



Designation: F 756 – 00

Standard Practice for Assessment of Hemolytic Properties of Materials¹

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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.

1.4 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 a specific application.

1.5 *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.*

1.6 *Identification of a supplier of materials or reagents is for the convenience of the user and does not imply single source. Appropriate materials and reagents may be obtained from many commercial supply houses.*

2. Referenced Documents

2.1 ASTM Standards:

F 619 Practice for Extraction of Medical Plastics²

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices²

3. Terminology

3.1 Definitions:

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

3.1.2 *% hemolysis*—the quotient of the free plasma hemoglobin (mg/ml) released as a result of contact with test material or extract divided by the total hemoglobin (mg/ml) present in the blood solution multiplied by 100. (This is synonymous with hemolytic index).

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

3.1.4 *direct contact test*—a test for the hemolytic property performed with the test material in direct contact with the blood.

3.1.5 *extract test*—a test for the hemolytic property performed with an isotonic extract of the test material, as described in F 619, 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*—a material, such as a 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*—a material capable of consistently producing a hemolysis of at least 8 % after subtraction of the results of the blank. Suggested materials include, but are not limited to, BUNA N (Aero Rubber and other suppliers), and plastisol (Plasti-Coat, Watertown CT).

3.1.9 *cyanmethemoglobin reagent*—a reagent to which whole blood, plasma, or test supernatant is added 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 (1), pH 7.0-7.4), is made with 0.14 g potassium phosphate, 0.05 g potassium cyanide, 0.2 g potassium ferricyanide, and 0.5-1 ml of nonionic detergent diluted to 1 L with distilled water. The conversion time of this reagent is 3-5 minutes. 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.

¹ This practice is under the jurisdiction of ASTM Committee F-4 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.16 on Biocompatibility.

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² *Annual Book of ASTM Standards*, Vol 13.01.

The first cyanmethemoglobin reagent used to measure total blood hemoglobin concentration was Drabkin's Reagent (1 g of sodium bicarbonate, 0.05g 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 versus the NCCLS cyanmethemoglobin reagent are that it has a conversion time of 15 minutes, and pH of 8.6 which may cause turbidity. However, it is still available as individual chemicals or kits such as Sigma 525-A.

The Drabkin's and cyanmethemoglobin reagents were developed to quantify the high hemoglobin concentration normally found in whole blood (e.g. 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 (2), (3)).

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*—absorbance value of cyanmethemoglobin reaction product measured at 540 nm.

3.1.12 *F*—slope of the hemoglobin 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 (i.e. no material added to this tube, only the isotonic medium).

3.1.17 *S*—sample (i.e. 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.

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 F 748.

6. Preparation of Test and Control Specimens

6.1 Specimens with a determinable surface area will be used at a ratio of 3 cm² surface area to 1 ml of test blood solution. For irregular materials or powders, a weight of 0.2 to 0.5 g per ml of liquid will be used. A minimum of 6 of each positive and negative control and each test sample should be prepared to be used in the direct contact test and the test with the extract.

6.2 The final sample should be prepared with a surface finish consistent with 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 Prepare a hemoglobin calibration curve consistent with the specifications of the International Committee for Standardization in Hematology (ICSH). For hemoglobin standardization use reference standards such as the Sigma Hemoglobin Reference Standard No 525 or equivalent and the suggested reagent diluent. The spectrophotometer used should be high resolution and provide at least 3 decimal places.

7.2 Prepare a standard curve from stock hemoglobin in 8 dilutions to accommodate the range of 1.4 to 0.03 mg/ml (140-3 mg/dl). 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 mg/ml Hb on the y axis and A₅₄₀ on the x-axis. The calibration coefficient (F) is the slope of this plot. The y-intercept should be approximately zero.

NOTE 1—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 3 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-ml sample of the pooled blood at 700-800 G in a standard clinical centrifuge for 15 min.

8.3.2 Add 0.5 ml of plasma to 0.5 ml of cyanmethemoglobin solution or validated diluent.