Designation: F 756 – 00

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

This standard is issued under the fixed designation F 756; 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.

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 Plastics[2]

F 748 Practice for Selecting Generic Biological Test Methods for Materials and Devices[2]

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.

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

[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.

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 supenatant 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 cm^2 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 A_{540} 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.

8.3.3 Read the absorbance of the resulting solution at 540 nm. 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):

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

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*:

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 minutes for Drabkins 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:

$$C = A^{C}*F*251 \quad (2)$$

8.4.4 Adjust the total hemoglobin content of the blood sample to 10 mg/ml ± l 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 400 µl of the diluted blood to 5 ml of reagent to remain on the standard curve. This is a dilution factor of 13.5.

$$T = A^{T}*F*13.5 \quad (3)$$

9. Procedure for the Test

9.1 *Extract*:

9.1.1 Prepare an extract of each of 3 replicate samples of each test, positive control, negative control material, and PBS blank according to Practice F 619 using the appropriate ratio of material to extractant. (The extractant is Mg Ca free PBS). A sample of 30 cm^2 surface area or 2-5 gram is recommended for use in 10 ml of extractant.

9.1.2 Use the highest temperature conditions of Practice F 619 that the material will withstand.

NOTE 2—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 ml of the resultant extract of each sample into individual screw capped test tubes of borosilicate glass or polystyrene approximately 16 x 125 or 16 x 150 mm.

9.1.4 Using an appropriate blank to zero the spectrophotometer, the absorbance of the extracts should be checked for background interference which could affect the supernatant hemoglobin concentration calculation.

9.2 *Direct Contact*:

9.2.1 Samples are cut into appropriate pieces. Transfer each of 3 nonextracted samples of test and control specimens into individual tubes as described in 9.1.3. For samples with a determinable surface area, the surface area should be 21 cm^2 or 1.4 to 3.5 g if the specimen is irregular or a powder. The recommended tube size is 16 x 125 mm. However the tube size may be any such that the specimen is covered by 7 ml of PBS liquid. Place 7 ml of PBS into each tube containing the nonextracted sample. Place 7 ml of PBS into each of 3 tubes to serve as the blank.

9.3 *Test*—Add 1 ml of blood prepared in 8.4.4 to each tube containing extract, each tube containing a specimen, and the blanks. Cap all tubes.

NOTE 3—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 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 700-800 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 to 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 min for Drabkin's or 3-5 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, dilute the 1 ml of the supernatant with 3 ml of diluent and repeat 9.8.

9.8.3.1 Determine the hemoglobin concentration in each supernatant from 9.8 using the calibration curve.

9.8.3.2 Multiply the results from 9.8.2 by 2 (or the results from 9.8.3 by 4) to correct for the dilution. Hemoglobin concentration of supernatant from test sample or control tubes:

$$S = A^{S}*F*2 \quad (4)$$

Hemoglobin concentration of the blank tube:

$$B = A^{B}*F*2 \quad (5)$$

9.8.3.3 Calculate the % hemolysis or hemolytic index as:

$$\%\ hemolysis = \frac{Concentration\ of\ hemoglobin\ released\ in\ supernatant*100\%}{Total\ hemoglobin\ concentration\ in\ Tube} \quad (6)$$

(To account for the dilution factor introduced by putting 1 ml of diluted blood into each tube that contains 7 ml of isotonic solution, the total hemoglobin concentration in each tube (W) is defined as W = T/8). Then,

$$\%\ hemolysis = \frac{S}{W}*100\% \quad (7)$$

In the above equation, the "total Hemoglobin in Tube" is calculated by dividing the total blood hemoglobin concentration obtained in 8.4.4 by 8 to account for the blood dilution in 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) which has been incubated along with the test sample tubes.

9.8.3.4 % hemolysis corrected for the blank:

$$\textit{Blank corrected \% hemolysis} = \frac{(A^S * F * 2) - (A^B * F * 2)}{(A^T * F * 13.5/8) - (A^B * F * 2)} * 100\% \quad (8)$$

The final equation can then be simplified:

$$\textit{Blank corrected \% hemolysis} = \frac{A^S - A^B}{(0.844)A^T - A^B} * 100\% \quad (9)$$

10. Report

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

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 % 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 following are suggested: 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.

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

In addition, if the mean 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 produce increased levels of blood cell lysis and 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.

REFERENCES

(**1**) International Committee for Standardization in Haematology. J Clin. Pathol, 1978, 31: 139-143, 1978.

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(**3**) Malinauskas, RA., Plasma hemoglobin measurement techniques for the in vitro evaluation of blood damage caused by medical devices. Artificial Organs 1997; 21:1255-1267.