



Designation: F2065 – 00 (Reapproved 2006)

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

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1. Scope

1.1 This practice provides a protocol for rapid, *in vitro* screening for alternative 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* alternative 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 C4-deficient guinea pig serum [C4(-)GPS], using 0.1-mL serum per 13 × 100-mm disposable glass test tubes. Sepharose² CL-4B is used as an example of test materials. Procedure B describes assaying the exposed serum for significant functional alternative pathway complement depletion as compared to control samples. The endpoint in procedure B is lysis of rabbit RBC in buffer containing EGTA and excess Mg⁺⁺.

1.4 This practice does not address function, elaboration, or depletion of individual complement components except as optional additional confirmatory information that can be acquired using human serum as the complement source. 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.

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 Sepharose is a registered trademark of Pharmacia, Inc. (now GE Healthcare), Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, U.K.

2. Referenced Documents

2.1 ASTM Standards:³

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

2.2 Other Document:

ISO 10993-4: Biological Evaluation of Medical Devices. Part 4: Selection of Tests for Interactions with Blood⁴

3. Terminology

3.1 Definitions of Terms Specific to This Standard:

3.1.1 *water*—distilled, endotoxin-free.

3.2 *Abbreviations*: 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-G-EGTA/Mg (Mg Buffer)*—barbital buffered saline – gelatin EGTA Mg⁺⁺

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

3.2.6 *C'*—complement

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

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

3.2.9 *EGTA*—ethylene glyco-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, tetrasodium salt

3.2.10 *HAGG*—heat aggregated gamma globulin

3.2.11 *HS*—human serum

3.2.12 *I*—control tube with serum but no material, kept on ice.

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

3.2.14 *NM*—tube containing serum but no material

³ 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.

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

3.2.15 *PVDF*—polyvinylidene fluoride

3.2.16 *RBC*—red blood cell(s)

4. Summary of Practice

4.1 Solid material specimens are exposed to a standard lot of C4(-)GPS complement under defined conditions, in parallel to appropriate controls (Procedure A). If the alternative complement pathway is activated by the material, complement components will be depleted from the serum. Exposed serum is then tested for remaining functional complement activity, by determining complement mediated lysis of rabbit RBC in buffer containing EGTA and excess Mg^{++} (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. Solid medical device materials may activate the complement directly by the alternative pathway. Unlike the classical complement activation pathway (see Practice F1984), antibodies are not required for alternative pathway activation. This practice is useful as a simple, inexpensive screening method for determining alternative whole complement activation by solid materials *in vitro*.

5.2 This practice is composed of two parts. In Part A (Section 10) C4(-)GPS is exposed to a solid material. Since C4 is required for classical pathway activation, activation of complement in C4(-)GPS can only occur by the alternative pathway (1).⁵ In principle, nonspecific binding of certain complement components to the materials may also occur. In Part B (Section 11), complement activity remaining in the serum after exposure to the test material is assayed by alternative pathway-mediated lysis of rabbit RBC.

5.3 Assessment of *in vitro* whole 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, including assays for specific complement components and their split products in human serum (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 outside the body.

6. Preparation of Buffers

6.1 Buffers are prepared in accordance with established protocols (1, 2). “Water” refers throughout to distilled, endotoxin-free H_2O . The use of barbital (veronal) buffer is recommended. In the United States, 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). These solutions are stable for one month at 4°C unless otherwise indicated.

6.2 The 5X stock BBS (barbital-buffered saline) is prepared by adding 20.75 g of NaCl plus 2.545 g of 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 $MgCl_2 \cdot 6 H_2O$ up to a final volume of 100 mL water), and a 0.3-M solution of $CaCl_2$ (4.41 g $CaCl_2 \cdot 2 H_2O$ up to a final volume of 100 mL of water), and combining the two solutions 1:1 (v:v).

6.4 The Ca buffer (BBS-GM working solution) is prepared daily, by dissolving 0.25 g of gelatin (type A: Porcine Skin, Approx. 300 Bloom, such as available from Sigma [G-1890]) in 50 mL of 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. The Ca buffer contains both Mg^{++} and Ca^{++} , which allows both classical and alternative pathway complement activation to occur.

6.5 The 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 $\cdot 2 H_2O$ to about 160 mL of 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 The 0.1 M EGTA (tetrasodium salt, EGTA $\cdot 4.5 H_2O$) is prepared by adding 4.683 g tetrasodium EGTA to about 80 mL of water, adjusting the pH to 7.35 (with 1 N NaOH or 1 N HCl), then bringing the volume to 100 mL in a volumetric flask.

6.8 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.

6.9 The Mg Buffer (BBS-G-EGTA/Mg 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 0.625 mL 2.0 M $MgCl_2$, plus 4 mL of 0.1 M EGTA, 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. The Mg buffer has EGTA to bind Ca^{++} . The presence of Mg^{++} allows the alternative pathway activation to proceed, while the absence of Ca^{++} prevents activation of the classical pathway.

7. Preparation of Sheep and Rabbit RBC

7.1 Commercially obtained sheep RBC preserved in Alsever's solution and defibrinated rabbit RBC are stored at 4°C. The sheep cells are discarded after eight weeks or when the supernatant from the second wash contains hemoglobin (red color) by visual inspection (as lots of RBCs age, they are more sensitive to complement lysis in parallel with increased spontaneous lysis). The rabbit cells are more fragile than the sheep cells, and should be discarded after four weeks or when the supernatant from the second wash contains hemoglobin by visual inspection.

⁵ The boldface numbers in parentheses refer to the list of references at the end of this specification.

NOTE 1—All centrifugations are at 4°C. Except when indicated, all reagents, tubes, and cell preparations are kept cold in chipped ice or an ice slurry.

7.2 Five millilitres of sheep or rabbit RBC are centrifuged at $1000 \times g$, 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 4°C 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) or BBS-G-EGTA/Mg (Mg buffer) (cells to be used in absorbing serum are washed in Ca buffer; cells to be used for detecting alternative pathway C' depletion, Procedure B, are washed and suspended in Mg buffer). Repeat twice (total of three washes.)

7.5 Adjust cell count spectrophotometrically (where an absorbance of 0.75 for sheep RBC and 1.30 for rabbit RBC corresponds to 2.0×10^8 RBC/mL, at a wavelength of 412 nm and a 1.0-cm light path for 1 volume of cells in BBS-GM or BBS-G-EGTA/Mg plus 24 volumes of water) or count with a hemocytometer. Prepare 10 mL of 2.0×10^8 cells/mL in 4°C 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 Although human serum would be preferable to guinea pig serum for alternative pathway complement activation by materials to be used in medical devices intended to contact patient blood, genetically deficient human sera are not routinely available. Human sera depleted of components by antibody absorption on columns are unsuitable for this purpose for the following two reasons: (1) specific component depletion is incomplete, so significant classical pathway activation remains; and (2) column material may activate the alternative pathway, depleting functional activity. Serum from guinea pigs genetically deficient in C4 [C4(-)GPS] has no classical pathway complement activity but is fully competent for alternative pathway complement activation. Although the titers are different (a greater concentration of C4(-)GPS is required to produce the same lysis of rabbit RBC as human serum)(5)C4(-)GPS gives equivalent results to human serum in detecting biomaterial complement activation (see Sepharose² example in Table 1). Thus, C4(-)GPS is suitable as a screen for subsequent confirmatory immunoassays that detect complement components and split-products indicative of alternative pathway activation in whole human serum. The C4(-)GPS suitable as a source of complement may be purchased from biological supply houses, and is generally labeled as reagent-grade complement.

8.2 Serum may be absorbed with sheep RBC and rabbit RBC in order to remove naturally occurring anti-sheep and anti-rabbit RBC hemolytic antibodies. The procedure is as follows:

8.3 Commercially available C4(-)GPS is stored at -70°C.

8.4 The serum is thawed on chipped ice or reconstituted (if lyophilized) with ice-cold water.

TABLE 1 Percent Lysis^A of Rabbit RBC in Mg Buffer by Human Serum or C4(-)GPS Pre-exposed 1 h at 37 °C, in 100-μL volumes, to Different Amounts of Sepharose² CL-4B

μL Sepharose, ² CL-4B ^B [HS/C4(-)GPS]	HS prep. 1 ^C	HS prep. 2 ^D	C4(-)GPS ^E
50/12.5	3.3 ± 1.2	3.4 ± 1.7	6.5 ± 2.6
25/6.25	14.2 ± 0.6	11.8 ± 0.8	21.3 ± 3.3
12.5/3.13	23.6 ± 5.2	19.6 ± 2.8	20.1 ± 0.9
6.25/1.57	33.2 ± 2.8	29.4 ± 3.8	27.3 ± 1.4
0/0 [37°C control]	49.9 ± 0.2	45.3 ± 4.6	41.3 ± 0.7
0/0 [ice control]	51.4 ± 1.6	52.9 ± 0.7	58.4 ± 0.5

^A Mean plus or minus standard deviation of three replicate tubes.

^B The indicated volume of Sepharose² CL-4B was added to 100 μL of HS or C4(-)GPS.

^C Whole human serum [Quidel (NHSC)], diluted 1:8 in Mg buffer.

^D Reconstituted lyophilized human serum [Sigma (S1764)], diluted 1:4 in Mg buffer.

^E Whole guinea pig serum, C4-deficient [Sigma (C1038)], 1:3 in Mg buffer.

8.5 All manipulations are done in chipped ice or in an ice slurry, with ice cold (4°C) reagents and cells. Centrifugations are carried out at $1000 \times g$ 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 4°C, Ca buffer-washed RBC (a 1:1 mixed volume of sheep and rabbit packed RBC) are gently mixed (by slow rocking), 0.1 mL of packed RBC/2.5 mL of serum, incubated for 10 min on ice, then centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant liquid is carefully transferred to a new container on ice.

8.7 The procedure in 8.6 is repeated twice.

8.8 The absorbed C4(-)GPS 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 in chipped ice or an ice slurry, used on the day of thawing, and not refrozen.

9. Whole Complement Titration to Determine Optimal Serum Dilution

9.1 If statistical evaluation of results is desired, all conditions should be assayed in triplicate, using three 13 × 100 mm round-bottomed, disposable glass test tubes per condition. Otherwise, single or duplicate tubes are sufficient. Tubes are numbered in advance. Conditions include “total lysis,” “no complement (RBC only)” (no C’), “tests” (dilutions of C4(-)GPS) with and without hemolysin, and “no RBC (serum color)” (complement color control, at highest concentration of serum used). All reagents, tubes, and manipulations are done at 4°C, with tubes held in a rack in an ice slurry.

9.2 Two sets of tubes are prepared in accordance with 9.3–9.4. One set contains diluted sera placed on Mg buffer-washed sheep RBC, and one set contains diluted sera placed on Mg buffer-washed rabbit RBC.

9.3 The Mg buffer-washed rabbit RBC are added to all tubes except “no RBC (serum color)” tubes (0.05 mL/tube of a 2.0×10^8 cells/mL suspension.) Since this is a small volume for the tube area, care should be taken to deliver the accurate volume to the center of the bottom of each tube. “No RBC (serum color)” tubes get 0.05 mL 4°C Mg buffer.

9.4 HS and C4(-)GPS at 4°C are diluted in 4°C Mg buffer to the desired concentrations (with mirroring of guinea pig