Designation: F 1984 – 99 (Reapproved 2003)

Standard Practice for Testing for Whole Complement Activation in Serum by Solid Materials[1]

This standard is issued under the fixed designation F 1984; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice provides a protocol for rapid, *in vitro* screening for whole complement activating properties of solid materials used in the fabrication of medical devices that will contact blood.

1.2 This practice is intended to evaluate the acute *in vitro* whole complement activating properties of solid materials intended for use in contact with blood. For this practice, the words "serum" and "complement" are used interchangeably (most biological supply houses use these words synonymously in reference to serum used as a source of complement).

1.3 This practice consists of two procedural parts. Procedure A describes exposure of solid materials to a standard lot of human serum, using a 0.1-mL serum/13 x 100-mm disposable test tube. Cellulose acetate powders and fibers are used as examples of test materials. Procedure B describes assaying the exposed serum for significant functional whole complement depletion as compared to control samples.

1.4 This practice does not address function, elaboration, or depletion of individual complement components, nor does it address the use of plasma as a source of complement.

1.5 This practice is one of several developed for the assessment of the biocompatibility of materials. Practice F 748 may provide guidance for the selection of appropriate methods for testing materials for other aspects of biocompatibility.

2. Referenced Documents

2.1 *ASTM Standards:* [2]

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

2.2 *ISO Document:*

ISO 10993-4: Biological Evaluation of Medical Devices, Part 4: Selection of Tests for Interactions with Blood[3]

3. Terminology

3.1 *Abbreviations:*

3.1.1 *Ab*—antibody (hemolysin).

3.1.2 *BBS*—barbital buffered saline.

3.1.3 *BBS-G*—barbital buffered saline—gelatin.

3.1.4 *BBS-GM*—barbital buffered saline—gelatin metals.

3.1.5 *C′*—complement.

3.1.6 *EDTA*—ethylenediaminetetraacetic acid, disodium salt: dihydrate.

3.1.7 *HS*—human serum.

3.1.8 *PVDF*—polyvinylidene fluoride.

3.1.9 *RBC*—red blood cell(s).

4. Summary of Practice

4.1 Solid material specimens are exposed to contact with a standard lot of complement under defined conditions (Procedure A). Exposed serum then is tested for significant functional complement depletion compared to controls under identical conditions (Procedure B).

5. Significance and Use

5.1 Inappropriate activation of complement by blood-contacting medical devices may have serious acute or chronic effects on the host. This practice is useful as a simple, inexpensive screening method for determining functional whole complement activation by solid materials *in vitro*.

5.2 This practice is composed of two parts. In Part A (Section 11), human serum is exposed to a solid material. Complement may be depleted by the classical or alternative pathways. In principle, nonspecific binding of certain complement components also may occur. The alternative pathway can deplete later acting components common to both pathways, that is components other than C1, C4, and C3 **(1)**.[4] In Part B (Section 12), complement activity remaining in the serum after exposure to the test material is assayed by classical pathway-mediated lysis of sensitized RBC.

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[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility Test Methods.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036.

[4] The boldface numbers in parentheses refer to the list of references at the end of this specification.

5.3 Assessment of *in vitro* whole complement activation, as described here, provides one method for predicting potential complement activation by medical materials intended for clinical application in humans when the material contacts the blood. Other test methods for complement activation are available, including assays for specific complement components and their split products (see X1.3 and X1.4).

5.4 This *in vitro* test method is suitable for adoption in specifications and standards for screening solid materials for use in the construction of medical devices intended to be implanted in the human body or placed in contact with human blood.

6. Preparation of Buffers

6.1 *Buffers*, are prepared according to detailed protocol **(2)**. "Water" refers throughout to distilled, endotoxin-free water. The use of barbital (veronal) buffer is recommended. Barbital is a class IV regulated substance and requires a DEA **(3)** license for purchase. The use of other buffer systems, such as, TRIS, is permissible if they have been demonstrated not to activate complement**(4)**.

6.2 *5X Stock BBS (barbital-buffered saline)*, is prepared by adding 20.75 g NaCl plus 2.545 g sodium barbital (sodium-5,5-diethyl barbiturate) to about 400 mL water. The pH is adjusted to 7.35 with 1 N HCl, then brought to a final volume of 500 mL in a volumetric flask.

6.3 *Metals Solution*, is prepared by making a 2.0 M solution of $MgCl_2$ (40.66 g $MgCl_2$• 6 H_2O into 100 mL distilled endotoxin-free water), and a 0.3 M solution of $CaCl_2$ (4.41 g $CaCl_2$• 2 H_2O into 100 mL distilled endotoxin-free water), and combining the two solutions 1:1 (v:v). These solutions are stable one month at 4°C.

6.4 *BBS-GM Working Solution*, is prepared daily, by dissolving 0.25 g gelatin in 50 mL endotoxin-free distilled water that is gently heated and stirred. The gelatin solution is added to 50 mL 5X stock BBS plus 0.25 mL metals solution, brought up to about 200 mL, then adjusted to pH 7.35 (with 1 N HCl or 1 N NaOH) before bringing the final volume to 250 mL in a volumetric flask.

6.5 *BBS-G Working Solution*, is prepared the same way, but the addition of the metals solution is omitted.

6.6 *10X Stock EDTA (0.1 M disodium dihydrate EDTA)*, is prepared by adding 7.44 g disodium EDTA•2 H_2O to about 160 mL water, adjusting the pH to 7.65 (with 1 N NaOH or 1 N HCl), then bringing the volume to 200 mL in a volumetric flask.

6.7 *BBS-G-EDTA (to be used in preparing RBC before being washed out)*, is prepared by adding 10 mL of stock 10X EDTA to 90 mL of BBS-G in a volumetric flask.

7. Preparation of Sheep RBC

7.1 Commercially-obtained sheep red blood cells (RBC) preserved in Alsever's solution are stored at 4°C. The cells are discarded after eight weeks or when the supernatant from the second wash contains hemoglobin by visual inspection.

NOTE 1—All centrifugations are at 4°C. Except where indicated, all reagents, tubes, and cell preparations are kept on ice.

7.2 Five mL of sheep RBC are centrifuged at 1 000 x g for 10 min.

7.3 The cell pellet is resuspended in 10 mL of cold BBS-G-EDTA and incubated for 10 min at 37°C. The cells are centrifuged, and the pellet resuspended in 10 mL of BBS-G-EDTA.

7.4 The cells are centrifuged, the supernatant discarded (first wash), and the pellet resuspended in 10 mL of cold BBS-GM. Repeat twice (total of three washes).

7.5 Adjust cell count spectrophotometrically (where an absorbance of 0.56 corresponds to 1.5 x 10^8 sheep RBC/mL, at a wavelength of 412 nm and a 1.0-cm light path for 1 volume of cells in BBS-GM plus 24 volumes of water) or count with a hemocytometer, preparing 10 mL of 1.5 x 10^8 cells/mL in cold BBS-GM.

7.6 The washed, diluted RBC can be held on ice and used for at least 12 h.

8. Absorption of Serum (Complement)

8.1 The use of human complement is required since there are species differences in the efficiency of complement activation and the test materials are to be used in humans. Human serum suitable as a source of complement may be purchased from biological supply houses, and generally, is labeled as reagent-grade complement.

8.2 Human serum may be absorbed with sheep RBC in order to remove naturally-occurring anti-sheep RBC hemolytic antibodies, though for most purposes, the amount of heterophile antibody in human serum is not enough to influence the reaction assuming the cells are optimally sensitized with hemolysin. The procedure is detailed in 8.3-8.8.

8.3 Fresh human serum or a commercial lot of human serum is obtained and stored at −70°C. Fresh serum is preferred as lyophilized complement often is not as active as fresh serum.

8.4 The serum is thawed on ice or reconstituted (if lyophilized) with ice-cold (4°C) distilled endotoxin-free water.

8.5 All manipulations are done on ice, with ice cold reagents and cells; centrifugations are carried out at 1000 x g at 4°C. It is critical that this entire procedure be done in the cold to avoid activation of complement in this step.

8.6 Cold serum and cold, packed, washed sheep RBC are mixed, 0.1 mL RBC/2.5 mL serum, incubated for 10 min on ice, then centrifuged at 1 000 x g for 10 min at 4°C. The supernatant is transferred carefully to a new container on ice.

8.7 The procedure in 8.6 is repeated twice.

8.8 The absorbed human serum is stored in 0.5–1.0-mL aliquots (convenient for one-experiment use), in cold snap-cap microfuge tubes and kept at −70°C until used. Aliquots should be thawed on ice, used on the day of thawing, and not be refrozen.

9. Determination of Optimal Hemolysin Concentration

9.1 Determination of optimal hemolysin concentration is necessary in order to conserve expensive reagents and to avoid prozone effects. Commercial rabbit anti-sheep RBC serum (Hemolysin) is obtained, thawed, or, if lyophilized, reconstituted with distilled endotoxin-free water), heat-inactivated at 56°C for 30 min to inactivate the rabbit complement, aliquoted in convenient volumes, and stored at −70°C until used.

9.2 To cold 13 x 100 mm disposable glass tubes, placed in a rack in an ice-bath, 0.1 mL of washed sheep RBC at 1.5 x 10^8 cells/mL is added. If statistical evaluation of the results is desired, three replicate tubes for each condition should be used. Otherwise, duplicates or even single dilution tubes are sufficient. One set of three replicate tubes receives only 0.1 mL of cold BBS-GM/tube (no RBC control, for complement color).

9.3 To the RBC-containing tubes, one set of three tubes gets 1.1 mL cold distilled H_2O/tube (total lysis control), another gets 0.1 mL BBS-GM (no hemolysin control), and the other sets get 0.1 mL each of 1:2 serial dilutions of hemolysin (tests). Dilutions between 1:400 to 1:25 600 antibody are recommended, with two sets of 1:400. The no RBC control receives 0.1 mL of additional BBS-GM.

9.4 Each tube is mixed quickly by gentle shaking to resuspend cells, the rack is placed in a 37°C water bath, incubated 10 min, then returned to the ice bath.

9.5 One of the two sets of 1:400 antibody gets 1.0 mL of cold BBS-GM (no-complement control). All other tubes besides the total lysis control set get 0.5 mL cold BBS-GM, then 0.5 mL of absorbed human serum (complement) diluted 1:100 or 1:200.

NOTE 2—For a particular lot of human serum, a 1:100 or 1:200 dilution should provide sufficient complement activity. Also, percent lysis in the no-hemolysin (complement only) control should not exceed 10 %. If lysis with the 1:100 dilution of complement exceeds 10 %, use 1:200. If the no-hemolysin control still exceeds 10 %, a different lot of serum will need to be tested.

9.6 Tubes are shaken manually to suspend cells, then the rack is incubated in a 37°C water bath for 1h, and intermittently shaken to keep cells in suspension.

9.7 At the end of 1h, the rack is placed on ice. The cold tubes then are centrifuged at 1 000 x g for 10 min at 4°C, and the supernatants decanted to correspondingly numbered 13 x 100-mm glass tubes.

9.8 Absorbance of the supernatants is measured at 412 nm. Percent lysis is calculated for each test and control tube by subtracting from the 412 nm absorbance the no RBC control (mean of the three replicate tubes), dividing by the total lysis control value (mean of the three replicate tubes), and multiplying by 100.

$$\% \text{ lysis} = \frac{\text{test absorbance} - \text{no RBC control absorbance}}{\text{total lysis absorbance}} \times 100 \quad (1)$$

9.9 Final % lysis for each condition is expressed as mean ± standard deviation of the three % lysis values for each three-replicate set.

9.10 A titration curve is obtained by plotting the inverse of the hemolysin concentration versus % specific lysis. Twice the concentration of hemolysin that is just on the plateau of the titration curve is used for sensitizing RBC for subsequent assays (optimal hemolysin concentration). Hemolysin is freshly diluted from stock each day of use.

10. Whole Complement Titration to Determine Optimal Serum Dilution

10.1 If statistical evaluation of results is desired, all conditions should be assayed in triplicate, using three 13 x 100 disposable glass test tubes per condition. Otherwise, duplicates or single tubes are sufficient. Tubes are numbered in advance. Conditions include total lysis, no complement (no C'), tests (dilutions of human serum—HS) with and without hemolysin, and no RBC (complement color control, at highest concentration of serum used). All reagents, tubes, and manipulations are done ice-cold, with tubes held in a rack in an ice slurry.

10.2 Washed RBC are added to all tubes except no RBC tubes (0.1 mL/tube of a 1.5 x 10^8 cells/mL suspension). No RBC tubes get 0.1 mL cold buffer.

10.3 Total lysis tubes get 1.1 mL distilled H_2O. The no C' and test with hemolysin tubes get 0.1 mL optimal hemolysin (see 9.10), and no RBC tubes get 0.1 mL cold BBS-GM. All tubes are shaken to resuspend cells, incubated in a 37°C water bath for 10 min, and placed back on ice.

NOTE 3—Another acceptable procedure is to make up one large batch of hemolysin-sensitized erythrocytes to cover all the tests planned within one week's time. These cells are made up at 5 x 10^8/mL and are stored at 4°C. They are washed each time they are used, and if hemolysis occurs, new sensitized cells are prepared. These sensitized cells are ready to use, making the addition of hemolysin to each tube unnecessary, which simplifies the experiment. Unsensitized RBC can be used as controls for nonspecific lysis.

10.4 To all but the total lysis tubes, a maximum volume of 1.0 mL of cold BBS-GM is added, reduced by the amount of diluted serum (see 10.5), which will be added at a maximum 0.5 mL volume. The no C' tubes get 1.0 mL BBS-GM.

10.5 The cold serum is diluted in cold BBS-GM to the desired concentration (with minimal agitation). It is recommended to test the HS initially at 1:50 to 1:300. The diluted serum is added to each test tube in a 0.5 mL volume. Final volume in each tube should be made up to 1.2 mL with BBS-GM.

10.6 The tubes then are treated as detailed in 9.6-9.9.

10.7 The optimal human serum dilution of a particular lot of human serum is defined as that in which the nonspecific lysis (HS + RBC, in absence of hemolysin antibody) is ≤10 %, while specific lysis (total lysis [RBC + Ab + HS] minus nonspecific lysis) is at least 20 % but not greater than 80 %, that is, the specific lysis is on the linear part of the complement titration curve. A typical optimal dilution for a lot of absorbed human serum is 1:200 added as a 0.5 mL volume in the assay.

11. Procedure A—Exposure of Material to Human Serum

11.1 *Powder*:

NOTE 4—An example of a powder is cellulose acetate. Centrifugation in a typical table-top clinical centrifuge is insufficient to pellet the powder following incubation with complement. Hence a filtration step, with appropriate control, is required.

11.1.1 Cold, absorbed human complement is placed onto the bottom of cold 13 x 100 disposable glass tubes on ice, 0.1 mL/tube. A minimal assay requires four tubes, labeled M (material), NM (no material control), I_1 (Ice-one, the filtration control), and I_2 (Ice-two, maximal complement activity control).

NOTE 5—Other controls might include a comparison to another material with same unit surface area or other appropriate measurable parameter, or a positive control for complement activation, such as zymosan or

heat-aggregated gamma globulin, HAGG, or both.

11.1.2 The upper portions of the M and NM tubes are warmed briefly by hand, to prevent powder from adhering to moisture on the sides. A defined quantity of powder is dropped onto the 0.1 mL serum at the bottom of the tube, such that the liquid is just covered, for example, 6 mg, by the powder. With no mixing, the two tubes are placed in a 37°C water bath and incubated for 1h. I_1 and I_2 are kept on ice.

11.1.3 Three syringe filters are prepared as follows. Recommended filters are low proteinbinding, such as hydrophilic PVDF membranes, with 0.22-µm pore size. Each filter is flushed with 2.0 mL cold BBS-GM, excess liquid expelled with air, and placed in holder on ice until needed.

11.1.4 At the end of 1h incubation, the M and NM tubes are put back on ice. Immediately, 4.9 mL of cold BBS-GM is added to each tube (a 1:50 dilution of the exposed serum). Using separate Pasteur glass pipettes, the contents of each tube are slowly drawn up and back down into the tube, insuring mixture of the serum and buffer.

11.1.5 The tubes then are centrifuged at 4°C, 2 000 x g, for 10 min. Place the pipette at midheight in the liquid, draw 3 mL, and transfer to another tube from which the contents are filtered through separate, prepared 0.22-µm syringe filters into fresh, cold tubes.

11.1.6 The contents of each tube then are diluted to the optimal human serum dilution (see 10.7) in cold tubes and kept on ice. Serum should be assayed within 1h for complement activity (see Section 12).

11.2 *Fibers or Solid Pieces*:

11.2.1 Assay for whole complement activation by solid fibers or pieces of material is similar to that detailed in 11.1 for powders, except that a defined amount of fiber or material (mg amounts, just enough to be covered fully by 0.1 mL serum) is put first into room temperature 13 x 100 tubes. Then 0.1 mL of serum is added to the M, NM, I_1, and I_2 tubes. Immediately the M and NM tubes are placed in a 37°C water bath while I_1 and I_2 are put on ice. At the end of 1h, the M and NM tubes are taken out of the 37°C water bath and also put on ice.

11.2.2 If the materials tested do not float, or if they form a firm pellet following the 2 000 x g centrifugation, the filtration step and the I_1 control may not be needed.

11.3 *Assay Size and Conditions Tested*:

11.3.1 The preceding general format can be used to test differing amounts of material to yield dose-response curves, the same quantity exposed to 37°C for various periods of time (time course), or to compare C' activation by various materials.

NOTE 6—This procedure does not preclude exposure of volumes of serum other than 100 µL to materials where the size or shape could not be tested in 13 x 100 disposable glass tubes.

11.3.2 Since each condition should be assayed for % specific hemolysis, which requires determination of % total hemolysis (Ab + RBC + HS) and % nonspecific hemolysis (RBC + HS), and each condition is assayed in triplicate, it is recommended that the total number of test samples to be assayed not exceed ten/experiment/technician (so as to not exceed final assay size of around 100 tubes.)

12. Procedure B—Assay of Serum for Complement Depletion

12.1 Procedure B is used to assay serum, which previously has been exposed to a material (Procedure A) for possible depletion of whole complement activity.

12.2 All conditions are assayed in triplicate, using three 13×100 disposable glass test tubes/condition. Tubes are numbered in advance. Conditions include total lysis, no complement (no C'), tests (dilutions of human serum—HS) with and without hemolysin (three tubes each), and no RBC (at highest concentration of serum used). All reagents, tubes, and manipulations are done ice-cold, with tubes held in a rack on ice.

12.3 Addition of washed RBC, and then hemolysin is conducted as directed in 10.2 and 10.3.

12.4 To the no-C' tubes, 1.0 mL of BBS-GM is added. To all except the "total lysis" tubes, 0.5 mL of cold BBS-GM is added. Then, 0.5 mL from each of the test or control condition tubes from the material exposure step, which are being held on ice and already are diluted to the optimal human serum concentration (see 10.7), is added to each of three tubes containing hemolysin-sensitized RBC and each of three tubes containing nonsensitized RBC.

12.5 The tubes then are treated as detailed in 9.6-9.9.

13. Report Section and Data Analysis

13.1 Incubation of serum not exposed to materials at 37°C (tube NM) may result in reduction in complement hemolytic activity compared to serum kept on ice (I_2). If a filtration step is needed (controlled for by the ice-1 tube, I_1), significant reduction from I_2 also may be seen in I_1.

13.2 At a minimum, materials should be tested in triplicate in Procedure A, with each of the three exposure tubes from Procedure A being assayed in triplicate in Procedure B. This allows demonstration of significant differences between the means of different conditions despite intertube assay variation. If small differences are being studied, the number of replicate tubes for each condition in Procedure A may need to be increased to five or more.

13.3 Significant depletion of control hemolytic activity in Procedure B denotes whole complement activation by test materials in Procedure A.

13.4 Differences in hemolysis are considered significant at $p \leq 0.05$, as calculated by an appropriate statistical test, such as ANOVA. Results may be presented as a bar graph displaying each condition as a mean and standard deviation.

14. Keywords

14.1 biocompatibility; blood compatibility; whole complement testing

APPENDIX

(Nonmandatory Information)

X1. RATIONALE

X1.1 The primary purpose of this practice is to describe a simple, inexpensive functional test to screen serum for complement activation by blood-contacting materials. Though serum is not the same as the plasma to which a material is exposed *in vivo*, artificially collected plasma, that is, with anticoagulant, is a poor choice because of the interference of these anticoagulants with the complement activation process.

X1.2 It is well recognized that complement activation is an important defense mechanism of the host; however, complement activation by material components of blood-contacting devices may be harmful to the host.

X1.3 Complementology has been an active research area for many years; however, the importance of chronic local complement activation on material/device function and actual impact on patient health is largely unknown. Many investigators have developed tests for whole complement functional activity, depletion of specific complement components, or generation of specific complement split products. Other validated test methods may be substituted for the functional whole complement-depletion assay described here. If immunological assays for individual complement pathway components are used, consideration should be given as to whether component depletion is by nonspecific binding to a material or by pathway activation.

X1.4 The procedure as presented is intended as a routine screening procedure. It is not to be represented as being the most sensitive nor the most specific procedure for assessing the complement-activation potential of all materials in all applications. Substances that activate weakly might still generate enough relevant split products (C3a, C5a, etc.) to cause a local inflammatory response but not be reflected by significant change in whole complement activity. The results obtained with this practice are intended to be used in conjunction with the results of other tests in assessing the blood compatibility of the test material.

REFERENCES

(**1**) Giclas, P.C., "Complement Tests," *Manual of Clinical Laboratory Immunology*, fifth edition, eds N.R. Rose, E.C. de Macario, J.D. Folds, H.C. Lane, and R.M. Nakamura, ASM Press, 1997, pp. 181–186.

(**2**) Gee, A.P., "Molecular Titration of Components of the Classical Complement Pathway," *Methods in Enzymology*, Vol 93, Immunochemical Techniques, eds J.J. Langone, H.V. Vunakis, Academic Press, 1983, pp. 339–375.

(**3**) United States Drug Enforcement Agency, Washington, DC.

(**4**) Lin, W-Q., White, Jr., K.L., "Complement Assays to Assess Immunotoxicity," *Methods in Immunotoxicology*, Vol I, eds G.R. Burleson, J.H. Dean, and A.E. Munson, Wiley-Liss, 1995, pp. 357–375.