American National Standard

ANSI/AAMI/ISO 10993-4:2002

Biological evaluation of medical devices— Part 4: Selection of tests for interaction with blood

AAMI Association for the Advancement of Medical Instrumentation

The Objectives and Uses of AAMI Standards and Recommended Practices

It is most important that the objectives and potential uses of an AAMI product standard or recommended practice are clearly understood. The objectives of AAMI's technical development program derive from AAMI's overall mission: the advancement of medical instrumentation. Essential to such advancement are (1) a continued increase in the safe and effective application of current technologies to patient care, and (2) the encouragement of new technologies. It is AAMI's view that standards and recommended practices can contribute significantly to the advancement of medical instrumentation, provided that they are drafted with attention to these objectives and provided that arbitrary and restrictive uses are avoided.

A voluntary *standard* for a *medical device* recommends to the manufacturer the information that should be provided with or on the product, basic safety and performance criteria that should be considered in qualifying the device for clinical use, and the measurement techniques that can be used to determine whether the device conforms with the safety and performance criteria and/or to compare the performance characteristics of different products. Some standards emphasize the information that should be provided with the device, including performance characteristics, instructions for use, warnings and precautions, and other data considered important in ensuring the safe and effective use of the device in the clinical environment. Recommending the disclosure of performance characteristics often necessitates the development of specialized test methods to facilitate uniformity in reporting; reaching consensus on these tests can represent a considerable part of committee work. When a drafting committee determines that clinical concerns warrant the establishment of *minimum* safety and performance criteria, referee tests must be provided and the reasons for establishing the criteria must be documented in the rationale.

A *recommended practice* provides guidelines for the use, care, and/or processing of a medical device or system. A recommended practice does not address device performance *per se*, but rather procedures and practices that will help ensure that a device is used safely and effectively and that its performance will be maintained.

Although a device standard is primarily directed to the manufacturer, it may also be of value to the potential purchaser or user of the device as a fume of reference for device evaluation. Similarly, even though a recommended practice is usually oriented towards health care professionals, it may be useful to the manufacturer in better understanding the environment in which a medical device will be used. Also, some recommended practices, while not addressing device performance criteria, provide guidelines to industrial personnel on such subjects as sterilization processing, methods of collecting data to establish safety and efficacy, human engineering, and other processing or evaluation techniques; such guidelines may be useful to health care professionals in understanding industrial practices.

In determining whether an AAMI standard or recommended practice is relevant to the specific needs of a potential user of the document, several important concepts must be recognized:

All AAMI standards and recommended practices are *voluntary* (unless, of course, they are adopted by government regulatory or procurement authorities). The application of a standard or recommended practice is solely within the discretion and professional judgment of the user of the document.

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Despite periodic review and revision (at least once every five years), a standard or recommended practice is necessarily a static document applied to a dynamic technology. Therefore, a standards user must carefully review the reasons why the document was initially developed and the specific rationale for each of its provisions. This review will reveal whether the document remains relevant to the specific needs of the user.

Particular care should be taken in applying a product standard to existing devices and equipment, and in applying a recommended practice to current procedures and practices. While observed or potential risks with existing equipment typically form the basis for the safety and performance criteria defined in a standard, professional judgment must be used in applying these criteria to existing equipment. No single source of information will serve to identify a particular product as "unsafe". A voluntary standard can be used as one resource, but the ultimate decision as to product safety and efficacy must take into account the specifics of its utilization and, of course, cost-benefit considerations. Similarly, a recommended practice should be analyzed in the context of the specific needs and resources of the individual institution or firm. Again, the rationale accompanying each AAMI standard and recommended practice is an excellent guide to the reasoning and data underlying its provision.

In summary, a standard or recommended practice is truly useful only when it is used in conjunction with other sources of information and policy guidance and in the context of professional experience and judgment.

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American National Standard

ANSI/AAMI/ISO 10993-4:2002
(Revision of ANSI/AAMI/ISO 10993-4:1993)

Biological evaluation of medical devices—
Part 4: Selection of tests for interactions with blood

Approved 1 October 2002 by
Association for the Advancement of Medical Instrumentation

Approved 31 October 2002 by
American National Standards Institute, Inc.

Abstract: This standard gives guidance to agencies, manufacturers, research laboratories, and others for evaluating the interactions of medical devices with blood.

Keywords: biological evaluation, blood, coagulation, *ex vivo*, hematology, medical devices, thrombosis

AAMI Standard

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Contents

Glossary of equivalent standards

International Standards adopted in the United States may include normative references to other International Standards. For each International Standard that has been adopted by AAMI (and ANSI), the table below gives the corresponding U.S. designation and level of equivalency to the International Standard.

NOTE—Documents are sorted by international designation.

Other normatively referenced International Standards may be under consideration for U.S. adoption by AAMI; therefore, this list should not be considered exhaustive.

International designation	U.S. designation	Equivalency
IEC 60601-1-2:2001	ANSI/AAMI/IEC 60601-1-2:2001	Identical
IEC 60601-2-21:1994 and Amendment 1:1996	ANSI/AAMI/IEC 60601-2-21 & Amendment 1:2000 (consolidated texts)	Identical
IEC 60601-2-24:1998	ANSI/AAMI ID26:1998	Major technical variations
ISO 5840:1996	ANSI/AAMI/ISO 5840:1996	Identical
ISO 7198:1998	ANSI/AAMI/ISO 7198:1998/2001	Identical
ISO 7199:1996	ANSI/AAMI/ISO 7199:1996/(R)2002	Identical
ISO 10993-1:1997	ANSI/AAMI/ISO 10993-1:1997	Identical
ISO 10993-2:1992	ANSI/AAMI/ISO 10993-2:1993/(R)2001	Identical
ISO 10993-3:1992	ANSI/AAMI/ISO 10993-3:1993	Identical
ISO 10993-4:2002	ANSI/AAMI/ISO 10993-4:2002	Identical
ISO 10993-5:1999	ANSI/AAMI/ISO 10993-5:1999	Identical
ISO 10993-6:1994	ANSI/AAMI/ISO 10993-6:1995/(R)2001	Identical
ISO 10993-7:1995	ANSI/AAMI/ISO 10993-7:1995/(R)2001	Identical
ISO 10993-8:2000	ANSI/AAMI/ISO 10993-8:2000	Identical
ISO 10993-9:1999	ANSI/AAMI/ISO 10993-9:1999	Identical
ISO 10993-10:2002	ANSI/AAMI BE78:2002	Minor technical variations
ISO 10993-11:1993	ANSI/AAMI 10993-11:1993	Minor technical variations
ISO 10993-12:1996	ANSI/AAMI/ISO/CEN 10993-12:1996	Identical
ISO 10993-13:1998	ANSI/AAMI/ISO 10993-13:1999	Identical
ISO 10993-14:2001	ANSI/AAMI/ISO 10993-14:2001	Identical
ISO 10993-15:2000	ANSI/AAMI/ISO 10993-15:2000	Identical
ISO 10993-16:1997	ANSI/AAMI/ISO 10993-16:1997	Identical
ISO 10993-17:2002	ANSI/AAMI/ISO 10993-17:2002	Identical
ISO 11134:1994	ANSI/AAMI/ISO 11134:1993	Identical
ISO 11135:1994	ANSI/AAMI/ISO 11135:1994	Identical
ISO 11137:1995 and Amdt 1:2001	ANSI/AAMI/ISO 11137:1994 and A1:2002	Identical

International designation	U.S. designation	Equivalency
ISO 11138-1:1994	ANSI/AAMI ST59:1999	Major technical variations
ISO 11138-2:1994	ANSI/AAMI ST21:1999	Major technical variations
ISO 11138-3:1995	ANSI/AAMI ST19:1999	Major technical variations
ISO TS 11139:2001	ANSI/AAMI/ISO 11139:2002	Identical
ISO 11140-1:1995 and Technical Corrigendum 1:1998	ANSI/AAMI ST60:1996	Major technical variations
ISO 11607:2002	ANSI/AAMI/ISO 11607:2000	Identical
ISO 11737-1:1995	ANSI/AAMI/ISO 11737-1:1995	Identical
ISO 11737-2:1998	ANSI/AAMI/ISO 11737-2:1998	Identical
ISO TR 13409:1996	AAMI/ISO TIR 13409:1996	Identical
ISO 13485:1996	ANSI/AAMI/ISO 13485:1996	Identical
ISO 13488:1996	ANSI/AAMI/ISO 13488:1996	Identical
ISO 14155:1996	ANSI/AAMI/ISO 14155:1996	Identical
ISO 14160:1998	ANSI/AAMI/ISO 14160:1998	Identical
ISO 14161: 2000	ANSI/AAMI/ISO 14161:2000	Identical
ISO 14937:2000	ANSI/AAMI/ISO 14937:2000	Identical
ISO 14969:1999	ANSI/AAMI/ISO 14969:1999	Identical
ISO 14971:2000	ANSI/AAMI/ISO 14971:2000	Identical
ISO 15223:2000	ANSI/AAMI/ISO 15223:2000	Identical
ISO 15223/A1:2002	ANSI/AAMI/ISO 15223:2000/A1:2001	Identical
ISO 15225:2000	ANSI/AAMI/ISO 15225:2000	Identical
ISO 15674:2001	ANSI/AAMI/ISO 15674:2001	Identical
ISO 15675:2001	ANSI/AAMI/ISO 15675:2001	Identical
ISO TS 15843:2000	ANSI/AAMI/ISO TIR15843:2000	Identical
ISO TR 15844:1998	AAMI/ISO TIR15844:1998	Identical
ISO TR 16142:1999	ANSI/AAMI/ISO TIR16142:2000	Identical

Committee representation

Association for the Advancement of Medical Instrumentation

Biological Evaluation Committee

The adoption of ISO 10993-4:2002 as an American National Standard was initiated by the AAMI Biological Evaluation Committee, which also functions as a U.S. Technical Advisory Group to the relevant work in the International Organization for Standardization (ISO). U.S. representatives from the AAMI Effects on Blood Working Group (U.S. Sub-TAG for ISO/TC 194/WG 9), chaired by Raju G. Kammula of the U.S. Food and Drug Administration, played an active part in developing the ISO standard.

At the time this document was published, the **AAMI Biological Evaluation Committee** had the following members:

Cochairs:
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Donald E. Marlowe

Members:
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Sumner A. Barenberg, PhD, Bernard Technologies
Eric R. Claussen, PhD, Becton Dickinson
Roger Dabbah, PhD, U.S. Pharmacopeial Convention, Inc.
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At the time this document was published, the **AAMI Effects on Blood Working Group** had the following members:

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NOTE—Participation by federal agency representatives in the development of this standard does not constitute endorsement by the federal government or any of its agencies.

Background of ANSI/AAMI adoption of ISO 10993-4:2002

As indicated in the foreword to the main body of this document (page viii), the International Organization for Standardization (ISO) is a worldwide federation of national standards bodies. The United States is one of the ISO members that took an active role in the development of this standard.

International Standard ISO 10993-4 was developed by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*, to provide general requirements for evaluating the interactions of medical devices with blood.

U.S. participation in this ISO TC is organized through the U.S. Technical Advisory Group for ISO/TC 194, administered by the Association for the Advancement of Medical Instrumentation on behalf of the American National Standards Institute. The U.S. made a considerable contribution to this International Standard.

AAMI encourages its committees to harmonize their work with International Standards in the area of biological evaluation of medical devices as much as possible in order to help reduce unnecessary repetition of testing.

Upon review of ISO 10993-4, the AAMI Biological Evaluation Committee and the AAMI Effects on Blood Working Group decided to adopt ISO 10993-4:2002 verbatim.

Several significant changes to the previous edition were made. One of the major changes was the addition of a new flowchart (Figure 1) to determine if the testing of a device for interaction with blood is necessary. In addition, Tables 1 and 2 were revised to aid in the selection of appropriate testing for various device categories. An informative annex (Annex C) was added on the evaluation hemolytic properties of medical devices. Finally, the bibliography was revised to include current publications.

AAMI (and ANSI) have adopted other ISO standards. See the glossary of equivalent standards for a list of ISO standards adopted by AAMI, which gives the corresponding U.S. designation and the level of equivalency with the ISO standard.

The concepts incorporated in this standard should not be considered inflexible or static. This standard, like any other, must be reviewed and updated periodically to assimilate progressive technological developments. To remain relevant, it must be modified as technological advances are made and as new data comes to light.

Suggestions for improving this standard are invited. Comments and suggested revisions should be sent to Standards Department, AAMI, 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-4795.

NOTE—Beginning with the ISO foreword on page viii, this American National Standard is identical to ISO 10993-4:2002.

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this part of ISO 10993 may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 10993-4 was prepared by Technical Committee ISO/TC 194, *Biological evaluation of medical devices*.

This second edition cancels and replaces the first edition (ISO 10993-4:1992), which has been technically revised.

ISO 10993 consists of the following parts, under the general title *Biological evaluation of medical devices*:

— *Part 1: Evaluation and testing*

— *Part 2: Animal welfare requirements*

— *Part 3: Tests for genotoxicity, carcinogenicity, and reproductive toxicity*

— *Part 4: Selection of tests for interactions with blood*

— *Part 5: Tests for in-vitro cytotoxicity*

— *Part 6: Tests for local effects after implantation*

— *Part 7: Ethylene oxide sterilization residuals*

— *Part 8: Selection and qualification of reference materials for biological tests*

— *Part 9: Framework for identification and quantification of potential degradation products*

— *Part 10: Tests for irritation and sensitization*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

— *Part 13: Identification and quantification of degradation products from polymeric medical devices*

— *Part 14: Identification and quantification of degradation products from ceramics*

— *Part 15: Identification and quantification of degradation products from metals and alloys*

— *Part 16: Toxicokinetic study design for degradation products and leachables*

— *Part 17: Establishment of allowable limits for leachable substances*

— *Part 18: Chemical characterization of materials*

Future parts will deal with other relevant aspects of biological testing.

Annexes A, B, and C of this part of ISO 10993 are for information only.

Introduction

The selection and design of test methods for the interactions of medical devices with blood should take into consideration device design, materials, clinical utility, usage environment, and risk benefit. This level of specificity can only be covered in vertical standards.

The initial source for developing this part of ISO 10993 was the publication, *Guidelines for blood/material interactions*, Report of the National Heart, Lung, and Blood Institute [29]; chapters 9 and 10. This publication has since been revised [32].

Biological evaluation of medical devices— Part 4: Selection of tests for interactions with blood

1 Scope

This part of ISO 10993 provides general requirements for evaluating the interactions of medical devices with blood.

It describes

a) a classification of medical and dental devices that are intended for use in contact with blood, based on the intended use and duration of contact as defined in ISO 10993-1;

b) the fundamental principles governing the evaluation of the interaction of devices with blood; and

c) the rationale for structured selection of tests according to specific categories, together with the principles and scientific basis of these tests.

Detailed requirements for testing cannot be specified because of limitations in knowledge and precision of tests for interactions of devices with blood. This part of ISO 10993 describes biological evaluation in general terms and may not necessarily provide sufficient guidance for test methods for a specific device.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this part of ISO 10993. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this part of ISO 10993 are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 10993-1:1997, *Biological evaluation of medical devices—Part 1: Evaluation and testing*.

ISO 10993-2:1992, *Biological evaluation of medical devices—Part 2: Animal welfare requirements*.

3 Terms and definitions

For the purposes of this part of ISO 10993, the terms and definitions given in ISO 10993-1 and the following apply.

3.1 blood/device interaction: Any interaction between blood or any component of blood and a device resulting in effects on the blood, or on any organ or tissue, or on the device.

NOTE—Such effects may or may not have clinically significant or undesirable consequences. Annex A contains further information on these interactions.

3.2 *ex vivo*: Term applied to a test system that shunts blood directly from a human subject or test animal into a test chamber located outside the body.

NOTE—If using an animal model, the blood may be shunted directly back into the animal (recirculating) or collected into test tubes for evaluation (single pass).

3.3 thrombosis: *In vivo* phenomenon resulting in the partial or complete occlusion of a vessel or device by a thrombus.

NOTE 1—Characterization of thrombosis includes *ex vivo* and *in vivo* methods, in either animals or the clinical setting.

NOTE 2—A thrombus is composed of a mixture of red cells, aggregated platelets, fibrin, and other cellular elements.

3.4 coagulation: Phenomenon that results from activation of the clotting factor cascade.

NOTE—Factors of the coagulation cascade and fibrinolytic systems can be measured following exposure to devices either *in vitro* or *in vivo*.

3.5 platelet: Anuclear, cellular body that is present in the circulation which adheres to surfaces and aggregates to form a hemostatic plug to minimize bleeding.

NOTE—Platelet testing includes quantification of platelet numbers as well as analysis of their structure and function. The testing can include analysis of platelet factors, or components on the platelet surface which are released from platelets or adherent to the device surface.

3.6 hematology: Study of blood, including quantification of cellular and plasma components of the blood.

3.7 complement system: Part of the innate immune system, consisting of several plasma proteins, including enzymes and cellular receptors.

NOTE—Effector molecules produced from complement components are involved in inflammation, phagocytosis, and cell lysis.

4 Abbreviated terms

Bb	product of alternate pathway complement activation
β-TG	beta-thromboglobulin
C4d	product of classical pathway complement activation
C3a, C5a	(active) complement split products from C3 and C5
CD62L	L-selectin
CH-50	50 % total hemolytic complement
CT	computerized tomography
D-Dimer	specific fibrin degradation products (F XIII cross-linked fibrin)
ECMO	extracorporeal membrane oxygenator
ELISA	enzyme/linked immunosorbent assay
E.M.	electron microscopy
FDP	fibrin/fibrinogen degradation products
FPA	fibrinopeptide A
$F1_{+2}$	prothrombin activation fragment 1 + 2
iC3b	product of central C complement activation
IVC	inferior vena cava
MRI	magnetic resonance imaging
PAC-1	monoclonal antibody which recognizes the activated form of platelet surface glycoprotein IIb/IIIa
PET	positron emission tomography
PF-4	platelet factor 4
PRP	platelet-rich plasma
PT	prothrombin time
PTT	partial thromboplastin time
P-selectin	receptor exposed during either platelet or endothelial cell release reaction
RIA	radioimmunoassay
S-12	monoclonal antibody, which recognizes the alpha-granule membrane component P-selectin exposed during the platelet release reaction
SC5b-9	product of terminal pathway complement activation

TAT thrombin-antithrombin complex

TCC terminal complement complex

TT thrombin time

VWF von Willebrand factor

5 Types of devices in contact with blood (as categorized in ISO 10993-1)

5.1 Non-contact devices

An *in vitro* diagnostic device is an example of a non-contact device.

5.2 External communicating devices

These are devices that contact the circulating blood and serve as a conduit into the vascular system. Examples include but are not limited to those in ISO 10993-1.

a) External communicating devices that serve as an indirect blood path include but are not limited to

- cannulae,
- extension sets,
- blood collection devices,
- devices for the storage and administration of blood and blood products (e.g., tubing, needles, and bags), and
- cell savers.

b) External communicating devices in contact with circulating blood include but are not limited to

- atherectomy devices,
- blood monitors,
- catheters,
- guidewires,
- intravascular endoscopes,
- intravascular ultrasound,
- intravascular laser systems,
- retrograde coronary perfusion catheters,
- cardiopulmonary bypass circuitry,
- extracorporeal membrane oxygenators,
- hemodialysis/hemofiltration equipment,
- donor and therapeutic apheresis equipment,
- devices for absorption of specific substances from blood,
- interventional cardiology and vascular devices, and
- percutaneous circulatory support systems.

5.3 Implant devices

Implant devices are placed largely or entirely within the vascular system. Examples include but are not limited to

- annuloplasty rings,
- mechanical or tissue heart valves,

— prosthetic or tissue vascular grafts,

— circulatory support devices (ventricular-assist devices, artificial hearts, intra-aortic balloon pumps),

— inferior vena cava filters,

— embolization devices,

— endovascular grafts,

— implantable defibrillators and cardioverters,

— stents,

— arteriovenous shunts,

— blood monitors,

— internal drug delivery catheters,

— pacemaker leads,

— intravascular membrane oxygenators (artificial lungs), and

— leukocyte-removal filters.

6 Characterization of blood interactions

6.1 General requirements

6.1.1 Figure 1 illustrates a decision tree that can be used to determine whether testing for interaction with blood is necessary.

Blood interactions can be classified into five categories based on the primary process or system being measured.

Tables 1 and 2 list examples of devices which contact circulating blood and the categories of testing appropriate to the device.

NOTE—Since this is a horizontal International Standard, good rationales can be developed to justify the choice of category based on the device being characterized. Thombosis testing is frequently the preferred method for device characterization. In many cases, rationales can be used to substitute some combination of coagulation, platelets, hematology, and complement system testing for thrombosis testing.

For medical devices where a specific International Standard (vertical standard) exists, the biological evaluation requirements and test methods set forth in that vertical standard shall take precedence over the general requirements suggested in this part of ISO 10993.

6.1.2 Where possible, tests shall use an appropriate model or system which simulates the geometry and conditions of contact of the device with blood during clinical applications, including duration of contact, temperature, sterile condition, and flow conditions. For devices of defined geometry, the ratio of test parameter (concentration per unit volume) to exposed surface area (cm^2) shall be evaluated.

Only blood-contacting parts should be tested. The selected methods and parameters should be in accordance with the current state of the art.

6.1.3 Controls shall be used unless their omission can be justified. Where possible, testing should include a relevant device already in clinical use or a well-characterized reference materials [7].

Reference materials used should include negative and positive controls. All materials and devices tested shall meet all quality control and quality assurance specifications of the manufacturer and test laboratory. All materials and devices tested shall be identified as to source, manufacturer, grade, and type.

6.1.4 Testing of materials which are candidates for components of a device may be conducted for screening purposes. However, such preliminary tests do not serve as a substitute for the requirement that the complete device or device component be tested under conditions which simulate or exaggerate clinical application.

6.1.5 Tests which do not simulate the conditions of a device during use may not predict accurately the nature of the blood/device interactions which can occur during clinical applications. For example, some short-term *in vitro* or *ex vivo* tests are poor predictors of long-term *in vivo* blood/device interactions [25], [26].

6.1.6 It follows from the above that devices whose intended use is *ex vivo* (external communication) should be tested *ex vivo* and devices whose intended use is *in vivo* (implants) should be tested *in vivo* in an animal model simulating as closely as possible conditions of clinical use.

6.1.7 *In vitro* tests are regarded as useful in screening external communicating devices or implants, but may not be accurate predictors of blood/device interactions occurring upon prolonged or repeated exposure or permanent contact (see 6.3.1). Devices intended for non-contact use only do not require evaluation of blood/device interactions. Devices which come into very brief contact with circulating blood (e.g., lancets, hypodermic needles, capillary tubes) generally do not require blood/device interaction testing.

6.1.8 The two recommendations in 6.1.5 and 6.1.6, together with clause 5, Figure 1, and Table 2, serve as a guide for the selection of tests listed in 6.2.1.

6.1.9 Disposable laboratory equipment used for the collection of blood and performance of *in vitro* tests on blood shall be evaluated to ascertain that there is no significant interference with the test being performed.

6.1.10 If tests are selected in the manner described and testing is conducted under conditions which simulate clinical applications, the results of such testing have the greatest probability of predicting clinical performance of devices. However, species differences and other factors may limit the predictability of any test.

6.1.11 Because of species' differences in blood reactivity, human blood should be used where possible. When animal models are necessary, for example, for evaluation of devices used for prolonged or repeated exposure or permanent contact, species' differences in blood reactivity shall be considered.

Blood values and reactivity in humans and non-human primates are very similar [26]. The use of animals such as the rabbit, pig, calf, sheep, or dog may also yield satisfactory results. Because species differences may be significant (for example, platelet adhesion, thrombosis [20], and hemolysis tend to occur more readily in the canine species than in the human), all results of animal studies shall be interpreted with caution. The species used and the number of species used shall be justified (see ISO 10993-2).

NOTE—The use of non-human primates for *in vivo* blood compatibility and medical device testing is prohibited by EU law (86/609/EEC) and some national laws.

6.1.12 The use of anticoagulants in *in vivo* and *ex vivo* tests should be avoided unless the device is designed to perform in their presence. The choice and concentration of anticoagulant used influence blood/device interactions, and their selection shall be justified. Devices that are used with anticoagulants should be assessed using anticoagulants in the range of concentrations used clinically.

6.1.13 Modifications in a clinically accepted device shall be considered for their effect on blood/device interactions and clinical functions. Examples of such modifications include changes in design, geometry, changes in surface or bulk chemical composition of materials, and changes in texture, porosity, or other properties.

6.1.14 A sufficient number of replications of a test including suitable controls shall be performed to permit statistical evaluation of the data. The variability in some test methods requires that those tests be repeated a sufficient number of times to determine significance. In addition, repeated studies over an extended period of blood/device contact provide information about the time-dependence of the interactions.

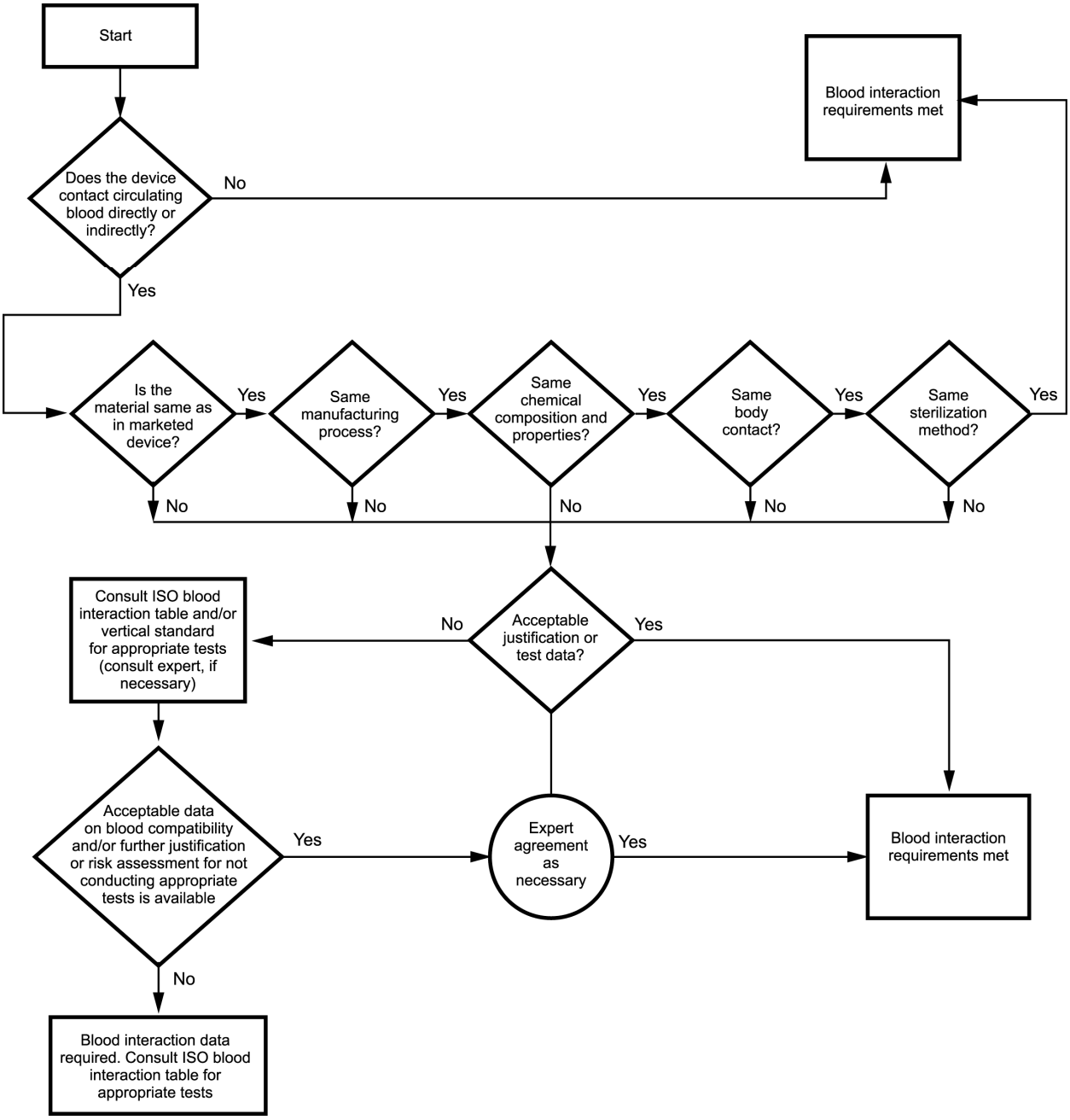

Figure 1—Decision tree that determines whether testing for interaction with blood is necessary

Table 1—Devices or device components which contact circulating blood and the categories of appropriate testing—External communicating devices

Device examples	Test category				
	Thrombosis	Coagulation	Platelets	Hematology	Complement System
Atherectomy devices				x[a]	
Blood monitors	x			x[a]	
Blood storage and administration equipment, blood collection devices, extension sets		x	x	x[a]	
Extracorporeal membrane oxygenator system, hemodialysis/ hemofiltration equipment, percutaneous circulatory support devices	x	x	x	x	x
Catheters, guidewires, intravascular endoscopes, intravascular ultrasound, laser systems, retrograde coronary perfusion catheters	x	x		x[a]	
Cell savers		x	x	x[a]	
Devices for absorption of specific substances from blood		x	x	x	x
Donor and therapeutic apheresis equipment		x	x	x	x

[a] Hemolysis testing only.

Table 2—Devices or device components which contact circulating blood and the categories of appropriate testing—Implant devices

Device examples	Test category				
	Thrombosis	Coagulation	Platelets	Hematology	Complement System
Annuloplasty rings, mechanical heart valves	x			x[a]	
Intra-aortic balloon pumps	x	x	x	x	x
Total artificial hearts, ventricular-assist devices	x			x	
Embolization devices				x[a]	
Endovascular grafts	x			x[a]	
Implantable defibrillators and cardioverters	x			x[a]	
Pacemaker leads	x			x[a]	
Leukocyte removal filter		x	x	x[a]	
Prosthetic (synthetic) vascular grafts and patches, including arteriovenous shunts	x			x[a]	
Stents	x			x[a]	
Tissue heart valves	x			x[a]	
Tissue vascular grafts and patches, including arteriovenous shunts	x			x[a]	
Vena cava filters	x			x[a]	

[a] Hemolysis testing only.

6.2 Categories of tests and blood interactions

6.2.1 Recommended tests for interactions of devices with blood

Recommended tests are organized on the basis of the type of device according to Tables 3 and 4.

The tests are classified into the following five categories based on the primary process or system being measured:

a) thrombosis (see 3.3),

b) coagulation (see 3.4),

c) platelets (see 3.5),

d) hematology (see 3.6), and

e) complement system (see 3.7).

The principles and scientific basis for these tests are presented in annex B.

6.2.2 Non-contact devices

These devices do not require blood/device interaction testing. Disposable test kits should be validated to rule out interference of materials with test accuracy.

6.2.3 External communicating devices

After using Tables 1 and 2 to determine the relevant blood interaction categories for a specific device type, Table 3 can be used as a guide to select the appropriate tests for external communicating devices as a function of the blood interactions appropriate to evaluate (see also 6.1.6). Test selection criteria will depend on the specific device evaluated.

6.2.4 Implant devices

After using Tables 1 and 2 to determine the relevant blood interaction categories for a specific device type, Table 4 can be used as a guide to select the appropriate tests for implant devices as a function of the blood interactions appropriate for evaluation (see also 6.1.6). Test selection criteria will depend on the specific device evaluated.

6.2.5 Indications and limitations

Immunoassays are available for human blood testing but are not generally available for other species. The human test kits usually do not cross-react with other species except for some non-human primates. Care should be taken when designing test systems to ensure that one is actually measuring activation due to the test material and not an artifact of the system. *In vitro* and *ex vivo* simulations with human blood often produce plasma levels of analytes that require a low, medium, or high level of dilution, depending on experimental conditions, for measurement in the valid range of the immunoassay. Care should be taken to report only those results measured within the valid ranges of the assays. Care should also be taken to ensure that a range of dilutions of the sample tested is measured.

Discrepancies in evaluating blood/device interactions may occur because of inadequate material characterization or inappropriate handling before blood tests are performed. For example, the studies may have relied on only one type of test or may have permitted the introduction of foreign material unrelated to the material or device under test. Materials to be used in a low-flow (venous) environment may interact with blood quite differently when used in high-flow (arterial) situations. Changes in design and/or flow conditions can alter the apparent *in vivo* hemocompatibility of a material.

Table 3—Test methods for external communicating devices

Test category	Evaluation method	Comments
Thrombosis	Percent occlusion	
	Flow reduction	
	Gravimetric analysis (thrombus mass)	
	Light microscopy (adhered platelets, leukocytes, aggregates, erythrocytes, fibrin, etc.)	
	Pressure drop across device	
	Labeled antibodies to thrombotic components	
	Scanning E.M. (platelet adhesion and aggregation; platelet and leukocyte morphology; fibrin)	
Coagulation	PTT (non-activated)	
	Thrombin generation—specific coagulation factor assays; FPA, D-dimer, F_{1+2}, TAT	
Platelets	Platelet count/adhesion	
	Platelet aggregation	
	Template bleeding time	
	Platelet function analysis	
	PF-4, β-TG, thromboxane B2	
	Platelet activation markers	
	Platelet microparticles	
	Gamma imaging of radiolabeled platelets, ^{111}In-labeled platelet survival	In-labeling is recommended for prolonged or repeated use (> 24 hours to 30 days) and permanent contact (> 30 days)
Hematology	Leukocyte count with or without differential	
	Leukocyte activation	
	Hemolysis	
	Reticulocyte count; activation-specific release products of peripheral blood cells (i.e., granulocytes)	
Complement system	C3a, C5a, TCC, Bb, iC3b, C4d, SC5b-9, CH50, C3 convertase, C5 convertase	

Table 4—Test methods for implant devices

Test category	Evaluation method	Comments
Thrombosis	Scanning EM (platelet adhesion and aggregation); platelet and leukocyte morphology; fibrin	
	Percent occlusion	
	Flow reduction	
	Labeled antibodies to thrombotic components	
	Autopsy of devices (gross and microscopic); histopathology	
	Autopsy of distal organs (gross and microscopic); histopathology	
Coagulation	Specific coagulation factor assay; FPA, D-dimer, F_{1+2}, PAC-1, S-12, TAT	
	PTT (non-activated), PT, TT; plasma fibrinogen; FDP	
Platelets	PF-4, β-TG, thromboxane B2	
	Platelet activation markers	
	Platelet microparticles	
	Gamma imaging of radiolabeled platelets, ^{111}In-labeled platelet survival	
	Platelet function analysis	
	Platelet count/adhesion	
	Platelet aggregation	
Hematology	Leukocyte count with or without differential	
	Leukocyte activation	
	Hemolysis	
	Reticulocyte count; activation-specific release products of peripheral blood cells (i.e., granulocytes)	
Complement system	C3a, C5a, TCC, Bb, iC3b, C4b, SC5b-9, CH 50, C3 convertase, C5 convertase	

6.3 Types of tests

6.3.1 *In vitro* tests

Variables that shall be considered when using *in vitro* test methods include hematocrit, anticoagulants, sample collection, sample age, sample storage, aeration and pH, temperature, sequence of test versus control studies, surface-to-volume ratio, and fluid dynamic conditions (especially wall shear rate). Tests shall be performed with minimal delay, usually within 4 h, since some properties of blood change rapidly following collection.

6.3.2 *Ex vivo* tests

Ex vivo tests shall be performed when the intended use of the device is *ex vivo*, for example, an external communicating device. *Ex vivo* testing can also be useful when the intended use is *in vivo*, for example, an implant such as a vascular graft. Such use should not, however, substitute for an implant test.

Ex vivo test systems are available for monitoring platelet adhesion, emboli generation, fibrinogen deposition, thrombus mass, white cell adhesion, platelet consumption, and platelet activation [20], [30], [48]. Blood flowrates can be measured with either Doppler or electromagnetic flow probes. Alterations in flowrates may indicate the extent and course of thrombus deposition and embolization.

Many *ex vivo* test systems use radiolabeled blood components to monitor blood/device interactions. Platelets and fibrinogen are components of blood which are most commonly radiolabeled. Alteration of platelet reactivity by the labeling procedure can be minimized by strict attention to technical detail [23], [24], [25].

The advantages of *ex vivo* tests over *in vitro* tests are that flowing native blood is used (providing physiological flow conditions), several materials can be evaluated since the chambers can be changed, and it is possible to monitor some events in real time. Some disadvantages include variability in blood flowrate from one experiment to another, variable blood reactivity from one animal to another, and the usually relatively short time intervals that can be evaluated. Positive and negative controls using the same animal are recommended in this regard.

6.3.3 *In vivo* tests

In vivo testing involves implanting the material or device in animals. Vascular patches, vascular grafts, prosthetic rings, heart valves, and circulatory assist devices are examples of configurations used in *in vivo* testing.

Patency (of a conduit) is the most common measure of success or failure for most *in vivo* experiments. The percent occlusion and thrombus mass are determined after the device is removed. The tendency of thrombi formed on a device to embolize to distal organs should be assessed by careful gross as well as microscopic examination of organs downstream from the device. In addition, histopathological evaluation of the surrounding tissue and organs is useful. The kidneys are especially prone to trap thrombi which have embolized from devices implanted upstream from the renal arteries (for example, ventricular-assist devices, artificial hearts, aortic prosthetic grafts) [19]. Methods to evaluate *in vivo* interactions without terminating the experiment are available. Arteriograms are used to determine graft patency or thrombus deposition on devices. Radioimaging can be used to monitor platelet deposition at various time periods *in vivo*; platelet survival and consumption can be used as indicators of blood/device interactions and passivation due to neointima formation or protein adsorption.

In some *in vivo* test systems, the material's properties may not be major determinants of the blood/device interactions. Rather, flow parameters, compliance, porosity, and implant design may be more important than blood compatibility with the material itself. As an example, low flowrate systems may give substantially different results when compared to the same material evaluated in a high flowrate system. In such cases, test system performance *in vivo* should carry more importance than *in vitro* test results.

Annex A
(informative)

Preclinical evaluation of cardiovascular devices and prostheses

A.1 General considerations

A.1.1 Background

This annex provides background for selecting tests to evaluate the interactions of cardiovascular devices with blood. Clause 6 of this part of ISO 10993 contains guidance on when testing is necessary, what blood interaction categories might be appropriate for specific devices, and a list of tests for evaluating blood/device interactions of non-contact, external communicating, and implant devices.

A.1.2 Classification

The following classification of blood/device interactions is provided as background.

a) Interactions which mainly affect the device and which may or may not have an undesirable effect on the subject are as follows:

   1) adsorption of plasma proteins, lipids, calcium, or other substances from the blood onto the surface of the device, or absorption of such substances into the device;

   2) adhesion of platelets, leukocytes, or erythrocytes onto the surface of the device, or absorption of their components into the device;

   3) formation of pseudointima or tissue capsule on the surface of the device; and

   4) alterations in mechanical and other properties of the device.

b) Interactions which have a potentially undesirable effect on the animal or human are as follows:

   1) activation of platelets, leukocytes, or other cells, or activation of the coagulation, fibrinolytic, or complement pathways;

   2) formation of thrombi on the device surface;

   3) embolization of thrombotic or other material from the device's luminal surface to another site within the circulation;

   4) injury to circulating blood cells resulting in anemia, hemolysis, leucopenia, thrombocytopenia, or altered function of blood cells;

   5) injury to cells and tissues adjacent to the device;

   6) intimal hyperplasia or accumulation of other tissue on or adjacent to the device, resulting in reduced flow or affecting other functions of the device; and

   7) adhesion and growth of bacteria or other infectious agents on or near the device.

A.1.3 Advantages and limitations of animal and *in vitro* testing

Animal models permit continuous device monitoring and systematic controlled study of important variables. However, the choice of an animal model may be restricted by size requirements, the availability of certain species, and cost. It is critical that the investigators be mindful of the physiological differences and similarities of the species chosen with those of the human, particularly those relating to coagulation, platelet functions, and fibrinolysis, and the response to pharmacological agents such as anesthetics, anticoagulants, thrombolytic and antiplatelet agents, and antibiotics. Because of species' differences in reactivity, subject differences in reactivity, and variable responses to different devices, data obtained from a single species should be interpreted with caution. Non-human primates such as baboons bear a close similarity to the human in hematologic values, blood coagulation mechanism, and cardiovascular system [30]. An additional advantage of a non-human primate is that many of the immunological probes for thrombosis developed for humans are suitable for use in other primates. These probes include PF-4, β-TG, FPA, TAT, and F_{1+2}. The dog is a commonly used species and has provided useful information; however, device-related thrombosis in the dog tends to occur more readily than in the human, a difference which can be

viewed as an advantage when evaluating this complication. The pig is generally regarded as a suitable animal model because of its hematological and cardiovascular similarities to the human. The effect of the surgical implant procedure on results should be kept in mind and appropriate controls included.

Because of species' differences in hemostatic and hematological factors and activities, it is preferable to use human blood in *in vitro* tests whenever possible.

Thrombus formation is a dynamic process. Therefore, *in vitro* testing is advisable to simulate as much as possible the dynamic conditions (for example, shear forces of the blood/material interface) in which thrombosis occurs. Static tests may be useful in some cases for evaluating the interactions of blood with materials.

Since patients with cardiovascular devices may be receiving anticoagulant or antithrombotic drugs, it is important to simulate these conditions *in vitro*.

A.1.4 Test protocols for animal testing

Thrombosis, thromboembolism, bleeding, and infection are the major deterrents to the use and further development of advanced cardiovascular prostheses. For devices with limited blood exposure (< 24 h), important measurements are related to the acute extent of variation of hematological, hemodynamic, and performance variables, gross thrombus formation, and possible embolism. With prolonged or repeated exposure, or permanent contact (> 24 h), emphasis is placed on serial measurement techniques that may yield information regarding the time course of thrombosis and thromboembolism, the consumption of circulating blood components, the development of intimal hyperplasia, and infection. In both of the above exposure and contact categories, assessment of hemolysis and platelet function is important. Thrombus formation may be greatly influenced by surgical technique, variable time-dependent thrombolytic and embolic phenomena, superimposed device infections, and possible alterations in exposed surfaces, for example, intimal hyperplasia and endothelialization.

The consequences of the interaction of artificial surfaces with the blood can range from gross thrombosis and embolization to subtle effects such as accelerated consumption of hemostatic elements; the latter may be compensated (the total number of platelets consumed by the device is so small it does not affect the total platelet count) or lead to depletion of platelets or plasma coagulation factors (the device surface area is large enough to consume enough platelets for the total platelet count to be affected).

A.1.5 Test protocols for *in vitro* testing

In vitro testing allows for the performance of a sufficient number of tests for statistical evaluation without the sacrifice of animals and with relatively low costs. Measurements are related to the acute extent of variation of hematological, hemodynamic and performance variables, gross thrombus formation, and complement activation. The *in vitro* approach permits the study of the kinetics of various factors and activities, by varying the duration of exposure of material or devices to blood.

A.2 Cannulae

Cannulae are typically inserted into one or more major blood vessels to provide repeated blood access. They are also used during cardiopulmonary bypass and other procedures. They can be tested acutely or chronically, and are commonly studied as arteriovenous (AV) shunts. The use of cannulae induces little alteration in the levels of circulating blood cells or factors in the coagulation or complement system. Cannulae, like other indirect blood path devices (5.2), generally require less testing than devices in direct contact with circulating blood (5.2, 5.3).

A.3 Catheters and guidewires

Most of the tests considered under cannulae are relevant to the study of catheters and guidewires. The location or placement of catheters in the arterial or venous system can have a major effect on blood/device interactions. It is advised that simultaneous control studies be performed using a contralateral artery or vein. Care should be taken not to strip off thrombus upon catheter withdrawal. *In situ* evaluation may permit assessment of the extent to which intimal or entrance site injuries contributed to the thrombotic process. In general, Doppler blood flow measurements are more informative than angiography. Kinetic studies with radiolabeled blood constituents are recommended only with chronic catheters.

A.4 Extracorporeal oxygenators, hemodialyzers, therapeutic apheresis equipment, and devices for absorption of specific substances from blood

The hemostatic response to cardiopulmonary bypass can be significant and acute. Many variables such as use of blood suction, composition of blood-pump priming fluid, hypothermia, blood contact with air, and time of exposure influence test values. Emboli in outflow lines may be detected by the periodic placement of blood filters *ex vivo*, or the use of ultrasonic radiation or other non-invasive techniques. Thrombus accumulation can be directly assessed during bypass by monitoring performance factors such as pressure drop across the oxygenator and oxygen transfer

rate. An acquired transient platelet dysfunction associated with selective alpha granule release has been observed in patients on cardiopulmonary bypass [31]; the template bleeding time and other tests of platelet function and release are particularly useful.

Complement activation is caused by both hemodialyzers and cardiopulmonary bypass apparatus. Clinically significant pulmonary leucostasis and lung injury with dysfunction can result. For these reasons, it is useful to quantify complement activation with these devices.

Therapeutic apheresis equipment and devices for absorption of specific substances from the blood, because of their high surface-to-volume ratio, can potentially activate complement, coagulation, platelet, and leukocyte pathways. Examination of blood/device interactions should follow the same principles as for extracorporeal oxygenators and hemodialyzers.

A.5 Ventricular-assist devices and total artificial hearts

These devices can induce considerable alteration in various blood components. Factors contributing to such effects include the large foreign surface area to which blood is exposed, the high flow regimes, and the regions of disturbed flow such as turbulence or separated flow. Tests of such devices may include measurements of hemolysis, fibrinogen concentration, thrombin generation, platelet survival and activation, complement activation, and close monitoring of liver, renal, pulmonary, and central nervous system function. A detailed pathological examination at surgical retrieval is an important component of the evaluation [40], [41].

A.6 Heart valve prostheses

Invasive, non-invasive, and *in vitro* hydrodynamic studies are important in the assessment of prosthetic valves.

One of the most effective means of screening for prosthetic valve dysfunction is auscultation [42]. 2D and M mode echocardiography makes use of ultrasonic radiation to form images of the heart. Reflections from materials with different acoustic impedances are received and processed to form an image. The structure of prosthetic valves can be examined. Mechanical prostheses emit strong echo signals and the movement of the occluder can usually be clearly imaged. However, the quality of the image may depend upon the particular valve being examined. Echocardiography can also be useful in the assessment of function of tissue-derived valve prostheses. Vegetations, clots, and evidence of thickening of the valve leaflets are elucidated. Using conventional and color flow Doppler echocardiography, regurgitation can be identified and semi-quantified [42].

Measurements of platelet survival and aggregation, blood tests of thrombosis and hemolysis, pressure and flow measurements, and autopsy of the valve and adjacent tissues are also recommended [41], [43].

A.7 Vascular grafts

Both porous and non-porous materials can be implanted at various locations in the arterial or venous system. The choice of implantation site is determined largely by the intended use for the prosthesis. Patency of a given graft is enhanced by larger diameter and shorter length. In general, for grafts of less than 4 mm inner diameter, length should exceed diameter by a factor of 10 (i.e., 40 mm for a 4 mm diameter graft) for a valid model. Patency can be documented by palpation of distal pulses in some locations and by periodic angiography. Ultrasonic radiation, MRI, and PET may also be useful. Results of serial radiolabeled platelet imaging studies correlate with the area of non-endothelialized graft surface in baboons [30]. Radiolabeled platelets facilitate non-invasive imaging of mural thrombotic accumulations. Serial measurements of platelet count, platelet release constituents, fibrinogen/fibrin degradation products, and activated coagulation species also are recommended. Autopsy of the graft and adjacent vascular segments for morphometric studies of endothelial integrity and proliferative response can provide valuable information. A systematic evaluation of longitudinal and cross-sectional sections of proximal and distal anastomoses and representative midgraft regions is necessary for a thorough evaluation of the device [43].

A.8 IVC filters, stents, and stented grafts

These devices can be studied by angiography and ultrasonic radiation. Other techniques useful for vascular graft evaluation (see A.7) are appropriate here as well [43].

Annex B
(informative)

Laboratory tests—Principles, scientific basis, and interpretation

B.1 General

B.1.1 Background

The principles and scientific basis of the tests listed in 6.2.1 are described here. Detailed methods are found in standard texts of laboratory medicine and clinical pathology. References [17] to [44], [46] to [49], and [59] describe tests which may be useful in the evaluation of blood/device interactions. Because of both biological variability and technical limitations, the accuracy of many of these tests is limited.

B.1.2 Principles for *in vitro* testing

Static and dynamic systems, e.g., circulatory loop and centrifugation systems, are used [50], [54].

B.1.3 Test conditions

In order for tests to be of use in the *in vitro* evaluation of blood/device interactions, anticoagulated blood or plasma collected from normal human subjects or experimental animals should first be exposed to the material or device under standardized conditions including time, temperature, and flowrate. An aliquot of the exposed blood or plasma is then tested shortly after exposure. Conditions of exposure should be based on the intended use of the device.

In the preparation of the test articles, it is essential to avoid activation or release of any component from blood before testing. However, the appropriate conditions depend on the device or material being tested and its intended end-use.

B.1.4 Classification

When evaluating externally communicating devices and implant devices while in their in-use position, blood is collected into an anticoagulant and the test is performed as described without a prior exposure stage. The tests are classified into five categories, as defined in 6.2.1, according to the process or system being tested: thrombosis, coagulation, platelets and platelet functions, hematology, and complement system.

B.2 Thrombosis

B.2.1 Percentage occlusion

Percentage occlusion is visually quantified after a device has been in use and has been removed. This is a measure of the severity of the thrombotic process in a conduit. Lack of occlusion does not necessarily eliminate the existence of a thrombotic process, since thrombi may have embolized or been dislodged before percentage occlusion is measured. Occlusion may be caused not only by thrombosis, but also by intimal hyperplasia, especially at perianastomotic sites in vascular grafts; microscopic examination is required to identify the nature of the occlusive process. Determinations of surface area covered by thrombus and thrombus-free surface area are semi-quantitative tests that can be used on a comparative basis.

B.2.2 Flow reduction

Flow (rate or volume) is measured after a period of use. Measurements may be performed either during use, or before and after use. Rationale and interpretation are the same as B.2.1.

B.2.3 Gravimetric analysis (thrombus mass)

This is conducted after removal of the device from the in-use position. Rationale and interpretation are as for B.2.1.

B.2.4 Light microscopy

By this technique, information can be obtained regarding the density of cells, cellular aggregates, and fibrin adherent to materials, as well as the geographic distribution of these deposits on the materials or device. The method is semi-quantitative.

B.2.5 Pressure drop across device

This is measured before and after a period of use. Rationale and interpretation are as for B.2.1.

B.2.6 Scanning electron microscopy (SEM)

Rationale and interpretation are the same as for B.2.4. This method has the advantage over B.2.4 of providing greater detail about the fine structure of components being examined. Quantitative conclusions require sufficient replicate determinations to establish a degree of reproducibility.

B.2.7 Antibody binding

Next to qualitative microscopic judgment of fibrin and platelet deposition on materials, a quantitative estimation is possible by measuring the amount of labeled antibody specific for fibrin(ogen) or platelet membrane receptors. For this purpose, materials are washed after exposure to blood to delete non-adherent blood components prior to labeled antibody binding.

B.2.8 Retrieval and examination of device

This method is of great importance in evaluating the biological responses to implanted devices. The distribution, size, and microscopic nature of cellular and proteinaceous deposits can best be determined by a careful and detailed examination. Proposed procedures have been published [40], [41].

B.2.9 Autopsy of distal organs

The rationale is to examine for distal effects of implanted devices. These effects include thromboembolism and embolization of components of the device [43].

B.2.10 Imaging techniques—Angiography, intravascular ultrasound, Doppler ultrasound, CT, and MRI

Choices can be made among these methods to determine patency or degree of narrowing of a graft or other conduit and to detect thrombus deposition on devices during their *in vivo* performance.

B.3 Coagulation

Coagulation methods are based on the use of native (fresh, non-anticoagulated) whole blood, anticoagulated whole blood (usually citrated), platelet-rich plasma, or platelet-poor plasma. Since most of the standard coagulation assays are designed to detect clinical coagulation disorders which result in delayed clotting or excessive bleeding, the protocols for evaluating blood/device interactions should be modified appropriately to evaluate accelerated coagulation induced by biomaterials. Reagents for tests based on the activated partial thromboplastin time include an activator such as kaolin, celite, or ellagic acid. Reagents with such activators should be avoided because they tend to mask the acceleration of coagulation which materials and devices cause. The material to be tested serves as the activator; controls (without the material) should be included.

Blood is exposed to test materials either in a static chamber, such as a parallel plate cell, or in a closed-loop system where the inner surface of the tubing is the test material. After a predetermined contact time with the test surface, tests of the surface and blood can be conducted [54].

B.3.1 Partial thromboplastin time (PTT)

The partial thromboplastin time [38] is the clotting time of recalcified citrated plasma on the addition of partial thromboplastin. Partial thromboplastin is a phospholipid suspension usually extracted from tissue thromboplastin, the homogenate from mammalian brain or lung. Shortening of the PTT following contact with a material under standard conditions indicates activation of the contact phase of blood coagulation. A prolonged PTT suggests a deficiency in any of the plasma coagulation factors: I (fibrinogen), II (prothrombin), V, VIII, IX, X, XI, or XII, but not VII or XIII. Heparin and other anticoagulants also cause a prolonged PTT.

Partial thromboplastin reagents using various activating substances such as kaolin or celite are commercially available. Using these reagents, the test is called the activated partial thromboplastin time (APTT). The APTT is of no value in the *in vitro* evaluation of blood/device interactions because the activating substances mask any activation caused by the device or its component materials.

B.3.2 Prothrombin time (PT)

This test measures prothrombin and accessory factors. In the presence of tissue thromboplastin, the clotting time depends on the concentrations of prothrombin, factor V, factor VII, and factor X (assuming fibrinogen, fibrinolytic, and anticoagulant activity to be normal). A prolonged prothrombin time generally indicates a deficiency of prothrombin or factor V, VII, X, or fibrinogen. Test kits are available commercially.

B.3.3 Thrombin time (TT)

The thrombin time [38] is the time required for plasma to clot when a solution of thrombin is added. The thrombin time is prolonged with a deficiency in fibrinogen (below 100 mg/dl), qualitative abnormalities in fibrinogen, and elevated levels of FDP or heparin. The test is useful for evaluating implant devices only.

B.3.4 Thrombin generation

Materials exposed to an intact coagulation system in the presence of phospholipids (see B.3.1) will generate thrombin which can be measured by conversion of a chromogenic substrate. This method has a much lower variability than the conventional coagulation tests.

B.3.5 Fibrinogen

Dysfibrinogenemia, afibrinogenemia, and hypofibrinogenemia cause prolonged PT, PTT, and TT results [21].

B.3.6 Fibrinogen and fibrin degradation products (FDP)

Normal physiological fibrinolysis yields the FDPs X, Y, C, D, and E in concentrations below 2 mg/mL of plasma. The normally low level of FDPs is maintained by the low rate of the degradation reaction and the high rate of clearance of FDPs from the circulation. Pathologic degradation of fibrin and fibrinogen, a result of increased plasminogen activation, yields FDP of 2 mg/mL to 40 mg/mL or more. The test is useful for evaluating implant devices only. The use of [51], [52] commercially available methods is recommended.

B.3.7 Specific coagulation factor assays

Significant reduction (e.g., to less than 50 % of the normal or control level) of coagulation factors following exposure of blood to a material or device under standard conditions suggests accelerated consumption of those factors by adsorption, coagulation, or other mechanisms.

B.3.8 FPA, D-dimer, F_{1+2}, TAT

Elevated levels of FPA, D-dimer, or F_{1+2} indicate activation of the coagulation mechanism. FPA and F_{1+2} indicate an activation of prothrombin to thrombin. Elevated TAT complexes indicate activation of blood coagulation and formation of a complex between thrombin being generated and circulating antithrombin. D-dimers are plasmin digested degradation products of F XIII cross-linked fibrin (coagulation and fibrinolysis). The use of ELISA and RIA is recommended.

B.4 Platelets and platelet functions

It is essential to avoid activation in the preparation of platelet suspensions.

B.4.1 Platelet count

It is important to determine the platelet count [21], [49], [59] because of the key role of platelets in preventing bleeding. A significant drop in platelet count of blood exposed to a device can be caused by platelet adhesion, platelet aggregation, platelet sequestration (for example, in the spleen), or blood coagulation on materials or devices. A reduction in platelet count during use of an implanted device may also be caused by accelerated destruction or removal of platelets from the circulation. Platelet count is performed using an EDTA suspension medium.

Blood collection techniques should be reproducible. Platelets can become hyperactive under a variety of conditions, including improper blood collection. Tests to verify normal platelet reactivity are usually performed with an aggregometer. Platelet preparations with reduced reactivity are easily detected using this method, but hyperactive platelets are not normally detected. Platelet aggregation tests can be modified (by appropriately reducing the concentration of platelets or aggregating agents) to determine if platelets become hyperactive following exposure to a material or device.

B.4.2 Platelet aggregation

Platelet aggregation [38] is induced by adding aggregating agents to PRP that is being stirred continually (e.g., ADP, epinephrine, collagen, thrombin, etc.). As the platelets aggregate, the plasma becomes progressively clearer. An optical system (aggregometer) is used to detect the change in light transmission, and a recorder graphically displays the variations in light transmission from the baseline setting. Delayed or reduced platelet aggregation can be caused by platelet activation and release of granular contents, increased FDP, or certain drugs (e.g., aspirin, nonsteriod anti-inflammatory drugs). It is important to bear in mind that platelet aggregation using some agents varies or may be absent in some animal species. Spontaneous platelet aggregation, occurring in the absence of added agonists, is an abnormal condition indicating activation of platelets. Platelet aggregates can also be screened automatically by the WU/HOAK method [52].

B.4.3 Blood cell adhesion

Blood cell adhesion [34] is a measure of the blood-compatibility of a material when considered in conjunction with distal embolization or evidence of activation of one or more hematological factors.

Various methods have been designed to measure the adhesion of cells to surfaces, for example, the Kuniki K-score [53]. Most of these methods are based on the observation that a certain proportion of platelets are removed from normal whole blood as a result of passage through a column of glass beads under controlled conditions of flow or pressure. This principle has been adapted to the quantification of the adhesion of other blood cells to polymers coated on glass beads. By such a method, it has been reported [34] that adhesion of canine species peripheral lymphocytes and polymorphonuclear leukocytes (PMNs) to beads coated with poly(hydroxyethyl methacrylate) (PHEMA) is lower than to beads coated with polystyrene and certain other polymers. Isolated lymphocytes and PMNs were used in this study.

An alternative method is the direct counting of platelets adherent to a test surface. Following exposure to blood or platelet-rich plasma under standardized conditions, the test surface is rinsed to remove non-adherent cells, fixed and prepared for either light or scanning electron microscopy. The number of adherent platelets per unit area is directly counted and their morphology (e.g., amount of spreading, degree of aggregate formation) is recorded. Alternatively, platelets prelabeled with ^{51}Cr or ^{111}In may be used [33], [55], [56].

B.4.4 Platelet activation

The use of certain materials or devices may cause platelet activation, which can result in

a) the release of platelet granule substances such as BTG (Beta thromboglobulin), platelet factor 4 (PF 4), and serotonin,

b) altered platelet morphology, and

c) the generation of platelet microparticles.

Activated platelets are pro-thrombotic. Platelet activation can be evaluated by various means: microscopic (light and electron microscopy) examination of platelet morphology of platelets adherent to the material or device; measurement of BTG, PF4, and serotonin; and the evaluation of platelet activation by flow cytometry (for microparticle generation, P-selectin [GMP-140] expression, or activated glycoprotein Ib and IIb/IIIa expression using monoclonal antibodies). Different epitopes of activated platelets are recognized by flow cytometry using two antibodies: one specific for platelets (i.e., GP Ib or GP IIb/IIIa) and one specific for platelet activation (P-Selectin) [60].

B.4.5 Template bleeding time

The commercial availability of a sterile disposable device for producing a skin incision of standard depth and length under standard conditions has significantly improved the reproducibility and value of this test. A prolonged result indicates reduced platelet function or reduced platelet count; the latter can be determined separately (B.4.1). A prolonged bleeding time combined with a normal platelet count has been observed in association with some external communicating devices with limited exposure (e.g., cardiopulmonary bypass) [31]. The test is suitable for use with some experimental animals. *In vitro* bleeding time measurements are also suitable.

B.4.6 Platelet function analysis

The classical template bleeding time has been used as the principle for an automated method. Whole blood is aspirated through a collagen filter with a 150 µm aperture. Platelets adhere and aggregate until the aperture is closed. Blood pressure and temperature are standardized; anticoagulation does not affect this test. The test is suitable for animal blood.

B.4.7 Gamma imaging of radiolabeled platelets

The high gamma emission of ^{111}In enables it to be used for this purpose [23], [30]. This method enables the localization and quantification of platelets deposited in a device. The technique is useful for external communicating, as well as implant devices.

B.4.8 Platelet lifespan (survival)

Platelets are obtained from the patient's blood and are labeled with ^{51}Cr or ^{111}In [23], [24], [32], [57]. Both of these agents label platelets of all ages present in the sample, do not elute excessively from the platelets, and are not taken up by other cells or reused during thrombopoiesis. $^{111}Indium$ has the advantage of being a high gamma emitter, requiring the labeling of fewer platelets and enabling surface body counting to assess localized platelet deposition to be combined with the lifespan study. A reduced platelet lifespan indicates accelerated removal from the circulation by immune, thrombotic, or other processes.

B.5 Hematology

B.5.1 Leukocytes

Leukocyte activation can be determined by the microscopic examination of the device surface or activated leukocytes and the use of flow cytometry for the evaluation of increased leukocyte markers such as L-selectin and CD 11b and quantitative disturbances in lymphocyte subpopulations.

B.5.2 Hemolysis

This is regarded as an especially significant screening test because of an elevated plasma hemoglobin level. If this test is properly performed, an elevated plasma hemoglobin level indicates hemolysis and reflects erythrocyte membrane fragility in contact with materials and devices (see annex C).

B.5.3 Reticulocyte count

An elevated reticulocyte count indicates increased production of erythrocytes in the bone marrow. This may be in response to reduced erythrocyte mass caused by chronic blood loss (bleeding), hemolysis, or other mechanisms [61].

B.6 Complement system—CH 50 and C3a, C5a, TCC, Bb, iC3b, C4d, SC5b-9

A decrease in CH 50 is an indicator of total complement consumption. Elevated levels of any of these complement components indicate activation of the complement system. Some materials activate complement, and activated complement components in turn activate leukocytes, causing them to aggregate and be sequestered in the lungs.

Measurement of complement split products has the disadvantage of species-specificity and high baseline levels when performed after *in vitro* testing. The classical CH50 method appears useful with human, bovine, porcine, and rabbit serum.

Another functional method of measurement of complement activation *in vitro* is the generation of complement C3- or C5-convertase, determined by substrate conversion.

ASTM F1984-99 and ASTM F2065-00 [13], [14] address complement activation.

Annex C
(informative)

Evaluation of hemolytic properties of medical devices and their components

C.1 General considerations

Extensive literature exists describing blood/material interactions. Unfortunately, very few methods exist which are reliable, reproducible, and predict clinical performance. This annex will review the known hemolysis test methods and discuss factors pertaining to their ability to characterize medical and dental materials and devices.

C.2 Terms and definitions

For the purposes of this annex to this part of ISO 10993, the following definitions apply.

C.2.1 anticoagulant: Agent which prevents or delays blood coagulation [62].

EXAMPLE—Heparin and citrate.

C.2.2 oncotic pressure, colloidal osmotic pressure: Total influence of the proteins or other large molecular mass substances on the osmotic activity of plasma [62].

C.2.3 hematocrit: Ratio of the volume of erythrocytes to that of whole blood in a given sample.

C.2.4 hemolysis: Liberation of hemoglobin from erythrocytes, either by destruction or through a partially damaged but intact cell membrane.

C.2.5 negative reference material: High density polyethylene, or similar validated alternative.

NOTE—See ISO 10993-12.

C.2.6 packed erythrocytes: component obtained by centrifugation from a unit of human blood following removal of plasma supernatant.

NOTE—Properties of human erythrocytes for transfusion: the erythrocyte volume fraction of the component is 0.65 to 0.80. The unit contains all of the original unit's erythrocytes, the greater part of its leukocytes (about 2.5 to 3.0 x 10^9 cells) and a varying content of platelets depending on the method of centrifugation.

C.2.7 washed erythrocytes: Erythrocyte suspension obtained from whole blood after removal of plasma and washing in an isotonic solution.

NOTE—This is an erythrocyte suspension from which most of the plasma, leukocytes, and platelets have been removed. The amount of residual plasma depends upon the washing protocol. Storage time should be as short as possible after washing, and certainly not longer than 24 h at 1 °C to 6 °C.

C.2.8 whole blood: Unfractionated blood, drawn from a selected donor, containing citrate or heparin as an anticoagulant.

C.3 Causes of hemolysis

C.3.1 Mechanical forces—Pressure

The erythrocyte membrane is a semipermeable membrane. A pressure differential will occur when two solutions of different concentrations are separated by such a membrane. Osmotic pressure occurs when the membrane is impermeable to passive solute movement. These pressure differentials can cause erythrocyte swelling and cell membrane rupture with release of free hemoglobin [62].

C.3.2 Mechanical forces—Rheology

Factors which influence blood flowrate, shear forces, and other forces that can deform the erythrocyte membrane can cause membrane rupture.

C.3.3 Biochemical factors

Changes to membrane structure on a molecular level can modify the strength and elastic properties of the erythrocyte membrane. A deficiency of nutritional factors or metabolic energy (ATP) can result in loss of the discoid

shape and microvesiculation of hemoglobin. Other chemicals, bacterial toxins, pH, and metabolic changes induced by temperature can compromise the erythrocyte membrane [63]. These changes may cause membrane rupture at lower than expected osmotic pressures. A test to determine the pressure at which an erythrocyte membrane ruptures (osmotic fragility) can be carried out.

C.4 Clinical significance of hemolysis

C.4.1 Toxic effects

Elevated levels of free plasma hemoglobin can induce toxic effects or initiate processes which can stress the kidneys or other organs [62]. The free plasma hemoglobin concentration is a convenient measure of injury to erythrocytes, but it is also an indirect indicator of damage to other blood elements as well.

C.4.2 Thrombosis

Intravascular hemolysis can promote thrombosis by liberating phospholipids [66]. When hemolysis causes a clinically significant drop in erythrocyte count, anemia and compromised oxygen carrying capacity with its subsequent effects on the brain and other organs or tissues can result.

C.5 Determining a Pass/Fail assessment for hemolysis

Hemolysis is a function of time and material properties such as surface energy, surface morphology, and surface chemistry. Hemolysis is also a function of shear stress, cell-wall interaction, character of adsorbed protein layers, flow stability, air entrainment, and variations of blood source, age, and chemistry [67], [68], [69]. These variables need to be adequately controlled for comparisons of hemolytic potential among materials and medical devices. The spectrum of methods for evaluating hemolysis varies from simplified to highly complicated models. Specific *in vitro* and *in vivo* models with flowing blood have been published. Studies of hemolytic potential are relative comparisons against materials or medical devices tested in the same model by a specific laboratory rather than absolute measures. *In vitro* test methods are able to quantify small levels of plasma hemoglobin which may not be measurable under *in vivo* conditions (e.g., due to binding of plasma hemoglobin to haptoglobin and rapid removal from the body). Measurement of lactate dehydrogenase and haptoglobin, as indicators of hemolysis in an *in vivo* test setting, should also be considered.

It is not possible to define a universal level for acceptable and unacceptable amounts of hemolysis for all medical devices and applications. The effect of a device on hemolysis can be masked in the short term by the trauma of the surgical procedure. A device can cause a substantial amount of hemolysis, but be the only treatment available in a life-threatening situation. Intuitively, a blood-compatible material is non-hemolytic. In practice, many devices cause hemolysis, but their clinical benefit outweighs the risk associated with the hemolysis. Therefore, when a device causes hemolysis, it is important to confirm that the device provides a clinical benefit and that the hemolysis is within acceptable limits clinically. Acceptance criteria should be justified based on some form of risk and benefit assessment. The following questions are suggestions for developing such an assessment:

a) What is the duration of exposure of the device to the patient?

b) How much hemolysis does the material or device cause? Does the hemolysis continue for the entire time the device is exposed to the patient? Does hemolysis continue after removal of the device?

c) What are the relative risks and benefits of other available methods for treating the condition?

d) What are the hemolytic properties of these known treatments? How does the device in question compare to these other treatments?

e) How effective is the test device compared with other forms of treatment? A more effective device can cause more hemolysis during use, but the additional effectiveness might increase the benefit to the patient.

C.6 Hemolysis testing—General considerations

C.6.1 Methods

C.6.1.1 General

In vitro tests are used to evaluate damage to erythrocytes. Direct methods determine hemolysis due to physical and chemical interactions with erythrocytes. Indirect methods determine hemolysis due to extractables from test articles. ASTM F756-00 is a standard that is specific for testing the hemolytic properties of materials (mainly due to chemical factors) and is not sufficient for testing whole medical devices. ASTM F756-00 [10] (and the hemolysis test listed in GB/T 16175-1996 [11]) is given as an example and possible starting point for developing a protocol for hemolysis testing for a specific device. In addition to material testing of devices, dynamic testing of whole medical devices to evaluate the effects of the structure, intended use, and hemodynamic factors should be considered.

In its simplest form, for highly diluted suspensions of erythrocytes in contact with test materials, hemolysis is often reported as a percentage of hemoglobin which has been liberated into the supernatant normalized by the total hemoglobin which was available at the beginning of the test [i.e., (free hemoglobin concentration/total hemoglobin concentration) x 100 %]. If all of the erythrocytes present at the beginning of the experiment are destroyed, there is 100 % hemolysis. For medical device testing, in which the use of highly diluted erythrocyte suspensions is not applicable, blood hematocrit and other factors must be accounted for in the normalization of a hemolysis index [63].

At a minimum, each laboratory shall be able to measure the total blood hemoglobin concentration and the plasma or supernatant hemoglobin concentration. The concentration of hemoglobin in plasma is significantly less than the total blood hemoglobin concentration. The free plasma hemoglobin concentration is normally 0 mg/dL to 10 mg/dL *in vivo*, whereas the normal range of total blood hemoglobin concentration is 11,000 mg/dL to 18,000 mg/dL. For this reason, different methods have been used to measure the great range of hemoglobin concentrations which are encountered during hemolysis testing.

Classically, three analytical methods have been used to determine total blood hemoglobin (Hb) concentrations [70].

NOTE—Researchers should be aware that hemolysis tests may be adversely affected by chemicals in medical materials or solutions which may alter erythrocyte fragility (e.g., by fixatives such as formaldehyde or glutaraldehyde), cause hemoglobin to precipitate (e.g., by copper or zinc ions), or alter the absorption spectra of hemoglobin (e.g., by polyethylene glycol or ethanol) [64], [65].

C.6.1.2 Total blood hemoglobin concentration measurements

C.6.1.2.1 Cyanmethemoglobin method

The first classical method, cyanmethemoglobin detection, was issued by the International Committee for Standardization in Hematology [71]. The cyanmethemoglobin (hemiglobincyanide; HiCN) analysis has the advantage of convenience, ease of automation, and the availability of a primary reference standard (HiCN). The method is based on the oxidation of Hb and subsequent formation of hemiglobincyanide, which has a broad absorption maxima at 540 nm. Lysing agents such as detergents are used, which, in addition to releasing Hb from the erythrocyte, decrease the turbidity (a source of interference as false absorbance at 540 nm) from protein precipitation. For the total hemoglobin concentration, the spectral interference due to plasma is minimal and the sample absorbance can be compared to the HiCN standard solution directly.

The broad absorption band of HiCN in this region enables the use of simple filter-type photometers as well as narrow band spectrophotometers for either manual or automated detection. The use of the HiCN reference standard provides comparability among all laboratories employing this method. The major disadvantage is the potential health risk in using the cyanide solutions. Cyano reagents are themselves toxic by various routes of exposure, and, additionally, release HCN upon acidification. Disposal of reagents and products has also become a considerable concern and expense.

C.6.1.2.2 Oxyhemoglobin method

The second classical method for determining the total hemoglobin concentration is not widely used today. The oxyhemoglobin method depends on the formation of HbO_2 during ammonia-hydroxide treatment, and spectrophotometric detection of this product. Quantification of oxyhemoglobin in dilute sodium carbonate solution has also been used. No stable reference preparation is available but this is not important because all that the method is required to measure is the percentage of the total hemoglobin in the original specimen which is present in the plasma. In any event, a short-term standard can be prepared from a fresh blood sample.

C.6.1.2.3 Iron method

The third classical method for determining the total hemoglobin concentration is based on determining the hemoglobin iron concentration in solution. Iron is first separated from Hb, usually by acid or by ashing. It is then titrated with $TiCl_3$ or complexed with a reagent to develop color that can be measured photometrically. This method is too complex for routine work, and is rarely used.

C.6.1.3 Plasma or supernatant hemoglobin concentration measurements

C.6.1.3.1 Direct optical and added chemical techniques

Due to many different factors (e.g., tradition, ease of use, disposal of waste chemicals, availability of standard solutions), there are currently about twenty different assays in use today for measuring plasma hemoglobin as an indicator of hemolysis, but no one method is widely accepted. The assays can be classified into two broad categories: those which are direct optical techniques (i.e., based on quantifying the oxyhemoglobin absorbance peaks at 415, 541, or 577 nm, directly or through use of derivative spectrophotometry) and those which are added chemical techniques (i.e., quantification of hemoglobin based on a chemical reaction with reagents such as

benzidine-like chromogens and hydrogen peroxide, or the formation of cyanmethemoglobin) [72]. All of the assays can be performed manually or can be automated.

A popular method for determining the concentration of hemoglobin is based on its catalytic effect on the oxidation of a benzidine derivative, such as tetramethylbenzidene, by hydrogen peroxide. The rate of formation of a colored product (photometrically detected at 600 nm) is directly proportional to the hemoglobin concentration. The advantages of this method are ease of automation (commercial equipment), elimination of potentially toxic and environmentally unsafe cyano reagents, and the availability of Hb standard sets which are calibrated against the HiCN primary reference standards. The detection limits of the assay (as low as 5.0 mg/dL) are comparable to the hemoglobin cyanide method [70]. The major disadvantages are that there is still a potential health risk in using benzidine dyes and an expense associated with disposal of reagents and products. Moreover, the reported dynamic range of this method is low (5 mg/dL to 50 mg/dL) [73], and possible reaction inhibition (by as much as 40 %) [74] may occur from calcium-chelating anticoagulants (e.g., citrates, oxalates, EDTA) [73], albumin [75], or other non-specific plasma components [74] which may interfere with H_2O_2 oxidation.

For these reasons, direct optical methods, such as those by Harboe [76], Cripps [77], or Taulier [78], with comparable sensitivity and reproducibility may be substituted. However, as noted above, chemically induced alterations to hemoglobin and its spectra can occur which may invalidate some of the hemoglobin assays. Moreover, compensation needs to be made for endogenous plasma background interference, since it can also alter the hemoglobin spectra [72]. The researcher should be aware of these limitations in the plasma hemoglobin assays and ascertain whether an appropriate technique [64], [65], [72], [75] is being used. This includes evaluating the test supernatant for the presence of a precipitate and comparing its optical spectra (e.g., 400 nm to 700 nm) to that of isolated oxyhemoglobin.

C.6.1.3.2 Immunonephelometric method

The immunonephelometric method is based on determination of plasma hemoglobin by means of nephelometry using a commercially available antibody. This method is for routine work. There is a good correlation and comparability to the optical techniques [79].

C.6.2 Blood and blood component preservation

This subclause presents the best demonstrated practices for the preservation of human blood components by the American Association of Blood Banks [80] and the Council of Europe [81]. In general, materials and devices should be tested using blood whose chemical condition mimics that which the device would experience clinically (e.g., proper choice of anticoagulant, minimal use of blood preservatives, and appropriate blood pH [63].

Anticoagulant solutions have been developed for use in blood collection that prevent coagulation and permit storage of erythrocytes for a certain interval of time. These solutions all contain sodium citrate, citric acid, and glucose; additionally, some contain adenine, guanosine, mannitol, sucrose, sorbitol, and/or phosphate, among others [82–87]. Although heparin is not used for blood preservation, it is often used for anticoagulation clinically with patients exposed to medical devices.

Blood clotting is prevented by citrate binding of calcium. Erythrocytes metabolize glucose during storage. Two molecules of adenosine triphosphate (ATP) are generated by phosphorylation of adenosine diphosphate (ADP) for each glucose molecule metabolized via the Embden-Myerhoff anaerobic glycolysis cycle. The ATP molecules support the energy requirements of the erythrocyte in maintenance of membrane flexibility and certain membrane transport functions. Conversion of ATP to ADP releases the energy necessary to support these functions. In order to prolong storage time, alkalinity must be reduced by addition of citric acid to the anticoagulant solution. This provides a suitably high hydrogen ion concentration at the beginning of erythrocyte storage at 4 °C. Increasing acidity during storage reduces the rate of glycolysis. The adenosine nucleotides (ATP, ADP, AMP) are depleted during storage and the addition of adenosine to the anticoagulant solution permits synthesis of replacement AMP, ADP, and ATP.

A considerable portion of glucose and adenine is removed with plasma when erythrocyte concentrates are prepared. Sufficient viability of the erythrocytes can only be maintained after removal of plasma if the cells are not over-concentrated. Normal citrate phosphate dextrose (CPD)-adenine erythrocyte concentrates should not have an erythrocyte volume fraction greater than 0.80. Even if more than 90 % of the plasma is removed, erythrocyte viability can be maintained by addition of an additive or suspension medium. Sodium chloride, adenine, and glucose are necessary for viability, while mannitol or sucrose can be used to further stabilize the cell membrane and prevent hemolysis [80].

The suitability of containers for the storage of blood products is evaluated by various methods that measure the quality of the blood product [70], [83]. The container with blood product containing an appropriate anticoagulant is stored upright at 1 °C to 6 °C under static conditions. At predetermined intervals, the amount of cell-free plasma hemoglobin is measured to assess the viability and quality of the stored product. The quality of the stored product can be enhanced by gentle mixing once a week. Evaluation of storage in the container indirectly evaluates the

permeability of the container to waste carbon dioxide from erythrocyte metabolism, in the absence of other confounding factors.

C.6.3 Protection of employees handling blood

Written procedures are necessary for protecting employees receiving, handling, and working with potentially contaminated human blood. Potentially contaminated materials include blood and other body fluids and products, equipment which has been or may have been in contact with blood or other body fluids, and materials used in the culturing of organisms causing blood-borne infections [88].

C.6.4 Blood collection (phlebotomy)

While it is not possible to guarantee 100 % sterility of the skin surface for phlebotomy, a strict, standardized procedure for preparation of the phlebotomy area should exist. It is especially important to allow the antiseptic solution to dry on the skin surface prior to venipuncture and that no further contact is made with the skin surface before the phlebotomy needle has been inserted [80].

A closed container system (i.e., one that does not contain room air) is preferred for blood collection for the prevention of microbial contamination. Needle punctures in the rubber seal of the specimen vial should be completely closed after withdrawal of the needles; otherwise, the partial vacuum created following cooling can draw in contaminated air [80].

NOTE—Use of a vacuum tube has the potential to cause slight hemolysis.

Blood collected in an open system can be contaminated by exposure to room air and is not considered sterile. Microbial contamination is a known cause of hemolysis.

C.6.5 Species selection

Ideally, hemolysis testing should be done with human erythrocytes. However, several factors can make such a choice difficult or impossible. In some countries, human blood supplies are limited and must be reserved for human transfusion. Health criteria for human and animal donors should also be considered. All blood has a limited “shelf life” and it may be more difficult to obtain human blood cells on a timely basis. If animal erythrocytes are used, attention should be paid to ensure 100 % hemolysis to obtain total hemoglobin content due to differences in membrane stability among animal species. Negative controls should cause minimal hemolysis so that the activity of the test material is not masked. Rabbit and human erythrocytes are reported to have similar hemolytic properties, whereas monkey erythrocytes are more sensitive and guinea pig erythrocytes less sensitive [89].

C.6.6 Evaluation of hemolysis—*in vitro*, *ex vivo*, and *in vivo* exposure to blood or blood components

Hemolysis can be evaluated by exposure of materials or devices under *in vitro*, *in vivo*, and *ex vivo* conditions. *In vitro* conditions are used to evaluate materials as well as devices. *Ex vivo* and *in vivo* conditions are used to evaluate devices which may contain more than one material.

In vivo and *ex vivo* assessments in animal models or during clinical trials are possible. Justification can be made for either of the following study designs. In the first case, the test device is compared to reference control marketed devices with known acceptable levels of hemolysis. In the second case, the test subject is evaluated for clinically significant consequences of hemolysis.

The purpose of *in vivo* or *ex vivo* tests is to characterize the hemolytic potential of a medical device. The preliminary studies may be *in vitro* and may use fresh or outdated human blood or blood from a nonhuman species. For medical devices indicated for *ex vivo* use, the general practice is to recirculate blood through the device using conditions that simulate the intended clinical usage. These investigations are followed by *ex vivo* simulations in an animal model for some medical devices or by limited, controlled studies in humans. The size of the medical device and the intended function influence the design of these studies.

C.6.7 Direct contact versus indirect methods

Extraction conditions to be used are outlined in ISO 10993-12. Some test methods call for direct contact of the device with erythrocytes, while other methods describe the preparation of an extract which is then exposed to erythrocytes. Test selection should be based upon the device itself and the conditions in which it will be used. Boundary conditions to be considered when elevated temperatures are used are outlined in ISO 10993-12.

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