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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 concernant 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.

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

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

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

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

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

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

— *Part 1: Evaluation and testing*

— *Part 2: Animal welfare requirements*

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

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

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

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

— *Part 7: Ethylene oxide sterilization residuals*

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

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

— *Part 10: Tests for irritation and sensitization*

— *Part 11: Tests for systemic toxicity*

— *Part 12: Sample preparation and reference materials*

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

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

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

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

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

— *Part 18: Chemical characterization of materials*

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

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

Introduction

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

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

Biological evaluation of medical devices —

Part 4:
Selection of tests for interactions with blood

1 Scope

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

It describes

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

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

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 interactions of devices with blood. This part of ISO 10993 describes biological evaluation in general terms and may not necessarily provide sufficient guidance for test methods for a specific device.

2 Normative references

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

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

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

3 Terms and definitions

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

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

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

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

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

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

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

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

3.4
coagulation
phenomenon that results from activation of the clotting factor cascade

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

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

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

3.6
haematology
study of blood, including quantification of cellular and plasma components of the blood

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

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

4 Abbreviated terms

Bb	product of alternative pathway complement activation
β-TG	beta-thromboglobulin
C4d	product of classical pathway complement activation
C3a, C5a	(active) complement split products from C3 and C5
CD62L	L-selectin
CH-50	50% total haemolytic complement
CT	computerized tomography
D-Dimer	specific fibrin degradation products (F XIII cross-linked fibrin)
ECMO	extracorporeal membrane oxygenator

ELISA	enzyme/linked immunosorbent assay
EM	electron microscopy
FDP	fibrin/fibrinogen degradation products
FPA	fibrinopeptide A
F_{1+2}	prothrombin activation fragment 1 + 2
iC3b	product of central C complement activation
IVC	inferior vena cava
MRI	magnetic resonance imaging
PAC-1	monoclonal antibody which recognizes the activated form of platelet surface glycoprotein IIb/IIIa
PET	positron emission tomography
PF-4	platelet factor 4
PRP	platelet-rich plasma
PT	prothrombin time
PTT	partial thromboplastin time
P-selectin	receptor exposed during either platelet or endothelial cell release reaction
RIA	radioimmunoassay
S-12	monoclonal antibody, which recognizes the alpha-granule membrane component P-selectin exposed during the platelet release reaction
SC5b-9	product of terminal pathway complement activation
TAT	thrombin-antithrombin complex
TCC	terminal complement complex
TT	thrombin time
VWF	von Willebrand factor

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

5.1 Non-contact devices

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

5.2 External communicating devices

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

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

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

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

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

5.3 Implant devices

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

- annuloplasty rings,
- mechanical or tissue heart valves,
- prosthetic or tissue vascular grafts,
- circulatory support devices (ventricular-assist devices, artificial hearts, intra-aortic balloon pumps),
- inferior vena cava filters,
- embolization devices,
- endovascular grafts,
- implantable defibrillators and cardioverters,
- stents,
- arteriovenous shunts,
- blood monitors,

— internal drug delivery catheters,

— pacemaker leads,

— intravascular membrane oxygenators (artificial lungs),

— leukocyte-removal filters.

6 Characterization of blood interactions

6.1 General requirements

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

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

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

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

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

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

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

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

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

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

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

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

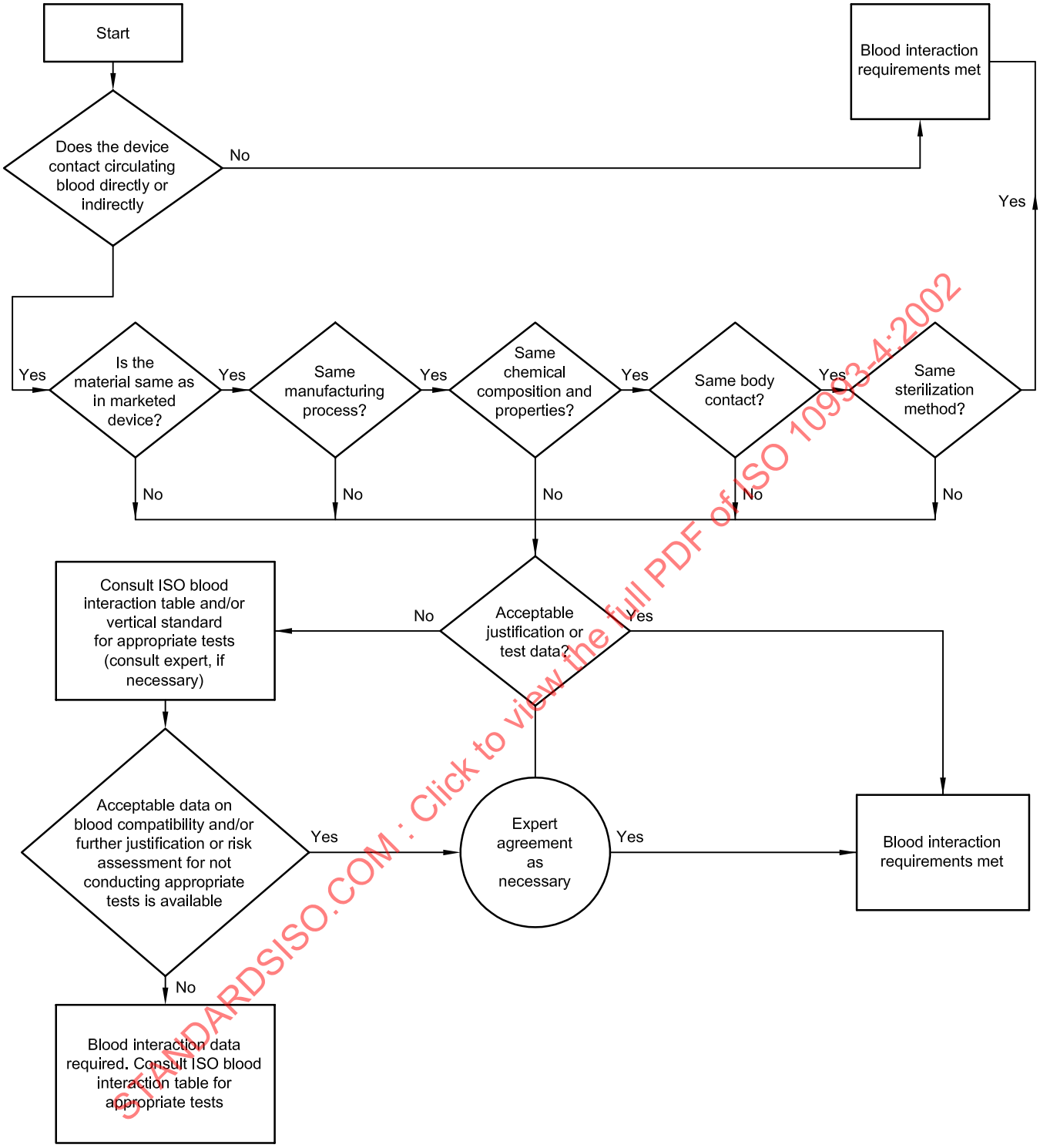

Figure 1 — Decision tree to determine whether testing for interaction with blood is necessary

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

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

[a] Haemolysis testing only.

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

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

[a] Haemolysis testing only.

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

6.1.8 The recommendations in 6.1.6 and 6.1.7, together with clause 5, Figure 1 and Table 2, serve as a guide for the selection of tests listed in 6.2.1.

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

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

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

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

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

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

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

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

6.2 Categories of tests and blood interactions

6.2.1 Recommended tests for interactions of devices with blood

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

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

a) thrombosis (see 3.3);

b) coagulation (see 3.4);

c) platelets (see 3.5);

d) haematology (see 3.6);

e) complement system (see 3.7).

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

Table 3 — Test methods for external communicating devices

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

Table 4 — Test methods for implant devices

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

6.2.2 Non-contact devices

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

6.2.3 External communicating devices

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

6.2.4 Implant devices

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

6.2.5 Indications and limitations

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

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

6.3 Types of test

6.3.1 In vitro tests

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

6.3.2 *Ex vivo* tests

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

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

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

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

6.3.3 *In vivo* tests

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

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

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

Annex A
(informative)

Preclinical evaluation of cardiovascular devices and prostheses

A.1 General considerations

A.1.1 Background

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

A.1.2 Classification

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

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

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

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

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

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

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

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

   2) formation of thrombi on the device surface;

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

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

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

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

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

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

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

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

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

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

A.1.4 Test protocols for animal testing

Thrombosis, thromboembolism, bleeding and infection are the major deterrents to the use and further development of advanced cardiovascular prostheses. For devices with limited blood exposure (< 24 h), important measurements are related to the acute extent of variation of haematological, haemodynamic and performance variables, gross thrombus formation and possible embolism. With prolonged or repeated exposure, or permanent contact (> 24 h), emphasis is placed on serial measurement techniques that may yield information regarding the time course of thrombosis and thromboembolism, the consumption of circulating blood components, the development of intimal hyperplasia and infection. In both of the above exposure and contact categories, assessment of 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, for example intimal hyperplasia and endothelialization.

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

A.1.5 Test protocols for *in vitro* testing

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

A.2 Cannulae

Cannulae are typically inserted into one or more major blood vessels to provide repeated blood access. They are also used during cardiopulmonary bypass and other procedures. They can be tested acutely or chronically, and are commonly studied as arteriovenous (AV) shunts. The use of cannulae induces little alteration in the levels of circulating blood cells or factors in the coagulation or complement system. Cannulae, like other indirect blood path devices (5.2.1), generally require less testing than devices in direct contact with circulating blood (5.2.2, 5.3).

A.3 Catheters and guidewires

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

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

The haemostatic response to cardiopulmonary bypass can be significant and acute. Many variables such as use of blood suction, composition of blood-pump priming fluid, hypothermia, blood contact with air and time of exposure influence test values. Emboli in outflow lines may be detected by the periodic placement of blood filters *ex vivo*, or the use of ultrasonic radiation or other non-invasive techniques. Thrombus accumulation can be directly assessed during bypass by monitoring performance factors such as pressure drop across the oxygenator and oxygen transfer rate. An acquired transient platelet dysfunction associated with selective alpha granule release has been observed in patients on cardiopulmonary bypass [31]; the template bleeding time and other tests of platelet function and release are particularly useful.

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

Therapeutic aphoresis equipment and devices for absorption of specific substances from the blood, because of their high surface-to-volume ratio, can potentially activate complement, coagulation, platelet and leukocyte pathways. Examination of blood/device interactions should follow the same principles as for extracorporeal oxygenators and 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, fibrinogen concentration, thrombin generation, platelet survival and activation, complement activation, and close monitoring of liver, renal, pulmonary and central nervous system functions. A detailed pathological examination at surgical retrieval is an important component of the evaluation [40], [41].

A.6 Heart valve prostheses

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

One of the most effective means of screening for prosthetic valve dysfunction is auscultation [42]. 2D and M mode echocardiography makes use of ultrasonic radiation to form images of the heart. Reflections from materials with

different acoustic impedances are received and processed to form an image. The structure of prosthetic valves can be examined. Mechanical prostheses emit strong echo signals and the movement of the occluder can usually be clearly imaged. However the quality of the image may depend upon the particular valve being examined. Echocardiography can also be useful in the assessment of function of tissue-derived valve prostheses. Vegetations, clots and evidence of thickening of the valve leaflets are elucidated. Using conventional and colour flow Doppler echocardiography, regurgitation can be identified and semi-quantified [42].

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 also recommended [41], [43].

A.7 Vascular grafts

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

A.8 IVC filters, stents and stented grafts

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

Annex B
(informative)

Laboratory tests — Principles, scientific basis and interpretation

B.1 General

B.1.1 Background

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

B.1.2 Principles for *in vitro* testing

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

B.1.3 Test conditions

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

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

B.1.4 Classification

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

B.2 Thrombosis

B.2.1 Percentage occlusion

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

B.2.2 Flow reduction

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

B.2.3 Gravimetric analysis (thrombus mass)

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

B.2.4 Light microscopy

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

B.2.5 Pressure drop across device

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

B.2.6 Scanning electron microscopy (SEM)

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

B.2.7 Antibody binding

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

B.2.8 Retrieval and examination of device

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

B.2.9 Autopsy of distal organs

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

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

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

B.3 Coagulation

Coagulation methods are based on the use of native (fresh, non-anticoagulated) whole blood, anticoagulated whole blood (usually citrated), platelet-rich plasma or platelet-poor plasma. Since most of the standard coagulation assays are designed to detect clinical coagulation disorders which result in delayed clotting or excessive bleeding, the protocols for evaluating blood/device interactions should be modified appropriately to evaluate accelerated

coagulation induced by biomaterials. Reagents for tests based on the activated partial thromboplastin time include an activator such as kaolin, celite or ellagic acid. Reagents with such activators should be avoided because they tend to mask the acceleration of coagulation which materials and devices cause. The material to be tested serves as the activator; controls (without the material) should be included.

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

B.3.1 Partial thromboplastin time (PTT)

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

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

B.3.2 Prothrombin time (PT)

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

B.3.3 Thrombin time (TT)

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

B.3.4 Thrombin generation

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

B.3.5 Fibrinogen

Dysfibrinogenaemia, afibrinogenaemia and hypofibrinogenaemia cause prolonged PT, PTT and TT results [21].

B.3.6 Fibrinogen and fibrin degradation products (FDP)

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

B.3.7 Specific coagulation factor assays

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

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

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

B.4 Platelets and platelet functions

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

B.4.1 Platelet count

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

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

B.4.2 Platelet aggregation

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

B.4.3 Blood cell adhesion

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

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

methacrylate) (PHEMA) is lower than to beads coated with polystyrene and certain other polymers. Isolated lymphocytes and PMNs were used in this study.

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

B.4.4 Platelet activation

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

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

b) altered platelet morphology,

c) the generation of platelet microparticles.

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

B.4.5 Template bleeding time

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

B.4.6 Platelet function analysis

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

B.4.7 Gamma imaging of radiolabelled platelets

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

B.4.8 Platelet lifespan (survival)

Platelets are obtained from the patient's blood and are labelled with ^{51}Cr or ^{111}In [23], [24], [32], [57]. 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 thrombopoiesis. ^{111}Indium 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.

B.5 Haematology

B.5.1 Leukocytes

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

B.5.2 Haemolysis

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

B.5.3 Reticulocyte count

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

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

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

Measurement of complement split products has the disadvantage of species-specificity and high baseline levels when performed after *in vitro* testing. The classical 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.

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

Annex C
(informative)

Evaluation of haemolytic properties of medical devices and their components

C.1 General considerations

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

C.2 Terms and definitions

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

C.2.1
anticoagulant
agent which prevents or delays blood coagulation

See [62].

EXAMPLE Heparin or citrate.

C.2.2
oncotic pressure
colloidal osmotic pressure
total influence of the proteins or other large molecular mass substances on the osmotic activity of plasma

See [62].

C.2.3
haematocrit
ratio of the volume of erythrocytes to that of whole blood in a given sample

C.2.4
haemolysis
liberation of haemoglobin from erythrocytes, either by destruction or through a partially damaged but intact cell membrane

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

NOTE See ISO 10993-12.

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

NOTE Properties of human erythrocytes for transfusion: the erythrocyte volume fraction of the component is 0,65 to 0,80. The unit contains all of the original unit's erythrocytes, the greater part of its leukocytes (about 2,5 to 3,0 $\times$ 10^9 cells) and a varying content of platelets depending on the method of centrifugation.

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

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

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

C.3 Causes of haemolysis

C.3.1 Mechanical forces — Pressure

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

C.3.2 Mechanical forces — Rheology

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

C.3.3 Biochemical factors

Changes to membrane structure on a molecular level can modify the strength and elastic properties of the erythrocyte membrane. A deficiency of nutritional factors or metabolic energy (ATP) can result in loss of the discoid shape and microvesiculation of haemoglobin. Other chemicals, bacterial toxins, pH and metabolic changes induced by temperature can compromise the erythrocyte membrane [63]. 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.

C.4 Clinical significance of haemolysis

C.4.1 Toxic effects

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

C.4.2 Thrombosis

Intravascular haemolysis can promote thrombosis by liberating phospholipids [66]. 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.

C.5 Determining a Pass/Fail assessment for haemolysis

Haemolysis is a function of 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 [67], [68], [69]. 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, should also be considered.

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 should be justified based on some form of risk and benefit assessment. The following questions are suggestions for developing such an assessment:

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

b) How much 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?

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

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

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

C.6 Haemolysis testing — General considerations

C.6.1 Methods

C.6.1.1 General

In vitro tests are used to evaluate damage to erythrocytes. Direct methods determine haemolysis due to physical and chemical interactions with erythrocytes. Indirect methods determine haemolysis due to extractables from test articles. ASTM F 756-00 is a standard that is specific for testing the haemolytic properties of materials (mainly due to chemical factors) and is not sufficient for testing whole medical devices. ASTM F 756-00 [10] (and the haemolysis test listed in GB/T 16175-1996 [11]) is given as an example and possible starting point for developing a protocol for haemolysis testing for a specific device. In addition to material testing of devices, dynamic testing of whole medical devices to evaluate the effects of the structure, intended use, and haemodynamic factors should be considered.

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. For medical device testing, in which the use of highly diluted erythrocyte suspensions is not applicable, blood haematocrit and other factors must be accounted for in the normalization of a haemolysis index [63].

At a minimum, each laboratory shall be able to measure the total blood haemoglobin concentration and the plasma or supernatant haemoglobin concentration. The concentration of haemoglobin in plasma is significantly less than the total blood haemoglobin concentration. The free plasma 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.

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

NOTE 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. by 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) [64], [65].

C.6.1.2 Total blood haemoglobin concentration measurements

C.6.1.2.1 Cyanmethaemoglobin method

The first classical method, cyanmethaemoglobin detection, was issued by the International Committee for Standardization in Haematology [71]. The cyanmethaemoglobin (haemoglobincyanide, 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 maxima at 540 nm. Lysing agents such as detergents are used which, in addition to releasing Hb from the erythrocyte, decrease the turbidity (a source of interference as false absorbance at 540 nm) from protein precipitation. For the total haemoglobin concentration, the spectral interference due to plasma is minimal and the sample absorbance can be compared to the HiCN standard solution directly.

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

C.6.1.2.2 Oxyhaemoglobin method

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

C.6.1.2.3 Iron method

The third classical method for determining the total 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.

C.6.1.3 Plasma or supernatant haemoglobin concentration measurements

C.6.1.3.1 Direct optical and added chemical techniques

Due to many different factors (e.g. tradition, ease of use, disposal of waste chemicals, availability of standard solutions), there are currently about twenty different assays in use today for measuring plasma haemoglobin as an indicator of haemolysis, but no one method is widely accepted. The assays can be classified into two broad categories: those which are direct optical techniques (i.e. based on quantifying the oxyhaemoglobin absorbance peak at 415, 541 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) [72]. 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 to the haemoglobin cyanide method [70]. The major disadvantages are that there is still a potential health risk in using benzidine dyes and an expense associated with disposal of reagents and products. Moreover, the reported dynamic range of this method is low (5 mg/dl to 50 mg/dl) [73], and possible reaction inhibition (by as much as 40 %) [74] may occur from calcium-chelating anticoagulants (e.g. citrates, oxalates, EDTA) [73], albumin, [75] or other non-specific plasma components [74] which may interfere with H_2O_2 oxidation.

For these reasons, direct optical methods, such as those by Harboe [76], Cripps [77], or Taulier [78] with comparable sensitivity and reproducibility may be substituted. However, as noted above, chemically induced alterations to 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 [72]. The researcher should be aware of these limitations in the plasma haemoglobin assays and ascertain wheher they are using an appropriate technique [64], [65], [72], [75]. 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.

C.6.1.3.2 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 [79].

C.6.2 Blood and blood component preservation

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

Anticoagulant solutions have been developed for use in blood collection that prevent coagulation and permit storage of erythrocytes for a certain interval of time. These solutions all contain sodium citrate, citric acid, and glucose; additionally, some contain adenine, guanosine, mannitol, sucrose, sorbitol and/or phosphate, among others [82], [83], [84], [85], [86], [87]. Although heparin is not used for blood preservation, it is often used for anticoagulation clinically with patients exposed to medical devices.

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

A considerable portion of glucose and adenine is removed with plasma when erythrocyte concentrates are prepared. Sufficient viability of the erythrocytes can only be maintained after removal of plasma if the cells are not over-concentrated. Normal citrate phosphate dextrose (CPD)-adenine erythrocyte concentrates should not have an erythrocyte volume fraction greater than 0,80. Even if more than 90 % of the plasma is removed, erythrocyte