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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)*

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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 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.

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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.

