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Designation: ~~F756 – 13~~ 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.*

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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:*~~ *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.

[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.

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

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 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~~ 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] 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.

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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 ~~subsections~~ 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.

[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.

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).

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.

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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 ~~which~~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~~ 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] Hemoglobin Standard, Pointe Scientific, 5449 Research Drive, Canton, MI, 48188.