give at least a 2+ reaction with C3d coated red cells and the serum shall have a potency titer value at least equal to that of the reference serum.

(3) For other complement or immunoglobulin antibodies claimed, the undiluted serum must give at least a 2+ reaction and a 1:4 dilution of the serum shall give at least a 1+ reaction with cells prepared by a method approved by OBRR.

III. METHODS FOR EVALUATING SPECIFICITY

The specificity of the anti-human globulin test serum shall be evaluated by the direct antiglobulin method described in the manufacturer's package insert with cells prepared by approved methods such as those described in part II.

Required tests:

Anti-Human Globulin	vs	Coated Cells
Anti-IgG,-C3d (Polyspecific)	IgG, 3Cb, C3d, C4b, C4d,	
Anti-IgG (Heavy Chain)	IgG, IgA, IgM, C3b, C3d, C4b, C4d	
Anti-IgG	IgG, C3b, C3d, C4b, C4d	
Anti-complement reagents	IgG, C3b, C3d, C4b, C4d	

Alternate protocols may be acceptable; submit a description of the alternate procedures to the OBRR for approval.

In addition, if the labeling claims the presence or absence of anti-C4b and/or anti-C4d, the appropriate coated cells must be tested.

If monospecific anti-C3d activity is claimed, the absence of anti-C3b activity must be confirmed by a method approved by the OBRR.

Testing with IgA coated cells is required if labeling claims that the product contains, or is free of, anti-IgA activity. If the presence or absence of Anti-IgM activity is claimed, this reactivity must be evaluated by methods approved by the Director, OBRR. Tests with IgM coated cells may serve to exclude the presence of antibody to light chains.

(A) Test for Heterospecific Antibodies.

The product shall be free of antibodies capable of agglutinating or hemolyzing untreated human red blood cells.

(1) A 2 percent red cell concentration in isotonic saline containing 1 percent autologous serum or plasma shall be prepared from blood from normal, healthy donors within seven days of collection. Group O, A_1 and B cells shall be prepared.

(2) The Test.

(i) Divide 9 test tubes into 3 sets each containing 3 tubes.

(ii) Into each tube place two drops of undiluted anti-human globulin.

(iii) To the first tube of each set, add 1 drop of the group O red cell suspension.

(iv) To the second tube of each set, add 1 drop of the group A_1 red cell suspension.

(v) To the third tube of each set, add 1 drop of group B red cell suspension. Mix all tubes.

(vi) Incubate the tubes:
Set No. 1 at 37° C for 30 minutes
Set No. 2 at 2° C to 8° C for 30 minutes
Set No. 3 at 20° C to 30° C for 30 minutes

(vii) Examine each tube for hemolysis after centrifugation. Gently dislodge the cell button, observe macroscopically and record reactions. The product is satisfactory when there is no agglutination or hemolysis in any of the tubes.

(B) Test for reactivity with normal cell samples

The product shall be free of antibodies capable of agglutinating non-treated, normal, stored, washed human red blood cells.

(1) Cell Suspensions

(i) Store 5 clotted, normal samples of whole blood at 2° C to 8° C for 24 hours or more. Group O, A_1, and B cells shall be used.

(ii) Wash the red blood cells 4 times in isotonic saline and resuspend to a 2 percent cell concentration in isotonic saline.

(2) The Test.

(i) Into 5 test tubes place 2 drops of undiluted anti-human globulin.

(ii) To the first tube, add 1 drop of the red blood cell suspension prepared from one of the 5 samples.

(iii) To the second tube, add 1 drop of the red blood cell suspension prepared from a second sample. Continue until cells from all 5 samples have been added to the appropriate tubes. Mix.

(iv) Centrifuge at approximately 1000 rcf for 20 seconds. Examine the contents of each tube macroscopically and microscopically and record results observed. Incubate 10 minutes at 20° C to 30° C, centrifuge, read and record as before.

The product is satisfactory when there is no agglutination visible macroscopically or microscopically in any of the tubes.

If the manufacturer would prefer to perform the test for heterospecific antibodies at an earlier stage in production, he may amend his product license appropriately. The test results should be reported on the protocol whether the test is performed on final containers or in-process product.

(C) Test with enzyme-treated cells

Anti-human globulin shall not agglutinate enzyme treated red blood cells.

(1) The anti-human globulin under test shall be non-reactive with trypsin treated red blood cells prepared by the following procedure.

(i) Add 0.4 mL of washed packed red cells to 1.6 mL of 0.1 percent trypsin.

(ii) Incubate tubes at 37° C for 30 minutes.

(iii) Immediately wash cells 4 times with large excesses of isotonic saline and resuspend to a 2 percent suspension.

(2) The Test.

(i) Place 2 drops of anti-human globulin into a tube.

(ii) Add 1 drop of the enzyme treated red cell suspension.

(iii) Centrifuge at 1000 rcf for 20 seconds. Examine the contents of each tube macroscopically and microscopically and record results observed.

The product is satisfactory when there is no agglutination visible macroscopically or microscopically in any of the tubes. Incubate 10 minutes at 20° C to 30° C, centrifuge, read and record results observed.

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CROSS FILE SHEET

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