



Designation: F2567 – 06

Standard Practice for Testing for Classical Pathway Complement Activation in Serum by Solid Materials¹

This standard is issued under the fixed designation F2567; 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* functional screening for classical pathway 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* classical pathway complement activating properties of solid materials intended for use in contact with blood. For this practice, “serum” is synonymous with “complement.”

1.3 This practice consists of two procedural parts. Procedure A describes exposure of solid materials to a standard lot of human serum [HS], using 0.1 mL serum per 13×100 mm disposable glass test tube. Procedure B describes assaying the exposed serum for significant functional classical pathway complement depletion (decrease in amount of C4) as compared to control serum samples not exposed to the material. The endpoint in Procedure B is lysis of sheep red blood cells (RBC) coated with antibody (hemolysin).

1.4 This practice does not 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 F748 may provide guidance for the selection of appropriate methods for testing materials for other aspects of biocompatibility. Practice F1984 provides guidance for testing solid materials for whole complement activation in human serum, but does not discriminate between the classical or alternative pathway of activation. Practice F2065 provides guidance for testing solid materials for alternative pathway complement activation in serum.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

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.

Current edition approved Oct. 1, 2006. Published October 2006. DOI: 10.1520/F2567-06.

2. Referenced Documents

2.1 ASTM Standards:²

F748 Practice for Selecting Generic Biological Test Methods for Materials and Devices

F1984 Practice for Testing for Whole Complement Activation in Serum by Solid Materials

F2065 Practice for Testing for Alternative Pathway Complement Activation in Serum by Solid Materials

2.2 Other Document:

ISO 10993-4 Biological Evaluation of Medical Devices, Part 4: Selection of Tests for Interactions with Blood³

3. Terminology

3.1 Definition of Term Specific to This Standard:

3.1.1 *water*—distilled, endotoxin-free.

3.2 Abbreviations:

3.2.1 *Ab*—antibody (hemolysin)

3.2.2 *BBS*—barbital buffered saline

3.2.3 *BBS-G*—barbital buffered saline–gelatin

3.2.4 *BBS-GM (Ca Buffer)*—barbital buffered saline–gelatin metals

3.2.5 *C'*—complement

3.2.6 *C4*—the fourth component of complement

3.2.7 *C4(-)GPS*—C4-deficient guinea pig serum [serum from guinea pigs genetically incapable of producing C4]

3.2.8 *EDTA*—ethylenediaminetetraacetic acid, disodium salt, dihydrate

3.2.9 *HAGG*—heat aggregated gamma globulin

3.2.10 *HS*—human serum

3.2.11 *I*—“ice” control tube with serum but no material, kept on ice

3.2.12 *M*—tube containing serum plus a test material

3.2.13 *NM*—tube containing serum but no material

3.2.14 *RBC*—red blood cell(s)

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, http://www.ansi.org.

4. Summary of Practice

4.1 This practice is based on a method published by Gaither et al, 1974 (1).⁴

4.2 Solid material specimens are exposed to a standard lot of human C' (specially-prepared, commercial human serum [HS]) under defined conditions, in parallel with appropriate controls (Procedure A). If the classical complement pathway is activated by the material, C4 will be depleted from the serum. Exposed serum is then tested for remaining C4 functional activity. An appropriate dilution of the HS, which by itself is too dilute to lyse sensitized sheep RBC, is added to hemolysin-coated sheep RBC in the presence of C4(-)GPS in which all complement components save the missing C4 are present in excess (Procedure B). Hemolysis in Procedure B provides a quantitative measure of the C4 remaining in HS exposed to test material in Procedure A. Depletion of hemolysis indicates specific classical pathway activation in the human serum caused by exposure to the test material.

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. Solid medical device materials may activate complement directly by the alternative pathway, or indirectly because of antigen-bound antibodies (as with immuno-adsorption columns) by the classical pathway. This practice is useful as a simple, inexpensive, function-based screening method for determining complement activation by solid materials *in vitro* by the classical pathway.

5.2 This practice is composed of two parts. In part A (Section 11), HS is exposed to a solid material. If complement activation occurs by the classical pathway, C4 will be depleted. Activation by the alternative pathway will not deplete C4. In part B (Section 12), C4 activity remaining in the serum after exposure to the test material is assayed by diluting the serum below the concentration needed to lyse antibody-coated sheep RBC on its own, then adding the diluted HS to C4(-)GPS (which is itself at a dilution where all complement components are in excess save the missing C4). Lacking C4, the C4(-)GPS does not lyse the antibody-coated sheep RBC unless C4 is present in the added HS. The proportion of lysis remaining in the material-exposed HS sample versus the 37°C control HS sample (which was not exposed to the test material) indicates the amount of C4 present in the HS, loss of which correlates with classical pathway activation.

5.3 This function-based *in vitro* test method for classical pathway complement activation 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 outside the body. It is designed to be used in conjunction with Practice F1984 for function-based whole complement activation screening, and Practice F2065 for function-based alternative pathway activation screening.

5.4 Assessment of *in vitro* classical complement activation as described here provides one method for predicting potential complement activation by solid medical device materials intended for clinical application in humans when the material contacts the blood. Other test methods for complement activation are available, such as immunoassays for specific complement components (including C4) and their split products in human serum (see X1.3 and X1.4).

5.5 If nonspecific binding of certain complement components, including C4, to the materials occurs in part A of this practice, a false positive for classical pathway activation will be observed in step B. Classical pathway complement activation by the test material may be confirmed by demonstrating an absence of C4 bound to the material following removal of the serum, and/or production of complement split-products such as C4d in the serum (as determined by immunoassay). Although immunoassay could be done in place of this screening procedure, determination of C4d production alone may not be functionally significant. This practice does not detect trivial amounts of classical activation unable to affect functional lysis of sensitized RBC.

6. Preparation of Buffers

6.1 *Buffers* are prepared according to established protocols (2, 3). "Water" refers throughout to distilled, endotoxin-free H₂O. The use of barbital (veronal) buffer is recommended. In the United States, barbital is a class IV regulated substance and requires a DEA (4) license for purchase. The use of other buffer systems (such as TRIS) is permissible if they have been demonstrated not to activate complement (5).

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 MgCl₂ (40.66 g MgCl · 6 H₂O into 100 mL water), and a 0.3 M solution of CaCl₂ (4.41 g CaCl₂ · 2 H₂O into 100 mL water), and combining the two solutions 1:1 (v:v). These solutions are stable for one month at 4°C.

6.4 *Ca Buffer (BBS-GM Working Solution)* is prepared daily, by dissolving 0.25 g gelatin in 50 mL 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 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. Ca buffer contains both Mg⁺⁺ and Ca⁺⁺, which allows both classical and alternative pathway complement activation to occur.

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

6.6 *10X Stock EDTA* (0.1 M disodium dihydrate EDTA) is prepared by adding 7.44 g disodium EDTA · 2 H₂O 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 with Ca buffer) is prepared by adding 10 mL of stock 10X EDTA to 90 mL of BBS-GM Working Solution.

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

7. Preparation of Sheep RBC

7.1 Commercially-obtained sheep RBC preserved in Alsever's solution are stored at 4°C. The sheep cells are discarded after eight weeks or when the supernatant liquid from the second wash contains hemoglobin by visual inspection (as lots of RBCs age, they increase in sensitivity to complement lysis in parallel with increased spontaneous lysis).

NOTE 1—All centrifugations are at 4°C. Except when indicated, all reagents, tubes, and cell preparations are kept on ice or in an ice slurry. In subsequent sections where the word “cold” is used, that denotes tubes in ice or sitting in an ice slurry.

7.2 Five mL of sheep RBC are centrifuged at 1000× g, at 4°C, 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 (Ca Buffer). This step is repeated twice more for a total of three washes.

7.5 Adjust cell concentration by counting with a hemocytometer, and prepare 10 mL of 3.0×10^8 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 Serum should be absorbed with sheep RBC in order to remove any naturally-occurring anti-sheep hemolytic antibodies. The procedure is as follows.

8.2 Commercially-available HS and C4(-)GPS are stored at -70°C. Both sera should be absorbed separately.

8.3 Serum is thawed on ice or reconstituted (if lyophilized) with ice-cold (4°C) water.

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

8.5 Sheep RBC are washed as in Section 7, centrifuged 1000× g for 10 min at 4°C, and the cold supernatant removed down to the pellet. The cold, packed RBC are then added to the serum in a glass tube on ice, 0.1 mL/2.5 mL serum. The cells are mixed thoroughly into the serum slowly inverting the capped tube several times. The cell/serum mixture is incubated for 10 min on ice, then centrifuged at 1000× g for 10 min at 4°C. The supernatant liquid is carefully transferred to a fresh glass tube on ice.

8.6 The procedure in 8.5 is repeated twice, exposing the cold serum to three fresh preparations of cold cells.

8.7 The absorbed HS is stored in 0.5 to 1.0 mL aliquots (convenient for one experiment), in pre-chilled, cold snap-cap microfuge tubes immediately placed at -70°C until used. Aliquots should be thawed cold, on ice (not allowed to warm higher than 4°C), used on the day of thawing, and not re-frozen.

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 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 13×100 mm disposable glass tubes, placed in a rack in an ice-slurry, 50 µL of washed sheep RBC at 3×10^8 cells/mL is added directly to the bottom of each tube. 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 50 µL of cold Ca buffer/tube (“no RBC” control, for complement color).

9.3 To the RBC-containing tubes, one set of three tubes gets 0.35 mL cold distilled H₂O/tube (“total lysis” control), another gets 50 µL mL Ca buffer (“no hemolysin” control), and the other sets get 50 µL mL each of 1:2 serial dilutions of hemolysin (“tests”). Dilutions between 1:200 to 1:25 600 antibody are recommended, with two sets of 3 tubes each for 1:200. The “no RBC” control receives 50 µL of additional BBS-GM instead of hemolysin. All tubes except “total lysis” controls should each contain at this point a total 0.1 mL.

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

9.5 One of the two 3-tube sets of 1:200 hemolysin gets 0.1 mL of cold Ca buffer (“no-complement” control). All other tubes besides the “total lysis” control set get 0.1 mL cold absorbed HS (C') 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 All tubes except the “total lysis” controls receive an additional 0.1 mL of Ca buffer. All tubes except for the “total lysis” control should at this point each contain a total 0.3 mL. Tubes are shaken manually to suspend cells, and the rack is placed in a 37°C water bath for 1 h. Each fifteen minutes into the incubation the rack is shaken to keep cells in suspension.

9.7 At the end of 1 h of 37°C incubation, the rack is placed in the ice-slurry. All tubes except the “total lysis” controls receive 0.1 mL BBS-G-EDTA. All tubes at this point should each contain 0.4 mL. The cold tubes are then centrifuged at 1000× g for 10 min at 4°C. Being careful not to disturb the pellets, 0.2 mL of the supernatant from each tube is transferred to a microtiter well plate and absorbance at 405 nm is measured.

9.8 “% Lysis” is calculated for each test and control tube by subtracting from the 405 nm absorbance the “no RBC” control (mean of the three replicate tubes), dividing by “total lysis” control value (mean of the three replicate tubes), and multiplying by 100.