
**Nanotechnologies — Characteristics of
working suspensions of nano-objects
for *in vitro* assays to evaluate inherent
nano-object toxicity**

*Nanotechnologies — Caractéristiques des suspensions de nano-objets
utilisées pour les tests in vitro évaluant la toxicité inhérente aux
nano-objets*



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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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Foreword

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

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

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

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

For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: [Foreword - Supplementary information](#)

The committee responsible for this document is ISO/TC 229, *Nanotechnologies*.

Introduction

Before nano-objects enter into the market, their possible impact on human health and the environment needs to be carefully evaluated.

In vitro toxicity assays using cultured cells are frequently used as a tool in screening hazardous materials. This testing provides essential information for understanding the mechanisms of biological effects induced by the materials. However, nano-objects require specific considerations with respect to the *in vitro* toxicity assays, because their behaviour is distinct from water soluble chemicals. For example, immediately after the introduction of nano-object samples into the culture medium, the nano-objects undergo changes, such as (a) dissolution, which is the dissolving of nano-objects into their ionic counterparts, (b) corona formation, which is the adsorption of the components of culture medium onto the nano-object surface, or (c) changes in aggregation/agglomeration state, leading to alteration in particles size and sedimentation. Therefore, it is critical to consider the aforementioned phenomena in clarifying if the observed effects are related to the tested nano-object itself or from other uncontrolled sources and to avoid false interpretation of assay results.

The rigorous characterization of the working suspension prior and during *in vitro* toxicity assays is essential to exclude the *in vitro* experimental artefacts. For example the corona formation, metal ion release from the nano-objects and impurities (residual from the nano-object synthesis process) can interfere with some *in vitro* assays,^[1] producing inaccurate results. Additionally, the formation of agglomerates and aggregates can alter the toxicity of a suspension. Therefore, it is important to carefully assess and describe the characteristics of the suspension of nano-objects being tested.

This Technical Specification describes the essential characteristics and applicable measurement methods of working suspension containing nano-object samples for *in vitro* toxicity assays. Intention is that reliable test results on nano-object toxicity could be shared and communicated among stakeholders of nano-objects, such as regulators, general public, manufacturers and end users. This Technical Specification does not describe a procedure for validation of working suspension.

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Nanotechnologies — Characteristics of working suspensions of nano-objects for *in vitro* assays to evaluate inherent nano-object toxicity

1 Scope

This Technical Specification describes characteristics of working suspensions of nano-objects to be considered when conducting *in vitro* assays to evaluate inherent nano-object toxicity. In addition, this Technical Specification identifies applicable measurement methods for these characteristics.

This Technical Specification is applicable to nano-objects, and their aggregates and agglomerates greater than 100 nm.

NOTE This Technical Specification intends to help clarify whether observed toxic effects come from tested nano-objects themselves or from other uncontrolled sources.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 29701, *Nanotechnologies — Endotoxin test on nanomaterial samples for in vitro systems — Limulus amoebocyte lysate (LAL) test*

3 Terms and definitions

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

3.1

culture medium

aqueous solution of nutrients required for cell growth

3.2

secondary particle

complex agglomerate/aggregate of primary particle(s), proteins and other medium components

3.3

stability

properties to remain unchanged over a given time under stated or reasonably expected conditions of storage and use for an *in vitro* toxicity assay

3.4

working suspension

suspension prepared for an *in vitro* assay that includes culture medium and nano-object sample

4 Abbreviated terms

For the purposes of this Technical Specification, the following abbreviated terms apply.

AAS	atomic absorption spectrometry
BCA	bicinchoninic acid
C-U/F	ultrafiltration assisted by centrifugation
DLS	dynamic light scattering
FFFF	flow field-flow fractionation
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma mass spectrometry
LD	laser diffraction
SLS	static light scattering
TFF	tangential flow filtration
TOC	total organic carbon
U/F	ultrafiltration
UV-Vis	ultraviolet-visible

5 Characteristics and measurement methods

5.1 General

To characterize the working suspension for *in vitro* toxicity assays, it is necessary to determine certain characteristics that might impact the biological system tested. This Clause specifies essential characteristics of the working suspension, listed below, and measurement methods that are applicable to them.

- Presence of endotoxins,
- stability of working suspensions,
- concentration of metal ions, and
- concentration of culture medium components.

Measurements of those characteristics shall be made for each dose of working suspensions. The measurement of endotoxin can be made alternatively for the stock nano-object suspension to be dosed. See [Annex A](#) for an example of flow of measurements.

5.2 Endotoxin

Contamination with endotoxins, part of the outer membrane of Gram-negative bacteria may significantly alter the results of the *in vitro* toxicity test. Therefore, it is critical to quantify the concentrations of endotoxins in the working suspension. The concentration of endotoxins in the working suspension shall be measured by Limulus amoebocyte lysate (LAL) test in accordance with ISO 29701 and the monocyte activation test (MAT).^{[2][3]}

5.3 Stability of working suspensions

5.3.1 General

Stability of working suspension is a key characteristic as it directly impacts the *in vitro* assay conditions in terms of the dose of the nano-objects to the cells.^{[4][5]} Aggregation/agglomeration and gravitational settling of the nano-objects are major issues that may affect the stability of the suspended nano-objects. The stability shall be evaluated for the two characteristics, i.e. the relative change of representative

size of secondary particles of nano-objects and the relative change of the concentration of nano-objects in the working suspension, resulting from gravitational settling during an *in vitro* toxicity assay, by considering experimental duration required for the *in vitro* toxicity assay. Evaluation results of the stability shall be expressed in the unit of per cent (%) over the timescale for *in vitro* toxicity assay.

NOTE ISO/TR 13097[6] is recommended as a comprehensive guidance for stability of working suspension.

5.3.2 Representative size change of secondary particles of nano-objects

An appropriate method shall be selected to directly measure the representative size change of secondary particles of nano-objects from among dynamic light scattering (DLS),[4][7] laser diffraction (LD)[8] and static light scattering (SLS).[9] Other methods deviating from this Technical Specification can be used and reported in accordance with 6.6.

See Annex B for measurements.

5.3.3 Concentration change of nano-objects

An appropriate method shall be selected to measure the concentration change of nano-objects suspended in the biological media from among the light scattering,[4][7][10] inductively coupled plasma mass spectrometry (ICP-MS),[11][12][13] ultraviolet-visible (UV-Vis) absorption, X-ray transmission[14] and the total organic carbon analysis.[15] Other methods deviating from this Technical Specification can be used and reported in accordance with 6.6.

See Annex B for measurements.

5.4 Concentration of metal ions

Metal ions, produced as a result of nano-object test sample dissolution, can contribute to test cell toxicity. The concentration of metal ions in the working suspension shall be measured after separation of particulate matter. Particulate matter can be separated from the ionic fraction by ultra-filtration (U/F), ultra filtration assisted by centrifugation (C-U/F) or tangential flow filtration (TFF). The measurement shall be made for all metallic elements that are included in the nano-object sample. An appropriate method shall be selected to measure the metal ion concentrations from among inductively coupled plasma-atomic emission spectrometry (ICP-AES), ICP-MS, atomic absorption spectrometry (AAS) and the colourimetric method. Other methods deviating from this Technical Specification can be used and reported in accordance with 6.6. Measurement results of concentrations shall be expressed in the unit of molarity, mass/mass or mass/volume. The measurements can be omitted when a toxic effect is not observed to the cells in the working suspensions. See Annex A for an example of flow of measurements.

See Annex C for measurements.

5.5 Concentration of culture medium components

5.5.1 General

A nano-object sample added to a culture medium to generate a working suspension may adsorb components of the culture medium.[1] This can result in starvation stress to the test cells. The concentration of protein components and calcium, as surrogates for the nutritional components in the solvent shall be measured by setting aside enough time after the addition of nano-object sample to the culture medium. If culture medium components other than protein and calcium that may significantly affect the stability of working suspension for *in vitro* toxicity assays are known, the concentration of those components shall be measured as well. The measurements can be omitted when a toxic effect is not observed to the cells in the working suspensions. See Annex A for an example of flow of measurements.

NOTE Nano-objects can affect pH, osmolality and other essential characteristics in the culture medium.

5.5.2 Proteins

An appropriate method shall be chosen for the protein concentration measurement from among bicinchoninic acid (BCA), Bradford, Lowry and ultraviolet, refractive index and SLS methods coupled with the flow field-flow fractionation (FFFF).^[9] When BCA,^[16] Bradford^[17], or Lowry^[18] method is chosen, the protein concentration in the solvent shall be measured after separation of particulate matter from the working suspension. Results of protein concentration measurement shall be expressed in the unit of mass/volume.

See [Annex D](#) for measurements.

5.5.3 Calcium

An appropriate measurement method shall be chosen for the calcium concentration measurement from among ICP-AES, ICP-MS, AAS and the colourimetric method. Results of calcium concentration measurement shall be expressed in the unit of molarity, mass/mass, or mass/volume.

See [Annex D](#) for measurements.

6 Reporting

6.1 General

Measurement and evaluation results obtained according to this Technical Specification shall be reported describing the source and the constituents of the nano-objects, culture medium and serum, as described in the following subclauses.

6.2 Name of nano-objects and manufacturer

Name and catalogue number of nano-objects and manufacturer information including name, address and contact information.

6.3 Metallic elements included in the nano-object sample

Principal and accessory materials, coating materials, catalytic materials and impurities, including their known or estimated quantity.

6.4 Culture medium and serum

Name and manufacturer of the medium, type and concentration of added serum (v/v %), pH values of original medium and pH values during assessment, and type and concentration of other additives, if any.

6.5 Measurement results

The following are required to report for individual doses of working suspensions. However, the results of the test for endotoxins can be reported for the stock suspensions of nano-objects instead of individual doses.

Reporting of metal ions and culture medium components are not required when toxicity was not observed for the individual doses of working suspension.

— Endotoxin

- a) Endotoxin positive/negative
- b) Date of measurement
- c) Employed test method

- d) Performing institution and data reliability information
- e) Other special supporting information, if any
- Stability of working suspension
 - a) Representative size change and concentration change
 - b) Date of measurement
 - c) Employed measurement methods for representative size change and concentration change
 - d) Performing institution and data reliability information
 - e) Supporting information on preparation method of working suspension
 - f) Other special supporting information, if any
- Metal ions
 - a) Names of metal ions and their concentrations
 - b) Date of measurement
 - c) Employed measurement method
 - d) Performing institution and data reliability information
 - e) Other special supporting information, if any
- Culture medium components
 - a) Protein and calcium concentrations
 - b) Date of measurement
 - c) Employed measurement methods for protein and calcium concentrations
 - d) Performing institution and data reliability information
 - e) Other special supporting information, if any

6.6 Deviation

Deviations from this Technical Specification shall be described in the report with the name of methods employed, its detailed information, reliability and justification.

Annex A (informative)

Flow of measurements

A schematic showing the stages at which measurements shall be made based on this Technical Specification is shown below.

Measurements of stability, metal ions and culture medium components are made for each dose of the working suspension prepared by mixing nano-object sample and cell culture medium. Measurements of endotoxin are made for a nano-object sample to be dosed, that can be alternatively made for a working stock suspension.

The working suspension is divided into two parts: one is for toxicity assay (assay sample) and the other for measurements of characteristics (characteristics measurement sample). Measurements of metal ions and culture medium components are carried out for the characteristic measurement samples when a toxicity effect is observed for the assay sample.

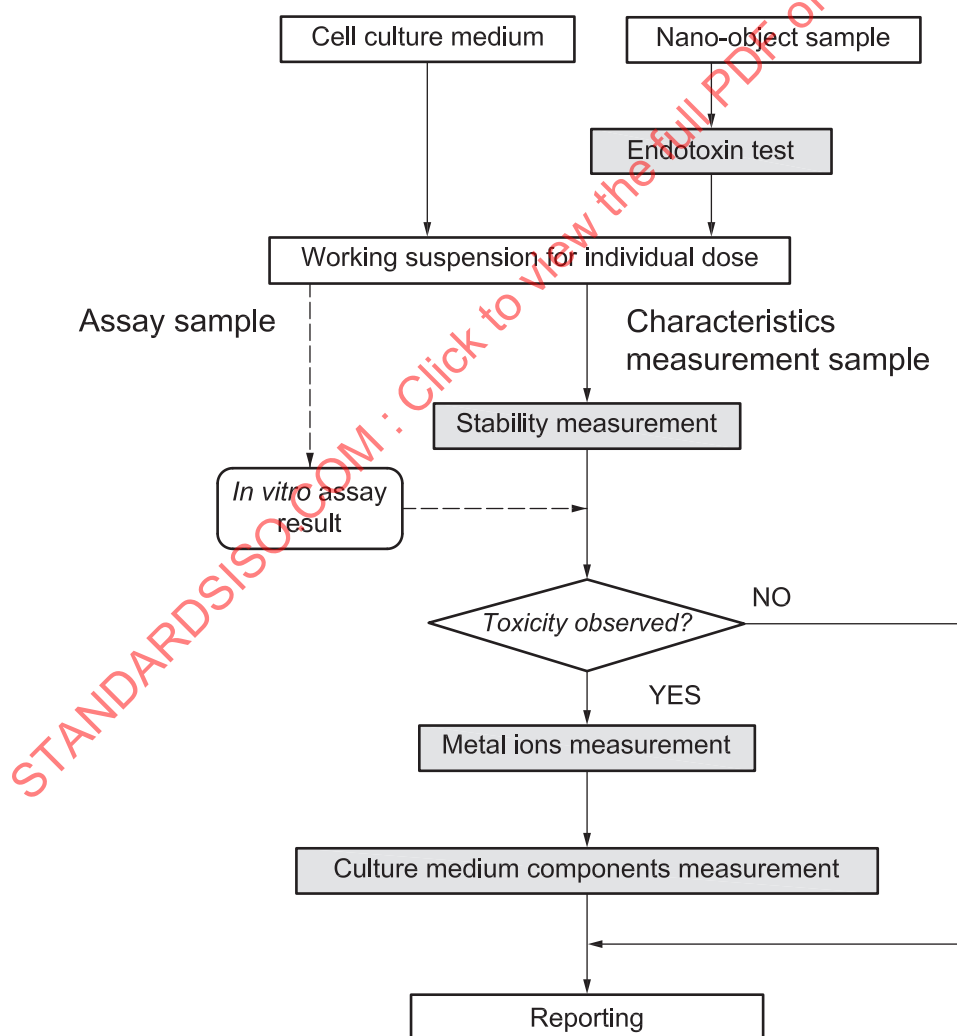


Figure A.1 — Schematic showing the states at which measurements are made

Annex B (informative)

Measurement and evaluation of stability

B.1 General

Examples of measurement methods of stability of nano-object samples are provided for the benefit of the user. The user is cautioned that these methods have not necessarily been validated for use in characterizing multiple types of nano-object samples. Due to the diversity of nano-object samples, the user should select an appropriate method to evaluate the stability of working suspension during *in vitro* toxicity assays.

All measurements should be performed on working suspension or the nano-object suspensions prepared in the same manner as the *in vitro* toxicity assay. The same sample stocks of the nano-object suspension should be used for the measurements and for the *in vitro* toxicity assessment experiments. Evaluated results of the change of values should be expressed in the unit of per cent (%) during an *in vitro* toxicity assay. It is recommended to perform multiple measurements (at least three times) and to present the data as average of the measurements.

B.2 Representative size change of secondary particles of nano-objects

In order to monitor the changes of the representative size of secondary particles of nano-objects, the user should select an appropriate method from the DLS, the LD and the SLS. The method should be selected by observable size range in the respective measurement principles.

B.3 Change of concentration of nano-objects

In order to monitor the changes of the concentration of a majority of the types of nano-objects, light scattering intensity monitoring methods, such as DLS or SLS can be applied, as long as the unit size of the nano-object is larger than protein molecules in the working suspensions. TOC method can be applicable to carbon-based nano-object analysis, when combustion temperatures or pyrolysis temperatures of the carbon-based nano-object and the culture media are different. In the case that the background carbon is subtracted accurately, TOC method can be also applicable. UV-Vis absorption method is applied to all groups of nano-objects when their UV-Vis absorbance can be decoupled from the culture medium background absorbance. X-ray transmission measurement is applicable to nano-object samples with high background in the UV-Vis absorption method, however, it is practically restricted to suspended nano-objects of atomic numbers less than carbon number.

Annex C (informative)

Measurement of metal ions

C.1 Separation of ions from particulate matter

C-U/F membrane with an optimal fractionation molecular weight is selected depending on the size of nanoparticles. Conditions of C-U/F, namely can be employed in order to separate particulate matter and metal ions. In the U/F, a relevant centrifugal acceleration and duration of centrifugation should be adequately set to fully recover the filtrate containing ions.^[19]

Alternatively, ultracentrifugation can be employed especially for metal nano-objects. Conditions of the centrifugation, namely centrifugal acceleration and duration of centrifugation should also be adequately set to fully recover the solution containing ions.

The filtrate or supernatant is analysed by the methods described in [C.2](#).

C.2 Measurements

C.2.1 Choice of methods

ICP-MS is a highly sensitive technique and can detect the level of parts per trillion (ppt). On the other hand, AAS is highly reliable.

If a chelating agent for the colourimetric measurement is suitable for detection of the expected metal ion, the colourimetric method can be employed. The chelating agent should have high specificity of substrate, and no cross-reactivity. When there is no suitable chelating agent for expected metal ion, colourimetric method should not be employed.

C.2.2 Inductively coupled plasma-atomic emission spectrometry (ICP-AES)

Calibration curves for the metal ions of interest are necessary using standard solutions of the metal ion.

Measurement of ICP-AES should follow the standard of ISO 11885.^[20]

C.2.3 Inductively coupled plasma mass spectrometry (ICP-MS)

Pre-treatment of the working suspension and generation of the calibration curves are conducted in accordance with [C.1](#).

The measurements are recommended to follow the relevant standards of ISO 17294-1^[21] and ISO 17294-2.^[22]

C.2.4 Atomic absorption spectrometry (AAS)

The calibration curves for the ions of interest are necessary using standard solutions of the ions.

C.2.5 Colourimetric method

If metal ions in the working suspension have characteristic absorption band in the visible or ultraviolet region, the colourimetric methods can be used to determine the concentration. Before the quantitative analysis, the absorption spectra are measured and identified for the ions. A characteristic absorption band that does not interfere with that of other ions is selected for the analysis.