Designation: F756 – 17

Standard Practice for Assessment of Hemolytic Properties of Materials[1]

This standard is issued under the fixed designation F756; 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 the assessment of hemolytic properties of materials used in the fabrication of medical devices that will contact blood.

1.2 This practice is intended to evaluate the acute *in vitro* hemolytic properties of materials intended for use in contact with blood.

1.3 This practice consists of a protocol for a hemolysis test under static conditions with either an extract of the material or direct contact of the material with blood. It is recommended that both tests (extract and direct contact) be performed unless the material application or contact time justifies the exclusion of one of the tests.

1.4 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 a specific application. Test Method E2524 provides a protocol using reduced test volumes to assess the hemolytic properties of blood-contacting nanoparticulate materials; this may include nanoparticles that become unbound from material surfaces.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

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.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E2524 Test Method for Analysis of Hemolytic Properties of Nanoparticles

F619 Practice for Extraction of Medical Plastics

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

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *plasma hemoglobin*—amount of hemoglobin in the plasma.

3.1.2 *% hemolysis*—free plasma hemoglobin concentration (mg/mL) divided by the total hemoglobin concentration (mg/mL) present multiplied by 100. This is synonymous with hemolytic index.

3.1.3 *comparative hemolysis*—comparison of the hemolytic index produced by a test material with that produced by a standard reference material such as polyethylene under the same test conditions.

3.1.4 *direct contact test*—test for hemolysis performed with the test material in direct contact with the blood.

3.1.5 *extract test*—test for hemolysis performed with an isotonic extract of the test material in contact with blood, as described in Practice F619.

3.1.6 *hemolysis*—destruction of erythrocytes resulting in the liberation of hemoglobin into the plasma or suspension medium.

3.1.7 *negative control*—material, such as polyethylene, that produces little or no hemolysis (<2 % after subtraction of the blank) in the test procedure. It is desirable that the control specimens have the same configuration as the test samples.

3.1.8 *positive control*—materials capable of consistently producing a hemolytic index (above the negative control) of at least 5 % (see 10.3). Although positive control materials have

[1] This practice is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devicesand 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.

not been validated for this practice, washed Buna N rubber (Aero Rubber Company; ARC-45010, 0.031 in. thick sheet) and vinyl plastisol (Plasti-Coat; 0.025 to 0.075 in. thick sheet, color: DB1541-medium blue 300)[3] produced hemolysis levels above 90 % when using extracts obtained at 121°C for 1 h during limited interlaboratory round robin evaluations.[4] In direct contact testing, Buna N rubber (ARC-45010) produced hemolysis levels of 14.5 ± 5.3 %.[4]

NOTE 1—The specific materials tested during the revision of this practice are available from Aero Rubber Company or Plasti-Coat. However, the materials are not certified for this application, their shelf life as positive controls has not been determined, and precision per Practice E691 has not been established. Hence, all available materials may not be suitable as positive control materials for this application. Materials considered for use in this application shall be checked for suitability in accordance with the requirements in this section. If you are aware of positive control materials, please provide this information to ASTM International Headquarters.

3.1.9 *cyanmethemoglobin reagent*—reagent to which is added whole blood, plasma, or test supernatant that quickly converts most of the forms of hemoglobin to the single cyanmethemoglobin form for quantification at its 540 nm spectrophotometric peak. The reagent (based on that by van Kampen and Zijlstra,[5] pH 7.0-7.4), is made with 0.14 g potassium phosphate, 0.05 g potassium cyanide, 0.2 g potassium ferricyanide, and 0.5 to 1 mL of nonionic detergent diluted to 1 L with distilled water. The conversion time of this reagent is 3 to 5 min. This reagent is recommended by the National Commission for Clinical Laboratory Studies (NCCLS) and may be made from the chemicals or purchased from supply houses.

3.1.9.1 *Discussion*—The first cyanmethemoglobin reagent used to measure total blood hemoglobin concentration was Drabkin's reagent (1 g of sodium bicarbonate, 0.05 g of potassium cyanide, 0.2 g of potassium ferricyanide and diluted with distilled water to 1 L). The disadvantages of using the Drabkin's reagent compared to the NCCLS cyanmethemoglobin reagent are that it has a conversion time of 15 min and pH of 8.6, which may cause turbidity. However, Drabkin's reagent is still available from commercial suppliers.

3.1.9.2 *Discussion*—The Drabkin's and cyanmethemoglobin reagents were developed to quantify the high hemoglobin concentration normally found in whole blood (for example, 15 000 mg/dL). By modifying the sample dilution volumes and accounting for background interference, these reagents can also be used to measure much lower plasma or supernatant hemoglobin concentrations as well (Moore et al, Malinauskas).[6,7]

3.1.10 *PBS*—phosphate buffered saline (Ca- and Mg-free). The use of phosphate buffered saline is preferable to the use of saline in order to maintain the pH. The use of magnesium- and calcium-free PBS is necessary to maintain the anticoagulant properties of the chelating agents used in collecting the blood. It is used as the background or "blank" for a hemolysis test.

3.1.11 A^x— absorbance value of cyanmethemoglobin reaction product measured at 540 nm, where "*x*" represents the specimen in 3.1.13 – 3.1.17.

3.1.12 *F*—slope of the hemoglobin standard curve. The units are [(mg/mL)/A] such that multiplication by an absorbance value yields a hemoglobin concentration. Implicit assumption: The *y*-intercept of the hemoglobin calibration curve is approximately zero and its effect on converting absorbance values to concentration values is negligible.

3.1.13 *PFH*—plasma free hemoglobin concentration.

3.1.14 *C*—total blood hemoglobin concentration.

3.1.15 *T*—diluted blood hemoglobin concentration.

3.1.16 *B*—blank (that is, no material added to this tube, only the isotonic medium).

3.1.17 *S*—sample (that is, test material sample, or negative and positive control sample).

4. Summary of Practice

4.1 Test and control material specimens or extracts are exposed to contact with rabbit blood under defined static conditions and the increase in released hemoglobin is measured. Comparisons are made with the control and test specimens tested under identical conditions. It is recommended that both tests (extract and direct contact) be performed unless the material application or contact time justifies the exclusion of one of the tests.

5. Significance and Use

5.1 The presence of hemolytic material in contact with the blood may cause loss of, or damage to, red blood cells and may produce increased levels of free plasma hemoglobin capable of inducing toxic effects or other effects which may stress the kidneys or other organs.

5.2 This practice may not be predictive of events occurring during all types of implant applications. The user is cautioned to consider the appropriateness of the method in view of the materials being tested, their potential applications, and the recommendations contained in Practice F748.

6. Preparation of Test and Control Specimens

6.1 Samples should be prepared in accordance with Practice F619. A minimum total of six positive and six negative controls, along with six test samples, should be prepared to be used in the direct contact test and the test with the extract (three samples per test).

[3] Aero Rubber Company, 8100 W. 185th St., Tinley Park, IL 60487, http://www.aerorubber.com; Plasti-Coat, 137 Brookside Dr., Waterbury, CT 06708, http://www.plasti-coat.com. See Note 1.

[4] Malczewski, R, Jackson, A, Lee, M, Malinauskas, R, Merritt, K, Peterson, L., "Standardizing an in vitro Hemolysis Assay for Screening Materials Used in Medical Devices ," Society for Biomaterials, Tampa, FL, Apr. 2002 (Extended abstract).

[5] International Committee for Standardization in Haematology. *J Clin. Pathol*, Vol 49, 1996, pp. 271–274.

[6] Moore, G. L., Ledford, M. E., Merydith, A., "A micromodification of the Drabkin hemoglobin assay for measuring plasma hemoglobin in the range of 5 to 2000 mg/dl," *Biochem. Med*, Vol 26, 1981 , pp. 167–173.

[7] Malinauskas, R. A., "Plasma hemoglobin measurement techniques for the in vitro evaluation of blood damage caused by medical devices," *Artificial Organs*, Vol 21, 1997, pp. 1255–1267.

6.2 The final sample should be prepared with a surface finish consistent with its end-use application.

6.3 The sample shall be sterilized by the method to be employed for the final product.

6.4 Care should be taken that the specimens do not become contaminated during preparation but aseptic technique is not required.

7. Hemoglobin Determination (Direct Method)

7.1 To create a hemoglobin concentration calibration curve using the cyanmethemoglobin method, use commercially available reference standards and reagents from clinical diagnostic companies that conform to the specifications of the International Committee for Standardization in Hematology (ICSH).[5] One commercial source is made by Pointe Scientific.[8] A spectrophotometer that provides absorbance readings to at least three decimal places, and is able to detect the entire hemoglobin concentration range (as specified in 7.2) should be used.

7.2 Prepare a standard curve from a suitable standard in six dilutions to accommodate the range of 0.03 to 0.7 mg/mL. It is acceptable to expand the range to 0.02 to 0.8 mg/mL. The cyanmethemoglobin reagent diluent serves as a zero blank in the spectrophotometer. Measure the absorbance at 540 nm. Plot a calibration curve from these values using hemoglobin concentration (mg/mL) on the *y*-axis and A_{540} on the *x*-axis. The calibration coefficient (F) is the slope of this plot. The *y*-intercept should be approximately zero.

NOTE 2—If local restrictions or other problems contraindicate use of these cyanmethemoglobin reagents, then another method for measuring total blood hemoglobin concentration, plasma free hemoglobin concentration, and supernatant hemoglobin concentration may be substituted provided that it is validated and shown to be substantially equivalent to the cyanmethemoglobin method. Methods which quantify oxyhemoglobin alone may not be appropriate since some materials can convert oxyhemoglobin to other forms or alter the absorbance spectrum. Investigators should be aware that their results of determining supernatant hemoglobin concentration may be compromised by absorption of hemoglobin by the test materials, precipitation of hemoglobin out of solution, or alteration of the spectrophotometric absorbance spectrum by material leachables.

8. Collection and Preparation of Blood Substrates

8.1 Obtain anti-coagulated rabbit blood from at least three donors for each test day. The preferred anticoagulant is citrate (0.13 M). Approximately 5 mL should be drawn from each rabbit. Store the blood at 4 ± 2°C and preferably use within 48 h. Blood may be used up to 96 h after collection if the plasma free hemoglobin is not excessive. Equal quantities of blood from each rabbit should be pooled.

8.2 Do not wash cells; use them suspended in the original plasma.

8.3 *Determination of Plasma Free Hemoglobin (PFH):*

8.3.1 Centrifuge a 3.0-mL sample of the pooled blood at 700 to 800 G in a standard clinical centrifuge for 15 min.

8.3.2 Perform a 1:1 dilution of the plasma with the cyanmethemoglobin reagent or validated diluent (for example, add 0.5 mL of plasma to 0.5 mL of cyanmethemoglobin reagent).

8.3.3 Read the absorbance of the resulting solution at 540 nm after 15 min. Obtain the concentration from the standard curve. Multiply by 2 to obtain, and record, the total plasma free hemoglobin concentration (PFH), although it has not been corrected for the plasma background interference. Plasma free hemoglobin (mg/mL) is calculated as follows:

$$PFH = A^{PFH} \times F \times 2 \quad (1)$$

8.3.4 Proceed with the testing if the value of the PFH is less than 2 mg/mL. If the PFH is 2mg/mL or greater, this sample should be discarded and another blood sample should be obtained.

8.4 *Determination of Total Blood Hemoglobin Concentration*—Note that the total blood hemoglobin concentration can be determined either by the cyanmethemoglobin method (detailed below) or by using a validated hemoglobinometer to replace steps 8.4.1 – 8.4.3. However, after dilution in step 8.4.4, the total blood hemoglobin concentration may be outside the valid range of a clinical hemoglobinometer.

8.4.1 Add 20 µL of well-mixed pooled whole blood specimen to 5.0 mL of cyanmethemoglobin solution or validated diluent.

8.4.2 Allow the resulting solution to stand 15 min for Drabkin's or 5 min for cyanmethemoglobin reagent and then read the absorbance of the solution with a spectrophotometer at a wavelength of 540 nm.

8.4.3 Determine blood hemoglobin concentration from the standard curve and multiply by 251 to account for dilution. This should be performed in duplicate. Total blood hemoglobin concentration is calculated as follows, where A^c is the absorbance value and F is the slope of the hemoglobin standard curve:

$$C = A^C \times F \times 251 \quad (2)$$

8.4.4 Adjust the total hemoglobin content of the blood sample to 10 ± 1 mg/mL by diluting with an appropriate amount of calcium- and magnesium-free PBS. Verify the hemoglobin concentration by repeating 8.4.1 – 8.4.3 in triplicate but using 300 µl of the diluted blood to 4.5 mL of reagent to remain on the standard curve. This is a dilution factor of 16.

$$T = A^T \times F \times 16 \quad (3)$$

where:

A^T = absorbance value of the diluted blood.

9. Procedure for the Test

9.1 *Extract:*

9.1.1 Prepare an extract of each of three replicate samples of each test, positive control, negative control material, and PBS blank according to Practice F619 using the appropriate ratio of material to extractant. (The extractant is Mg- and Ca-free PBS). Samples shall be prepared in accordance with Practice F619.

9.1.2 Use the highest temperature conditions of Practice F619 that the material will withstand.

[8] Hemoglobin Standard, Pointe Scientific, 5449 Research Drive, Canton, MI, 48188.

NOTE 3—If the extraction is done at 121°C, borosilicate tubes must be used and any volume lost should be noted. At lower temperatures, either polystyrene or glass tubes may be used.

9.1.3 Transfer 7.0 mL of the resultant extract of each sample into individual screw capped test tubes of borosilicate glass or polystyrene (or equivalent) approximately 16 × 125 or 16 × 150 mm.

9.1.4 After zeroing the spectrophotometer, the absorbance of the extracts should be checked for background interference, which could affect the supernatant hemoglobin concentration calculation. Any background absorbance should be recorded and used to correct the absorbance of the test article as determined in 9.8.

9.2 *Direct Contact:*

9.2.1 It is important to note that the direct contact procedure calls for test article preparation consistent with Practice F619, but for a fluid volume of 7.0 mL; therefore, prepare three replicate samples of each test, positive control, and negative control material according to Practice F619 using the following table as guidance:

Test Article Thickness	Surface Area to Volume Ratio (Practice F619)	Surface Area per 7.0 mL PBS (for Practice F756 test)
≤0.50 mm	120 cm^2 : 20.0 mL	42 cm^2 : 7.0 mL
>0.50 mm	60 cm^2 : 20.0 mL	21 cm^2 : 7.0 mL
>1.0 mm or intricate geometry	4.0 g : 20.0 mL	1.4 g : 7.0 mL

9.2.2 Samples are cut into appropriate pieces. Transfer each of three nonextracted samples of test and control specimens into individual tubes as described in 9.1.3. The recommended tube size is 16 × 125 mm. However the tube size may be any size as long as the specimen is covered by 7.0 mL of PBS liquid. Place 7.0 mL of PBS into each tube containing the nonextracted sample. Place 7.0 mL of PBS into each of three tubes to serve as the blank.

9.3 *Test*—Add 1.0 mL of blood prepared according to 8.4.4 to each tube containing extract, each tube containing a specimen, and the blanks. Cap all tubes.

NOTE 4—This procedure calls for preparing the sample, adding the diluent to the sample and then adding the blood, which minimizes the time difference for contact of the sample with blood. Alternatively, the blood may be added to the diluent and then the sample added to the prepared solution. Whichever method is chosen must be used for the controls as well as the test specimens.

9.4 Maintain tubes in a suitable test tube rack for at least 3 h at 37 ± 2°C in a water bath. Gently invert each tube twice approximately every 30 min to maintain contact of the blood and material. In some cases of samples with complicated configurations, it may be necessary to do more inversions to adequately mix the sample.

9.5 At the end of the specified incubation time, transfer the fluid to a suitable tube and centrifuge at 700 to 800 G for 15 min in a standard clinical centrifuge.

9.6 Remove the supernatant carefully to avoid disturbing any button of erythrocytes which may be present. Place the supernatant into a second screw cap tube. Record the presence of any color in the supernatant and any precipitate.

9.7 Analyze the samples from 9.6 for supernatant hemoglobin concentration using the method in 9.8.

9.8 *Supernatant Hemoglobin Determination:*

9.8.1 Add 1.0 mL of supernatant to 1.0 mL of cyanmethemoglobin reagent, or validated diluent.

9.8.2 Allow the sample to stand for 15 to 30 min[9] for Drabkin's or 3 to 5 min for cyanmethemoglobin reagent. Read the absorbance of the solution with a spectrophotometer at a wavelength of 540 nm.

9.8.3 In the unlikely event that A_{540} exceeds 2, this may signify a procedural or background problem; the problem should be identified and addressed, and the testing repeated.

9.8.3.1 Determine the hemoglobin concentration in each supernatant using the calibration curve.

9.8.3.2 The hemoglobin concentration of supernatant from the test sample or control tubes is calculated as follows (using the absorbance value obtained in 9.8.2 and correcting for the dilution factor of 2):

$$S = A^S \times F \times 2 \tag{4}$$

The hemoglobin concentration of the blank tube is calculated as follows:

$$B = A^B \times F \times 2 \tag{5}$$

9.8.3.3 Calculate the % hemolysis (hemolytic index) as:

$$\%\ hemolysis = \frac{supernatant\ hemoglobin\ concentration \times 100\,\%}{total\ hemoglobin\ concentration\ in\ tube} \tag{6}$$

In Eq 6, the "total hemoglobin concentration in tube" is calculated by dividing the total blood hemoglobin concentration obtained in 8.4.4 by 8 to account for the blood dilution by PBS in the test tubes. Use of this equation assumes that background interference from endogenous plasma and free hemoglobin, and from the extracts, is negligible. This assumption can be verified by measuring the supernatant absorbance of the extract solutions and of blood diluted in a test tube containing 7 mL of PBS and 1 mL of diluted blood (10 mg/mL) that has been incubated along with the test sample tubes.

9.8.3.4 The percent hemolysis is calculated by correcting for the background from the blank sample:

$$Blank\ corrected\ \%\ hemolysis = \frac{S - B}{(T/8) - B} \times 100\,\% \tag{7}$$

By following the dilution factors set out in subsections 8.4.4 and 9.8.1, Eq 7 can be simplified as follows:

$$Blank\ corrected\ \%\ hemolysis = \frac{A^S - A^B}{A^T - A^B} \times 100\,\% \tag{8}$$

It should be noted that Eq 8 is only applicable if the dilutions as set out in subsections 8.4.4 and 9.8.1 are strictly followed; otherwise, corrective dilution factors need to be introduced into Eq 7.

10. Report

10.1 Express results in the form of the corrected % hemolysis index as described in 9.8.3.4.

[9] van Kampen E. J., Zijlstra W. G., *Adv Clin Chem*, 1983;23:199-257. PMID: 6398614, p. 211.

10.2 The final report, as a minimum shall include the following:

10.2.1 Detailed sample and control preparations including generic or chemical names, catalog number, lot or batch number, and other pertinent available designations or descriptions.

10.2.2 Detailed sample and control preparations, including sample size, thickness, configuration of test specimens, and method of sterilization.

10.2.3 Age of blood and type and concentration of anticoagulant used.

10.2.4 Method of hemoglobin determination.

10.2.5 Tabulation of total supernatant hemoglobin levels.

10.2.6 Percent hemolysis for the test samples, the negative controls, the positive controls, and the blanks. Include mean and standard deviation for each of the replicate samples, blanks, and positive and negative controls.

10.2.7 Other pertinent observations of the experiment.

10.3 *Conversion of % Hemolysis for reporting purposes*—This practice provides a method for determining the propensity of a material to cause hemolysis. Pass/fail criteria for the material are subject to consideration of the nature of the tissue contact, duration of contact, and surface area-to-body ratios, and the nature of the device. Historically a hemolytic grade had been assigned. However, the hemolytic grade is an arbitrarily derived scale, has not been validated, and is based on previous results using a slightly different procedure. If the assignment of a hemolytic grade is required, the mean hemolytic index of the blank should be subtracted from the mean hemolytic index of the controls and the test samples. The results of the test sample should be compared to the results of the negative control, using the following table as a guide:

Hemolytic Index above the negative control	Hemolytic Grade
0–2	nonhemolytic
2–5	slightly hemolytic
>5	hemolytic

In addition, if the mean hemolytic index from the replicate test samples is less than 5 but one or more samples gave a hemolytic index of greater than 5, then the test should be repeated with double the number of test articles.

11. Precision and Bias

11.1 *Precision*—The precision of this test method is being established. Although this method has been shown to have intralaboratory repeatability, especially with regards to classification of hemolytic response, interlaboratory variation is still significant.

11.2 *Bias*—The bias of this test method includes the quantitative estimates of the uncertainties of the calibration of the test equipment and the skill of the operators. At this time, statements of bias should be limited to the documented performance of particular laboratories.

12. Keywords

12.1 biocompatibility; blood compatibility; direct contact; extract; hemoglobin; hemolysis testing

APPENDIX

(Nonmandatory Information)

X1. RATIONALE

X1.1 The presence of hemolytic material in contact with blood may increase blood cell lysis and produce increased levels of plasma hemoglobin. This may induce toxic effects or other effects which may stress the kidneys or other organs.

X1.2 This practice is presented as a screening procedure for comparing the hemolytic potential of a material with that of a negative control material which is generally acknowledged to be appropriate for blood contact applications. Materials with a hemolytic potential above that of the specified negative control material, which is known to have excellent performance in blood-contacting situations, should be carefully considered for use since they may or may not be a potential cause of *in vivo* hemolysis.

X1.3 The procedure as presented is intended as a routine reproducible screening procedure. It is not to be represented as being the most sensitive nor the most specific procedure for assessing the hemolytic potential of all materials in all use applications. The results obtained with this procedure are intended to be used in conjunction with the results of other tests in assessing the blood compatibility of the test material.