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Nanotechnologies — *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles

Nanotechnologies - Analyse du MTS in vitro pour la mesure de l'effet cytotoxique des nanoparticules

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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.

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

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Introduction

The field of nanotechnologies continues to advance rapidly through the development of new materials, products and applications. At the same time, many questions have been raised relating to the potential impact on human health and on the environment of some of these materials. Internationally, a large program of research is underway to better understand and quantify potential hazards. Also the chemicals used to coat the surface of nanoparticles in processing or in products can affect the toxicity of nanoparticles, even more so due to their large surface to volume ratio.

Cellular systems are a fundamental element of living biological systems. It is likely that monitoring toxic response of cellular model systems to nanoparticle exposure will provide insight into the "modes-of-action" of nanoparticles and which of them would need to be further investigated for risk assessment.

In 2008, a number of international researchers concluded that some published results of nanomaterial toxicity could not be replicated across laboratories and that accurate and reproducible nanotoxicology tests were needed. As a result, the International Alliance for NanoEHS Harmonization (IANH) was formed with the goal of developing testing protocols that would accurately assess toxicity and biological interactions of nanoparticles in cellular systems and that these results be reproducible in any laboratory. The IANH performed round robin characterization of particle size distributions in liquid suspensions, and *in vitro* interactions of nanomaterials with cells with the several common cytotoxicity assays (Annex A). This group identified a number of factors that increased variability and developed techniques to reduce it. Research funded by the US NIEHS NanoGo further assessed some of these protocols, in particular, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay protocol[1]. A third team extended the IANH protocol and performed experiments that employed a systematic plate layout to achieve improved analysis and consistency of results (Annex B)[2]. Importantly, each of these protocols used interlaboratory testing between multiple laboratories to identify sources of variability and improve the assay protocols.

This document is a method to assess *in vitro* cell viability with the MTS assay.[3] This assay produces a colourmetric change (absorption peak at 490 nm) in a culture well due to generation of a formazan product in the presence of cytoplasmic reductase enzymes. In general, changes in absorption intensity is directly proportional to cell number although assay conditions that alter reductase activity or reagent availability can result in colourmetric changes that are not directly due to changes in cell viability (i.e. cell number). The MTS reagents are directly added to cell culture well which allows rapid evaluation of potential intrinsic toxicity of nanoparticles. Due to the potential interference effects that can occur with nanoparticles and colourmetric assays, it is important control experiments with the nanoparticles and the MTS reagents are performed before the assay results are accepted. Direct microscopic observation of cells after treatment also provides an orthogonal method to validate an MTS assay result. The normalized protocol presented here is limited to adherent cell types, but it could be modified to be used with suspension cells.

This measurement of toxicity in this assay is a first-tier measurement of nanoparticle effects on individual cellular systems. The normalized method presented here is based on the three MTS assay protocols described above. Differences between the experimental systems are described in Table 1.

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Table 1 — Summary of the studies used to develop a normalized MTS assay protocol

Study ID	Cell line[a]	Nanoparticle tested[b]	Positive and negative control materials	Centrifuge step
IANH	RAW-264.7	+PS-NP, CeO_2	$CdSO_4$,no-particle treatment	No
NanoGo	BEAS-2B, RLE-6TN and THP-1	ZnO, TiO_2, MWCNT	No-particle treatment	Yes
EMPA-NIST[c]	A549	+PS-NP	$CdCl_2$, no-particle treatment	No

a ATCC Cell Bank Name

b +PS-NP is a positively charged polystyrene nanoparticle, CeO_2 is cerium oxide, ZnO is zinc oxide, TiO_2 is titanium dioxide, and MWCNT is a multiwall carbon nanotube.

c EMPA is the Swiss Federal Laboratories for Material Science and Technology.

As a result of these differences, some parts in the normalized protocol contains optional steps that were presented in three interlaboratory studies.

Several methods can be used for determining cell viability, including MTS,[3] 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT[4]), (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT[5]), lactate dehydrogenase (LDH[6]), trypan blue exclusion[7] and neutral red assay[8], The MTS assay was used in a multi-group round robin characterization. The MTS assay is an improved version of the MTT assay and provides a simple high throughput characterization for cell viability[1][9]. The optical density of the MTS assay solution increases upon its reduction by the functioning cell enzymes in live cells.

Control experiments are required to determine a baseline optical density of cell viability for untreated cells, and to verify that cells have an expected response to known non-toxic nanoparticles, toxic chemicals and toxic nanoparticles as measured with the assay [10]. Furthermore, it is important to determine whether nanoparticles interfere with the optical readout of the assay and potentially invalidate assessment of the nanoparticle cytotoxicity response. [11]

It is important to note that the MTS assay described here is one of many commercially assays available to assess the cytotoxicity of nanomaterials. Although assays such as the LDH assay which assesses plasma membrane integrity, the ATP assay which evaluates energy metabolism and the BrdU assay for DNA synthesis are not discussed here, the results from these assays in addition to the MTS assay allow for a more comprehensive evaluation of the overall impact of nanoparticles on cells.

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Nanotechnologies — *In vitro* MTS assay for measuring the cytotoxic effect of nanoparticles

1 Scope

This document specifies a method for evaluating the effects of nano-objects and their aggregates and agglomerates (NOAA) on cellular viability using the MTS assay. The assay design includes performance requirements and control experiments to identify and manage variability in the assay results.

This document is applicable to the use of a 96-well plate.

2 Normative references

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

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/TS 80004-2 and the following apply.

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

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

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

3.1
culture vessel
example assay vessel described in this document based a 96-well tissue culture-grade plate format

Note 1 to entry: Other tissue culture grade vessels (i.e. 384 well plates, 24 well plates, 6 well plates) can be used interchangeably in these methods provided that they meet the requirements of tissue culture grade and are suitable for use with mammalian cells.

Note 2 to entry: Adjustments to the protocol such as cell seeding volumes, cell rinsing volumes, and cell dosing volumes may be required if other tissue culture grade vessels are used during this procedure.

[SOURCE: ISO 10993-5:2009, 3.1]

3.2
dispersion
microscopic multi-phase system in which discontinuities of any state (solid, liquid or gas: discontinuous phase) are dispersed in a continuous phase of a different composition or state

Note 1 to entry: If solid particles are dispersed in a liquid, the dispersion is referred to as a suspension. If the dispersion consists of two or more liquid phases, it is termed an emulsion. A superemulsion consists of both solid and liquid phases dispersed in a continuous liquid phase.

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3.3
endotoxin
part of the outer membrane of the cell envelope of Gram-negative bacteria

Note 1 to entry: The main active ingredient is lipopolysaccharides (LPS).

[SOURCE: ISO 29701:2010, 2.3]

3.4
negative control material
material or chemical which, when tested in accordance with this document, does not produce a cytotoxic response

Note 1 to entry: The purpose of the negative control is to demonstrate the basal level response of the cells. This control is often composed of the vehicle solvent used to store the nanomaterial in stock concentrations.

[SOURCE: ISO 10993-5:2009, 3.4]

3.5
positive control material
material or chemical which, when tested in accordance with ISO 10993-5, provides a reproducible cytotoxic response

Note 1 to entry: The purpose of the positive control is to demonstrate an appropriate test system response. For example, a nanomaterial positive control would be positively charged polystyrene.

[SOURCE: ISO 10993-5:2009, 3.2, modified]

3.6
sedimentation
settling (separation) of the dispersed phase due to the higher density of the dispersed particles compared to the continuous phase

Note 1 to entry: The accumulation of the dispersed phase at the bottom of the container is evidence that sedimentation has taken place.

[SOURCE: ISO/TR 13097:2013, 2.13]

3.7
test sample
material that is subjected to biological or chemical testing or evaluation

[SOURCE: ISO 10993-5:2009, 3.5]

4 Symbols and abbreviated terms

cells/mL	cells/mL (cells per millilitre)
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NPS	nanoparticle suspension
PMS	phenazine methosulfate
PS	polystyrene

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