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Biological evaluation of medical devices —

Part 4: Selection of tests for interactions with blood

Évaluation biologique des dispositifs médicaux —

Partie 4: Choix des essais pour les interactions avec le sang

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 194, *Biological and clinical evaluation of medical devices.*

This third edition cancels and replaces the second edition (ISO 10993-4:2002), which has been technically revised.

It also incorporates the Amendment ISO 10993-4:2002/Amd 1:2006.

The following changes were made:

a) some definitions have been revised and new definitions have been added;

b) Tables 1 and 2 have been consolidated into a single new Table 1 with test categories and headers reorganized to emphasize and include material and mechanical-induced haemolysis testing and *in vitro* and *in vivo* testing for assessment of risk for thrombosis;

c) Tables 3 and 4 have been consolidated into a single new Table 2 with a simplified list of suggested and most common tests;

d) Annex B has been updated to cover only the most common practiced tests for assessing blood interactions;

e) Annex C has been added to cover the topic of *in vivo* thrombosis and methods for testing;

f) Annex D, which was Annex C in the previous edition, has been updated and now includes added information on mechanically-induced haemolysis;

g) Annex E has been added to cover the topic of complement testing and best test method practices;

h) Annexes F and G have been added to present the less common tests used to assess interactions with blood and the tests that are not recommended for preclinical assessment of medical device blood interaction, respectively. Many of these methods were previously included in Annex B;

i) subtle language refinements can be found throughout the revised document;

j) the Bibliography has been reorganized by common subjects of interest and updated with additional and more current references.

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 document was the publication, *Guidelines for blood/material interactions*, Report of the National Heart, Lung, and Blood Institute[14] chapters 9 and 10. This publication was subsequently revised[15].

Biological evaluation of medical devices —

Part 4:
Selection of tests for interactions with blood

1 Scope

This document specifies general requirements for evaluating the interactions of medical devices with blood.

It describes

a) a classification of medical 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,

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 the knowledge and precision of tests for evaluating interactions of devices with blood. This document describes biological evaluation in general terms and may not necessarily provide sufficient guidance for test methods for a specific device.

The changes in this document do not indicate that testing conducted according to prior versions of this document is invalid. For marketed devices with a history of safe clinical use, additional testing according to this revision is not recommended.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10993-1, *Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process*

ISO 10993-12, *Biological evaluation of medical devices — Part 12: Sample preparation and reference materials*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 10993-1, ISO 10993-12 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
anticoagulant
agent which prevents or delays blood coagulation

EXAMPLE Heparin, ethylenediaminetetraacetic acid (EDTA), sodium citrate.

3.2
blood/device interaction
interaction between blood or a blood component and a device

3.3
coagulation
phenomenon that results from activation of the clotting (coagulation) factor cascade

Note 1 to entry: Factors of the coagulation cascade and fibrinolytic systems can be measured following exposure to devices either *in vitro* or *in vivo*.

3.4
complement system
part of the innate immune system consisting of over 30 distinct plasma proteins, including enzymes, cofactors, and cellular receptors which may be involved in the promotion of thrombosis

Note 1 to entry: Effector molecules produced from complement components are possible components in the phenomena of inflammation, phagocytosis and cell lysis. Complement activation related to immunotoxicity, hypersensitivity and generation of anaphylatoxins is not covered in this document. (See ISO/TR 10993-20.)

Note 2 to entry: The focus in this document is complement activation as it can promote and accelerate haemolysis, platelet and leukocyte activation and thrombosis on device material surfaces. (See also Annex E on complement activation.)

3.5
direct blood contact
term used when the device or device material comes into physical contact with blood or blood constituents

3.6
embolization
process whereby a blood thrombus, or foreign object, is carried in the bloodstream and which may become lodged and cause obstructed blood flow downstream

3.7
***ex vivo* test system**
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 1 to entry: If using an animal model, the blood may be shunted directly back into the animal (recirculating) or collected in test tubes for evaluation (single pass). In either case, the test chamber is located outside the body.

3.8
haematology
study of blood that includes quantification of cellular and plasma components of the blood

3.9
haematocrit
ratio of the volume of erythrocytes to that of whole blood in a given sample

3.10
haemolysis
liberation of haemoglobin from erythrocytes, either by destruction or through a partially damaged but intact cell membrane

3.11
haemocompatible
<device or device material> able to come into contact with blood without any appreciable clinically-significant adverse reactions such as thrombosis, *haemolysis* (3.10), platelet, leukocyte, and complement activation, and/or other blood-associated adverse event occurring

3.12
indirect blood contact
nature of devices that contact the patient's blood path at one point and serve as a conduit for entry into the vascular system

EXAMPLE Drug and parenteral nutrition solution delivery devices.

3.13
legally-marketed comparator device
LMCD
approved, or cleared long-established, and recognized-to-be-safe medical device used as a reference control in an *in vitro* or *in vivo* safety evaluation of a test device of similar design, material(s), and clinical use

Note 1 to entry: It may be necessary that the LMCD be legally marketed in the same region as the regulatory submission for the test device.

3.14
non-blood-contact
nature of the device or material contact with the patient's body where the device or potentially extracted material does not have direct or indirect contact with blood

3.15
colloidal osmotic pressure
total influence of the proteins or other large molecular mass substances on the osmotic activity of plasma

3.16
platelets
anuclear, cellular bodies that are present in blood and contribute to the process of thrombosis by adhering to surfaces, releasing factors, and/or aggregating to form a haemostatic plug

3.17
platelet adherent
<material or device> having the tendency to allow or promote *platelets* (3.16) to attach to its surface

Note 1 to entry: This is often characterized relative to a negative control, positive control, and/or LMCD upon blood contact due to its surface properties.

Note 2 to entry: Platelet adherent does not necessarily mean platelet activating, i.e. platelets on a surface may or may not be activated.

3.18
thrombin generating
<material or device> due to its surface properties, having the tendency to promote or show increased thrombin formation

Note 1 to entry: This is often characterized relative to a negative control, positive control, and/or LMCD upon blood contact.

3.19
thrombogenic
<material or device> due to its surface properties, having the tendency to form or promote thrombus formation

Note 1 to entry: This is often characterized relative to a negative control, positive control, and/or LMCD upon blood contact.

3.20
thromboembolization
process where a dislodged *thrombus* (3.21) is carried downstream, where it may cause subsequent vascular blockage or occlusion

3.21
thrombus
coagulated mixture of red blood cells, aggregated *platelets* (3.16), fibrin and other cellular elements

3.22
thrombosis
formation of a *thrombus* (3.21) under *in vivo*, *ex vivo*, or *in vitro* simulated conditions, caused by activation of the coagulation system and *platelets* (3.16) in flowing whole blood

Note 1 to entry: Thrombosis can also occur in regions of a blood vessel or device where there is stasis.

3.23
whole blood
unfractionated blood drawn from a human donor or test animal

Note 1 to entry: The blood may be non-anticoagulated or anticoagulated, e.g. contain sodium citrate or heparin as an anticoagulant.

4 Abbreviated terms

Bb	enzymatically active fragment of Factor B produced by cleavage (by Factor D) in the activation of the alternative pathway
β-TG	beta-thromboglobulin
C4d	degradation product of C4 by classical pathway complement activation
C3a, C5a	complement split products from C3 and C5
CH-50	amount of complement required to lyse 50 % of a RBC suspension
D-Dimer	specific fibrin degradation products (F XIII cross-linked fibrin) consisting of D-fragment dimer
ELISA	enzyme-linked immunosorbent assay
FDP	fibrin/fibrinogen degradation products
FPA	fibrinopeptide A
F1.2	the non-catalytic fragment split off from prothrombin in its conversion to thrombin (also referred to as F1+2)
iC3b	inactive form of C3b, a sub-fragment of C3
IFU	instruction for use
IVC	inferior vena cava
MRI	magnetic resonance imaging
PET	positron emission tomography
PF-4	platelet factor 4

PRP	platelet-rich plasma
PT	prothrombin time
PTT	partial thromboplastin time
SC5b-9	product of terminal pathway complement activation
SEM	scanning electron microscopy
TAT	thrombin-antithrombin complexes
TCC	terminal complement complex; also called membrane attack complex (MAC); estimated by measuring SC5b-9
TT	thrombin time
TxB2	thromboxane B2

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

5.1 Non-blood-contact devices

Non-blood-contact devices are devices that do not have direct or indirect contact with either blood or blood constituents that reside in the body or that are returned to the body. An *in vitro* diagnostic device and a blood-collection tube are examples of non-blood-contact devices. Some devices, such as introducer systems for implants, may contain both blood-contacting and non-blood-contacting components.

5.2 External communicating devices

5.2.1 General

These are devices that contact the circulating blood and serve as a conduit into the vascular system. Some devices may have components or portions with different types of contact (direct and indirect). Examples include but are not limited to the following.

5.2.2 External communicating devices that serve as an indirect blood path

— blood collection devices;

— cannulae;

— cell savers;

— devices for the storage and administration of blood and blood products (e.g. tubing and bags);

— extension sets;

— intravascular catheters.

5.2.3 External communicating devices directly contacting circulating blood

— atherectomy devices;

— blood monitoring devices with direct or indirect blood contact;

— cardiopulmonary bypass circuitry;

— devices for adsorption of specific substances from blood;

— donor and therapeutic apheresis equipment;

— extracorporeal membrane oxygenators;

— haemodialysis/haemofiltration devices;

— interventional cardiology and vascular devices;

— intravascular catheters (balloon, imaging, laser, ultrasound);

— leukocyte removal filters;

— percutaneous circulatory support devices;

— retrograde coronary perfusion catheters;

— vascular guide wires.

5.3 Implant devices

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

— annuloplasty rings;

— arteriovenous shunts;

— blood monitors (implantable);

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

— embolization devices;

— endovascular synthetic vascular grafts;

— implantable defibrillator and cardioverter leads;

— inferior vena cava filters;

— internal drug delivery catheters;

— intravascular oxygenators (artificial lungs);

— mechanical or tissue heart valves;

— pacemaker leads;

— surgical synthetic or tissue vascular grafts;

— vascular stents.

6 Characterization of blood interactions

6.1 General requirements

IMPORTANT — Since this is a horizontal International Standard, sound rationales can be supplied to justify the choice of test category(ies) based on the device being characterized. For example, *in vivo* testing for evidence of thrombosis is frequently the preferred method for device characterization in the thrombosis category. However, in some cases, written rationales that include a combination of tests from the categories of coagulation, platelets, haematology and complement can be used as a substitute for thrombosis testing.

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 divided into several categories based on the primary process or system being measured. Table 1 lists examples of devices which contact circulating blood and the categories of testing appropriate to each device. The list is not all inclusive and sound judgement shall be applied to devices not listed in the tables.

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 document.

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. The simulation should include an appropriate duration of contact, temperature, sterile condition, anticoagulant (and level; see 6.1.12) and flow conditions. For example, for devices of defined geometry such as a vascular stent, the surface area used in the test, in cm^2, shall be given consideration relative to the fluid volume of the *in vitro* test system. For devices with undefined or complicated geometry (such as a dispersion of PVA particles used as an embolization agent), mass should be used instead of surface area to determine the amount of sample used in test system.

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

Appropriate type and level of anticoagulant may be case specific depending on both the device use indication and the type of test conducted. Include information on the specific type and level of anticoagulation used and provide a discussion on the ability to discern positive and negative responses. For further information, see 6.1.6 and C.2 for animal studies, 6.1.12 for *in vivo* and *ex vivo* tests, 6.3.1 for *in vitro* tests and A.3 for catheters and guide wires.

As many tests for haemocompatibility are recognized to be strictly surface-contact dependent, such tests (e.g. complement activation) will not apply to indirect contact applications.

6.1.3 Controls (positive and negative) shall be used unless their omission can be justified. Where possible, testing should include a relevant predicate device already in clinical use (i.e. a LMCD) or a well-characterized material[6].

Controls should include negative and positive reference materials. All materials and LMCDs 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 sterilized device or device component should be tested under conditions which simulate or exaggerate clinical application.

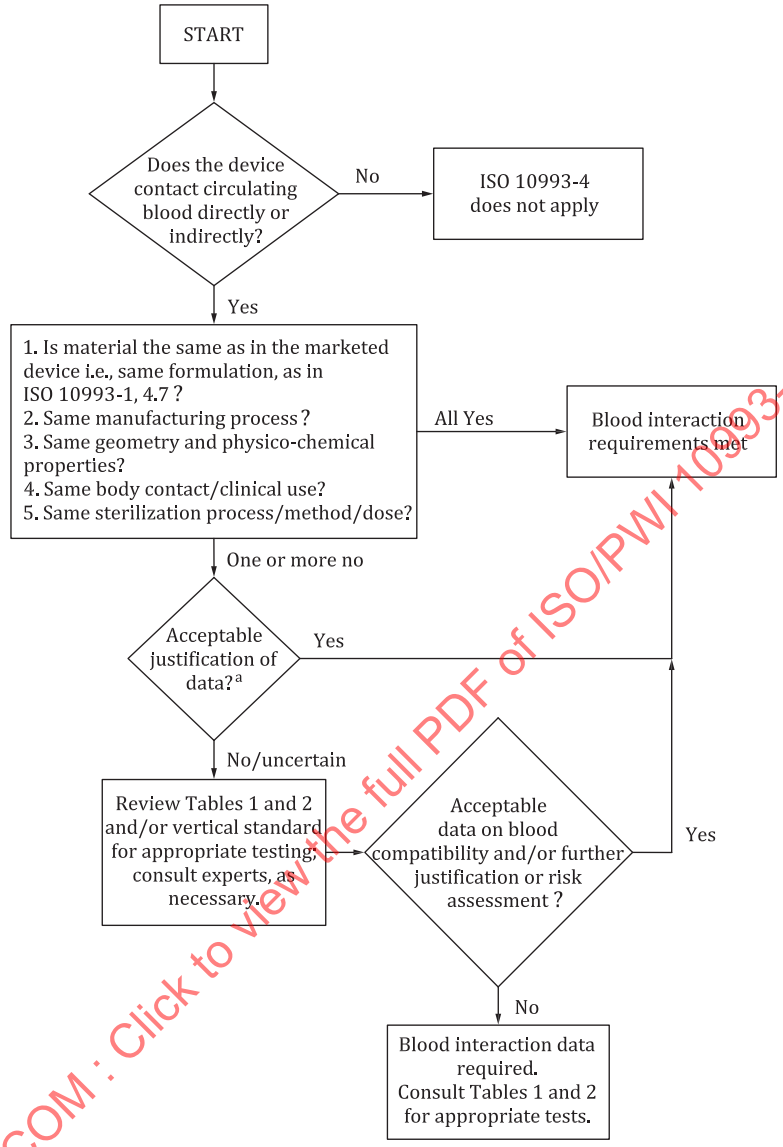
NOTE 1 Changes in manufacturing process (including use of manufacturing aids) that could affect the surface properties, or chemistry of the complete sterilized device, could also impact haemocompatibility.

NOTE 2 Where aging could impact the final device properties, use of aged samples can also be necessary. (For example, the properties of biologically active coatings such as heparin could change over time.)

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. In addition, the capacity of short-term *in vitro* or *ex vivo* tests to predict performance in actual clinical applications is thought to be higher when the clinical application involves limited exposure rather than prolonged or permanent exposure.

NOTE Simplified testing of candidate device materials (e.g. surface geometric and functional chemical modifications) can serve as a crucial step in device material identification, optimization and selection.

6.1.6 If an animal study is to be conducted, 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. Protocols in such investigations should specifically call out each test category (see 6.2.1) being evaluated and describe the specific method(s) of assessment.



a For direct and indirect contact devices, the necessity for haemocompatibility testing should be considered based upon appropriate risk analysis, including prior haemocompatibility testing, clinical data, extractable/leachable data, and/or information on surface characteristics. For example, for devices with direct contact, extractable/leachable testing may not be sufficient if the surface morphology is changed, even if the extractable/leachable chemistry is the same (see ISO 10993-1).

Figure 1 — Decision tree to help determine whether testing for interaction with blood is necessary

Table 1 — Circulating blood-contacting devices or device components and the categories of appropriate testing for consideration — External communicating devices and implant devices

Device examples	Test category						
	Haemolysis		Thrombosis				
			in vitro				*In vivo*/ *Ex vivo*[a]
	Material-induced	Mechanically-induced	Coagulation	Platelet activation	Complement [d]	Haematology	
External communicating devices							
Blood monitors (temporary/*ex vivo*)[b]	X		X	X		X	
Blood storage and administration equipment (e.g. infusion/transfusion sets), blood collection devices, extension sets	X		X	X		X	
Catheters in place for less than 24 h (e.g. atherectomy devices, intravascular ultrasound catheters, antegrade/retrograde coronary perfusion catheters, guide wires); cannulae	X		X[c]	X[c]		X[c]	X[c]
Catheters in place for more than 24 h (e.g. parenteral nutrition catheters, central venous catheters); cannulae	X		X[c]	X[c]		X[c]	X[c]
Cell savers[b]	X		X	X			
Devices for adsorption of specific substances from blood[b]	X	X	X	X	X		
Donor and therapeutic aphaeresis equipment and cell separation systems[b]	X	X	X	X	X		
Cardiopulmonary bypass system[b]	X	X	X[c]	X[c]	X	X[c]	X[c]
Haemodialysis/haemofiltration equipment[b]	X	X	X[c]	X[c]	X	X[c]	X[c]
Leukocyte removal filter[b]	X		X[c]	X[c]	X	X[c]	X[c]
Percutaneous circulatory support devices[b]	X	X	X[c]	X[c]	X	X[c]	X[c]
Implant devices							
Annuloplasty rings, mechanical heart valves	X	X					X
Embolization devices	X						X
Endovascular grafts	X						X
Implantable defibrillator and cardioverter leads	X						X
Intra-aortic balloon pumps[b]	X	X					X
Pacemaker leads	X						X
Prosthetic (synthetic) vascular grafts and patches, including arteriovenous shunts	X						X

[a] Thrombosis is an *in vivo* or *ex vivo* phenomenon, but can be simulated with *in vitro* conditions. *In vivo* or *ex vivo* testing might not be necessary if clinically relevant *in vitro* thrombosis testing is performed.

[b] Direct or indirect blood-contacting components only. For components that have only indirect blood contact, *in vivo* thrombogenesis and mechanical haemolysis or complement activation might not be necessary.

[c] It is recognized that coagulation, platelet and leucocyte responses are primarily involved in the process of thrombosis. Therefore, it is up to the manufacturer to decide if specific testing in the coagulation, platelet and haematology test categories is appropriate as an alternate to *in vivo* testing.

[d] See also ISO/TS 10993-20 for information on when complement activation should be considered for other end points such as anaphylaxis.

Table 1 *(continued)*

Device examples	Test category							
	Haemolysis		Thrombosis					
			in vitro					
	Material-induced	Mechanically-induced	Coagulation	Platelet activation	Complement [d]	Haematology	*In vivo/ Ex vivo* [a]
Stents (vascular)	X						X
Tissue heart valves, vascular grafts and patches and AV shunts	X						X
Total artificial hearts	X	X					X
Vena cava filters	X						X
Ventricular-assist devices [b]	X	X					X

[a] Thrombosis is an *in vivo* or *ex vivo* phenomenon, but can be simulated with *in vitro* conditions. *In vivo* or *ex vivo* testing might not be necessary if clinically relevant *in vitro* thrombosis testing is performed.

[b] Direct or indirect blood-contacting components only. For components that have only indirect blood contact, *in vivo* thrombogenesis and mechanical haemolysis or complement activation might not be necessary.

[c] It is recognized that coagulation, platelet and leucocyte responses are primarily involved in the process of thrombosis. Therefore, it is up to the manufacturer to decide if specific testing in the coagulation, platelet and haematology test categories is appropriate as an alternate to *in vivo* testing.

[d] See also ISO/TS 10993-20 for information on when complement activation should be considered for other end points such as anaphylaxis.

6.1.7 *In vitro* tests are regarded as useful in screening external communicating devices or implants and potential early interactions between devices/materials with blood, but may not be accurate predictors of blood/device interactions occurring upon prolonged or repeated exposure or permanent contact (see 6.3.1).

NOTE For new devices or devices where there is a change in geometry, testing under physiologic flow can be needed. For long-term catheters or permanent implants, *in vitro* test systems might not be sufficient due to blood stability issues.

6.1.8 Devices or device components which come into very brief/transient contact with circulating blood (e.g. lancets, hypodermic needles, capillary tubes that are used for less than 1 min) generally do not require blood/device interaction testing.

NOTE 1 For products made with materials such as coatings that could be left in contact with blood after the device is removed, blood/device interaction testing might be necessary.

NOTE 2 If some device components (e.g. syringe bodies) are in contact with fluids that will ultimately be injected into the patient, and the storage time is unspecified or greater than 1 min, haemolysis testing of the fluid-contacting component would be needed, even though the device itself would be in contact with circulating blood for less than 1 min.

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. For devices that operate over a range of conditions, the extreme and the average conditions should be considered. 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 (with the exception of established test methods with animal blood, such as some haemolysis tests). 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[204]. The use of animals, such as the rabbit, pig, calf, sheep or dog, can also be acceptable for a particular type of test. However, since species differences may be significant (for example, platelet adhesion[148][150], thrombosis[44] and haemolysis[47] tend to occur more readily in the canine than in the human), all results of animal studies shall be interpreted with caution. The species selected and the number of animals used shall be justified (see also 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 type 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 and/or described in the product IFU or other appropriate literature. Species differences should also be considered when determining the appropriate level of anticoagulation.

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. An *in vitro* flow model with application-consistent exposure conditions and relevant measurements can be used to evaluate the effect of modifications to a clinically accepted device.

6.1.14 A sufficient number of replications of a test including suitable controls should 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 blood-device interactions[213]–[216]. Balance should be considered between statistical evaluation and animal welfare when applying *in vivo* testing; see ISO 10993-2.

6.1.15 The recommendations within 6.1, together with Figure 1 and Table 1, serve as a guide for the selection of tests listed in Table 2. Further guidance on pre-clinical evaluations is given in Annexes A to G. In summary, the following procedure shall be performed:

a) determine which potential blood interaction categories (see 6.2) are appropriate for consideration to establish safety of the particular device (see examples in Table 1);

b) evaluate the *existing information* in each test category for the device;

c) where *sufficient safety information exists*, prepare an appropriate rationale to support this conclusion and that further testing is not necessary;

NOTE Any difference in formulation, geometry, surface properties, fabrication methods, sterilization technique and/or clinical use could limit the use of safety information on a similar product.

d) where *insufficient information exists* under a test category(ies), select appropriate tests, based upon examples in Tables 1 and 2, to supply the additional safety information.

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 (see examples in Table 1). The tests are divided into the following categories based on the primary process or system being measured:

- haemolysis
  - material-induced
  - mechanically-induced
- thrombosis
  - *in vitro*
    - coagulation
    - platelet activation
    - complement
    - haematology
  - *in vivo/ex vivo*

The principles and scientific bases for these tests are given in Annexes A to E.

Table 2 — Common tests used to assess interaction with blood

Tests by categories	
Haemolysis	Material-induced (e.g. ASTM[17], NIH[28], MHLW[22])
	Mechanical-induced
Thrombosis (*in vivo*, *ex vivo*)	Gross analysis[a], percentage occlusion, light microscopy, SEM
***In vitro* thrombosis**	
Coagulation	Thrombin (e.g. TAT, F1.2), fibrin (e.g. FPA) assays, PTT assay
Platelet activation	Platelet count (% loss) and some indicator of activation (e.g. release products or platelets surface markers such as βTG, PF4, TxB2) or SEM (platelet morphology)
Haematology	Complete blood count (CBC), leucocyte activation
Complement system	SC5b-9 (C3a optional)

[a] Included in all animal studies (see B.2.1 and ISO 10993-6).

Not all tests are needed for each category and testing in each category might not be equivalent.

6.2.2 Non-contact devices

These devices do not require blood/device interaction testing.

6.2.3 External communicating devices and implant devices

After using Table 1 to align a new device under investigation with similar existing devices and noting the test categories for consideration, use Table 2, Annexes A and E to guide the selection of appropriate tests for assessing blood interactions.

6.2.4 Limitations

Testing and study design parameters may present certain practical limitations/considerations based upon science, technology and the particular application. For example:

a) materials/devices in a high blood flow (arterial) environment may interact with blood differently in a low blood flow (venous) environment;

b) blood interactions may occur with all materials, i.e. the test materials/test devices *and* the non-test materials (e.g. test system). Caution shall be taken to not confound blood interactions associated with the test materials to those contributed by other factors;

c) studies that rely on just one type of test for blood interactions may be less predictive of the true response than studies that include several different tests for blood interactions;

d) immunoassays for detecting protein indicators of haemocompatibility, e.g. TAT, C3a, etc., are often available for human blood testing but are not generally available for use or functional with blood from other species.

6.3 Types of tests

6.3.1 *In vitro* tests

In vitro testing (models) should consider designs to simulate the anticipated worst-case clinical use conditions of each device application. Variables that shall be considered when using *in vitro* test methods

include haematocrit, anticoagulant (type and amount), test sample preparation, test sample age, blood/blood component age, test sample storage, aeration and pH, temperature, proper randomization, test sample surface area to blood volume ratio and for dynamic studies, fluid flow conditions, especially flow rate, wall shear rate and pressure(s). Tests shall be started with minimal delay, usually within 4 h of blood draw, since some properties of blood change rapidly following collection. Alternatives to the latter may be feasible if validated. In some cases, the resulting samples can also be frozen using appropriate techniques for future analysis if the freeze/thaw process does not affect the analyte being assessed.

NOTE Clinically relevant types and amounts of anticoagulant may or may not be appropriate, depending on the test system and the ability to discern positive and negative responses.

When used to evaluate the haemocompatibility of device modifications, *in vitro* testing for haemolysis, thrombus formation, platelet and coagulation responses may be assessed and compared between the modified device and the clinically accepted device (see A.1.4).

6.3.2 *Ex vivo* tests

Ex vivo tests shall be performed when the intended use of the device is *ex vivo*, e.g. an external communicating device. *Ex vivo* testing can also be useful when the intended use is *in vivo*, e.g. to assess the acute response to 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[44][46][47][50][54][70][78][80]. Blood flow rates can be measured with either Doppler or electromagnetic flow probes. Alterations in flow rates may indicate the extent and course of thrombus deposition and embolization. Simple thrombus build-up can be assessed by gross and or microscopic visualization. Other more advanced and technically demanding tools have also been used[53][69][73][74][79].

6.3.3 *In vivo* tests

In vivo testing involves implanting the material or device in animals. Vascular patches, vascular catheters, vascular grafts, vascular stents, annuloplasty rings, heart valves and circulatory assist devices are examples of devices tested *in vivo*. Given the diversity of blood-contacting medical device applications, *in vivo* test models are expected to be equally diverse, in order to appropriately mimic each clinical application.

"Patency of a conduit or device (i.e. the unimpeded flow of blood through the device)" is a common measure of success or failure for some *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 (e.g. ventricular-assist devices, artificial hearts, aortic prosthetic grafts)[184][187][236][237].

Methods to evaluate *in vivo* interactions without terminating the experiment are available. Arteriograms or imaging from intravascular ultrasound (IVUS) catheters are used to determine 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[46][72][79].

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 flow rate systems may give substantially different results when compared with the same material evaluated in a high flow rate system. In such cases, test system performance *in vivo* should carry more importance than *in vitro* test results.

In vivo test protocols should contain precise and stand-alone sections stating how each test category identified for testing, i.e. haemolysis, thrombosis, coagulation, platelets, haematology and complement system, will be evaluated.

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 contains guides to determine when testing is necessary, which 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. The classification of blood/device interactions in A.1.2 is provided as background.

A.1.2 Classification

A.1.2.1 Interactions which mainly affect the device and which may or may not have an undesirable effect on the animal or human are as follows:

a) 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;

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

c) formation of pseudointima or neointima on the blood contacting surface and tissue capsule on the surface of the device;

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

A.1.2.2 Interactions which have a potentially undesirable effect on the animal or human are as follows:

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

b) formation of thrombus on the device surface;

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

d) injury to circulating blood cells resulting in anaemia, haemolysis, leucopoenia, thrombocytopenia or altered function of blood cells;

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

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

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

NOTE For items b), c) and d) above, some devices such as embolization coils require thrombus formation to be functional.

A.1.3 Advantages and limitations of animal models

Animal models permit the closest end use simulation of clinical devices prior to actual testing in humans. They permit continuous device monitoring and a systematic controlled investigation of important variables. However, the choice of an animal model may be restricted by size requirements, the availability of certain species and cost. For example, a device may not be operated under the full range of clinical use conditions in an animal model due to anatomical limitations. 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 anaesthetics, 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 exhibit a close similarity to the human in haematological values, blood coagulation mechanism and cardiovascular system[50]. An additional advantage of a non-human primate is that many of the immunological probes for thrombosis assays developed for humans are suitable for use in primates. These probes include PF-4, β-TG, FPA, TAT and F1.2. 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 (as a challenging or accelerated model) when evaluating this complication. Pigs and sheep are generally regarded as suitable animal models because of their haematological and cardiovascular similarities to the human[71][148][149][150]. The effect of the surgical implant procedure on results should be kept in mind and appropriate controls included. The final decision on the use of an animal or *in vitro* model ultimately involves consideration of the availability and ethical use of animals (see ISO 10993-2), the availability and limits of *in vitro* blood models and the applicability of proper statistics for sound conclusions[213][214][215][216].

A.1.4 Advantages and limitations of *in vitro* models

In vitro blood-exposure models are attractive approaches to testing the haemocompatibility of medical materials and cardiovascular devices because they allow

a) avoidance of costly animal models,

b) high replication testing of test objects alongside controls and reference materials using the same batch of blood and at the same time,

c) use of human or animal blood where flow, temperature and anticoagulation is standardized,

d) worst case scenario testing, where activation products accumulate without clearance by kidneys or liver or other organs and activation-inhibiting functions of endothelial cells are absent, and

e) isolation from confounding factors associated with device implantation/tissue injury associated with *in vivo* usage.

Such testing of medical materials and devices should simulate as best as possible the range of clinical conditions of blood exposure to the device, since testing on blood under clinically inapplicable conditions, e.g. non-clinical anticoagulation (types or levels) and flow conditions, can make interpretation of results difficult. Whenever possible, consult product IFU brochures or common medical practice literature for applicable anticoagulant type(s) and amount(s). When appropriate, testing over the full range of labelled use conditions for the device should be considered. For example, to evaluate mechanically-induced haemolysis and platelet activation, testing is often performed at the highest blood flow rate. For thrombosis testing, the minimum labelled blood flow rate may be important to characterize the safety of the device. Since it has been shown that responses in blood may differ considerably between various species[47][148][149][150], the use of human blood is more relevant to the interpretation of results. Another advantage in using human blood is that it offers a more detailed array of test methods, since most contemporary bioanalytical methods are based on human blood components/epitopes. Conversely, there are certain limitations in the volume of blood that can be obtained from a single human donor. Thus, the use of blood from a single large animal may be more practical in cases where the model designed to simulate clinical-relevant conditions presents a large volume capacity.

To test for general material/device haemocompatibility, the classical Chandler loop *in vitro* test model[43] or modifications thereof[193][194][195][199][200][203] to impart physiological and/or quasi-physiological flow have been used. Alternatively, blood-material (device) exposure using gentle agitation may also be useful in some cases for evaluating the interactions of blood with materials. To gauge the impact of the model on blood, haemolysis and general cell blood count can be monitored to check for blood normalcy. These models appear effective for screening studies, in particular for those applications involving short-term blood exposure.

A.1.5 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 extent of acute variation of haematological, haemodynamic and performance variables, gross thrombus formation and possible embolism. With prolonged or repeated exposure or permanent contact (>24 h and >30 d, respectively), 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 and the development of intimal hyperplasia and infection. In both of these exposure and contact categories, assessment of haemolysis 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, e.g. intimal hyperplasia, fibrotic encapsulation and endothelialization. Importantly, anticoagulation type(s) and amount(s) can have a profound impact on results. For example, at clinically relevant levels, anticoagulation and antiplatelet drugs may substantially reduce or abolish platelet, coagulation and thrombotic responses.

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 elements involved in normal haemostasis. The latter may be clinically insignificant, e.g. platelet consumption by the device could be so small that it does not affect the total platelet count. Alternatively, a device with a large surface area could lead to depletion of platelets or plasma coagulation factors such that the total platelet count may be significantly affected and normal haemostasis may become altered.

Regardless of the animal model used and the particular test category under evaluation, i.e. haemolysis, thrombosis, coagulation, platelets, haematology and complement system, the *in vivo* study protocol should provide sufficient detail in the methods and criteria to be used for evaluation for each test category under investigation. A retrospective report on results for a particular test category, without supporting original plans within the protocol, is often considered unacceptable as regulatory submission documentation.

A.2 Cannulae used for direct vascular access and cannulae used for indirect access

The term "cannulae" has been generally used in two rather different clinical applications. In one application, cannulae are inserted directly through the skin and into one or more major blood vessels. This is done to provide continuous and direct high-volume access to blood. For example, this type of large-diameter cannulae is used during cardiopulmonary bypass surgery as a limited-exposure access device that shunts blood to and from the body for blood oxygenation. Cannula testing, in this example, should take place using exposure conditions that closely replicate clinical use, as such devices can potentially induce some alteration in the levels of circulating blood cells as well as increase factors in the coagulation or complement system. The particular response is often multifactorial as it depends on a variety of factors such as implantation site, insertion technique, subject factors and anticoagulation regimen. The term cannulae has also been used to describe much smaller diameter tubes that are inserted only subcutaneously, and may be used for limited (<24 h) or prolonged (<30 d) indirect exposure to blood. These cannulae, for example, are used for infusion of insulin from drug pumps and in subcutaneous sensing for blood glucose levels. These later type of cannulae, like other indirect blood path devices (see 5.2.2), generally require less testing than devices with direct contact with circulating blood (see 5.2.3 and 5.3).

A.3 Catheters and guide wires

Most of the tests considered under blood-contacting cannulae are relevant to the study of blood-contacting catheters and guide wires. 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, using a clinically approved device of similar dimensions and material(s), be performed using a contralateral artery or vein. Care should be taken not to strip off thrombus upon catheter withdrawal. Evaluation of the device *in situ* may permit assessment of the extent to which intimal or entrance site injuries contribute to the thrombotic process. In general, Doppler blood flow measurements are more informative than angiography. A venous or arterial implant model with anticoagulation pertinent to the clinical application may be a useful tool for evaluating device blood-contact responses, particularly when assessing a new device material or a coating developed to present anti-thrombogenic properties[143][161][162][163]. See C.3. Alternatively, an appropriate *in vitro* model may be more sensitive to detecting such material surface differences.

In cases where anticoagulation is called for, the rationale for the type and level of anticoagulation used in testing should be based upon the clinical application, yet be able to provide sufficient evidence that the test is able to distinguish between positive and negative responses. For example, following simple dose-response kinetics, the thromboresistance of a medical device heparin coating can be completely masked by normal (clinical) levels of solution heparin anticoagulant. However, under a reduced/challenging level of solution heparin, the effectiveness of the heparin coating to reduce thrombus formation becomes more apparent. In cases where the application may not involve use of anticoagulation, testing should be conducted without anticoagulation.

Validation information for testing with a specific type and level of anticoagulation should demonstrate the ability to discern between positive and negative responses.

A.4 Extracorporeal blood oxygenators, haemodialysis/haemofiltration devices, donor and therapeutic apheresis equipment, devices for adsorption of specific substances from blood

The blood responses 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 ultrasound 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[158]; other tests of platelet function and release are particularly useful.

Complement activation is caused by both haemodialysers and cardiopulmonary bypass equipment. Clinically significant pulmonary leucostasis and lung injury with dysfunction can result[5][11][16][129]–[147]. For these reasons, it is useful to quantify complement activation or leukopenia with these devices. See also Annex E.

Therapeutic apheresis equipment and devices for adsorption 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 in these and any other high surface area devices should follow the same principles as for extracorporeal oxygenators and haemodialysers.

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 haemolysis, thrombus formation, fibrin formation, thromboembolization, thrombin generation, platelet survival and activation, complement activation and close monitoring of liver,

renal, pulmonary and central nervous system effects. A detailed pathological examination at surgical retrieval is an important component of the evaluation[236][237].

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[186]. Two-dimensional 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, thrombus and evidence of thickening of the valve leaflets are elucidated. Using conventional and colour flow Doppler echocardiography, regurgitation can be identified and semi-quantified[2][185][186][187].

Measurements of platelet survival and aggregation, blood tests of thrombosis and haemolysis, pressure and flow measurements, and autopsy of the valve and adjacent tissues are recommended[205][206].

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 anatomical considerations of the model and the clinical site of use. Patency of a given graft is enhanced by larger diameter and shorter length. Patency can be documented by palpation of distal pulses in some locations and by periodic angiography. Ultrasound, MRI and PET may also be useful. Serial measurements of platelet count, platelet release constituents, fibrinogen/fibrin degradation products and activated coagulation proteins also are recommended. Autopsy of the graft and adjacent vascular segments for vascular tissue responses 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[4][205]. As with many vascular devices, appropriate clinical anticoagulation regimens are critical to device function and performance.

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[205].

Annex B
(informative)

Recommended laboratory tests — Principles, scientific basis and interpretation

B.1 General considerations

B.1.1 Background

The general principles and scientific bases of the *more commonly* used tests to evaluate the categories of haemolysis, thrombosis, coagulation, platelets, haematology and complement system (see 6.2) are described in B.1 to B.3. See Annexes C, D and E for further information on the test categories of thrombosis, haemolysis and complement.

Additional, albeit less common methods, that may be of further value in the evaluation of particular blood/device interactions are described in Annex F. Because of biological variability and technical limitations, the accuracy and predictivity of many of these tests require careful attention to methodology and caution in interpretation of results. Annex G lists tests which are not recommended.

B.4 presents methodology considerations for testing of plasma factors specific to coagulation, platelet and leucocyte activation and complement activation using ELISA (or other similar) techniques.

All references in the bibliography describe in more detail and give examples of various standards, tests and models for consideration.

B.1.2 *In vitro* versus *ex vivo* versus *in vivo* testing

B.1.2.1 A host of *in vitro*, *ex vivo* and *in vivo* models have been used extensively to estimate blood-material interactions[1]–[30][42]–[147][157]–[237]. It is appropriate to select the model most suitable for the device application and test objective and to consult ISO 10993-12 regarding proper sample preparation and control group considerations.

No single *in vitro*, *ex vivo* or *in vivo* model will be appropriate for all applications. Thus, the appropriateness of the model to the application under consideration should be justified.

In vivo tests present a more realistic end-use simulation, yet are complicated by factors such as:

— choice of appropriate animal model;

— interspecies and intersubject variability in responses[47][71][148][149][150];

— scarcity of species-specific commercial test kits for common indicators of thrombosis and coagulation[58][59][60];

— heightened costs and ethical and statistical concerns involved in using animal models.

B.1.2.2 Consult with vertical standards for preferred models[1]–[41]. See also Reference [186] for the juvenile sheep as an accelerated model to study bio-prosthetic valve calcification, Reference [187] for the adult pig or sheep to investigate transvascular-placed valves and surgically implanted valves, References [217] to [231] for the adult canine and sheep femoral replacement models used in testing on small and large diameter vascular graft model, and References [232] to [235] for the porcine coronary model used extensively to investigate stent designs.

B.1.2.3 As described in other parts of ISO 10993, carefully conducted *in vitro* tests offer valid screening tools to assess the biological safety of medical devices and materials.

Important factors in an *in vitro* model that require specification are

— volume of whole blood in the basic test system, e.g. tube, loop, or other model,

— blood exposure time(s),

— blood temperature,

— blood flow condition,

— anticoagulant type and level,

— exposure ratio, i.e. ratio of material/device surface area (cm^2) to volume of whole blood in the system (ml), and

— blood-contacting surface area of the test system itself (cm^2).

NOTE 1 The "blood exposure phase" of an *in vitro* investigation requires a precise definition of the exposure conditions of the test material/device to blood. The closer the test conditions mimic the clinical application, the greater the predictivity becomes of the model and the response(s) being evaluated.

NOTE 2 A "testing phase" follows the exposure phase where specific tests are conducted on the exposed blood, blood plasma or the material/device itself. A test in the testing phase is usually targeted at one or more of the general categories, i.e. haemolysis, thrombosis, coagulation, platelets, haematology and complement system.

B.2 and B.3 examine the common methods used to assess the main categories of blood-materials/device interaction (see Table 2).

B.2 Thrombosis

B.2.1 Gross analysis — Retrieval and examination of device and autopsy of distal organs

Gross analysis should always be included as part of a basic device evaluation, as this segment of a device evaluation is of central importance in evaluating the *in vivo* biological responses to implanted devices. The distribution, visible size and nature of cellular and proteinaceous deposits, and any emboli, can best be determined by a careful and detailed gross examination. Proposed procedures have been published[7][205][206][207].

The rationale behind necropsy of distal organs is to examine for distal effects (such as emboli) of implanted devices. The importance of this analysis varies with device application and is restricted to applications where risk for thromboembolism or material/device embolization, for example with mechanical heart valves and intra-aortic balloon pumps, is intermediate to high[206].

In this type of investigation, low- and/or high-magnification high-resolution colour film or digital images at key points of interest (of the device, and the surrounding tissues, etc.) are taken and labelled appropriately.

B.2.2 Percentage occlusion, surface area covered by thrombus and thrombus-free surface area

Percentage occlusion may be quantitatively assessed during the in-life portion of the study using contrast radiography and ultrasonography imaging techniques. Percentage occlusion can also be visually assessed after an implanted device has been removed. The percentage of occlusion may be a measure of the severity of the thrombotic process in a conduit. However, 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 peri-anastomotic sites in vascular grafts. Thus, a supporting microscopic examination is useful to identify the nature of the occlusive process. Determinations of

surface area covered by thrombus and thrombus-free surface area are semi-quantitative or quantitative tests that can be used on a comparative basis with test and/or control devices.

B.2.3 Light microscopy

By this technique, information can be obtained regarding the density of cells, presence of cellular aggregates, composition of encapsulating tissue, intensity of foreign body response and thrombus or fibrin adherent to materials. The evaluation of geographic distribution of these deposits on the materials or device is also possible. The method is semi-quantitative.

For polymeric or biologically-derived materials and devices, paraffin wax embedding methods and special stains may be used to assess the device-biological interface.

For metal and ceramic materials and devices, more sophisticated hard plastic embedding and sectioning techniques are useful to capture the intact material/device-biological interface[207]–[211].

B.2.4 Scanning electron microscopy (SEM)

For SEM, rationale and interpretation are the same as for light microscopy (see B.2.3). This method has the advantage over light microscopy of providing greater detail about fine structure of components being examined. Quantitative conclusions require sufficient replicate determinations to establish degree of reproducibility. This type of microscopy best reflects what can be seen at surfaces. Cross-sectional analyses can also be used to support the surface observations if additional details regarding the cell and thrombus surface interactions are informative[70][71][143][205][206]. The morphological evaluation of platelet and leukocyte activation, fibrin and thrombus formation after blood or blood component (e.g. platelet-rich plasma) dynamic exposure with comparison to reference controls is valuable[143][173].

B.3 *In vitro* haemocompatibility

B.3.1 Haemolysis — Methods for testing

Haemolysis is regarded as a significant screening test because an elevated *in vivo* plasma haemoglobin level is abnormal and may be indicative of an underlying haemopathology or vascular problem. Properly performed, an elevated plasma haemoglobin level indicates haemolysis, i.e. the release of contents of red blood cells (RBCs), and may reflect erythrocyte membrane fragility or damage to RBCs. In the assessment of blood-material/device interactions, haemolysis may result due to:

a) direct blood contact with the material(s)/device surface(s) (material-induced);

b) indirect contact from exposure to extractable chemicals from the device materials (material induced);

c) exposure to turbulence and elevated (i.e. non-physiological) shear stresses from the device operation (mechanically-induced).

See Annex D for more extensive details on testing for haemolysis.

B.3.2 Coagulation — Methods for testing

B.3.2.1 General

The coagulation cascade has two parallel pathways, the *contact activation pathway* (the intrinsic pathway) and the *tissue factor pathway* (the extrinsic pathway) that merge to form a common pathway. The latter includes the protein thrombin, which catalyses the formation of *fibrin*, a main component of a thrombus. While it is known that the primary pathway for the initiation of blood coagulation is the *tissue factor* pathway, coagulation associated with blood contacting devices and materials occurs through the contact activation pathway. The pathways themselves are a series of reactions in which successive inactive enzyme precursors (referred to as zymogens) interact with their glycoprotein

co-factors to become active components in a cascade of activation events. The reactions culminate in the formation of active thrombin that then catalyses the formation of fibrin. Coagulation factors are generally indicated by Roman numerals, with a lowercase "*a*" appended to indicate the active form. See Figure B.1.

Assessment of coagulation activity, i.e. the degree of change in blood levels of proteins leading to thrombin and fibrin formation (see Figure B.1), has long relied on clinical assays that measure plasma levels of key proteins in the coagulation cascade. Normal resting (homeostasis) levels of coagulation activity are well established, as are some elevated levels observed in various clinical coagulopathies. The presumption for such testing with medical devices is that appropriate materials and device designs should not be associated with excessive coagulation activity that could bring risk to the patient. High levels of coagulation activity may be an indicator of a higher tendency for the material or device to induce or be associated with acute thrombosis or thromboembolism. To measure coagulation activity, clinical laboratories often rely upon test kits that use common enzyme-linked immunosorbent assay technique. In a basic study, which may be either *in vivo* or *in vitro* and will depend upon availability of appropriate antibodies to species-specific target coagulation protein epitopes, blood samples are retrieved under defined conditions and prepared and analysed per assay instructions. Typical defined conditions or factors important in an *in vitro* model are described in B.1.2.3. Comparison of results to appropriate controls such as negative controls (e.g. baseline levels or no material/device exposure) and results on a predicate device(s)/materials(s) is critical. Coagulation activity in blood that is statistically-significantly and biologically-significantly higher than controls may be an indicator of a material/device design that presents a higher risk of coagulation-related complications. Example coagulation activation proteins for which commercially-available ELISA kits are available include TAT (thrombin-antithrombin complexes), F.1.2 (protein fragment released from prothrombin upon formation of thrombin) and FPA (protein fragment released from fibrinogen upon formation of fibrin).

Proteins indicating coagulation activation generally exhibits an initiation, propagation and termination phase[56][57]. This reflects the initial formation reaction(s), a cascade/feedback amplification period and a slowdown/deactivation period where critical precursors may be consumed or the measured protein deactivated by negative control feedback proteins. Thus, order-of-magnitude differences in levels of coagulation activation proteins are to be expected over time. Consequently, an important factor to consider is when the activation phase actually occurs during the time of the material/device-blood contact. For example, the impact of test materials when mixed with blood may be quite different at each phase. In addition, as coagulation protein activation is generally proportional to blood-contacting surface area, surface area (SA) of a device or device material can be very influential on results. For this reason it is important to specify the test SA-to-blood-volume ratio (exposure ratio) in each study. If possible, the exposure ratio may be treated as a variable to aid in understanding the specificity of the material effect. Exposure ratios of 3,0 cm^2 to 6,0 cm^2/ml blood (based on device thickness) are consistent with ISO 10993-12. Other exposure ratios such as 1,5 and 2,0 times this ratio may be worth considering as higher surface areas will theoretically increase the sensitivity of the coagulation responses to the test material.

There will be a physical limitation on the amount of test material that can be tested due to the volume of the test system, e.g. a test tube, and the target exposure ratio. In this case, it may be appropriate to use cut sections of device materials. If the device contains more than one material, the proportion of each in the complete device should be maintained. Care should also be made to avoid introducing cut sections that result in exposure of significant amounts of non-blood contact surfaces.

Naturally, there are a number of mechanisms that have evolved to keep the coagulation cascade in check. One of those mechanisms involves the protein antithrombin. Antithrombin is a serine protease inhibitor that can bind to and deactivate the serine proteases thrombin, FIXa, FXa, FXIa and FXIIa. While antithrombin is constantly active, its interaction with heparin alters its conformation, which greatly accelerates its rate of inhibition of the proteases.

NOTE ELISA testing for blood coagulation factors represents the "testing phase" discussed in B.1.2, i.e. for testing on the blood samples procured *following in vitro* or *in vivo* blood exposure to the medical device or material.

Many standard coagulation assays are designed to detect clinical coagulation disorders which result in *delayed* clotting or excessive bleeding, rather than conditions that *enhance* clotting/thrombosis.

Protocols for evaluating blood/device interactions shall be modified appropriately to evaluate accelerated coagulation induced by biomaterials.

Interactions between the coagulation and complement systems are recognized[143]–[147].

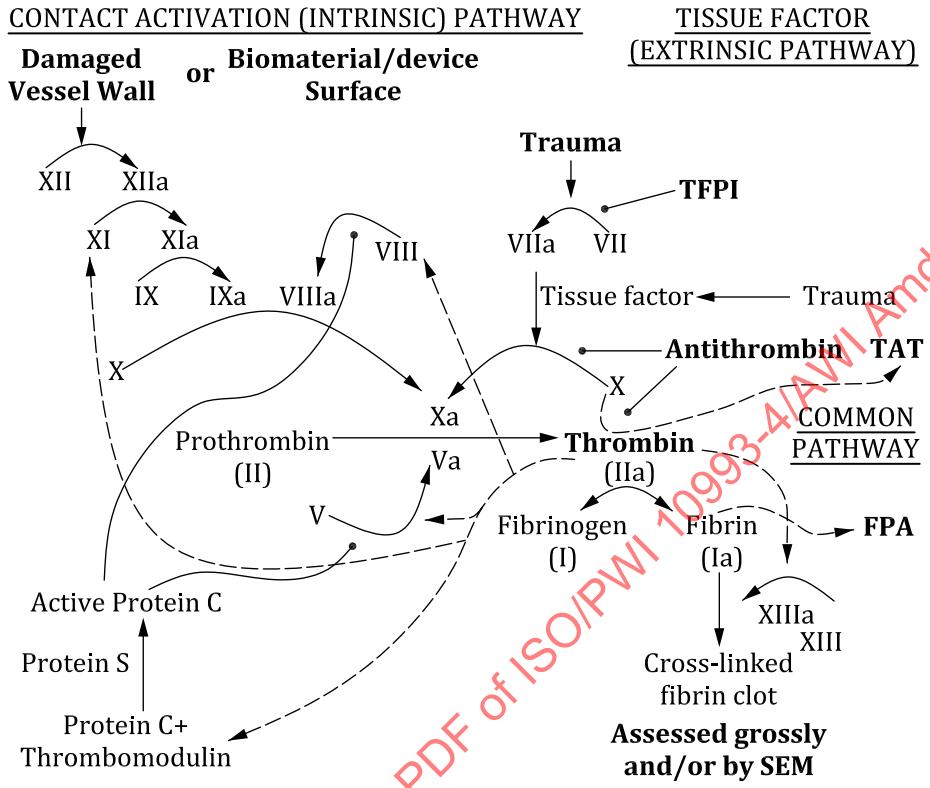


Figure B.1 — Coagulation cascade

B.3.2.2 Thrombin-antithrombin (TAT), F1.2 and fibrin (FPA) ELISA assays

These ELISA assays that directly reflect thrombin (TAT, F1.2) and fibrin (FPA) formation are commercially available. The output is a quantitative estimate of the amount of thrombin present and the amount of fibrin being formed, both of which are reflective of the level of coagulation activity taking place and may be reflective of thrombosis taking place. See B.4 for details on general ELISA methodology.

B.3.2.3 Partial thromboplastin time (PTT)

The partial thromboplastin time is the clotting time of recalcified citrated plasma upon the addition of partial thromboplastin which does not contain an activator. 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 intrinsic coagulation pathway of blood coagulation. Heparin and other anticoagulants cause a prolonged PTT. See Reference [23].

Reagents for tests based on the activated partial thromboplastin time (APTT) include an activator, such as kaolin, celite or ellagic acid. Reagents with such activators should be avoided when assessing the effects of blood-contacting devices or device materials because they mask the coagulation caused by materials or devices.

In coagulation testing of medical materials and devices, it is the device or material itself that serves as the activator of coagulation. Appropriate positive and negative control materials should be used whenever available. A negative control, the blood plasma itself without the material/device, should be included.

B.3.3 Platelets — Methods for testing

B.3.3.1 General

Assessment of platelets and their state of activation has been described throughout the literature, e.g. see References [69] to [94] as a partial list. However, for blood-contacting medical devices and materials, the most commonly used methods have arguably been simple counting of platelets and measurement of platelet degranulation proteins following controlled exposure of medical devices or materials to blood. Normal resting (homeostasis) levels of platelets and degranulation proteins are well established (see commercial ELISA kit product literature), as are some abnormal levels observed in various clinical thrombocytopathies. The presumption for such testing with medical devices is that appropriate materials and device designs should not be associated with excessive platelet consumption and/or activation that could bring risk to the patient. High levels of platelet loss and/or degranulation may be an indicator of a tendency for the material/device to induce or promote these conditions which can give rise to complications of bleeding or thrombosis. To measure platelet counts and platelet degranulation, counting is performed using a routine differential cell counter and the degranulation is assessed using standard enzyme-linked immunosorbent assays (ELISAs) for well-recognized platelet alpha-granule proteins. Clinical laboratories often rely upon test kits that use common enzyme-linked immunosorbent assay technique to measure the alpha-granule proteins. In a basic study, which may be either *in vivo* or *in vitro* and will depend upon availability of appropriate antibodies to species-specific target platelet granule protein epitopes, blood samples are retrieved under defined conditions and prepared and analysed per assay instructions. Typical defined conditions or factors important in an *in vitro* model are described in B.1.2.3. Comparison of results to appropriate controls such as negative controls (e.g. baseline levels or test system with no material/device exposure) and results on a predicate device(s)/materials(s) is critical. Platelet count decreases and blood degranulation protein increases that are statistically-significantly and biologically-significantly different than controls may be an indicator of a material/device design that presents a higher risk for platelet consumption and activation. Example alpha-granule proteins for which commercially-available ELISA kits are available include

— PF4 (platelet factor 4, a 70-amino acid protein that binds with high affinity to heparin; PF4's major physiologic role appears to be neutralization of heparin-like molecules on the endothelial surface of blood vessels, thereby inhibiting local antithrombin III activity and promoting coagulation), and

— βTG (beta-thromboglobulin, a chemokine for fibroblasts and neutrophils).

NOTE Thrombin from the coagulation cascade is a potent platelet agonist that can readily cause platelet degranulation. Thus, high levels of thrombin will correlate with high levels of platelet degranulation.

As in coagulation, platelet consumption (loss) is generally seen to be affected by blood-contacting surface area. Thus, surface area (SA) of a device or device material can influence platelet count data. For this reason, it is important to specify the SA-to-blood-volume ratio (exposure ratio) in each study. If possible, the exposure ratio may be treated as a variable to aid in understanding the specificity of the material effect. Exposure ratios of 3,0 cm^2 to 6,0 cm^2/ml blood (based on device thickness) are consistent with ISO 10993-12. Other exposure ratios such as 1,5 and 2,0 times this ratio may be worth considering as higher surface areas will theoretically increase the sensitivity of the platelet responses to the test material.

Platelet activation is a process that occurs over a time (minutes to hours) and is recognized to be potentially reversible up to a point, or once initiated, it can progress non-reversibly to the point of presenting significant shape deformation, loss of cytoplasmic constituents and shedding of microparticles and complete destruction. This process is dependent on stimulus type and amount. There are numerous known potent stimulants, called platelet agonists, examples of which are thrombin, ADP and collagen. Foreign surfaces themselves, such as blood-contacting medical devices, can also behave like agonists since they can cause thrombin generation. In addition, platelets can adhere to these surfaces, remain adhered or detach in activated or non-activated states and go through shape changes leading to platelet destruction. Thus, assessing the overall state of platelet activation at any point in time may benefit from the use of agents that help to "arrest" the platelets in their physical and biochemical state at a particular point in time. Consequently, if platelet assessment cannot be made immediately after removing the test material or device from the test system, a number of agents

have been suggested to counteract further platelet activation and to stabilize platelets[88][89][90][91]. Examples of these agents are acid citrate dextrose (ACD), citrate, theophylline, adenosine, dipyridimol (CTAD) and other platelet-stabilizing reagents, such as ThomboFix™[1]. To the extent that such stabilizers can minimize artefactual platelet activation, and not obscure interpretation of biomaterial-specific platelet activation responses, use of such stabilizers is worth consideration, if validated to confirm that relevant endpoints are not adversely impacted.

B.3.3.2 Platelet count

It is important to determine the platelet count[45][121] because of the key role platelets have in preventing bleeding and in the general process of thrombosis. 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 thrombus formation 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. Various anticoagulants may be suitable for enumerating platelets[151]–[156].

Blood collection techniques should be reproducible. Platelets can become hyperactive/activated under a variety of conditions, including improper blood collection. Tests such as platelet aggregometry and flow cytometry may be considered to verify normal platelet reactivity and activation.

B.3.3.3 Platelet activation: Platelet granule-release proteins beta-thromboglobulin (ß-TG) and platelet factor 4 (PF4), thromboxane B2 (TxB2) and platelet morphological changes

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

a) release of platelet granule substances, such as βTG, PF4, TxB2 and serotonin;

b) altered platelet morphology;

c) generation of platelet microparticles.

Activated platelets are pro-thrombogenic. Platelet activation can be evaluated by various means, such as microscopic (light and electron microscopy) examination of morphology of platelets adherent to the material or device and measurement of βTG, PF4 and TxB2 released from activated platelets.

βTG and PF4 are proteins that are stored in alpha-granules of platelets and released in large amounts after platelet activation[85][86][87][106]. Both of these proteins can be assessed by commercially-available ELISA assays. Increases in platelet activation can occur through multiple paths associated with medical devices and materials. The device/material itself may be platelet activating, turbulence and excessive shear forces can cause platelet activation and platelet activation can be caused by potent agonists such as thrombin which may form as a result of thrombosis associated with the material/device or local injury. High levels of TxB2, also measurable by ELISA, indicate high levels of its precursor compound thromboxane A2, a potent platelet agonist thought to be produced by activated platelets; TxB2 is also thought to be reliable species-independent marker of platelet activation. See B.3.3.1 and B.4 for details on general ELISA methodology. It may also be valuable to assess platelet activation through the evaluation of the morphological changes platelets undergo when activated on a material/device surface[70][71][173].

B.3.4 Haematology — Methods for testing

B.3.4.1 Complete blood count (CBC)

The electronic complete blood count analysis (often referred to as CBC) is a vital test used every day in hospital haematology laboratories. Its primary purpose is to quickly and accurately enumerate the concentration of the various cell populations in the patient, where abnormal readings can provide early and vital information on a host of potential disorders. The CBC is used to determine the number or

1) ThomboFix™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute and endorsement by ISO of this product.

proportion of white and red blood cells in the body. The analysis includes platelet counting. In analyses on blood-material/device interactions, CBC data provides basic information on the impact of the device/material interaction with formed blood elements. Counts of platelets and leukocytes pre- and post-blood exposure to material/device are valuable in deducing the loss of activated platelets and leukocytes taken up in clot formation by thrombogenic surfaces and therefore provide an estimate of the surface's thrombogenic potential[24].

B.3.4.2 Leukocyte activation

Leukocyte activation can be determined by microscopic examination of the device surface for activated leukocytes. A simple more quantitative method involves use of a commercial ELISA assay to determine the amount of polymorphonuclear leukocyte (PMN) elastase released to plasma following activation from interaction of a material or device with blood. Another approach based on the principle that thrombi adhering to material will contain a large number of platelets and leukocytes involves assessing the decrease in their counts in blood[24].

B.3.5 Complement system — Methods for testing for C3a and SC5b-9

The complement system resides in blood plasma in the form of a biochemical cascade that functions as a defence mechanism designed to supplement or "complement" the ability of antibodies to clear pathogens from the body. It is a part of the immune system referred to as the "innate immune system". Here, unlike antibody protection, the response activity is neither acquired nor adaptable over time. The complement system forms a fundamental line of defence that works alongside and can mediate specific antibody mechanisms. The complement system can, however, also be brought into action by the surface(s) of materials foreign to the body, including blood-contacting medical devices[129][138].

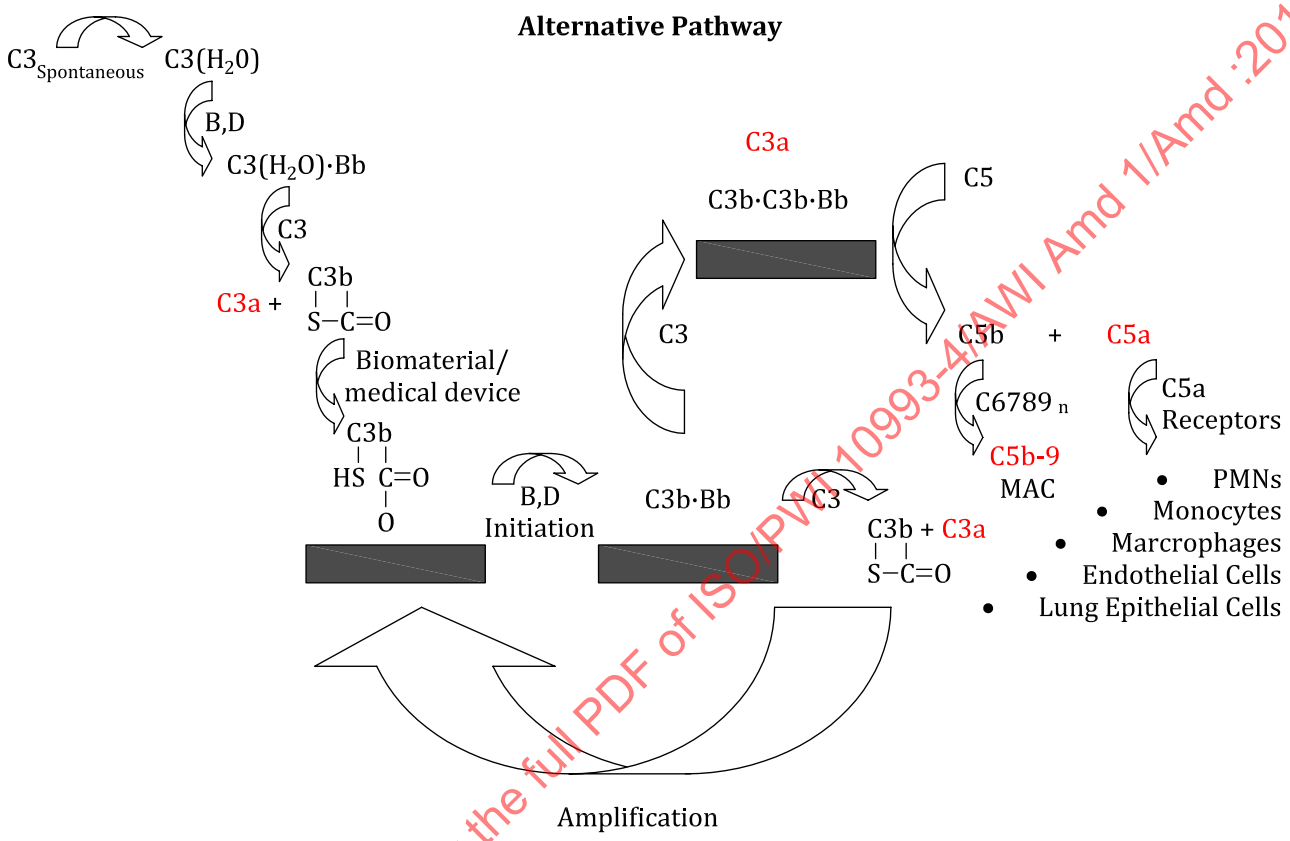
The system consists of a number of proteins found in blood that normally circulate as inactive precursors. The nomenclature for the complement proteins is "C" followed by a simple Arabic number for the native protein, and if cleaved, a small "a" or "b" to indicate the fragment. In the presence of a low level of spontaneously formed reactive C3b, the presence of a biomaterial can trigger an amplification response whose end result is production of inflammatory mediators, e.g. C5a and cytotoxic protein complexes, e.g. membrane attack complex (MAC), which can stimulate an array of inflammatory responses including white blood cell (WBC) chemotaxis, reactive oxygen species (ROS) production and cytokine expression[129][130]. See Figure B.2.

There are numerous proteins and protein fragments that make up the complement system and these can be divided into three distinct activation pathways: the classical complement pathway, the alternative complement pathway and the mannose-binding lectin pathway. It is the alternative pathway that is most regarded as being affected by and reactive to the presence of medical materials.

A number of commercial ELISA assays are available to assess the amount of complement protein in blood. As C3a is an ubiquitous fragment amplified during activation, this complement protein is considered a good *general indicator* of complement activation. In addition, a soluble form of the terminal MAC abbreviated SC5b-9 can also be assessed by ELISA assay. SC5b-9 is generally considered a more important marker representative of the *full extent of complement activation*. Elevated levels of any of complement components indicate activation of the complement system. High surface area devices such as haemodialysis filters and cardiopulmonary bypass devices have been associated with high levels of activated complement components[129]–[138][143] and this phenomenon has been linked to activate leukocytes and leukocyte sequestration in the lungs[130][137].

Measurement of complement fragments has several disadvantages. First, ELISA kits only assay complement components in the fluid phase (serum or plasma); they do not measure the complement components which are activated and adhere to the device surface. Depending on the nature of the device material, there could be significant amounts of activated complement on the device/material surface which is undetected by commercial ELISA kits. Second, there is species-specificity for many of the commercially-available kits and high baseline levels are observed in typical *in vitro* testing. Thus, appropriate controls need to be included and compared. The classical CH-50 method appears useful with human, bovine, porcine and rabbit serum. However, sensitivity of CH-50 for detecting complement activation following contact with materials/devices is low, given the CH-50 test measures

residual complement activity and most often only a small portion of complement system is activated by materials/devices. Another functional method of measurement of complement activation *in vitro* is the generation of complement C3- or C5-convertase determined by substrate conversion. References [18] and [19] also address complement activation. Annex E provides further information on considerations for complement testing of medical materials and devices. See B.4 for details on general ELISA methodology.



NOTE Factors that are shown in red are measurable by commercially available assay kits.

Figure B.2 — Alternative complement pathway

B.4 Methodology considerations for testing of plasma factors specific to coagulation, platelet and leucocyte activation and complement activation using ELISA (or other similar) techniques

B.4.1 General

Assessment of coagulation, platelet, blood cell and complement activity has long relied on clinical assays that measure plasma levels of key proteins formed in activation cascades or released from activation or damage to various cells types. Here, clinical laboratories often rely upon test kits that use common enzyme-linked immunosorbent assay technique. In a basic study, which may be either *in vivo* or *in vitro*, implementation will depend upon availability of appropriate antibodies/test kits to species-specific target protein epitopes. Additionally, blood samples will be retrieved under defined conditions and prepared and analysed per assay instructions. Defined conditions may include blood exposure time, anticoagulation and anticoagulant level, temperature, flow, haematocrit and other factors. Comparison of results to appropriate controls, such as negative controls (e.g. baseline levels or no material/device exposure) and a predicate LMCD, is critical. Activation markers in blood that

are statistically-significantly and biologically-significantly higher than controls may indicate a material/device design that presents a higher level of coagulation-, platelet- or complement-mediated risk to the patient. Example coagulation proteins for which ELISA kits are commercially available include TAT (thrombin-antithrombin complexes), F1.2 (fragment released from prothrombin upon formation of thrombin) and FPA (fibrinopeptide A released from fibrinogen upon formation of fibrin). Similarly, commercial ELISA kits are available for assessing platelet activation (e.g. alpha granule release of BTG and PF4) and complement activation (e.g. C3a and SC5b9 formation).

B.4.2 General assay methods and documentation

B.4.2.1 General

Include the following with any test reports.

B.4.2.1.1 Reference documents

Manufacturer ELISA protocol IFU in kit.

B.4.2.2 Storage and stability

Describe all storage and stability conditions of the kit reagents and the blood/blood plasma being used.

B.4.2.3 Procedure

B.4.2.3.1 Sample preparation

Provide general instructions.

Results of such testing are invariably dependent upon surface area (SA) of a device or device test material being tested. For this reason, the SA-to-blood-volume ratio (exposure ratio) should be specified in each study. Exposure ratios of 3,0 cm^2 to 6,0 cm^2/ml blood (based on device thickness) are consistent with ISO 10993-12. Other exposure ratios such as 1,5 and 2,0 times this ratio may be worth considering as higher surface areas will theoretically increase the sensitivity of the response to the test surface.

It is important to specify the means of quenching the reaction following the incubation period, i.e. list name and concentration(s) of the quenching agent(s).

B.4.2.3.2 Dilution factor (DF)

Provide details on dilution factors used, diluents used, etc.

WARNING — Considerable inter and intra donor-to-donor variability can be observed, making a single ideal DF difficult to identify. A minimum of two test sample dilution factors is therefore recommended to capture all sample values on the standard curve. The sample absorbances shall be in the range defined by the lowest and the highest standards. If not, the samples should be retested with a new DF so that results fall within the range of the standard curve.

B.4.2.3.3 Data selection in cases of testing with multiple DFs

If all or some samples are tested under conditions of no dilution, low dilution and high dilution, report the values requiring the smallest DF that are on the standard curve. It is also acceptable that if the "no dilution" samples contain some off-the-curve values and all "low dilution" samples are on the curve and the "high dilution" samples contain some below-the-curve values, to simply report the "low dilution" readings where all values are on the curve under the same DF.

B.4.2.3.4 Preparation of standards

Describe in detail how the standard curve and controls are prepared.

B.4.2.3.5 Method

Describe in detail the overall methods in the order followed, including highlighting any deviations from the ELISA kit instructions.

B.4.2.3.6 Evaluation

Describe in detail the calculations made to determine the plasma levels of the protein being measured.

B.4.2.3.7 Statistical analysis

Describe in detail the methods of statistical analysis.

B.4.2.3.8 Limitations and interferences

List all limitations or interfering factors such as incorrect blood collection technique, e.g. inadequate mixing of the sample and citrate solution (or other anticoagulant such a heparin) may lead to falsely elevated coagulation protein values; a wrong anticoagulant may lead to elevation of all background values, etc.

B.4.2.3.9 Reference interval

List known normal and abnormal plasma levels of the protein being measured and expected values of controls.

B.4.3 Attachments

Include suggested dilution factor information, ELISA assay data sheet forms, checklists for the ELISA procedure, etc.

Annex C
(informative)

Thrombosis — Methods for *in vivo* testing

C.1 General considerations

Numerous approaches have been applied to assess the process and events of thrombosis. However, for blood-contacting medical devices and materials, the *in vivo* nature of thrombosis has led to the methods described in C.2 and C.3 being more commonly used to assess device-associated thrombosis. These methods are used in evaluation of permanent- and temporary-contact devices. As pointed out in this document, the diversity of blood-contacting medical device applications dictates that *in vivo* test models will be necessarily diverse, in order to appropriately mimic each clinical application.

The method in C.2 is accepted to be the most relevant manner in which to assess devices for thrombosis, as here the actual device is evaluated in its intended clinical implant configuration in an animal model. The main elements in this work are alignment of the animal protocol according to ISO 10993-2, accurate simulation of the human clinical application and inclusion in the protocol of a detailed method(s) and analyses to be used to assess the degree of thrombosis.

The method in C.3 is used less commonly and is not widely accepted throughout the world. However, it may be required or requested by certain regulatory authorities. The method has been referred to as the non-anticoagulated venous implant (NAVI) model (when anticoagulation is not used) and the anticoagulated venous implant (AVI) model (when anticoagulation is included). The method itself involves insertion of catheter-shaped devices, or device materials formed into catheter shapes, into the veins of animals for up to 4 h followed by gross assessment of amount of thrombus on the material/catheter surface. The caveats with the methodology are shown in Table C.3 along with noted advantages provided in Table C.4. Because of these caveats, and to avoid the false labelling of materials and devices as thrombogenic in nature, data generated using this model requires extreme caution in interpretation[143]. See also A.3.

When the test device is a catheter-type device used in a venous application, the methods in C.2 and C.3 are equivalent.

C.2 *In vivo* implant study of final device in pre-clinical animal study

As stated in this document, *in vivo* testing should be performed on devices intended for *in vivo*/implant applications (see 6.1.6, 6.3.2 and 6.3.3). Protocols should include detailed methods for appropriate assessment of blood/device interactions, e.g. analyses on

a) the device itself,

b) blood samples from the test subject, and

c) susceptible tissues and end organs.

Such preclinical testing should use models that simulate actual in-use application conditions, e.g. same implant site, geometry, flow, contact duration, temperature, sterility, etc. (see 6.1.2), and include appropriate traceable controls, e.g. a predicate device (see 6.1.3). Importantly, testing should only be done on actual complete (finished) devices or components (see 6.1.4), as testing on non-finished products and testing in poorly simulated use conditions will not be highly predictive of performance in clinical applications (see 6.1.6).

Accordingly, devices with *ex vivo* application and devices with *in vivo* application should be tested in appropriate *ex vivo* and *in vivo* models, respectively. Anticoagulant use in *ex vivo*/*in vivo* models should

be consistent with the type and quantity used in the routine clinical device application and the product IFU (see 6.1.12). Use of appropriate replication, statistical design and analysis methods should be considered in animal studies (see 6.1.14 and References [213], [214], [215] and [216]).

Consider tests, as appropriate, in these categories: thrombosis, coagulation, platelets, haematology and complement activation, as described in Table 2. By way of example, one analysis might consist of gross examination and SEM on the device to assess degree of device-associated thrombosis. Inspection of susceptible downstream organs, e.g. lung and kidney, for evidence of thromboemboli will aid in assessing potential for device-associated thromboembolism. If appropriate antibodies are available, coagulation may be assessed by measuring blood plasma levels of indicators of coagulation and fibrin formation, e.g. TAT and FPA measured via ELISA technique. Likewise, simple platelet counting and or measurement of platelet activation markers, e.g. βTG, can be used to assess the impact of the device on platelets. Routine differential blood cell counting and plasma-free haemoglobin can be used for a general assessment of haematology factors and assessment of blood cell physical damage. Finally, for large surface area devices, providing availability of antibodies, plasma levels of various complement factors may be used to assess activation of the alternative complement pathway.

Generally speaking, it is desired that the test device has no, low or equivalent impact on the factor being measured relative to the results observed in a predicate device. Results higher than in the predicate device may be justified based on a risk/benefit analysis. Devices without predicates should use an appropriate control, with justification provided for selection of that control. Such evaluations are preferred over the use of the method described in C.3, as this method most appropriately mimics the *in vivo* application. As always, vertical standards should be consulted in the various device areas. For the latter, numerous examples can be found among references.

NOTE 1 For catheter-shaped devices intended to be implanted in the *venous* environment under no anticoagulation, or anticoagulation, the NAVI and AVI implant models, respectively as described in C.3, describe the appropriate *in vivo* study approach for these devices.

NOTE 2 For catheter-shaped devices intended to be implanted in an *arterial* environment under no anticoagulation, or anticoagulation, a non-anticoagulated arterial implant (NAAI) or anticoagulated arterial implant (AAI) model, in analogous appropriate arterial implant positions to those described in C.3, describe the ideal study approach for arterial implant devices.

See also 6.3.3 and Annexes A and B.

C.3 *In vivo* NAVI and AVI thrombogenicity tests

The NAVI and AVI test involves inserting a catheter (or other appropriately-shaped) device, or a device material made into a catheter shape, into the vein of a large animal. It has been called for in situations, for example, of characterizing a new catheter, evaluating a new device coating, characterizing a new material and in circumstances of changing a vendor or material/device processing step. In the model itself, a number of venous positions have been used (see Figures C.1 and C.2). In the absence (NAVI) or presence (AVI) of anticoagulants, duplicate or triplicate implants are allowed to incubate *in situ* for a period of up to 4 h. (Instances of less and more time may be justified, given the specific application.) The implants are then removed and assessed for the amount of apparent thrombus on the surface. The canine femoral or jugular vein model is most commonly used, where a test material/device is positioned in one vein and a control material or predicate device is placed in the contralateral site. The test requires two to three large animals, with alternating test and control implant locations to avoid bias, and assessment of thrombus on the devices using a scoring method such as those shown in Tables C.1 and C.2. Results may be supplemented with gravimetric analysis of the observed thrombus and observations of vessel patency. Results of the test device should be equivalent to, or less thrombogenic than, an appropriate LMCD, if available or unless otherwise justified. It is important to make sure that all clinically blood contacting device components are evaluated. Various worldwide regulatory authorities have suggested that up to 15 cm of the test and control be implanted in order to ensure adequate exposure during testing. Depending on the device configuration, this may require that a custom device be created to reduce the length of the components, while maintaining the same material ratios as the complete device. As an alternative, a device can be subdivided into multiple test samples if necessary.

For devices and materials intended for arterial implantation, methods may be adjusted accordingly to use arterial implant positions.

CAUTION — Variation in results has been observed between

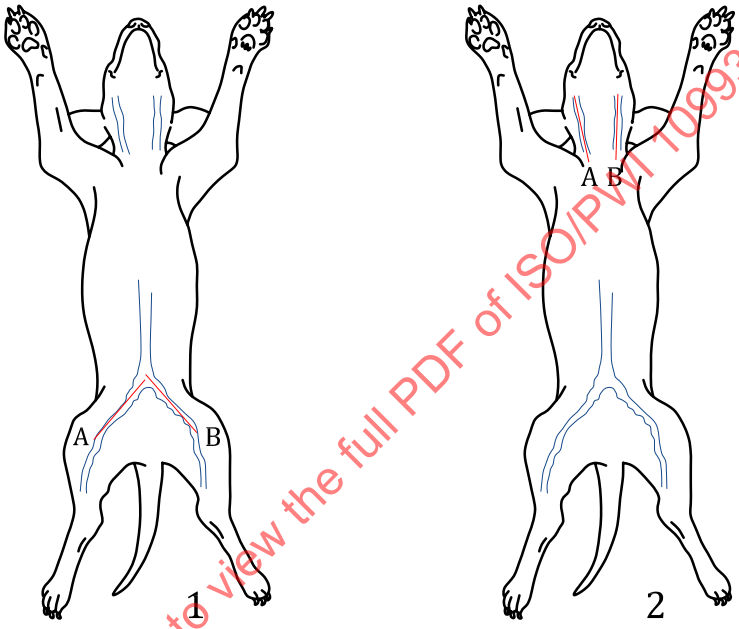
a) test facilities,

b) test evaluators,

c) replicates on the same material, and

d) scores obtained on controls[143].

Table C.3 gives a summary of the main controversies of the NAVI and AVI models. Table C.4 provides some noted advantages of the NAVI and AVI models. The greatest utility of the model may be in assessment of test materials intentionally modified to reduce acute thrombus formation, for example in evaluating heparin coatings.

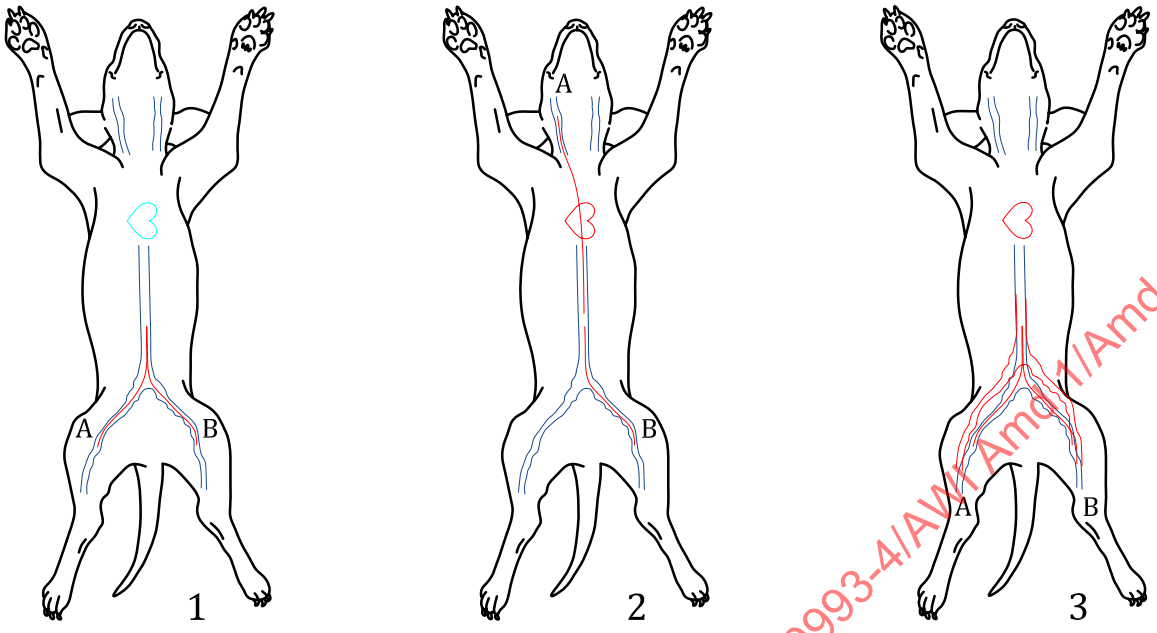


Key

1 femoral

2 jugular

Figure C.1 — Main implant positions used in the NAVI/AVI model



Key

1	IVC-IVC
2	SVC-IVC
3	IVC-AA
AA	abdominal aorta
IVC	inferior vena cava
SVC	superior vena cava

CAUTION — Use of these implant positions requires careful consideration for artefact due to device-device interaction and/or bias for positional differences in thrombus formation.

Figure C.2 — Other less-frequently used NAVI and AVI implant positions

Table C.1 — NAVI/AVI scoring scheme A

Thrombus formation score description	Score
No significant thrombosis (a very small clot is acceptable at insertion).	0
Minimal thrombosis, one location.	1
Minimal thrombosis, multiple locations.	2
Significant thrombosis, > 1/4 to ≤ 1/2 the surface of the implant, vessel patent.	3
Significant thrombosis, > 1/2 the surface of the implant, vessel patent.	4
Vessel completely occluded.	5

Table C.2 — NAVI/AVI scoring scheme B

Thrombus formation score description	Score
Thrombus non-existent or minimal and, if present, appears to be associated with implant venotomy site.	0
Thrombus minimal, observed to be covering 1 % to 25 % of material surface.	1
Thrombus moderate, observed to be covering 26 % to 50 % of material surface.	2
Thrombus severe, observed to be covering 51 % to 75 % of material surface.	3
Thrombus extensive, covers 76 % to 100 % of material surface.	4

Table C.3 — Main caveats in using the NAVI or AVI models[143]

Factor	Description	Concern
1	Implant position	High flow environments lead to low levels of surface-associated thrombus and vice versa. Therefore, slight differences in anatomical factors such as target vessel diameters and/or venous valve positions can have potential impact on the amount of thrombus observed. Additionally, if the implant is placed at an angle or position which alters blood flow to create eddy currents leading to stasis, thrombus formation may occur unrelated to the material properties of the test device.
2	Implant technique	Each test and control should be identically and precisely inserted into the target veins with each situated in an identical position (preferably centrally-located with no vessel wall contact).
3	Extent of device-vessel wall contact	This factor relates to vessel wall injury/endothelial denudation during the implantation and incubation periods. The implant itself can contribute to thrombosis as a result of mechanical contact with the vessel wall, which induces injury and tissue factor activation. Here, the material factor may play a minor role in the extent of observed thrombus and device geometry can play a major role.
4	Time/incubation period	The main measured response of extent of surface-associated thrombus tends to be intense within the first 1/2 h to 2 h. Sometime after this period, the thrombolytic (fibrinolytic) system can initiate and biochemically remove some of the associated thrombus.
5	Explant technique	Depending on the makeup and the extent of the surface-associated thrombus on the sample, the thrombus material being measured/scored can be fragile and readily slough off during the device retrieval/exposure. Without special precautions, this material, often referred to a “sleeve thrombus”, can be “squeegee off” if the sample is retracted from or disturbed in its implant site. Some investigators use of *in situ* perfusion fixation to flush away non-adherent blood elements and concomitantly crosslink the device/thrombus/vessel into a tougher (and more physiological) geometry.
6	Material/ material surface	This model has been used to assess the thrombogenic potential of new materials, evaluate process changes on existing approved materials and to qualify new vendors. Often a legally-marketed comparator device (LMCD) or material is used as a control, which yields equally variable results.
7	Non-thrombo adherent materials get labelled non-thrombogenic	Extensive work by experts in the field[177][178][179] has demonstrated that hydrophilic surfaces can be thrombogenic (and thromboembolic) yet not be thromboadherent. This test will give passing scores to devices and materials that are thrombogenic and non-thromboadherent.
8	Recipient/ subject thrombotic potential	Papers on this topic[56][57][180] indicate that test subjects can have significantly different “thrombotic potentials”, i.e. differing capacity to form thrombus upon exposure to medical implants and other stimuli. This can result in significant differences in scoring between test subjects.
9	Statistical power	In complying with ISO 10993-2, combined with the variability in responses seen between predicate devices and materials, and between test subjects, obtaining a statistically-meaningful conclusion is often not possible.
10	Evaluator expertise	The training and skill of the evaluator assigning thrombus scores is extremely important. Some may have difficulty differentiating between true *in vivo* thrombus and false thrombus [post/ante-mortem (agonal) clot formations].
11	Anticoagulation impact	Nearly all devices and materials tested in the AVI model receive scores of zero and pass the test. This brings into question the purpose and rationale of using the AVI model. In the NAVI model, most hydrophobic materials show varying degrees of thrombus while hydrophilic materials show minute levels of thrombus.
12	Implant size to vessel diameter	Besides potentially causing vessel wall damage, if the size of the implant relative to the vessel diameter is too large, blood flow can be affected (stagnation), predisposing to thrombosis. In general, the cross-sectional area of the implant should not occupy more than 50 % of the vessel lumen.

Table C.4 — Advantages in using the NAVI or AVI models

Factor	Description	Concern
1	Studying thrombus formation	The NAVI model has been known to consistently show substantial *in vivo* thrombus formation on hydrophobic polymers, particularly after 1 h or less blood exposure. As such, the NAVI model is a good model to study thrombus formation. For example, it may be a useful tool to study the impact of thrombus on devices, such as intravascular sensors[143].
2	Evaluating coatings intended to reduce thrombogenicity	Coatings applied to device surfaces to reduce thrombogenicity should show measurably different and consistent responses, i.e. lower than non-modified controls, when tested in the NAVI model. Caution: Testing should include steps to assess thromboembolic potential, as non-thrombo-adherent surfaces may still be thrombogenic.
3	Evaluating coatings intended to enhance thrombogenicity	Coatings applied to device surfaces to enhance thrombogenicity should show measurably different and consistent responses, i.e. higher than non-modified controls, when tested in the NAVI or AVI model. Caution: Testing should include steps to assess thromboembolic potential, as thrombogenic surfaces may shed emboli.
4	Screening of materials for cardiovascular applications	The NAVI and AVI models are most appropriate for testing catheter-type devices intended to be introduced into the vasculature for short periods of time. Multiple materials and device designs can be tested in this model with the contralateral vessel being used to assess a control or non-modified version of test devices.

Annex D
(informative)

Haematology/haemolysis — Methods for testing — Evaluation of haemolytic properties of medical devices and medical device materials

D.1 General considerations

Extensive literature is available that describes blood/material interactions. Unfortunately, very few methods exist which are reliable, reproducible and predict clinical performance. This annex will review the known haemolysis test methods and discuss factors pertaining to their ability to characterize medical materials and devices. Haemolysis is a function of blood-material exposure time and material properties such as surface energy, surface morphology and surface chemistry. Haemolysis is also a function of local mechanical forces and biochemical factors.

D.2 Causes of haemolysis

D.2.1 Osmotic pressure (osmotic pressure-induced haemolysis)

The erythrocyte membrane is a semi permeable 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, yet it allows passage of a pure solvent, such as water. Such a pressure differential can cause erythrocyte swelling and cell membrane rupture with release of free haemoglobin[175]. It should also be noted that the osmotic fragility between mammalian red blood cells can vary[95]–[98].

D.2.2 Mechanical forces (mechanically-induced haemolysis)

Fluid dynamic factors such as blood flow rate, turbulence and non-physiological shear forces can deform the erythrocyte membrane and potentially cause membrane rupture. The latter can potentially be exacerbated by devices with mechanical operation and/or complicated flow paths. Examples of such devices are

— apheresis and cell separation systems,

— arterial blood filters[11][36],

— blood pumps[29][30][124][157],

— cardiopulmonary bypass systems[5][10][11][13][37][41],

— cardiotomy/venous reservoir systems[10][11],

— circulatory support devices[9],

— haemodialysis systems[16][28][41],

— mechanical heart valves[2], and

— ventricular-assist devices[236][237].

D.2.3 Biochemical factors (material-induced haemolysis)

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 haemoglobin. Other chemicals, e.g. extractables from a medical device, bacterial toxins, pH and metabolic changes induced by temperature, can compromise the erythrocyte membrane[95]. These changes can 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.

D.3 Clinical significance of haemolysis

D.3.1 Toxic effects

Elevated levels of plasma-free haemoglobin can induce toxic effects or initiate processes which can stress the kidneys or other organs[175]. The plasma-free haemoglobin concentration is a convenient measure of injury to erythrocytes, but it is an indirect indicator of damage to other blood elements as well.

D.3.2 Thrombosis and anaemia

Intravascular haemolysis can promote thrombosis by a cascade of events involving liberated RBC ADP and phospholipids[106] causing platelet activation and degranulation of prothrombotic agents. When haemolysis causes a clinically significant drop in erythrocyte count, anaemia and compromised oxygen-carrying capacity with its subsequent effects on the brain and other organs or tissues can result.

D.4 Determining a pass/fail assessment for haemolysis

Haemolysis is a function of exposure time and material properties such as surface energy, surface morphology and surface chemistry. Haemolysis 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[108][112][113]. These variables need to be adequately controlled for comparisons of haemolytic potential among materials and medical devices. The spectrum of methods for evaluating haemolysis varies from simplified to highly complicated models. Specific *in vitro* and *in vivo* models with flowing blood have been published. Studies of haemolytic 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 haemoglobin which may not be measurable under *in vivo* conditions (e.g. due to binding of plasma haemoglobin to haptoglobin and rapid removal from the body). Measurement of lactate dehydrogenase and haptoglobin, as indicators of haemolysis in an *in vivo* test setting, may be applicable.

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

— What is the duration of exposure of the device to the patient?

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

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

— What are the haemolytic properties of these known treatments? How does the device in question compare to these other treatments?

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

D.5 Haemolysis testing — General considerations

D.5.1 Methods

D.5.1.1 General

Haemolysis of red blood cells (erythrocytes) is assessed using *in vitro* tests. Direct methods determine haemolysis due to physical and chemical interactions with erythrocytes. Indirect methods determine haemolysis due to extractables from test articles. Reference [17] is one standard that is specific for testing the haemolytic properties of materials (mainly due to chemical factors) and, depending on device size and complexity, it may not be sufficient for testing whole intact medical devices. References [17], [22] and [28] are examples of methodologies specifically developed for haemolysis testing of medical devices and their component materials. Reference [20] was developed to assess haemolysis caused by medical nanoparticles. In its simplest form, for highly diluted suspensions of erythrocytes in contact with test materials, haemolysis is often reported as a percentage of haemoglobin which has been liberated into the supernatant normalized by the total haemoglobin which was available at the beginning of the test, i.e. (free haemoglobin concentration/total haemoglobin concentration) × 100 %. If all of the erythrocytes present at the beginning of the experiment are destroyed, there is 100 % haemolysis.

In addition to material testing of devices, dynamic testing of whole medical devices under clinical use conditions to evaluate the effects of the device structure, mechano-physical interactions of blood with materials, range of clinically relevant use conditions (e.g. blood flow rate, rpm, pressure, exposure time), intended use and haemodynamic factors on haemolysis should be considered. For many devices, haemolysis caused by hydrodynamic forces and dynamic interaction with surfaces exceeds that caused by the chemical effects of the material. To appropriately simulate the clinical use conditions, blood haematocrit and other factors should be accounted for during the dynamic haemolysis testing[30][37][41][124]. To ascertain a worst case amount of haemolysis that may occur, *in vitro* testing is often conducted at the highest blood flow rate for which the device is expected to be used. References that provide protocols for mechanical haemolysis testing of devices include References [5], [37], [41] and [124].

The concentration of haemoglobin in plasma is significantly less than the total blood haemoglobin concentration. The plasma-free haemoglobin concentration is normally 0 mg/dl to 10 mg/dl *in vivo*, whereas the normal range of total blood haemoglobin 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 haemoglobin concentrations which are encountered during haemolysis testing. A note of caution:

CAUTION — Some common haemolysis assays suggest a cut-off level for material-induced haemolysis below which there is no/low risk of concern based on historically accepted but non-validated values[14][17][28]. However, higher acceptable levels of material-induced haemolysis may be justified based on a suitable risk/benefit analysis.

Researchers should be aware that haemolysis tests may be adversely affected by chemicals in medical materials or solutions which may alter erythrocyte fragility (e.g. certain buffers and fixatives, such as formaldehyde or glutaraldehyde), cause haemoglobin to precipitate (e.g. by copper or zinc ions) or alter the absorption spectra of haemoglobin (e.g. by polyethylene glycol or ethanol)[115][170].

D.5.1.2 Total blood haemoglobin concentration measurements

Classically, the analytical methods outlined in D.5.1.2.1 and D.5.1.2.2 have been used to determine total blood haemoglobin (Hb) concentrations[106]. Total blood haemoglobin concentration can also be measured using calibrated complete blood cell counters and haemoglobinometers.

D.5.1.2.1 Cyanmethaemoglobin method

The first classical method, cyanmethaemoglobin detection, was issued by the International Committee for Standardization in Haematology[121]. The cyanmethaemoglobin (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 haemoglobincyanide which has a broad absorption maximum 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 haemoglobin concentration, the spectral interference due to plasma is minimal and the sample absorbance can be compared with 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.

D.5.1.2.2 Iron method

The second classical method for determining the total haemoglobin concentration is based on determining the haemoglobin 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 colour that can be measured photometrically. This method is too complex for routine work and is rarely used.

D.5.1.3 Plasma or supernatant haemoglobin concentration measurements

The following two methods have been used to measure plasma or supernatant haemoglobin concentrations.

D.5.1.3.1 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 have been a host of different assays used for measuring plasma haemoglobin as an indicator of haemolysis, with no one method being widely accepted. The assays can be classified into two broad categories: those which are direct optical techniques (i.e. based on quantifying the oxyhaemoglobin absorbance peak at 415 nm, 541 nm or 577 nm, directly or through use of derivative spectrophotometry) and those which are added chemical techniques (i.e. quantification of haemoglobin based on a chemical reaction with reagents such as benzidine-like chromogens and hydrogen peroxide or the formation of cyanmethaemoglobin)[109]. All of the assays can be performed manually or can be automated.

A popular method for determining the concentration of haemoglobin 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 coloured product (photometrically detected at 600 nm) is directly proportional to the haemoglobin 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 with the haemoglobin cyanide method[106]. 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)[116] and possible reaction inhibition (by as much as 40 %)[117] may occur from calcium-chelating anticoagulants (e.g. citrates, oxalates, EDTA)[116], albumin[104] or other non-specific plasma components[106] which may interfere with H_2O_2 oxidation.

For these reasons, direct optical methods, such as those by References [102], [105] or [118] with comparable sensitivity and reproducibility may be substituted. However, as noted above, chemically induced alterations to haemoglobin and its spectra can occur which may invalidate some of the haemoglobin assays. Moreover, compensation needs to be made for endogenous plasma background interference, since it can also alter the haemoglobin spectra[109]. The analyst should be aware of these limitations in the plasma haemoglobin assays and ascertain whether they are using an appropriate technique[104][109][115][170]. 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 oxyhaemoglobin.

D.5.1.3.2 Immunonephelometric method

The immunonephelometric method is based on determination of plasma haemoglobin 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[107].

D.5.2 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[99] and the Council of Europe[101]. 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[151]–[156].

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[151]–[156]. 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-Parnas 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 should 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 haemolysis[99].

The suitability of containers for the storage of blood products is evaluated by various methods that measure the quality of the blood product[103][106]. 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 haemoglobin 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.

D.5.3 Protection of employees handling blood

Written procedures are necessary for protection of 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[114].

D.5.4 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[99].

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[99].

NOTE Use of a vacuum tube has the potential to cause slight haemolysis[125][126][127].

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 haemolysis.

D.5.5 Species selection

Ideally, haemolysis 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 should 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 % haemolysis to obtain total haemoglobin content due to differences in membrane stability among animal species. Negative controls should cause minimal haemolysis so that the activity of the test material is not masked. Rabbit and human erythrocytes are reported to have similar haemolytic properties whereas monkey erythrocytes are more sensitive and guinea pig erythrocytes are less sensitive[95][96][97][98][123].

D.5.6 Evaluation of haemolysis — *In vitro*, *ex vivo* and *in vivo* exposure to blood or blood components

Haemolysis 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 with reference control marketed devices with known acceptable levels of haemolysis. In the second case, the test subject is evaluated for clinically significant consequences of haemolysis.

The purpose of *in vivo* or *ex vivo* tests is to characterize the haemolytic potential of a medical device. The preliminary studies may be *in vitro* and may use fresh or outdated human blood or blood from a non-human species. For medical devices indicated for *ex vivo* use, the general practice is to recirculate blood through the device using conditions that simulate the most clinically relevant and intended worse-case (e.g. highest blood flow rate) 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.

D.5.7 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.* Extraction conditions to be considered when elevated temperatures are used are outlined in ISO 10993-12.

Annex E
(informative)

Complement — Methods for testing

E.1 Background information

Complement activation has been implicated in certain adverse reactions during clinical extracorporeal therapies, in particular in haemodialysis[129][130][131][132] and cardiopulmonary bypass applications[133][134][135][136]. These therapies notably involve devices with high blood contact surface areas and relatively short contact times. Complement activation occurs usually in the early stage shortly after blood contacts the device material surface; it has yet to be seen to continue for a longer period. Here, activation is recognized to start with blood contact with the device material(s) and the deposition of numerous plasma proteins, including the critical complement proteins C3 and C3b. The contact of these specific proteins leads to the alternative pathway formation of the crucial C3 and C5 convertase enzymes (C3b•Bb, C3b•C3b•Bb, respectively; see Figure B.2). The C5 convertase protein catalyses the cleavage of C5 resulting in C5a and C5b generation. The C5a protein is a recognized effector of receptor-mediated neutrophil and monocyte activation and the C5b fragment is the recognized initial complement component that leads to formation of the complement membrane attach complex (MAC) which binds to and activates and/or destroys bystander cells by causing lysis. WBCs can detect material surface-bound C3 and C4 fragments, which results in their subsequent surface adhesion and activation. Neutrophil and monocyte activation, MAC formation and activity, and WBC adhesion and activation on materials account for the archetypical pathophysiology seen clinically in high surface area device applications[136]. Importantly, Reference [138] have shown the critical role of the fragment C5a in mediating many of these adverse reactions. Here, dose-dependent responses identical to those seen under actual dialysis were observed in simulated haemodialysis and infusion of purified C5a. Continued work in this field has shown that materials whose surfaces are highly nucleophilic (hydroxyl- and amine-containing) present the highest complement activating potential and that various surface modifications that reduce this type of surface chemistry greatly nullify the classic clinical sequelae. This work is supported by other investigators studying the relationship of complement activation by medical device materials and the biological response[136][137].

Fortunately, the discovery of hydroxyl- and amine-containing blood-contact materials as the source of complement activation led to development of new materials that eliminated or masked these groups. This material modification greatly reduced complement activation in the larger surface area/acute contact applications. This attention to complement activation in these large device areas however has driven such testing to become more common on all devices regardless of blood-contact surface area and implant duration. To date, no scientific papers or clinical reports of complement-related adverse events have been identified by this Working Group in other device applications, i.e. all medium to small surface-area devices. Thus, appropriate references that link device-associated complement activation to adverse events in humans, along with a threshold device surface area of concern are not available. It may be noteworthy that classic anaphylactic reactions have occurred in association with use of medical devices. Here, however, this reaction has been generally attributed to an agent being delivered, rather than to a device or device material[139]–[142]. Some false associations of complement activation to the devices involved may have arisen from such reports[143].

As in assessing coagulation, thrombosis and platelet activation, a number of molecular biology tools (e.g. ELISA assays) exist to monitor the levels of complement pathway activation in blood. Example complement proteins for which commercially-available ELISA kits are available include but are not limited to C3a, C5a and SC5b9. Despite the strong link of complement-mediated pathophysiology to the C5a fragment, classic complement testing has focused on assessing C3a and SC5b9 complex. In conjunction with these tools, methods exist to assess related responses such as WBC adhesion (using SEM) and WBC activation, for example, using bioassays for PMN elastase release.

E.2 Complement activation tests and documentation (suggested to consider in reporting complement test results for scientific or regulatory purposes)

The general methods and documentation used in complement testing using ELISA assays are described in B.4.

Like other biological reactions, such as coagulation protein formation, complement formation generally exhibits an initiation, propagation and termination phase[56][57][137]. This reflects the initial C3 and C5 convertase formation reactions, cascade/feedback amplification and a slowdown/deactivation period where critical precursors may be consumed or the active proteins lose activity due to short half-life or deactivation by negative control feedback proteins. Thus, order-of-magnitude differences in levels of complement proteins are to be expected over time. Consequently, the phase of the complement cascade in blood at the time the material/device-blood contact actually occurs is an important factor. That is, the impact of test materials when mixed with blood may be quite different at each phase. In addition, as complement activation is generally proportional to blood-contacting surface area, surface area (SA) of a device or device material can be very influential on results. For this reason, the SA-to-blood (whole, plasma or serum) volume ratio (exposure ratio) should be specified in each study. If possible, the exposure ratio may be treated as a variable to aid in understanding the specificity of the material effect. Exposure ratios of 3,0 cm^2 to 6,0 cm^2/ml blood (based on device thickness) are consistent with ISO 10993-12. Other exposure ratios such as 1,5 and 2,0 times this ratio may be worth considering as higher surface areas will theoretically increase the sensitivity of the response to the test material.

NOTE There will be a physical limitation on the amount of test material that can be tested due to the volume of the test system, e.g. a test tube, and the target exposure ratio.

E.3 Complement activation tests method considerations

A review of multiple test laboratory complement testing methodologies has shown that use of commercially available test kits is common. However, a number of inconsistencies between laboratory methods were observed. These were the following.

a) **Blood preparation and anticoagulation use:** the blood used for test material exposure varied considerably between laboratories. For example, special commercially available human serum, fresh and frozen citrated human plasma, fresh human serum and directly exposed fresh heparinized whole human blood were used. The impact of these various preparations on complement results has not been evaluated, with the exception of use of EDTA as anticoagulant. For the latter, it is generally well known that complement activation through the classical pathway is calcium-dependent and through the alternative pathway is magnesium-dependent. Thus, use of potent calcium/magnesium chelators such as EDTA will bind up available calcium and magnesium and shut off complement activation, making most measurements using these anticoagulants result in close to background levels of complement proteins.

   With the above said, there is no standard blood or blood preparation currently identified for use in complement testing. However, when serum is used, serum should be functionally intact and retain the capacity of showing complement activation. If whole blood or plasma is used, the type of anticoagulant should be carefully selected to ensure that it does not inhibit or potentiate complement activation caused by the test device itself.

b) **Ratio of test article surface area to blood (whole, plasma or serum) volume:** some laboratories specify following ISO 10993-12 ratios where the blood (whole, plasma or serum) volume is used in place of the extract fluid. Some laboratories do not specify a ratio and/or a variable ratio is used. As complement activation is influenced by surface area (SA), standardized and reported SAs are important to inter- and intralaboratory interpretation of results.

c) **Use of controls:** use of a negative biomaterial control, such as polypropylene, and a positive biomaterial control, such as latex or cellulose acetate, was somewhat consistent; use of a liquid negative control, such as saline, and a positive liquid control, such as cobra venom factor, was inconsistent; use of negative and positive controls provided in the commercially available kits was fairly consistent.

d) Complement activation on blood-contacting medical devices is recognized to be a surface phenomenon. As such, testing with a device extract is not appropriate. Rather, complement testing on devices or materials should always be conducted using a [blood or blood-constituent] direct-contact method. A negative control material like polyethylene (PE) should be included in the assay along with a positive control material such as non-modified cellulose, e.g. Cuprophan[2)] (if available). Correspondingly, the use of a liquid negative control like saline is not an appropriate negative control for medical device complement activation testing. On the other hand, a potent liquid complement activator such as cobra venom factor may be used to demonstrate that the test system is working at specified conditions.

e) **Standard curve preparation/dilutions:** laboratories used different dilutions of the standard to generate a standard curve that captured most levels of test and control samples. For example, dilutions of 1:100, 1:200, 1:1 000 and 1:10 000 on the highest standard were reported. The most important factor here is that all samples ultimately lie between the low and high values on the standard curve.

f) **Incubation period for test articles and controls:** incubation periods varied between laboratories, with laboratories reporting use of 60 min, 90 min or 30 min and 60 min and 90 min incubation periods.

g) **Incubation time following addition of chromogenic substrate:** 15 min to 30 min incubations periods were reported.

h) **Test sample evaluation:** the method of assessing whether a test material gave a positive or negative result was inconsistent between laboratories. Most laboratories made a statistical comparison of test sample results to positive and negative biomaterial and/or liquid controls. Some laboratories included a comparison with historical values, results on a predicate device and/or a special mathematical formula involving negative and positive controls as part of the final assessment.

There are no established pass/fail criteria for a clinically acceptable level of complement activation. Inclusion of a legally marketed comparator device in the complement activation testing would be helpful for data interpretation. The comparator device data could be used to evaluate the clinical relevance of the test device data.

If the comparator device is not legally marketed in the regulatory region where the device will be submitted for marketing, regional regulatory authorities may request comparator testing for a device already legally marketed in that region.

2) Cuprophan is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute and endorsement by ISO of this product.

Annex F
(informative)

Less common laboratory tests

F.1 General

This annex and Table F.1 describe tests that have been used primarily in research to assess device/material interaction with blood. These tests have not, however, seen widespread use in regulatory device submissions. The tests mentioned here are for informational purpose with the caveat that they may not be standardized nor correlated for clinical relevance. As a medical device preclinical biological evaluation strategy should focus on the most meaningful and widely accepted tests (see Annex B), caution is advised in including any Annex F methods in device submissions. Laboratory tests that are clearly not recommended can be found in Annex G.

Table F.1 — Less common tests used to assess interactions with blood

Tests by categories[a]	
Thrombosis	Flow reduction, gravimetric analysis, pressure drop across device, adsorbed protein analysis, imaging techniques
***In vitro* thromobosis**	
Coagulation	thrombin generation assay using chromogenic substrates, fibrinogen and fibrin degradation products (FDP), D-dimer
Platelets	platelet adhesion assessments, flow cytometry analysis of platelet activation, platelet microparticle formation, gamma imaging of radiolabelled platelets, platelet aggregometry
Haematology	Leucocyte activation by flow cytometry, blood cell adhesion assessments, platelet-leucoyte complexes (PLCs)
Complement system	Bb, C3bBb, C5a
[a] Because of biological variability and technical limitations, the accuracy and predictivity of many of these tests, which are most commonly used for research purposes, requires careful attention to methodology and caution in interpretation of results.	

F.2 Thrombosis

F.2.1 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 for B.2.1.

F.2.2 Gravimetric analysis (thrombus mass)

This is conducted after removal of the device from the in-use position. Rationale and interpretation are as described in B.2.1. Here, the difference in weight between the pre-implant weight of the device and the weight immediately post-implant may reflect the amount of thrombus present. An important caveat is that all tissue present may not be thrombus.

F.2.3 Pressure drop across device

This is measured before and after a period of use.

F.2.4 Adsorbed protein analysis (via antibody binding)

The protein adsorption onto test materials or devices that occurs after contact with blood, e.g. the first layer or the surface layer upon reaching equilibrium, is thought to potentially impact device performance and/or clinical outcome. Next to qualitative microscopic judgement of fibrin and platelet deposition on materials, a quantitative estimation of surface protein is possible by measuring the amount of labelled antibody specific for proteins such as fibrinogen or platelet membrane receptors. For this purpose, materials are first washed after exposure to blood to remove non-adherent proteins and blood components. The surfaces or extracts of the surfaces are then combined with labelled antibody binding for qualitative or quantitative analysis. Additionally, it is possible to measure the total amount of adsorbed protein directly without using antibodies[164][181][182][183].

F.2.5 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.

F.3 Coagulation

F.3.1 Thrombin generation assay using chromogenic substrates

Materials exposed to an intact coagulation system in the presence of phospholipids will generate thrombin which can be measured by conversion of a chromogenic substrate[61][62][63].

F.3.2 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[64]. The test is mainly useful for evaluating implant devices. The use of commercially available methods such as ELISA is recommended.

Dysfibrinogenaemia, afibrinogenaemia and hypofibrinogenaemia cause prolonged PT, PTT and TT results[45].

F.3.3 D-dimer

An elevated level of D-dimer indicates activation of the coagulation mechanism. D-dimers are plasmin digested degradation products of FXIII cross-linked fibrin (coagulation and fibrinolysis). The use of ELISA and/or RIA assay is recommended for quantifying such proteins[65][66].

F.4 Platelets

F.4.1 Platelet adhesion assessments

Blood cell adhesion[167] 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 haematological factors.

Various methods have been designed to measure the adhesion of cells to surfaces, for example the Kunicki K-score[75]. 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.

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 pre-labelled with 51Cr or 111In may be used[70][71][73]. An alternative non-isotope method, the LDH and the acid phosphatase methods, which assess enzyme activity in the bulk after lysis of adhered platelets, has also been reported as a useful tool to assess platelets on surfaces[83][84].

F.4.2 Flow cytometry analysis of platelet activation

The use of certain materials or devices may cause platelet activation and the expression of activation markers at the platelet surface or the generation of platelet micro-particles[173]. Platelet surface activation markers have been evaluated by flow cytometry for 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)[69].

F.4.3 Gamma imaging of radiolabelled platelets

The high gamma emission of 111In enables it to be used for this purpose[46][47][50][72]. This method enables the localization and quantification of platelets deposited on a device. The technique is useful for external communicating as well as implant devices.

F.4.4 Platelet aggregometry

Platelet aggregation[74] is induced by adding aggregating agents (e.g. ADP, epinephrine, collagen and thrombin) to platelet-rich plasma (PRP) that is being stirred continually. As the platelets aggregate, the plasma becomes progressively clearer. An optical system (platelet 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 by the WU/HOAK method[79].

F.5 Haematology

F.5.1 Leucocyte state and morphology

Change in leukocyte state of activation can be determined by flow cytometry for the evaluation of increased leukocyte markers, such as L-selectin and CD 11b, and by quantitative disturbances in lymphocyte subpopulations. It is also possible to assess leukocyte activation through evaluation of morphological changes leukocytes undergo when activated on a medical device surface. This is usually performed via SEM[173].

F.5.2 Blood cell adhesion assessments

Blood cell adhesion[167] 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 haematological factors. By such a method, it has been reported[167] that adhesion of canine species peripheral lymphocytes and 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.

F.5.3 Platelet leucocyte complexes (PLCs)

PLCs can be measured by flow cytometry and may be an indicator of white blood cell and platelet activation following exposure to medical devices and materials[85].

F.6 Complement system

F.6.1 Complement activation assessment of Bb, C3bBb and C5a

Of these three complement proteins, the C5a fragment is considered one of the most critical complement factors in blood-contacting device-related complement activation[145]. However, routine testing for C5a is not required given the low sensitivity of commercially available ELISA kits for *in vitro* assessment of this protein.

Annex G
(informative)

Tests which are not recommended

G.1 General

The tests described in Table G.1 and below are tests that are generally not used or accepted by regulatory authorities as part of a preclinical assessment of a blood-contacting medical device safety evaluation. These tests are considered either outdated or of insufficient/not applicable scientific merit for such evaluations.

Table G.1 — Tests not used in preclinical assessment of medical device safety

Tests by categories	
***In vitro* haemocompatibility**	
Coagulation	APTT, PT and TT
Platelets	template bleeding time, platelet lifespan (survival)
Haematology	reticulocyte count
Complement system	CH-50, C3 convertase, C5 convertase

G.2 Coagulation

G.2.1 Activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT)

These tests generally measure coagulation disorders normally associated with abnormal levels of patient clotting factors.

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 rarely useful in the *in vitro* evaluation of the thrombogenic properties of blood-contacting devices/materials because the activating substances mask any activation caused by the device or its component materials.

They are not in general used in the assessment of medical devices and/or materials that contact blood.

G.3 Platelets

G.3.1 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[67]. A prolonged result indicates reduced platelet function or reduced platelet count; the latter can be determined separately. 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)[158]. The test is suitable for use with some experimental animals. *In vitro* bleeding time measurements are also suitable. This test is not used in the assessment of medical devices and/or materials that contact blood.

G.3.2 Platelet lifespan

The high gamma emission of 111In platelets are obtained from the patient's blood and are labelled with 51Cr or 111In[46][15][72][148]. Both 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 thrombogenesis. 111In has the advantage of being a high gamma emitter, requiring the labelling 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. This test is not recommended in the non-clinical assessment of medical devices and/or materials that contact blood.

G.4 Haematology

G.4.1 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), haemolysis or other mechanisms[55][77][111]. This test is not used in the assessment of medical devices and/or materials that contact blood.

G.5 Complement system

G.5.1 Complement activation assessment of CH-50, C3 convertase, C5 convertase

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[129][130][132][137].

Measurement of complement split products has the disadvantage of species specificity and high baseline levels when performed after *in vitro* testing. The classical CH-50 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. Commercially available ELISA kits for key complement components also exist.

These tests are in general not used in the assessment of complement activation of blood contacting medical devices and/or materials.