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DRAFT

RECOMMENDED METHODS FOR
EVALUATING POTENCY, SPECIFICITY, AND REACTIVITY
OF ANTI-HUMAN GLOBULIN

March 1992

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PROPOSED REVISION

RECOMMENDED METHODS FOR
ANTI-HUMAN GLOBULIN EVALUATION

MARCH 1992

In follow-up to the 7-9 November 1990 workshop "REAGENTS FOR THE 1990's", the comments received from licensed manufacturers of Blood Grouping Reagents, users, and other interested parties concerning "Proposed Revised Performance Criteria for Anti-Human Globulin Reagents" were reviewed and incorporated into this document. A summary of the changes that have been made is included on page (ii).

Subsequent to the publication of the Federal Register Notice of Availability, all licensed manufacturers and other interested parties will have the opportunity to review and comment on the draft.

Specific references to the pages which your comments relate to or photocopies of the pages with your comments written on them would be helpful and appreciated. Responses are requested within 60 days of the date of publication of Federal Register Notice of Availability, however, comments are welcome at any time.

These recommended methods are provided to help assist manufacturers in pursuing new product license applications and amendments to existing product license applications. The methods described herein do not bind the agency, and manufacturers may consider use of other methods. In cases where manufacturers wish to use methods other than those described herein, FDA recommends that the matter be discussed with FDA in advance. The methods, potency titer values, specificity results, and other matters referred to in this document are intended to assist manufacturers in preparing submissions to FDA. The information is based on current knowledge and is not meant to be all inclusive and should not be viewed as ensuring approval or the only means of achieving FDA approval. Following the methods provided in this document will assist in the approval process, but does not guarantee approval. FDA will review applications on an individual basis and approvals will be granted when supported by scientific evidence.

PROPOSED REVISION
RECOMMENDED METHODS FOR
ANTI-HUMAN GLOBULIN EVALUATION
MARCH 1992

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SUMMARY OF COMMENTS INCORPORATED AND CHANGES MADE

**BASED ON WORKSHOP DISCUSSIONS CONCERNING
"PROPOSED REVISED PERFORMANCE CRITERIA FOR ANTI-HUMAN GLOBULIN REAGENTS"**

1. Correction and clarification of centrifugation times and speeds.
2. The potency tests against cells sensitized with anti-D and anti-Fy^a were revised to limit the number of tubes being read at a given time.
3. The warm low ionic methods of coating red blood cells with C3b/C4b and C3d/C4d have been included as alternative methods for complement coating red blood cells.
4. The test against enzyme treated cells has been modified to allow the use of papain and/or ficin.
5. Typographical errors were corrected.

SUMMARY OF COMMENTS RECEIVED BUT NOT INCORPORATED

1. The suggested changes to terminology, i.e. to change C3b to C3c and C4b to C4c, were not made because the workshop indicated there was incomplete agreement on this issue. FDA would appreciate additional consideration of this point because common terminology for the future would certainly be an asset and it is hoped that current revision efforts will serve international standardization objectives.
2. Several people suggested deleting some of the control cells instituted in the 1st draft. Those deletions have not been made because it is believed they are valid and necessary controls. Additionally, the various suggestions for deletion of control cells were not in agreement.
3. The suggestion to include a test to detect prozones needs further definition from the manufacturers.
4. The suggestion to include a test for resistance to inhibition also requires additional data to ensure effectiveness before general use.

NOTE: Suggested methods for both items 3 and 4 above will be incorporated and voluntary performance encouraged. Licensed manufacturers are requested to perform these tests for prozone and inhibition on 10 lots of product within the next 12 months and submit data to FDA for evaluation. Manufacturers who do routinely test each lot and obtain satisfactory results may so state in package insert labeling.

RECOMMENDED METHODS FOR ANTI-HUMAN GLOBULIN EVALUATION

GENERAL INFORMATION

These recommended methods are provided to help assist manufacturers in pursuing new product license applications and amendments to existing product license applications. The methods described herein do not bind the agency, and manufacturers may consider use of other methods. In cases where manufacturers wish to use methods other than those described herein, FDA recommends that the matter be discussed with FDA in advance. The methods, potency titer values, specificity results, and other matters referred to in this document are intended to assist manufacturers in preparing submissions to FDA. The information is based on current knowledge and is not meant to be all inclusive and should not be viewed as ensuring approval or the only means of achieving FDA approval. Following the methods provided in this document will assist in the approval process, but does not guarantee approval. FDA will review applications on an individual basis and approvals will be granted when supported by scientific evidence.

I. REFERENCE PREPARATIONS

- A. The Reference Anti-Human Globulin preparations listed below can be obtained from:

Center for Biologics Evaluation and Research
Food and Drug Administration
8800 Rockville Pike
Bethesda, MD. 20892
USA

Anti-IgG

Anti-C3d

NOTE: FDA Reference Anti-Human Globulin preparations are not routinely available to anyone except U.S. licensed manufacturers and amounts issued will be proportional to lots released in the previous year.

- B. Reference Anti-Human Globulin preparations are to be used according to the accompanying package insert only for determining the potency of anti-IgG and anti-C3d as part of their final lot release testing. These reference preparations should be run in parallel with the reagent under test.

In-house reference materials should be developed for all stability testing, in process testing or product development purposes.

II. GENERAL CONSIDERATIONS

A. RED BLOOD CELLS

1. Red blood cells for use in control testing should be used within seven days of collection or prepared from red blood cells frozen within seven days of collection. Blood samples should be collected in approved anticoagulants.
2. Red blood cells should be washed at least twice with isotonic saline or until a clear supernate is obtained.
3. Throughout these methods the diluent for cell suspensions should be isotonic saline containing 1-2% bovine albumin (unless otherwise specified) and the concentration of red blood cell suspensions should be 2%.
4. Uncoated red blood cells should give negative direct antiglobulin tests with polyspecific Anti-Human Globulin reagents.
5. Red blood cells coated with immunoglobulin or complement by one of the methods detailed herein may be frozen and thawed for use in potency and specificity testing, unless otherwise noted.

Frozen red blood cells should be used on the day of thawing unless data demonstrating that longer storage is effective has been accepted by the FDA. To ensure that the correct sample was thawed, appropriate controls should be used to demonstrate the desired reactivity and identity of the thawed red blood cells.

The method of freezing, storing and thawing red blood cells, including a description of the cryoprotective medium, should be described in detail and should be approved by the Director, Center for Biologics Evaluation and Research, before use in control testing of licensed products.

B. SEROLOGIC CONTROLS

1. Red blood cells coated with complement or immunoglobulin should be tested each day of use with the following positive and negative control reagents to assure reactivity.

| CELLS WITH | POSITIVE CONTROLS | NEGATIVE CONTROLS |
|------------|------------------------------|--|
| C3b | Anti-C3b or Anti-C3b,-C3d | Anti-C4b,-C4d; Anti-IgG |
| C3d | Anti-C3d | Anti-C3b; Anti-C4b,C4d; Anti-IgG |
| C4b | Anti-C4b | 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 (Heavy Chain Spec.) | Anti-C3b,-C3d; Anti-C4b,- C4d; Anti-IgA |

When specific control reagents are unavailable, approval to use alternative procedures may be obtained from the Director, Center for Biologics Evaluation and Research.

For the purpose of these methods, the Center for Biologics Evaluation and Research has defined the antibody which reacts only with C3b coated red blood 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 have called this antibody "anti-C3c". C3b coated red blood cells prepared by the Fruitstone method will react with Anti-C3d reagents. These red blood cells may also be coated with trace amounts of IgG.

Red blood 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 Rh₀(D) Immune Globulin (Human) diluted 1:1000.

C. REAGENT DILUTIONS

1. The diluent for reagent dilutions should be 1-2% bovine albumin in isotonic saline.
2. Master, serial, two-fold dilutions for potency titrations should be made in tubes large enough to facilitate adequate mixing of the contents (i.e. 12 X 75 mm or larger tubes).
3. A separate clean pipette or plastic tip should be used for each dilution to avoid carryover.
4. The last tube should contain diluent only and serve as a diluent control.

D. CENTRIFUGATION

1. Unless otherwise specified, all tests are centrifuged for 15 seconds at approximately 3400 rpm (900-1000 rcf) or at a time and speed appropriate for the centrifuge being used.

E. REACTION GRADING

1. Cell buttons should be gently dislodged from the tube and observed macroscopically. The reactions should 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 should be recorded as doubtful. For the purpose of potency testing, doubtful reactions are deemed to be negative.
 - 0 Negative - cell button dislodges with no visible clumps.

III. POTENCY TEST METHODS

A. TEST PROCEDURE FOR DETERMINATION OF ANTI-IgG USING RED BLOOD CELLS COATED WITH ANTI-D.

1. Select an anti-D serum for coating test red blood cells that meets the criteria listed below.

The undiluted anti-D should not directly agglutinate a 2% suspension of Group O, Dce or DCce (R₀r or R₁r) red blood cells. If undiluted single donor serum is not available, approval for the use of pooled or single donor antisera which are diluted may be requested from the Director, Center for Biologics Evaluation and Research. Monoclonal anti-D should not be used.

The anti-D serum used for cell coating should have a 1+ titration endpoint no less than 16 and no greater than 64 by the following test procedure.

- a. Beginning with undiluted anti-D serum, prepare separate master, serial, two-fold dilutions (1:2, 1:4 etc.) of the serum.
- b. To 0.1 ml of each anti-D serum dilution add 0.1 ml of a 2% suspension of Dce or DCce (R₀r or R₁r) red blood cells.
- c. Mix and incubate at 37°C for 30 minutes.
- d. Wash four times with large volumes of isotonic saline.
- e. To each cell button add 0.1 ml of a released lot of licensed Anti-Human Globulin containing anti-IgG. Mix.
- f. Centrifuge and record reactions.

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 Anti-Human Globulin reagent under test.

2. Coat the red blood cells with anti-D as follows.
 - a. Beginning with the anti-D serum or an approved dilution of the serum meeting the criteria for cell coating (III.A.1), prepare master, serial, two-fold dilutions (1:2, 1:4, etc.) of

this serum. Include at least two dilutions of anti-D immediately following the endpoint that was observed when selecting the serum.

- b. Add to the dilutions of anti-D an equal volume of the 2% red cell suspension used in selecting the anti-D serum and mix thoroughly.
 - c. Incubate at 37°C for 30 minutes.
 - d. After incubation, wash the coated red blood cells four times with large excesses of isotonic saline and resuspend to the original 2% red cell concentration.
3. Prepare dilutions of the Anti-Human Globulin reagent under test as well as the Reference Anti-IgG preparation as follows.

Beginning with undiluted reagent, prepare separate master, serial, two-fold dilutions (undiluted through 1:8) of the Anti-Human Globulin under test and the Anti-IgG reference preparation.

4. Perform the test for potency as follows.
- a. For both the reagent under test and the Reference Anti-IgG preparation, prepare at least four sets of test tubes, each containing no fewer than seven tubes. Each set should represent a dilution of the Anti-Human Globulin as prepared in III.A.3. To each tube of the first set place 0.1 ml of undiluted Anti-Human Globulin; to each tube of the second set place 0.1 ml of Anti-Human Globulin diluted 1:2, etc.
 - b. To the last tube of each set add 0.1 ml of red blood cells which are not coated with anti-D. Mix, centrifuge and record reactions observed.
 - c. To the second to the last tube of each set, add 0.1 ml of red blood cells coated with the highest dilution of the anti-D. Mix, centrifuge and record reactions observed.
 - d. Continue the serial addition of red blood cells coated with increasing amounts of anti-D until all sets are completed.

B. TEST PROCEDURE FOR DETERMINATION OF ANTI-IgG USING RED BLOOD CELLS COATED WITH ANTI-Fy^a

1. Select an anti-Fy^a for coating test red blood cells that meets the criteria listed below.

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 Center for Biologics Evaluation and Research 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 red blood cells should have a 1+ titration endpoint of no less than 8 and no greater than 32 using a 2% suspension of group O, Fy(a+b+) red blood cells by the following procedure.

- a. To 0.5 ml of anti-Fy^a add 0.07 ml of 0.11 M (4.45%) dipotassium ethylenediamine-tetraacetic acid (K₂EDTA). Incubate the serum-EDTA mixture at room temperature (20°C to 30°C) for 15 to 20 minutes.
- b. Beginning with undiluted anti-Fy^a serum, prepare separate master, serial, two-fold dilutions (1:2, 1:4, etc.) of the serum.
- c. To 0.1 ml of each anti-Fy^a serum dilution add 0.1 ml of the 2% suspension of Fy(a+b+) red blood cells.
- d. Mix and incubate at 37°C for 30 minutes.
- e. Wash four times with large volumes of isotonic saline.
- f. To each cell button add 0.1 ml of a released lot of licensed Anti-Human Globulin containing anti-IgG. Mix.
- g. Centrifuge and record reactions observed.

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 cell donor source are

used to determine the anti-IgG potency of the Anti-Human Globulin reagent under test.

2. Coat the red blood cells with anti-Fy^a as follows.
 - a. To three ml of anti-Fy^a serum or approved serum dilution meeting the criteria for cell coating (III.B.1), add 0.38 ml of 0.11 M (4.45%) K₂EDTA. Incubate the serum-EDTA mixture at room temperature (20°C to 30°C) 15 to 20 minutes.
 - b. Using the anti-Fy^a serum-EDTA mixture, prepare master, two-fold, serial dilutions (undiluted, 1:2, 1:4, etc.). Include at least two dilutions immediately following the end point observed when selecting the anti-Fy^a serum in III.B.1.
 - c. Add to the dilutions of anti-Fy^a an equal volume of the red cell suspension used in selecting the anti-Fy^a serum.
 - d. Incubate at 37°C for 30 minutes.
 - e. After incubation, wash the coated red blood cells four times with large excesses of isotonic saline and resuspend to the original 2% red cell concentration.
3. Prepare dilutions of the Anti-Human Globulin under test as well as the Reference Anti-IgG preparation as follows.

Beginning with undiluted reagent, prepare separate master, serial, two-fold dilutions (undiluted through 1:8) of the Anti-Human Globulin under test and the Anti-IgG Reference preparation.

4. Perform the test for potency as follows.
 - a. For both the reagent under test and the Reference Anti-IgG preparation, prepare at least four sets of test tubes, each containing no fewer than six tubes. Each set should represent a dilution of the Anti-Human Globulin as prepared in III.B.3. To each tube of the first set, place 0.1 ml of undiluted Anti-Human Globulin; to each tube of the second set, place 0.1 ml of Anti-Human Globulin diluted 1:2, etc.

- b. To the last tube of each set add 0.1 ml of red blood cells which are not coated with anti-Fy^a Mix, centrifuge and record reactions observed.
- c. To the second to the last tube of each set, add 0.1 ml of red blood cells coated with the highest dilution of the anti-Fy^a Mix; centrifuge and record reactions observed.
- d. Continue the serial addition of red blood cells coated with increasing amounts of anti-Fy^a until all sets are completed.

C. TEST PROCEDURE FOR DETERMINATION OF ANTI-C3b.^{1,2,6}

Please note that the amount of complement coated to the red blood cells *in vitro* may vary from donor to donor. It is recommended that initial testing include at least five donors to determine the best donor for use. Each donors' red blood cells should be sensitized with its own plasma or serum.

1. Solution J and Solution H are needed for this procedure. (See the appendix of Stock Solutions).
2. Prepare red blood cells coated with C3b as follows.

At least two normal clotted or anticoagulated whole blood samples should be collected and treated within one hour of collection as follows:

- a. Place 19.8 ml of Solution J in a 0°C ice bath and stir gently with magnetic stirring bar.
- b. Add 0.5 ml of washed, packed group O red blood cells.
- c. Add 0.5 ml of the same donor's fresh serum or plasma diluted 1:50 with isotonic saline.
- d. Immediately add 0.1 ml of Solution H to each of the above mixtures.
- e. Incubate for 30 minutes at 0°C with constant, gentle, stirring. Monitor the temperature to ensure 0°C is maintained.
- f. Wash the red blood cells four times with large excesses of isotonic saline and remove supernatant from the packed red blood cells.
- g. Resuspend each preparation to a 2% suspension.

- h. Store at 4°C. Use within three days or aliquot and freeze immediately.
3. Prepare "sucrose control" red blood cells for each donor in the same manner but substitute isotonic saline for the diluted serum or plasma in step III.C.2.c.
4. Prepare dilutions of the Anti-Human Globulin under test as follows.

Beginning with undiluted reagent, prepare master, serial, two-fold dilutions (1:2, 1:4, through 1:8) of the Anti-Human Globulin under test.

5. Perform the test for potency as follows.
 - a. Place 0.1 ml of each dilution of the test reagent into a separate test tube for each donor of the prepared C3b coated test cell suspensions.
 - b. Add 0.1 ml from each of the C3b coated test cell suspensions to each test dilution of Anti-Human Globulin.
 - c. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - d. Centrifuge and record reactions observed.
6. Perform a "sucrose control" test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of the "sucrose control" cell suspension prepared with the C3b coated red blood cells.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
7. Perform a negative control test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of untreated red blood cells from the same donors as in III.C.2 and III.C.3.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.

c. Centrifuge and record reactions observed.

D. TEST PROCEDURE FOR DETERMINATION OF ANTI-C3d.^{3,6}

1. Solution S is needed for this procedure. (See the appendix of Stock Solutions).
2. Prepare the C3d coated red blood cells as follows.

These red blood cells should be prepared from the same blood sample used to prepare the C3b test red blood cells.

- a. Prepare C3b coated packed red blood cells from two blood samples as previously outlined in III.C.
 - b. Place 0.5 ml of each of the C3b coated red blood cells in a test tube.
 - c. Add 1.0 ml of Solution S to each of the tubes.
 - d. Mix and incubate at 37° C for 30 minutes with occasional further mixing.
 - e. Wash the red blood cells four times with large excesses of isotonic saline and resuspend to a 2% concentration.
 - f. Store at 4°C. Use within three days or aliquot and freeze immediately.
3. Prepare "trypsin control" red blood cells for each donor in the same manner but substitute the "sucrose control" red blood cells from step III.C.3 for the washed, packed red blood cells in III.D.2.b.
 4. Dilute the Anti-Human Globulin under test and the Reference Anti-C3d preparation as follows.

Beginning with undiluted reagent, prepare master, serial, two-fold dilutions (1:2, 1:4, through 1:8) of the Anti-Human Globulin under test and the Reference Anti-C3d preparation.

5. Perform the test for potency as follows.
 - a. Place 0.1 ml of each dilution of the Anti-Human Globulin reagent under test and the Reference Anti-C3d preparation into a separate

test tube for each donor of the C3d coated test cell suspensions.

- b. Add 0.1 ml from each of the C3d coated test cell suspensions to each test dilution of Anti-Human Globulin.
 - c. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - d. Centrifuge and record reactions observed.
6. Perform a "trypsin control" test as follows.
- a. To 0.1 ml of the undiluted Anti-Human Globulin add 0.1 ml of the "trypsin control" cell suspension prepared with the C3d coated red blood cells.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
7. Perform a negative control test as follows.
- a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of untreated red blood cells from the same donors as in III.C.2 and III.C.3.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.

E. TEST PROCEDURE FOR DETERMINATION OF ANTI-C4b.^{4,6}

1. Solution K and Solution N are needed for this procedure. (See the appendix of Stock Solutions.)
2. Prepare the C4b coated test red blood cells as follows.

At least two normal clotted blood samples should be collected and treated within one hour of collection as follows:

- a. Place 10 ml of Solution K in a large tube.
- b. Add 0.15 ml of Solution N.

- c. Add 0.5 ml of washed, packed group O red blood cells.
 - d. Add 0.5 ml of fresh serum.
 - e. Mix well and incubate at 37°C for 15 minutes.
 - f. Wash the red blood cells four times with isotonic saline.
 - g. Resuspend the red blood cells to a 2% concentration.
 - h. Store at 4°C. These red blood cells should be used within 3 days or aliquot and freeze immediately.
3. Prepare "sucrose control" red blood cells for each donor in the same manner but substitute isotonic saline for the fresh serum in step III.E.2.d.
 4. Dilute the Anti-Human Globulin under test as follows.
 - a. Beginning with undiluted reagent, prepare master, serial, two-fold dilutions (1:2, 1:4 through 1:8) of the reagent under test.
 5. Perform the test for potency as follows.
 - a. To 0.1 ml of each Anti-Human Globulin dilution, add 0.1 ml from each of the C4b coated test cell suspensions.
 - b. Mix, thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
 6. Perform a "sucrose control" test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin add 0.1 ml of the "sucrose control" cell suspension prepared with the C4b coated red blood cells.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.

7. Perform a negative control test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of untreated red blood cells from the same donors as in III.E.2 and III.E.3.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.

F. TEST PROCEDURE FOR DETERMINATION OF ANTI-C4d.^{4,6}

1. Solution S is needed for this procedure. (See the appendix of Stock Solutions).
2. Prepare the C4d coated red blood cells as follows.

These red blood cells should be prepared from the same blood sample used to prepare the C4b test red blood cells.

 - a. Prepare C4b coated packed red blood cells from two blood samples as previously outlined in III.E.
 - b. Place 0.5 ml of each of the C4b coated red blood cells in a test tube.
 - c. Add 1.0 ml of Solution S to each of the tubes.
 - d. Mix and incubate at 37°C for 30 minutes with occasional further mixing.
 - e. Wash the red blood cells four times with large excesses of isotonic saline and resuspend to a 2% concentration.
 - f. Store at 4°C. Use within three days or aliquot and freeze immediately.
3. Prepare "trypsin control" red blood cells for each donor in the same manner but substitute the "sucrose control" red blood cells from step III.E.3 for the washed, packed red blood cells in III.E.2.c.

4. Dilute the Anti-Human Globulin under test as follows.
 - a. Beginning with undiluted reagent prepare master, serial, two-fold dilutions (1:2, 1:4, through 1:8) of the reagent under test.
 5. Perform the test for potency as follows.
 - a. To 0.1 ml of each dilution of the test reagent, add 0.1 ml of the C4d coated cell suspensions.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
 6. Perform a "trypsin control" test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin add 0.1 ml of the "trypsin control" cell suspension prepared in III.F.2.c. above.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
 7. Perform a negative control test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of untreated red blood cells from the same donors as in III.E.2 and III.E.3.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.
- G. TEST PROCEDURE FOR DETERMINATION OF ANTI-C3b/C4b⁶
1. Solution A and Solution B are needed for this procedure. (See the appendix of Stock Solutions).
 - a. Prepare Solution A1 by diluting 5.0 ml of Solution A to 1000 ml with DI water.
 - b. Prepare Solution B1 by diluting 5.0 ml of Solution B to 1000 ml with DI water.

- c. Place Solution A1 in a 1000 ml beaker and add Solution B1 until pH 6.1 is achieved.
 - d. Weigh out 92.4 gm of sucrose and dissolve in and QS to 1000 ml with the buffer prepared in G.1.c. above. (This solution may be aliquoted and stored at -20° C or below.)
2. Prepare red blood cells coated with C3b/C4b as follows.
- At least two normal clotted whole blood samples should be collected and treated within 18 hours of collection as follows:
- a. Place 8.5 ml of the buffer from III.G.1.d. into a 20 - 25 ml container.
 - b. Add 0.5 ml of fresh group O serum and mix.
 - c. Immediately add 1.0 ml of washed group O cells (from the same donor) at a concentration of 50% in isotonic saline.
 - d. Mix and incubate at 37°C for 30 minutes with occasional further mixing.
 - e. Centrifuge the red blood cells and discard the supernatant. Wash the red blood cells 4 times with large excesses of isotonic saline, discarding the last wash.
 - f. Resuspend each preparation to a 2% suspension.
 - g. Store at 4° C. Use on the day of preparation only.
3. Prepare "sucrose control" red blood cells for each donor in the same manner but substitute isotonic saline for the serum in step III.G.2.b.
4. Prepare "negative control" red blood cells by making a 2% suspension of untreated red blood cells.
5. These coated red blood cells and their control red blood cells may be used in the same manner as those prepared in III.C. and III.E.

H. TEST PROCEDURE FOR DETERMINATION OF ANTI-C3d/C4d⁶

1. Solution S is needed for this procedure. (See the appendix of Stock Solutions).
2. Prepare C3d/C4d coated red blood cells as follows.

These red blood cells should be prepared from the same blood sample used to prepare the C3b/C4b test red blood cells.

- a. Prepare C3b/C4b coated packed red blood cells from the two blood samples as previously outlined in III.G.
 - b. In a 20 - 25 ml container, mix 1.0 ml of Solution S and 0.5 ml of washed, packed C3b/C4b red cells.
 - c. Incubate at 37°C for 30 minutes with occasional further mixing.
 - d. Centrifuge the red blood cells and discard the supernatant. Wash the red blood cells 4 times with large excesses of isotonic saline, discarding the last wash.
 - e. Resuspend each preparation to a 2% suspension.
 - f. Store at 4° C. Use on the day of preparation only.
3. Prepare "trypsin control" red blood cells for each donor in the same manner but substitute "sucrose control" red blood cells for the C3b/C4b cells in step III.H.2.b.
 4. Prepare "negative control" red blood cells by making a 2% suspension of untreated red blood cells.
 5. These coated red blood cells and their control red blood cells may be used in the same manner as those prepared in III.D. and III.F.

I. TEST PROCEDURE FOR IgA DETERMINATION⁵.

1. Prepare the following reagents.
 - a. 1% Chromic Chloride Stock Solution (store in dark bottle at 4°C). Dilute 1:20 in unbuffered isotonic saline for use.

- b. Purified IgA myeloma protein (0.1 mg/ml in unbuffered isotonic saline).
2. Prepare the IgA coated red blood cells as follows.

Obtain group O red blood red blood cells from at least two donors and wash them four times with unbuffered saline.

 - a. Place 0.1 ml of washed packed red blood cells in a test tube.
 - b. Add 0.1 ml of diluted IgA protein. Mix.
 - c. Add 0.1 ml of diluted CrCl₃ and mix immediately.
 - d. Mix continually for four minutes at room temperature.
 - e. Wash the red blood cells four times with large excesses of unbuffered isotonic saline and discard supernatant from the packed red blood cells.
 - f. Resuspend the red blood cells to a 2% concentration.
3. Prepare "chromic chloride control" red blood cells for each donor in the same manner but substitute isotonic saline for the IgA in step III.G.2.b.
4. Perform the test for potency as follows.
 - a. To 0.1 ml of each Anti-Human Globulin reagent under test add 0.1 ml from each of the IgA coated red blood cells.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record macroscopic reactions observed.
4. Perform a "chromic chloride control" test as follows.
 - a. To 0.1 ml of the undiluted Anti-IgA add 0.1 ml of a 2% suspension of the "chromic chloride control" red blood cells prepared in III.G.3.
 - b. Incubate for 5 minutes at 20°C to 30°C.

- c. Centrifuge and record reaction.
5. Perform a negative control test as follows.
 - a. To 0.1 ml of the undiluted Anti-Human Globulin under test, add 0.1 ml of untreated red blood cells from the same donors as in III.G.2.
 - b. Mix thoroughly and incubate at 20°C to 30°C for 5 minutes.
 - c. Centrifuge and record reactions observed.

J. POTENCY TEST VALUES

The following potency values should apply to all Anti-Human Globulin specificities claimed to be present.

1. Potency titer values for the anti-IgG component.

Each dilution of the reagent tested in parallel should give reactions equal to or greater than the correspondingly diluted Reference Anti-Human Globulin preparation when tested against red blood cells coated with the smallest amounts of IgG detectable by the Reference Anti-Human Globulin preparation. The reagent under test should not prozone-i.e., no dilution of the reagent under test should give significantly stronger reactions than those observed with the undiluted reagent under test.

2. Potency titer values for the Anti-C3d component.

The undiluted Anti-Human Globulin should give at least a 2+ reaction with C3d coated red blood cells and the reagent should have a potency titer value at least equal to that of the Reference Anti-C3d preparation.

3. For other complement or immunoglobulin antibodies claimed, the undiluted reagent should give at least a 2+ reaction and a 1:4 dilution of the reagent should give at least a 1+ reaction with red blood cells prepared by a method approved by Center for Biologics Evaluation and Research.

IV. METHODS FOR EVALUATING SPECIFICITY

A. TESTS AGAINST IMMUNOGLOBULIN AND/OR COMPLEMENT COATED RED BLOOD CELLS

The specificity of the Anti-Human Globulin reagent under test should be evaluated by the direct antiglobulin method described in the manufacturer's package insert with red blood cells prepared by approved methods such as those described in part III.

1. The following tests are recommended.

| ANTI-HUMAN GLOBULIN REAGENT | VS | CELLS COATED WITH |
|---------------------------------|----|-----------------------------------|
| Anti-IgG,-C3d (polyspecific) | | IgG, C3b, C3d, C4b, C4d |
| Anti-IgG (Heavy Chain Specific) | | IgG, IgA, IgM, C3b, C3d, C4b, C4d |
| Anti-IgG | | IgG, C3b, C3d, C4b, C4d |
| Anti-Complement reagents | | IgG, C3b, C3d, C4b, C4d |

2. Alternative protocols may be acceptable; submit a description of the alternative procedures to the Center for Biologics Evaluation and Research for approval.
3. In addition, if the labeling claims the presence or absence of anti-C4b and/or anti-C4d, the appropriate coated red blood cells should be tested.
4. If monospecific anti-C3d activity is claimed, the absence of anti-C3b activity should be confirmed by a method approved by the Center for Biologics Evaluation and Research.
5. Testing with IgA coated red blood cells is recommended if labeling claims that the product contains, or is free of, anti-IgA activity.
6. If the presence or absence of Anti-IgM activity is claimed, this reactivity should be evaluated by methods approved by the Director, Center for Biologics Evaluation and Research.

7. Tests with IgM coated red blood cells may serve to exclude the presence of antibody to light chains.

B. TEST FOR HETEROSPECIFIC ANTIBODIES

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

1. A 2% red cell concentration in isotonic saline containing 1% autologous serum or plasma should be prepared from blood from normal, healthy donors within seven days of collection. Group O, A₁ and B red blood cells should be prepared.

2. The Test

- a. Divide nine test tubes into three sets each containing three tubes.
- b. Into each tube place 2 drops (or the amount recommended in the manufacturer's package insert) of undiluted Anti-Human Globulin under test.
- c. To the first tube of each set, add one drop of the group O red cell suspension.
- d. To the second tube of each set, add one drop of the group A₁ red cell suspension.
- e. To the third tube of each set, add one drop of group B red cell suspension. Mix all tubes.
- f. 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
- g. Centrifuge and examine each tube for hemolysis after centrifugation. Gently dislodge the cell button, observe macroscopically and record reactions.

NOTE: If the manufacturer recommends microscopic interpretation of results then this test should be examined microscopically.

The product is satisfactory when there is no agglutination or hemolysis 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 FOR REACTIVITY WITH NORMAL CELL SAMPLES

The product should be free of antibodies capable of agglutinating human red blood cells obtained from donor segments collected in FDA approved anticoagulants and stored at 2°C to 8°C. The age of the segments should span the approved shelf life of donor blood.

1. Cell Suspensions

- a. Obtain six group A₁, B and O donor segments as outlined above.
- b. Wash the red blood cells from the segments four times in isotonic saline and resuspend one portion to a 2% cell concentration in isotonic saline and another portion to a 2% cell concentration in LISS* solution.

2. Obtain six serum samples which contain no alloantibodies and were collected within 24 hours of product testing. These donors should be ABO group compatible with the six donor segments in IV.C.1.

* Refer to the manufacturer's package insert for the low ionic additive reagent being used. The recommended volumes for cells, serum and low ionic additive reagents should be followed to achieve the desired molarity.

3. Perform the test as follows.

- a. Place one drop of serum from IV.C.2 into two separate tubes for each of the six serum donors.
- b. To the first tube add one drop of a compatible donor's isotonic saline suspended red blood cells and to the second tube, add one drop of the same donor's red blood cells suspended in LISS*. Continue until red blood cells from all six donor samples have been added to the appropriate compatible serum samples. Mix.

- c. Centrifuge. Examine the supernatants for hemolysis and record macroscopic agglutination.
- d. Incubate the test for 15 minutes at 37°C. Centrifuge. Examine the supernatants for hemolysis. Record macroscopic agglutination.
- e. Wash the red blood cells four times in isotonic saline. Add 2 drops (or the amount recommended in the manufacturer's package insert) of the Anti-Human Globulin under test to the contents of each tube.
- f. Centrifuge. Examine the contents of each tube macroscopically and microscopically and record results observed.
- g. The product is satisfactory when there is no hemolysis and no agglutination visible macroscopically or microscopically in any of the tubes.

* Refer to the manufacturer's package insert for the low ionic additive reagent being used. The recommended volumes for cells, serum and low ionic additive reagents should be followed to achieve the desired molarity.

D. TEST WITH ENZYME-TREATED RED BLOOD CELLS

Anti-Human Globulin should not agglutinate enzyme treated red blood cells.

1. Obtain red blood cell samples from at least one group A₁, one group B, and one group O donor.
2. Enzyme treat the red blood cells as follows.
 - a. Add 0.4 ml of washed packed red blood cells to 1.6 ml of 0.1 % enzyme (trypsin, papain, or ficin).
 - b. Incubate the tubes at 37°C for 30 minutes.
 - c. Immediately wash the red blood cells four times with large excesses of isotonic saline and resuspend to a 2% suspension.

3. Perform the test as follows.
 - a. Place 2 drops (or the amount recommended in the manufacturer's package insert) of the Anti-Human Globulin under test into a tube.
 - b. Add one drop of the enzyme treated red cell suspension.
 - c. Centrifuge. Examine the contents of each tube macroscopically and microscopically and record results observed.
 - d. Incubate the tubes for 10 minutes at 20°C to 30°C, centrifuge, read and record results observed.

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

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APPENDIX I

STOCK SOLUTIONS⁶

FOR USE IN PREPARATION OF CELLS COATED WITH COMPLEMENT COMPONENTS

SOLUTIONS FOR PREPARATION OF C3b and C3b/C4b COATED CELLS

Solution A (1.0M Dipotassium phosphate) Store at 4°C

| | |
|--|------------|
| K ₂ HPO ₄ -3H ₂ O | 228.2 gm |
| DI Water | to 1000 ml |

Solution B (1.0M Potassium phosphate) Store at 4°C

| | |
|---------------------------------|------------|
| KH ₂ PO ₄ | 136.1 gm |
| DI Water | to 1000 ml |

Solution L (0.2M Disodium EDTA)

| | |
|--|-----------|
| Na ₂ EDTA-2H ₂ O | 7.45 gm |
| DI water | to 100 ml |

Solution F (buffered sucrose)

| | |
|------------|-----------|
| Solution A | 1.25 ml |
| Solution L | 5.25 ml |
| Sucrose | 23.1 gm |
| DI Water | to 250 ml |

Solution G (buffered sucrose)

| | |
|------------|-----------|
| Solution B | 1.1 ml |
| Solution L | 5.25 ml |
| Sucrose | 23.1 gm |
| DI Water | to 250 ml |

Solution H (0.4M Magnesium chloride)

| | |
|-------------------|------------|
| MgCl ₂ | 38.09 gm |
| DI Water | to 1000 ml |

Solution J (pH 5.1 buffered sucrose)

Add Solution F to Solution G until pH 5.1 is attained.

SOLUTIONS FOR PREPARATION OF C4b COATED CELLS

Solution K (10% Sucrose) Aliquot and store at -20°C or below

| | |
|----------|-----------|
| Sucrose | 25 gm |
| DI Water | to 250 ml |

Solution L (0.2M Disodium EDTA)

| | |
|--|-----------|
| Na ₂ EDTA-2H ₂ O | 7.45 gm |
| DI Water | to 100 ml |

Solution M (0.2M Tetrasodium EDTA)

| | |
|----------------------|-----------|
| Na ₄ EDTA | 8.32 gm |
| DI Water | to 100 ml |

Solution N (0.2M Trisodium EDTA)

| | |
|------------|--------|
| Solution L | 100 ml |
| Solution M | 100 ml |

SOLUTIONS FOR PREPARATION OF C3d, C4d AND C3d/C4d COATED CELLS

Solution A (1.0M Dipotassium phosphate) Store at 4°C

| | |
|--|------------|
| K ₂ HPO ₄ -3H ₂ O | 228.2 gm |
| DI Water | to 1000 ml |

Solution B (1.0M Potassium phosphate) Store at 4°C

| | |
|---------------------------------|------------|
| KH ₂ PO ₄ | 136.1 gm |
| DI Water | to 1000 ml |

Solution Q (1.0% Trypsin) Aliquot and store at -20°C or below

| | |
|-----------|-----------|
| Trypsin* | 1.0 gm |
| 0.05N HCl | to 100 ml |

Solution R (pH 7.7 phosphate buffer, dilute) Aliquot and store at -20°C or below

| | |
|------------|----------|
| Solution A | 5.0 ml |
| DI Water | to 50 ml |

| | |
|------------|----------|
| Solution B | 1.0 ml |
| DI Water | to 10 ml |

Add Diluted Solution A to Diluted Solution B to pH 7.7.

Solution S (0.1% Trypsin)

| | |
|------------|--------|
| Solution Q | 0.1 ml |
| Solution R | 0.9 ml |

* Trypsin, twice crystallized
