INTERNATIONAL STANDARD

ISO 29701

First edition
2010-09-15

Nanotechnologies — Endotoxin test on nanomaterial samples for *in vitro* systems — *Limulus* amebocyte lysate (LAL) test

Nanotechnologies — Essai de détection d'endotoxines sur des échantillons de nanomatériaux pour des systèmes in vitro *— Essai au lysat d'amébocyte de* Limule *(LAL)*

Reference number
ISO 29701:2010(E)

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

Contents

Page

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 2.

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 document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 29701 was prepared by Technical Committee ISO/TC 229, *Nanotechnologies*.

Introduction

Endotoxins (lipopolysaccharides LPS) are part of the outer membrane of the cell wall of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*. Endotoxins can cause a variety of systemic reactions in mammals, including humans, such as fever, disseminated intravascular coagulation, hypotension, shock and death: the responses are mediated by production of various kinds of cytokines, activation of the complement cascade, activation of the coagulation cascade, etc. Endotoxins are present in the ordinary environment. Since most test samples of nanomaterials intended for *in vitro* and *in vivo* test systems require various preparation procedures, endotoxins might contaminate the test nanomaterials if the samples are prepared without special care.

For the purpose of toxicity screening or biocompatibility testing of nanomaterials, or mechanism studies on the possible toxicity induced by nanomaterials, various cell-based *in vitro* test systems and *in vivo* animal models are being developed and employed. In *in vitro* test systems, macrophages and other relevant mammalian cells are frequently used as the test cells especially for nanomaterials because they are primarily the responsible surveillance cells in the body. However, these cells are highly reactive to endotoxins; therefore it is difficult to distinguish the response to endotoxins from that to nanomaterials. Consequently, contamination by endotoxins would confound the result of tests *in vitro*.

Contamination by endotoxins of test samples may be reduced if appropriate precautions are followed in preparation of the test sample. Therefore the preliminary detection of endotoxins is required to minimize the contamination by endotoxins or confirm the insignificant levels of endotoxins in the test sample. It is also important to quantify endotoxin levels for the adequate interpretation of data obtained by *in vitro* biological test systems.

Since endotoxins may contaminate medical devices and medicines for parenteral use, quantitative and semi-quantitative assay methods to test for endotoxins both *in vivo* and *in vitro* have been developed and used for regulatory purposes as well as laboratory standard operational procedures for nanomaterials (see Reference [6]). The bacterial endotoxin test using *Limulus* amebocyte lysate (LAL) reagent has been developed as an *in vitro* assay method to test for the presence of endotoxin contamination as an alternative to the pyrogenicity test using rabbits, and methods are described in the pharmacopoeia of many countries.

This International Standard provides considerations for the application of the LAL test to nanomaterial samples intended for *in vitro* biological tests.

Nanotechnologies — Endotoxin test on nanomaterial samples for *in vitro* systems — *Limulus* amebocyte lysate (LAL) test

1 Scope

This International Standard describes the application of a test using *Limulus* amebocyte lysate (LAL) reagent for the evaluation of nanomaterials intended for cell-based *in vitro* biological test systems. The test is suitable for use with nanomaterial samples dispersed in aqueous media, e.g. water, serum or reaction medium, and to such media incubated with nanomaterials for an appropriate duration at 37 °C.

This International Standard is restricted to test samples for *in vitro* systems, but the methods can also be adapted to nanomaterials to be administered to animals by parenteral routes.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
coagulogen
clottable protein in LAL which is known to play a central role in gel-clot formation by endotoxins

NOTE Coagulogen derived from Japanese horseshoe crab (*Tachypleus tridentatus*) consists of a total 175 amino acids with the molecular weight of 19,723 (see Reference [7]).

2.2
coagulin
resulting fragments of coagulogen after limited proteolysis of clotting enzyme in LAL

NOTE A coagulin derived from Japanese horseshoe crab (*Tachypleus tridentatus*) consists of the N-terminal fragment peptides (Ala1 – Arg18) and the C-terminal fragment peptides (Gly47 – Phe175) (see Reference [7]).

2.3
endotoxin
part of the outer membrane of the cell envelope of Gram-negative bacteria

NOTE The main active ingredient is lipopolysaccharides (LPS).

2.4
endotoxin unit
EU
standard unit of endotoxin activity

NOTE 1 The endotoxin unit was defined by the World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) in 1996, relative to the activity of 0,1 ng of WHO reference standard endotoxin (RSE) from *Escherichia coli* 0113:HK10:K(-) or 10 EU/ng (see Reference [8]).

NOTE 2 EU is equal to international unit (IU) of endotoxin.

2.5
lambda
λ
labelled sensitivity of LAL for gel-clot method or the lowest endotoxin concentration on the standard curve for chromogenic or turbidimetric methods, expressed in EU/mL

2.6
***Limulus* amebocyte lysate**
LAL
aqueous extract of the blood corpuscle of horseshoe crabs, *Limulus polyphemus* or *Tachypleus tridentatus*

2.7
***Limulus* amebocyte lysate test**
LAL test
test for measuring bacterial endotoxins using *Limulus* amebocyte lysate reagent

NOTE The LAL test is called “bacterial endotoxin test (BET)” in pharmacopoeia.

2.8
optical density
OD
optical absorbance of an optical element for a given wavelength per unit distance

2.9
test sample
aqueous dispersion or aqueous extract of nanomaterials under investigation

3 Abbreviated terms

BET	bacterial endotoxin test
CSE	control standard endotoxin
ECBS	expert committee on biological standardization
EF	endotoxin-free
EU	endotoxin unit
I/EC	inhibition/enhancement control
LAL	*Limulus* amebocyte lysate
LPS	lipopolysaccharide
OD	optical density
RSE	reference standard endotoxin
WHO	World Health Organization

4 Pre-test considerations

4.1 Storage of nanomaterials

Nanomaterials because of their high surface area can collect many of the contaminants including endotoxins from the environment. For this reason, nanomaterials shall be collected and stored in endotoxin-free, sealable containers (e.g. glassware) upon arrival until use. Suitable blanks such as endotoxin-free metal oxide powders like titanium dioxide, silicon dioxide, etc. shall be used to verify the absence of endotoxin contamination.

NOTE 1 It is advisable that plastics like polypropylene be avoided for the storage of nanomaterials, due to the possible interference with the LAL test as shown in Annex A.

NOTE 2 Endotoxin-free metal oxide powders can be obtained by heat-treatment (see 4.2).

4.2 Storage containers

Glassware and other heat stable storage containers for storage of nanomaterials and test samples should be treated by heating to a temperature of greater than 250 °C for at least 30 min or other validated combinations of temperature and time (e.g. 180 °C for at least 3 h, or 650 °C for 1 min) to eliminate endotoxins. Commercially available sterile endotoxin-free polystyrene containers can be used.

4.3 Handling of nanomaterials

Dust found in the indoor environment usually contains significant amounts of endotoxins. Special attention shall be paid to avoid contact between dust and nanomaterials during sampling and handling. A clean air laboratory condition is required (recommended in 6.5).

5 Test sample

5.1 Aqueous dispersion

Nanomaterials which are dispersed in aqueous liquid may be subjected to the LAL test directly or after dilution with endotoxin-free water.

5.2 Aqueous extract

Endotoxin-free reaction medium, physiological saline solution or other extraction vehicles incubated with nanomaterials may be used as test sample for the LAL test.

6 Preparation of test sample

6.1 Dispersion method

Test dispersions might be prepared by one or more of the following:

— hand grinding;

— mechanical milling;

— ultrasonication.

The dispersion medium will depend on the purpose and the particular *in vitro* test.

NOTE Nanomaterials can have high surface area, porosity, hydrophobicity and other properties that can make this step difficult. Therefore, methods development might be required.

6.2 Extraction method

The extraction conditions, such as extraction medium, incubation time, incubation temperature and the concentration of the test sample may simulate the incubation condition of the *in vitro* test concerned. Reaction medium without pH indicator (e.g. phenol red) or buffered saline are preferable for the extraction medium to avoid the interference with colour. The extraction medium should be certified endotoxin-free or reconstituted from endotoxin-free reagents. Addition of antibiotics and antimycotics to the extraction medium might be effective to prevent respectively bacterial and fungi growth. Interference effects of the added anti-biotic agents to the LAL test should be validated (see 7.3.3). After extraction, the extraction mixture shall be centrifuged to remove the particulates, and the supernatant, which shall serve as a test sample for the LAL test, should be collected to endotoxin-free tubes or containers with endotoxin-free pipette tips. The extraction conditions including centrifugation shall be justified and recorded. In particular, centrifugation condition shall be determined according to the nanomaterials concerned.

NOTE 1 0,05 % polysorbate 20 is proposed as an extraction vehicle for airborne endotoxin from glass fibre filters (see Reference [9]).

NOTE 2 0,1 % vitamin E surfactant (vitamin E *d*-α-tocopheryl polyethylene glycol-1000 succinate) was found to improve the extraction of endotoxin from carbon nano-objects (see Reference [10]).

NOTE 3 For more information on the extraction methods, see ISO 10993-12:2007.

6.3 Concentration

Test sample shall be reconstituted in endotoxin-free water to the highest concentration in the cell-based *in vitro* test concerned, if necessary.

6.4 Storage of test sample

The test sample shall, if possible, be tested as soon as possible after preparation because degradation of endotoxins in the test sample or bacterial growth during storage can occur. The test sample shall be stored in an endotoxin-free, sealable container (see 4.2) at a temperature of between 2 °C and 8 °C. If the test sample is stored longer than 24 h, the stability and homogeneity of the test sample under the storage conditions shall be verified.

6.5 Laboratory environment

6.5.1 Tap water and air cleanliness

Tap water and dust found in the indoor environment usually contain significant amounts of endotoxins. Nanomaterials shall be processed with endotoxin-free medium and endotoxin-free laboratory-ware to ensure aseptic sample preparation. A clean room, a clean air hood, or an equivalent clean air device with an air cleanliness of ISO Class 5 (see ISO 14644-1) shall be used in laboratory circumstances where airborne endotoxin contamination is a demonstrated problem, unless otherwise justified. For guidance on air cleanliness, see ISO 14644-1, ISO 14644-2 and ISO 14644-7.

6.5.2 Equipment and laboratory-ware

Equipment and laboratory glassware used for the preparation of the test sample should be treated by heating to a temperature of greater than 250 °C for at least 30 min or other validated combinations of temperature and time (e.g. 180 °C for at least 3 h, and 650 °C for 1 min) to eliminate endotoxins. Heat-labile or other materials which are not suitable for heat-treatment shall be treated with measures other than heat treatment to reduce endotoxins. Rinsing with endotoxin-free water after soaking the materials in strong alkali or oxidizing solution is a reliable method to remove endotoxins. If strong alkali or an oxidizing solution is used, the method needs to be validated to ensure that the method reduces the presence of endotoxins and that no residuals remain after treatment that interfere with the test. With respect to heat-labile laboratory-ware such as containers, tubes, tips for micropipettes, endotoxin-free plastic products are commercially available.

NOTE It is advisable to use polystyrene products when plastic products are used.

6.5.3 Rinse water

Water is one of the sources of endotoxins detected in equipment and laboratory-ware. Distilled water may be used for rinsing the equipment and laboratory-ware after endotoxin reduction treatments. However, distilled water prepared in-house might be contaminated with endotoxins due to inadequate equipment or inappropriate handling, although distillation has been shown to be effective in removing endotoxins from contaminated water (see Reference [11]). The endotoxin level in the distilled water prepared in-house shall be measured periodically to validate that it contains insignificant levels of endotoxins. If endotoxin contamination in distilled water is unavoidable, commercially available endotoxin-free water should be used.

7 Test methods

7.1 Principle

Endotoxins activate a factor in the LAL and trigger a proteolytic cascade (see Reference [12]). The clotting enzyme, which is released from the proclotting enzyme by one of the activated factors, catalyses a proteolysis of coagulogen in the LAL and the resulting fragments, coagulins, spontaneously bind to each other through disulfide linkage to develop the turbidity of the LAL and finally form a gel-clot. The gel-clot formation is principally determined by visual inspection after inverting test tubes. This method requires no optical reader and the procedures are easy to perform. The most sensitive gel-clot method using commercially available reagents measures 0,015 EU/mL.

NOTE One of the practical procedures for the gel-clot method is described in Annex B.

7.2 Alternative test methods

7.2.1 Endpoint photometric methods

The optical density (OD) of the reaction mixture is measured after a certain period of reaction time. With regard to endpoint photometric methods, there are two techniques; the turbidimetric technique measuring the turbidity of the reaction mixture and the chromogenic technique measuring *p*-nitroaniline (*p*-NA) liberated from a synthetic substrate, such as Boc-Leu-Gly-Arg-*p*-NA or Boc-Thr-Gly-Arg-*p*-NA for the clotting enzyme. Due to the low sensitivity and a technical difficulty stopping the progress of turbidity generation at a designated time point, the simple turbidimetric method is replaced with the kinetic turbidimetric method described in 7.2.2. There are at least two procedures for measuring *p*-NA in the reaction mixture:

— one measures the OD of *p*-NA directly at a wavelength of 405 nm, and

— the other measures the diazotized magenta derivative of *p*-NA photometrically at a wavelength of between 540 nm and 550 nm.

The sensitivity of endpoint photometric method using commercially available reagents by measuring the OD at a wavelength of 405 nm is 0,01 EU/mL while that of the diazo-coupling method is 0,001 EU/mL.

NOTE One of the practical procedures for the endpoint photometric method is described in Annex C.

7.2.2 Kinetic methods

The time required to reach the predetermined OD of the reaction mixture or the rate of colour or turbidity development is determined by an optical reader. With regard to kinetic procedures, the OD of *p*-NA liberated from the synthetic peptide stated above or turbidity of the reaction mixture is read at multiple time points as the reaction proceeds, and thus several types of automated instruments have been developed. To detect endotoxins more precisely and accurately with kinetic methods, sophisticated automated instruments are necessary. The best sensitivity of the kinetic method using a commercially available automated instrument is 0,001 EU/mL.

NOTE One of the practical procedures for the kinetic method is described in Annex D.

7.3 Selection and validation of the test method

7.3.1 Considerations of minimum required sensitivity

The critical concentration for endotoxins in *in vitro* test systems to produce biological reactions varies according to the *in vitro* test systems employed; 0,01 ng of endotoxin/mL induced IL-1β and TNF-α in human macrophages (see Reference [13]) whereas 0,048 ng of endotoxin induced insignificant biological responses in human dendritic cells or peripheral blood mononuclear cells (see Reference [13]). It is highly desirable to know the allowable contamination level of endotoxins in *in vitro* test systems to select the appropriate LAL test. The sensitivity of the LAL reagent should be better than or at least equivalent to the allowable contamination level in the *in vitro* test concerned.

7.3.2 Inhibition/enhancement potential to test by test sample

7.3.2.1 It is reported that several materials interfere with the LAL test. Some nanomaterials may have been recently synthesized and have unknown inhibition/enhancement potential on the LAL test due to their properties.

NOTE Examples of potential interferences are available in Annex A.

7.3.2.2 Interference by colour or turbidity of the test sample is a matter of concern when endpoint photometric methods or kinetic methods are employed. The test method should be selected according to the optical feature of the test sample.

7.3.3 Validation of test method

7.3.3.1 The interfering effects of nanomaterials on the endotoxin test can be examined by testing a series of dilutions of the test sample with and without a known amount of spike endotoxin.

NOTE 1 The guidance procedures for the validation of test methods are described in Annexes B, C and D.

NOTE 2 For information on the validation of test methods, see pharmacopoeia (e.g. References [26], [27] and [28]).

7.3.3.2 The optimum pH for the LAL reaction is between 6 and 8. A pH measurement shall be taken on the reaction mixture consisting of the test sample and lysate using an appropriate pH test system when the test is inhibited. If pH adjustment is necessary, adjust the pH of the test sample so that the pH of the reaction mixture falls within the above pH range.

NOTE 1 For pH adjustment purposes, endotoxin-free tris hydroxymethyl aminomethane (TRIS) buffer, 0,1N NaOH or 0,1N HCl may be used.

NOTE 2 Salts resulting through pH adjustment can interfere with the LAL reaction (see Annex A).

7.4 Test procedures

One of the test methods described as “Bacterial endotoxin tests” or “Bacterial endotoxins” in the current version of the European, Japanese or United States Pharmacopoeia, shall be used.

8 Assessment of results

8.1 General

When assessing the results of the LAL test on nanomaterials, good scientific judgment should be applied, keeping in mind the limitation of the test. It is especially important to consider the nature of any interference of the test nanomaterials on the LAL test. In such cases, the acceptable limitation of the interfering effects shall be discussed and careful interpretation of data shall be made.

8.2 Guidance on application of test

8.2.1 If interference cannot be successfully overcome by dilution of the test sample or by other measures, the LAL test cannot be applied to such test sample.

8.2.2 When contamination by endotoxins is inevitable, treatment of the test sample with reagents such as polymyxin B can be applied to eliminate the effects by endotoxins (see References [15] and [16]).

8.2.3 It is well known that serum may be contaminated with β-1,3-glucan which originates from yeast, fungi or other microbes and that an ordinary LAL reagent reacts with β-1,3-glucan in addition to endotoxins (see Reference [12]). Thus, it is highly recommended to use β-1,3-glucan-free serum for the preparation of test samples, if necessary. Endotoxin-specific LAL reagents which do not react with β-1,3-glucan should be used when β-1,3-glucan contamination is suspected.

9 Test report

9.1 The test report shall be in accordance with the test procedures used.

9.2 The test report shall include the following:

a) test results;

b) test procedures;

c) complete identification of the nanomaterials tested;

d) procedures for test sample preparation, storage condition of the test sample and information on the classification of the laboratory environment used;

e) identification of the LAL reagent (trade-name, manufacturer's code, catalogue or formulation number, batch number or date of manufacture, sensitivity, etc.);

f) identification of standard endotoxin (trade-name, manufacturer's code, catalogue or formulation number, batch number or date of manufacture, potency, etc.);

g) validity of the test including interference by the test sample.

NOTE For information on the records, see ISO 10993-12:2007.

Annex A
(informative)

Examples of potential interferences to LAL test

A.1 Inhibition

A.1.1 Salts (see Reference [17]): CH_3COONa (0,3M), $NaHCO_3$ (0,1M), KCl (50mM), NaCl (0,6M).

NOTE Reversible: dilution of test sample overcomes the inhibition.

A.1.2 Heparin (see Reference [18]).

NOTE Reversible (addition of Na^+ and Ca^{2+} overcomes the inhibition). A commercial endotoxin-free cation-buffer can be used to overcome the inhibition.

A.1.3 Metal ions (see References [19] and [20]): Fe^{2+}, Fe^{3+}, Cr^{3+}, Al^{3+}.

NOTE Irreversible: addition of EDTA-Na partly recovers the activity.

A.1.4 Plastics (polypropylene) (see Reference [21]).

A.1.5 Particles functionalized with quaternary ammonia (see Reference [22]).

A.1.6 Particle filters: Cellulose ester (see Reference [23]).

A.1.7 Protease inhibitors (see Reference [24]).

A.1.8 Polysorbate 20 (see Reference [25]).

NOTE Reversible: dilution of test sample overcomes the inhibition.

A.2 Enhancement

A.2.1 EDTA-4Na (see Reference [19]).

NOTE 1 Mechanism has not been elucidated yet.

NOTE 2 Although a high concentration of EDTA-Na can inhibit the LAL reaction, due to its chelating activity with divalent cations which are needed in the test reaction, no interference (inhibition or enhancement) with the LAL test was observed by EDTA-2Na or -3Na up to 0,5 mM in the reaction mixture (see Reference [19]).

Annex B
(informative)

Gel-clot method

B.1 General

This annex describes an example of the procedures for the gel-clot method using LAL reagent-preformulated test tubes.

B.2 Reagents

B.2.1 LAL reagent-preformulated test tubes for the gel-clot method with a sensitivity of λ.

B.2.2 Endotoxin-free water (EF-water).

B.2.3 Lyophilized control standard endotoxin (CSE)[1)].

NOTE Lyophilized LAL reagent for dispensing is commercially available or users can prepare their own.

B.3 Equipment

B.3.1 Endotoxin-free glassware or **endotoxin-free polystyrene tubes or containers** for making dilutions of endotoxin standard or test sample.

NOTE 1 For the preparation of endotoxin-free equipment and laboratory-ware, see 6.5.2.

NOTE 2 Endotoxin-free products are labelled as pyrogen-free, in general.

B.3.2 Test tube racks to hold, to incubate, or to hold and incubate reaction tubes.

B.3.3 Pipettes, **automatic pipettors with pipette tips**, or **repeating pipettors with plastic syringe barrels**, all endotoxin-free.

B.3.4 Non-circulating water bath or **dry block incubator** capable of maintaining (37 ± 1) °C.

B.3.5 Vortex-type mixer.

B.3.6 Timer.

1) Official endotoxin preparations that have been standardized against WHO or US Food and Drug Administration (FDA) reference standard endotoxin (RSE).

B.4 Preparation of the standard endotoxin solution

B.4.1 Preparation of the stock solution

A control standard endotoxin (CSE) as a lyophilized powder shall be used. The contents of the vial containing the CSE shall be reconstituted with EF-water to make a CSE stock solution. The actual concentration of the vial will be determined by the value stated on the accompanying certification.

B.4.2 Preparation of the calibration standards

Calibration standards shall be prepared by dilution of the CSE stock solution with EF-water. The concentration in the calibration standards shall consist of four concentrations including 0,25λ, 0,5λ, λ and 2λ. The dilution factor of the CSE stock solution to any concentration should not exceed 10 to minimize the pipetting error.

B.5 Preparation of the inhibition/enhancement control (I/EC)

An I/EC should be prepared by dilution of the CSE solution with undiluted test sample. The concentration in the I/EC shall consist of four concentrations including 0,25λ, 0,5λ, λ and 2λ. In order to avoid a significant dilution of the test sample, the amount of the CSE solution for spike should not exceed 5 % (e.g. the amount of the CSE solution for spike should not exceed 50µL in a total I/EC volume of 1,0 mL).

B.6 Dilution of the test sample

A series of twofold dilutions (e.g. ×2, ×4, ×8 or more, if necessary) of the test sample with EF-water shall be prepared.

B.7 Experimental procedures

B.7.1 Confirmation of the labelled sensitivity of LAL reagent

B.7.1.1 A manufacturer specified volume of EF-water (negative control) or the calibration standard (0,25λ, 0,5λ, λ, or 2λ in EF-water) shall be dispensed carefully into a test tube preformulated with the LAL reagent for gel-clot assay, and mixed. Quadruplicate measurements shall be performed.

B.7.1.2 The test tubes shall be kept in a non-circulating water bath or a dry block incubator maintained at (37 ± 1) °C for (60 ± 2) min, avoiding vibration.

B.7.1.3 To test the integrity of the gel after incubation, each tube shall be inverted through 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, the result shall be recorded as positive. A result shall be negative if either a firm gel is not formed, or if a fragile gel has formed but flows out upon inversion.

B.7.1.4 The test is valid when both the negative control (EF-water) and the lowest concentration (0,25λ) of the calibration standard are negative.

B.7.1.5 The lowest concentration which yields a positive result is defined as the endpoint concentration. The geometric mean of the four endpoint concentrations, c, in the calibration standard is considered as the sensitivity of the LAL reagent in EF-water, as follows:

$$c = \text{antilog}\left(\frac{\sum e}{f}\right)$$

where

$\sum e$ is the sum of the log endpoint concentrations of the dilution series used;

f is the number of measurements.

B.7.1.6 The sensitivity of the LAL reagent in EF-water should be within the range of 0,5λ and 2λ.

NOTE It is advisable that the labelled sensitivity be confirmed for each lot of LAL reagent.

B.7.2 Validation of the test method

B.7.2.1 A manufacturer specified volume of EF-water (negative control), the calibration standards (0,25λ, 0,5λ, λ, or 2λ in EF-water), I/EC (0,25λ, 0,5λ, λ, or 2λ in the test sample) or the test sample shall be dispensed carefully into a test tube preformulated with the LAL reagent for gel-clot assay, and mix. Duplicate measurements for the calibration standard and negative control, and quadruplicate measurements for I/EC and the test sample shall be performed.

B.7.2.2 The test shall be performed as described in B.7.1.2 and B.7.1.3.

B.7.2.3 The test is valid when the negative control (EF-water) and the test sample are negative and the result of the calibration standard confirms the labelled sensitivity of the LAL reagent within the range of 0,5λ and 2λ.

NOTE The test sample is diluted to a dilution containing no detectable endotoxin if the test sample is contaminated with endotoxins.

B.7.2.4 The geometric mean of the endpoint concentrations in I/EC shall be calculated as described in B.7.1.5, and the value should be confirmed within the range of 0,5λ and 2λ.

B.7.2.5 If the geometric mean of the endpoint concentrations in I/EC is out of the above range, the test shall be repeated with a greater dilution of the test sample. The labelled sensitivity of the LAL reagent should be corrected by multiplying the dilution factor which overcomes interference.

B.7.2.6 When interference with the test sample is not overcome by the dilution of the test sample, other measures such as filtration, neutralization, dialysis or heat treatment of the test sample may be applied to eliminate interference.

B.7.2.7 When interference is not overcome by dilution of the test sample or other plausible measures mentioned above, the LAL test by the gel-clot method should not be applied to the test sample.

B.7.3 Assay with the test sample

B.7.3.1 A designated amount (according to the manufacturer's instructions) of EF-water (negative control), I/EC (e.g. 2λ in the test sample), the calibration standard (0,25λ, 0,5λ, λ, or 2λ in EF-water), or the test sample (undiluted, x2 diluted, x4 diluted, or x8 diluted in EF-water) shall be dispensed carefully into a test tube preformulated with the LAL reagent for gel-clot assay and mixed. Duplicate measurements shall be performed.

B.7.3.2 The test shall be performed as described in B.7.1.2 and B.7.1.3.

B.7.3.3 The test is valid when the following conditions are met:

a) the replicates of the negative control (EF-water) are negative,

b) the replicates of I/EC are positive, and

c) the geometric mean endpoint concentration of the calibration standard is within the range of 0,5λ and 2λ.

B.7.3.4 The highest dilution factor which yields the last positive result is defined as the endpoint, and the endpoint concentration is calculated by multiplying the endpoint by the labelled sensitivity (λ) or the corrected sensitivity to obtain the endotoxin concentration in the undiluted test sample.

B.7.3.5 The geometric mean of endotoxin concentration shall be reported as the endpoint concentration in the test sample

B.7.3.6 If none of the dilutions of the test sample is positive, the endotoxin concentration in the test sample shall be reported to be less than the labelled sensitivity (λ) or the corrected sensitivity.

B.7.3.7 If all dilutions are positive, the endotoxin concentration in the test sample shall be reported to be equal to or greater than the highest dilution factor multiplied by the labelled sensitivity (λ) or the corrected sensitivity.

Annex C
(informative)

Endpoint photometric method

C.1 General

This annex describes an example of the procedure for the endpoint photometric method using a synthetic substrate, such as Boc-Leu-Gly-Arg-*p*-NA or Boc-Thr-Gly-Arg-*p*-NA. The amount of *p*-NA in the reaction mixture is measured by the optical density (OD) of *p*-NA at a wavelength of 405 nm.

C.2 Reagents

C.2.1 **Lyophilized LAL reagent** for chromogenic method with a sensitivity of λ.

C.2.2 **Reconstitution buffer** for the LAL reagent.

C.2.3 **Lyophilized control standard endotoxin** (CSE).

C.2.4 **Endotoxin-free water** (EF-water).

C.2.5 **Stop solution** (see the LAL reagent manufacturer's direction).

NOTE Reagents are commercially available as a pre-packed kit.

C.3 Equipment

C.3.1 **Endotoxin-free glass tubes** or **endotoxin-free polystyrene microtiter plates** (microplates).

NOTE 1 For the preparation of endotoxin-free equipment and laboratory-ware, see 6.5.2.

NOTE 2 Endotoxin-free products are labelled as pyrogen-free, in general.

C.3.2 **Endotoxin-free glassware** or **endotoxin-free polystyrene tubes or containers** with adequate capacity for making dilutions of endotoxin standard or test sample.

NOTE 1 For the preparation of endotoxin-free equipment and laboratory-ware, see 6.5.2.

NOTE 2 Endotoxin-free products are labelled as pyrogen-free, in general.

C.3.3 **Test tube racks** to hold, to incubate, or to hold and incubate reaction tubes.

C.3.4 **Pipettes**, **automatic pipettors with pipette tips**, or **repeating pipettors with plastic syringe barrels**, all endotoxin-free.

C.3.5 **Water bath** or **dry block incubator** capable of maintaining (37 ± 1) °C.

C.3.6 **Vortex-type mixer**.

C.3.7 **Optical reader** (spectrophotometer) or **microplate reader**, suitable for measurement at a wavelength of 405 nm.

C.3.8 **Timer**.

C.4 Preparation of the standard curve

C.4.1 Preparation of the stock solution

A control standard endotoxin (CSE) as a lyophilized powder shall be used. The contents of the vial containing CSE should be reconstituted with EF-water to make a CSE stock solution. The actual concentration of the vial will be determined by the value stated on the accompanying certification.

C.4.2 Preparation of the calibration standards

The calibration standards shall be prepared by dilution of the CSE stock solution with EF-water. The calibration standards shall consist of at least three concentrations including the labelled sensitivity (λ) of the LAL reagent. The dilution of the CSE solution shall be made in a serial dilution according to the manufacturer's instructions. When diluting the CSE stock solution to any concentration, the dilution factor should not exceed 10 to minimize the pipetting error.

C.5 Preparation of the inhibition/enhancement control (I/EC)

An I/EC shall be prepared by dilution of the CSE stock solution with undiluted test sample. There are several options for the endotoxin concentration in the I/EC (e.g. 2λ, 4λ or the middle concentration of the standard curve). Generally the middle concentration of the standard curve is employed. In order to avoid a significant dilution of test sample, the amount of the CSE solution for spike should not exceed 5 % (e.g. the amount of CSE solution should not exceed 50µL in a total I/EC volume of 1,0 mL).

C.6 Experimental procedure

C.6.1 Lyophilized LAL reagent containing a synthetic substrate shall be dissolved with EF-water or a suitable buffer to make the LAL reagent solution according to the manufacturer's instructions.

C.6.2 A manufacturer specified volume of LAL reagent shall be mixed with a specified volume of EF-water (negative control), the calibration standard, I/EC or the test sample in a reaction tube or a microtiter plate.

C.6.3 The test tubes or microtiter plate shall be placed in the incubating device and incubated for a specified period of time at (37 ± 1) °C, according to the manufacturer's instructions.

C.6.4 A manufacturer specified volume of the stop solution shall be added to the reaction mixture and the OD of the reaction mixture shall be read at a wavelength of 405 nm by an optical reader or a spectrophotometer.

C.6.5 For an automated system, the equipment and test procedure shall be modified to that specified for the system.

C.7 Assay acceptance criteria

C.7.1 The standard curve shall be constructed by plotting the OD reading against the concentration of the calibration standards.

C.7.2 The negative control should be non-reactive (i.e. the endotoxin value calculated for the negative control should not eclipse the bottom point on the standard curve).

C.7.3 A linear regression algorithm is used to build the standard curve.

C.7.4 The correlation coefficient of the standard curve should be at least 0,980. If the standard curve fails to meet the acceptance criterion, the run should be repeated.

C.7.5 Precision (percent coefficient of variation, %CV) of the study sample should be within 25 %.

C.8 Calculation of the endotoxin concentration in the test sample

C.8.1 The assay acceptance criteria should be achieved.

C.8.2 The arithmetic mean of OD of each test level shall be fitted to the standard curve to read the endotoxin concentration.

C.8.3 The endotoxin concentration of each test level should be multiplied by the corresponding dilution factor to obtain the endotoxin concentration of the undiluted test sample.

C.8.4 The endotoxin concentrations shall be averaged arithmetically and the mean value shall be considered as the endotoxin concentration of the undiluted test sample.

C.9 Validation of the test method

C.9.1 The recovery of endotoxin in the I/EC shall be calculated by dividing observed endotoxin concentration in the test sample by the nominal endotoxin concentration. Recovery is expressed in percentage.

C.9.2 The recovery of endotoxin concentration added in the I/EC should be within 50 % to 200 % of the nominal value. If the recovery of added endotoxin concentration is outside of the above range, the study sample is interfering with the assay. If interference is detected, the test should be repeated using a test sample without significant interference. Dilution or other measures such as filtration, neutralization, dialysis or heat treatment of the test sample may eliminate interference. Accordingly, I/EC should be prepared using the test sample without interference.

Annex D
(informative)

Kinetic method

D.1 General

This annex describes an example of the procedure for the kinetic methods using turbidimetric or chromogenic techniques using an incubating optical reader or microplate reader[2)].

D.2 Reagents

D.2.1 **Lyophilized LAL reagent** for turbidimetric or chromogenic method with a sensitivity of λ.

D.2.2 **Endotoxin-free water** (EF-water) or **reconstitution buffer** for the LAL reagent.

D.2.3 **EF-water**.

D.2.4 **Lyophilized control standard endotoxin** (CSE).

NOTE Reagents are commercially available as a pre-packed kit.

D.3 Equipment

D.3.1 Endotoxin-free glass tubes or endotoxin-free polystyrene microtiter plates (microplates).

NOTE 1 For the preparation of endotoxin-free equipment and laboratory-ware, see 6.5.2.

NOTE 2 Endotoxin-free products are labelled as pyrogen-free, in general.

D.3.2 Endotoxin-free glassware or endotoxin-free polystyrene tubes or containers, with adequate capacity for making dilutions of endotoxin standard or test sample.

NOTE 1 For the preparation of endotoxin-free equipment and laboratory-ware, see 6.5.2.

NOTE 2 Endotoxin-free products are labelled as pyrogen-free, in general.

D.3.3 Pipettes, automatic pipettors with pipette tips, or repeating pipettors with plastic syringe barrels, all endotoxin-free.

D.3.4 An incubating optical reader or a microplate reader.

2) Equipment which maintains the test tubes or microtiter plate at a designated temperature and initiates photometrical measurement continuously at certain intervals during the incubation time.