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Nanotechnologies — In vitro acute nanoparticle phototoxicity assay

Nanotechnologies — Essai in vitro de phototoxicité aiguë des nanoparticules

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Foreword

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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 document 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).

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

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Phototoxicity (PT) is defined as a toxic response to an organism exposed to a substance, where the response is either elicited or increased (apparent at lower dose levels) after subsequent exposure to light, or that is induced by skin irradiation after systemic administration of a substance. The increasing use of nanomaterials in various industries also leads to increased exposure, especially to skin. Furthermore, some nanomaterials are used in commercial sunscreens. Hence, possible impacts on human health including detrimental chemical reactions in the presence of light (both natural and artificial) or photo-protective effects, is of interest.

PT is based on a quantum phenomenon. The absorption of a photon with sufficient energy generates an electron-hole pair that can migrate to the nanoparticle (NP) surface and react with water and oxygen, thus forming extremely reactive radicals and reactive oxygen species (ROS). Generation of the ROS by some wide-bandgap materials, such as TiO_2, ZnO, WO_3, CeO_2, carbon nanotubes, quantum dots and some metal NPs when illuminated by UV-VIS light, can cause oxidative stress, resulting in toxic effects in living organisms. Absorption of a photon with sufficient energy is the necessary condition for photochemical reactions to induce phototoxic response. Material PT is closely related to photocatalytic activity (PCA). Measurement of PCA under physiological conditions allows for an assessment of its phototoxicity potency (see ISO 20814).

A wide variety of light sources, light exposure levels, cell lines, incubation times, viability assays, used for nanomaterial PT measurement hamper data comparison. Existing PT standard test methods for soluble chemical substances (e.g. OECD 432) are not directly applicable to determine nanomaterial PT. It states that DMSO or EtOH should be used as cosolvents in case the material is not soluble in water with additional tests for cosolvent toxicity. The method is not applicable for particulate materials.

The in vitro NP PT test is intended to evaluate the nanomaterial acute phototoxicity when exposed to a near ultraviolet (UVA) light. Cell viability is assessed at a fixed NP concentration after exposure to six doses of the UVA light.

Nanotechnologies — In vitro acute nanoparticle phototoxicity assay

1 Scope

This document specifies a procedure to evaluate acute phototoxicity of nanoparticles (NPs), suspended in cell culture media, by measuring the relative reduction in cellular viability under near ultraviolet (UVA) exposure (315 nm to 400 nm).

The measurement is intended to assess the potential for acute phototoxicity of NPs by comparing NP photoactivity in vitro to a positive control chlorpromazine. It is not designed to predict other joint effects of nanomaterials and light, such as genotoxicity, photo-allergy or photo-mutagenicity. The method is also applicable to NP aggregates and agglomerates.

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 80004-1:2023, *Nanotechnologies – Vocabulary — Part 1: Core vocabulary*

3 Terms, definitions, symbols and abbreviated terms

For the purposes of this document, the terms and definitions given in ISO 80004-1 and the following apply.

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

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

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

3.1 Terms and definitions

3.1.1
phototoxicity
acute light-induced response, which occurs when photoreactive chemicals are activated by light and transformed into toxic products to cells.

3.1.2
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

[SOURCE: ISO/TR 13097:2013, 2.5, modified — Note to entry deleted and "in general" deleted from the definition.]

3.1.3
endotoxin
part of the outer membrane of the cell envelope of Gram-negative bacteria

3.1.4
positive control
material or chemical which, when tested in accordance with this document, provides a reproducible phototoxic response

3.1.5
negative control
material or chemical which, when tested in accordance with this document, does not exhibit phototoxic response

[SOURCE: ISO 10993-5:2009, 3.4, modified — "or chemical" added, "this part of ISO 10993 " changed to " this document", "cytotoxic" changed to "phototoxic" and Note to entry deleted.]

3.1.6
test sample
material that is subjected to biological or chemical testing or evaluation

[SOURCE: ISO 10993-12:2021, 3.14, modified — "medical device, component or material (or a representative sample thereof, manufactured and processed by equivalent methods), or an extract or portion thereof" replaced by "material".]

3.1.7
ultraviolet A
UVA
type of electromagnetic radiation with wavelengths between 320 and 400 nanometers

3.2 Abbreviated terms

BSA	Bovine Serum Albumin
CPZ	Chlorpromazine
DIW	deionized water with ≥ 18 MΩ·cm resistivity
FBS	Fetal Bovine Serum
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH	nicotine adenine dinucleotide hydrate
NIH/3T3	embryonic mouse fibroblast cell line
NP	nanoparticle
OD	optical density
PT	phototoxicity
ROS	reactive oxygen species
UVA	ultraviolet A
UV-Vis	ultraviolet and visible

4 Test method

This document describes a set of procedures for the phototoxicity measurement method of NPs present in an aqueous suspension. NPs that are phototoxic cause the photocatalytic action after successive irradiation with artificial UV light, resulting in a decrease in cell survival level. The cell survival level is affected by the concentration of NPs and the dose of the received UVA light. Cell survival level is measured at a fixed NP concentration and six UVA light doses. The resulting cell survival levels are then plotted vs the UVA

dose. The slopes of these dependences are calculated for negative control (media only), positive control (chlorpromazine), and test NP. The multiplexed assay utilizes a 96-well plate, UVA source and a multiple-plate optical reader leading to a fast and accurate measurement. A separate 96-well plate is utilized to account for possible NP interferences with the cell viability assay.

5 Materials and equipment

5.1 Materials

5.1.1 Reagents

5.1.1.1 DIW

5.1.1.2 Dulbecco modified eagle medium (DMEM) with or without phenol red

5.1.1.3 Phosphate-buffered saline (PBS, pH 7,4)

5.1.1.4 Fetal bovine serum (FBS)

5.1.1.5 Trypsin-EDTA (2,5 g/l)

5.1.1.6 1X Penicillin/Streptomycin

5.1.1.7 Bovine serum albumin (BSA)

5.1.1.8 Trypan blue solution (4 g/l)

5.1.1.9 MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

5.1.1.10 Chlorpromazine hydrochloride (CPZ)

5.1.2 Cell line

NIH/3T3 cell line (mouse fibroblast): e.g. ATCC® CRL-1658™.[1)]

Established cell lines are preferred and, where used, shall be obtained from recognized repositories.

If a stock culture of a cell line is stored, storage shall be at −80 °C or below in the corresponding culture medium but containing a cryoprotectant, e.g. dimethylsulfoxide or glycerol. Long-term storage (several months up to many years) is only possible at −196 °C or below.

Only cells free from mycoplasma shall be used for the test. Before use, stock cultures should be tested for the absence of mycoplasma.[1]

Human skin keratinocytes (HaCaT) may also be used with the test procedure with culture conditions adapted for them and appropriate phototoxicity response to CPZ can be demonstrated.

1) ATCC® CRL-1658™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5.1.3 Controls

A known phototoxic chemical shall be tested concurrently with each in vitro 3T3 phototoxicity test. Chlorpromazine hydrochloride (CPZ, CAS Number: 69-09-0) should be used as a positive control according to 8.2. Cell media should be used as a negative control.

The procedure is as follows:

— Prepare 1 ml 20 mg/ml solution of CPZ by dissolving 20 mg of the dry CPZ in 1 ml of 100 % ethyl alcohol (by volume).

— Store the solution at –20 °C.

— For preparation of CPZ at 5 µg/ml, transfer 1 µl of 20 mg/ml CPZ solution into the 15 ml tube with 4 ml of DMEM-FBS.

5.2 Apparatus

5.2.1 Laminar flow cabinet, standard biological hazard

5.2.2 Incubator (37 °C, 95 % humidified, 5 % CO_2/air)

5.2.3 Inverted phase contrast microscope

5.2.4 Centrifuge

5.2.5 Water bath

5.2.6 Multiple well plate reader

5.2.7 Tissue culture flasks

5.2.8 24 multi-well plates with flat bottom

5.2.9 Flat bottom 96-well black polystyrene microplate

5.2.10 8-channel pipette, 20-200 µl Hemocytometer

5.2.11 Conical tube

5.2.12 Reservoir

5.2.13 Vortex mixer

5.2.14 Refrigerator

5.2.15 Freezer

5.2.16 UV crosslinker (capable of light emission at λ=365 nm), as a UVA source. Irradiation of the test plate should be between 4 and 6 mW/cm^2.

5.2.17 UV-VIS spectrophotometer, capable of measurements in the wavelength range from 300 to 800 nm.

5.2.18 UV power meter, capable of measurements in the wavelength range from 315 to 400 nm.

6 Sample preparation

Following the basic principle of sample preparation, nanoparticles shall be dispersed in a biologically compatible fluid with a reproducible procedure. These can include sonication and mixing by vortexing. NP suspension for phototoxicity testing should be homogeneous and stable in aqueous solution for the duration of the measurement (48 h). For dispersion, it may contain dispersants such as BSA. It is recommended to use the newly dispersed NP for each test. Specific dispersing techniques are not discussed in this document. Details for dispersion can be found in the references cited in the notes in Clause 6 and in Annex C.

NOTE 1 Several procedures have been published that identify methods to reproducibly disperse nanoparticles and characterize nanosuspensions and their stability, see References [2], [3], [4], [5], [6], and [7].

NOTE 2 For information regarding biologically compatible chemical stabilizers, see Reference [8]. For information regarding coatings such as albumin, see Reference [9].

The size distribution of NP suspension and its stability over 24 h must be verified in culture medium. The dispersion state of NP suspension may be characterized using DLS as described in ISO 22412.

NOTE 3 The biomolecules and protein corona in the culture medium can increase the average size of NP and may change the cellular uptake pathway.[11]

NPs can be contaminated with endotoxin (lipopolysaccharides, LPS) during production or handling. Contamination by endotoxins confounds the result of tests in vitro. Therefore, the preliminary detection of endotoxins is required in order to minimize the contamination by endotoxins or to confirm the insignificant levels of endotoxins in the test sample (see ISO 29701).

7 Preparations

7.1 General

All procedures must be carried out under sterile conditions and in a laminar flow cabinet (biological hazard standard).

7.2 Culture medium

The culture medium shall be sterile and meet the growth requirements of the NIH/3T3 cell line.

Storage conditions such as refrigerator temperature shall be validated.

NOTE The stability of the culture medium varies with the composition and storage conditions.

7.2.1 10 % Fetal bovine serum (FBS)

7.2.2 90 % Dulbecco modified eagle medium

7.2.3 High glucose

7.2.4 L-glutamine

7.2.5 Phenol red

7.2.6 Sodium pyruvate

7.2.7 100 µg/ml streptomycin

7.2.8 100 IU/ml penicillin

7.3 Preparation of cell stock culture

If the cells are to be grown from cultures taken from storage, remove the cryoprotectant if present. Subculture the cells at least once before use. When subculturing cells, remove and resuspend the cells by enzymatic and/or mechanical disaggregation using a method appropriate for the cell line.

7.4 Verify viable cell growth

Prior to performing experiments on nanoparticles, characterize viability and doubling rates of the cells. Cell growth rates: viability and doubling rates shall be characterized and monitored. Cell viability should remain > 95 % by using a trypan blue exclusion assay.

7.4.1 Grow the cells in 24 well plates for 24 h and 48 h.

7.4.1.1 Transfer 200 000 cells/ml in 500 µl of culture medium per well with eight replicates per time period.

7.4.1.2 Use one plate for each time period (24 h and 48 h).

7.4.1.3 Gently move the plates into the incubator without agitation to avoid disturbing cell attachment resulting in non-uniform deposition.

7.4.1.4 Verify that incubators have been recently calibrated for: temperature, humidity, CO_2 concentration. Record metrics in a laboratory notebook to establish charting metrics.

7.4.1.5 At each time point (24 h and 48 h), remove one plate from the incubator.

7.4.1.6 Make note of the apparent health and morphology of the cells with a stereo-microscope.

7.4.2 Assess cell number and viability with trypan blue.

7.4.2.1 Remove culture medium from the wells with gentle pipetting.

NOTE Tilt the plate at an angle of approximately 45 degrees and carefully slide the pipette along the well wall to the corner of the well.

7.4.2.2 Wash cells with PBS.

7.4.2.3 Add detaching agent (e.g., trypsin). Incubate at 37 °C until cells are fully detached from the dish (2 min to 20 min depending on the cell line).

7.4.2.4 Resuspend cells in fresh culture medium and collect the culture medium containing cells in a centrifuge tube.

7.4.2.5 Spin the supernatant with the added cells in a centrifuge at 400 g for 5 min to form a pellet.

7.4.2.6 Discard the supernatant.

7.4.2.7 Add 25 µl (4 g/l trypan blue in PBS) to 100 µl culture medium.

7.4.2.8 Resuspend the pellet in trypan blue or culture medium with a pipette.

7.4.2.9 Deposit the cells on a hemacytometer.

7.4.2.10 Record the total number of live and dead (blue) cells and the percent viability (live/total) by counting the cells in the hemacytometer with a stereomicroscope. See Reference [13] for details.

7.4.2.11 Cell doubling times should be consistent with those expected for the cell line and the percentage of viable cells through 48 h should be > 95 % prior to continuing with nanoparticle exposure experiments.

7.5 Irradiation conditions

7.5.1 UVA source

A UV crosslinker with light emission maximum at 365 nm wavelength and area larger or equal to the 96-well plate. See Annex A. UV crosslinker should provide the irradiance at the test plate position between 4 mW/cm^2 and 6 mW/cm^2 as measured with a UV power meter.

NOTE The position of the plate must be marked so that it can always be placed in a consistent position.

7.5.2 Light dose (insolation) measurement

The light intensity shall be checked regularly prior to phototoxicity testing using a suitable broadband UV meter. Light intensity I (irradiation) should be reported in units of W/cm^2. Actual insolation D (total amount of UVA energy received on a given surface area over a given time) is calculated from:

$$D = I \times t \tag{1}$$

where

D insolation in joules per square centimetre;

I light intensity in watts per square centimetre;

t exposure time in seconds.

NOTE Light attenuation by the 96-well plate lid window should be accounted for while measuring light intensity.

7.6 Multiple well plate reader

Ensure that the instrument is operating properly prior to performing the measurement. It should be able to measure absorbance in the range of 300 nm to 800 nm. Reader uniformity should be tested according to Annex B.

8 Measurement procedure

All measurement procedures follow the steps of Figure 1.

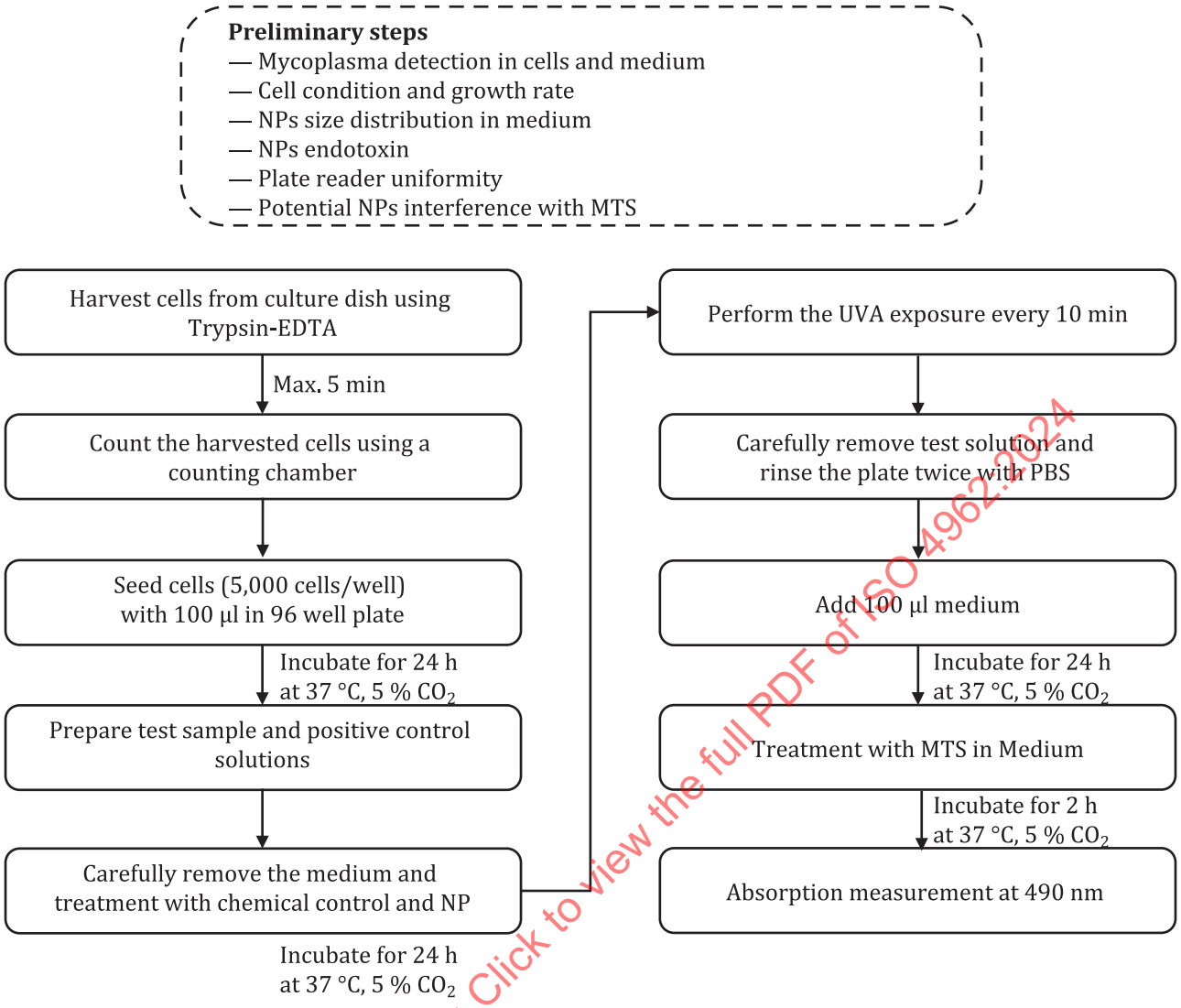
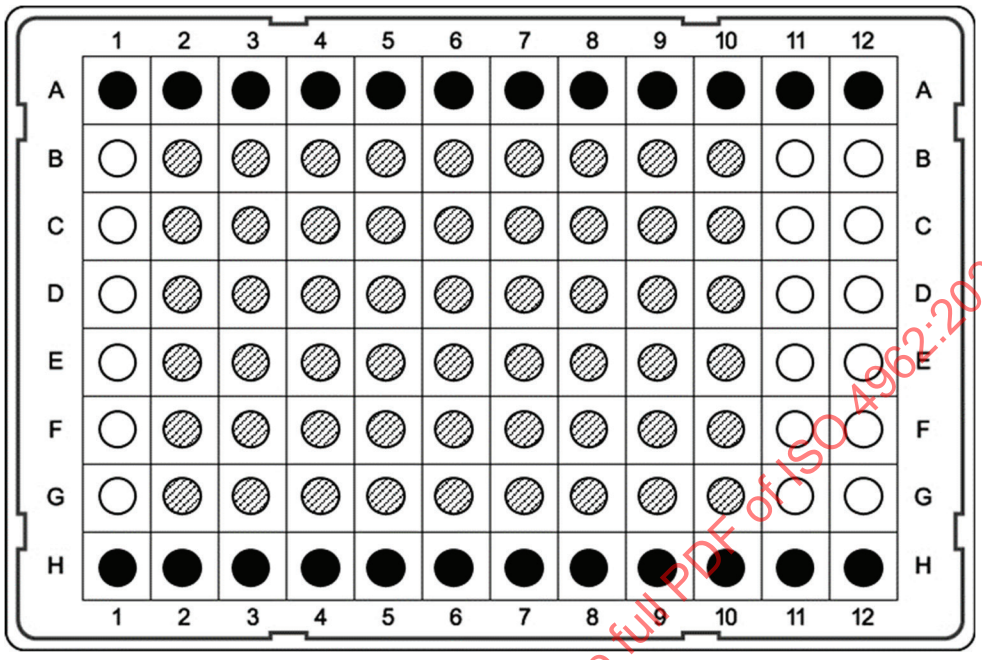


Figure 1 — Screening process overview for the acute phototoxicity of NP

8.1 Cell seeding (Day 1)

8.1.1 Fill 100 µl of PBS into the black colour wells and fill 100 µl of DMEM medium only into the white colour wells of a 96-well plate (see Figure 2). In the gray colour wells, fill 100 µl of a cell suspension, consisting of 5 x 10^4 cells/ml (= 5 x 10^3 cells/well) in the DMEM medium (10 % FBS).



Key

- PBS only
- cell-dispensing area
- medium only

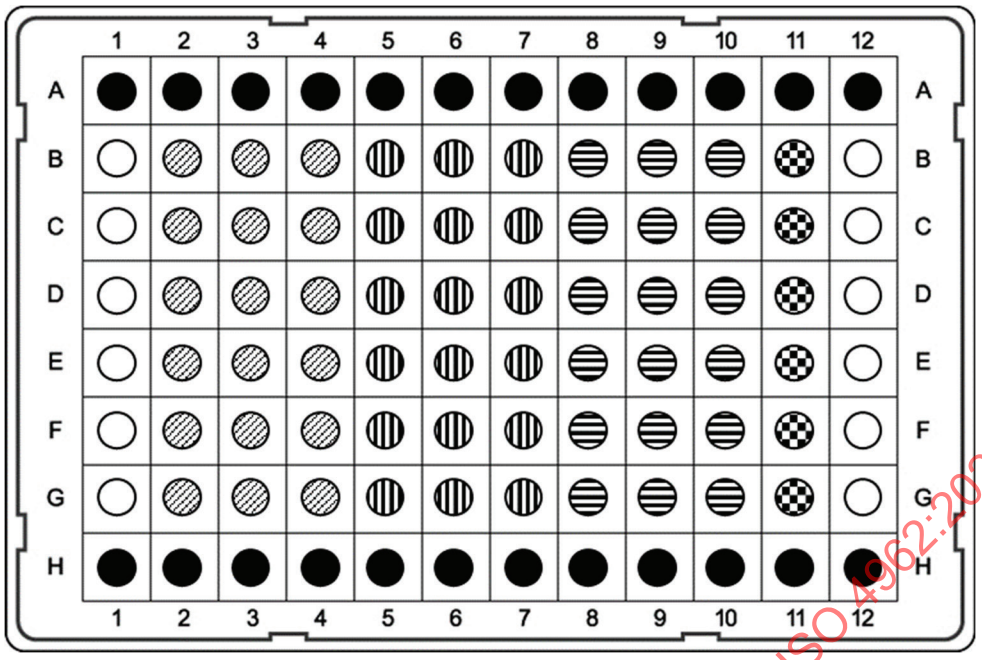
Figure 2 — Schematic diagram of a 96-well plate for cell seeding

8.1.2 Incubate cells in 5 % CO_2, 95 % humidity at 37 °C for 24 h.

8.2 Incubation of cells with the positive control and NP suspension (Day 2)

8.2.1 After incubation, discard culture medium from the cells by suction and add 100 µl of DMEM medium (phenol red free) containing CPZ (the positive control) and NP suspension. Only add DMEM medium to no treatment wells (please see Figure 3). Here, the concentration of CPZ at 5 µg/ml and the concentration of the tested NP suspension should be adjusted to match OD = 1 ± 0,1 at λ = 365 nm. (e.g. for TiO_2 NP the concentration is about 100 µg/ml). Incubate cells with CPZ and NP suspension in 5 % CO_2, 95 % humidity at 37 °C for 24 h.

NOTE Some NPs can be inherently toxic to test cells in the dark and can preclude the phototoxicity measurement. Compare cell viability between wells B2, B3, B4 and B8, B9, B10 and confirm that the average viability in columns B8, B9, and B10 is above 50 % (relative to B2, B3, and B4). The phototoxicity assay is not applicable to NPs which demonstrate significant (above 50 %) cytotoxicity in the dark.



Key

- no treatment
- positive control
- NPs
- NP interference control
- no cells/no treatment

Figure 3 — Schematic diagram of the phototoxicity test plate loading

8.3 UVA exposure (Day 3)

8.3.1 To perform the UVA exposure, centre the plate in the UV crosslinker, as shown in Figure A.1. Next, cover the plate with light shield (black paper and metal foil) leaving the first loaded row (row G) exposed. Expose that row for 10 min. The UVA light dose should be 2,4 J/cm^2 < D < 3,6 J/cm^2, see 7.5.1 and 7.5.2. Turn off the UVA light and move the shield to expose 2 rows (row F and G) again for the same period. Repeating this over the whole plate will result in 5 rows at decreasing exposure levels with the last row (row B) left with no UVA exposure (dark reading). See Annex A for further information.

The maximum UVA dose (row G, 50 min total) should be adjusted in order not to exceed more than 20 % of the viability decrease in the untreated cells (G2, G3, G4).

8.3.2 Upon expiration of the irradiation period, turn off the UV source, discard test solution by suction, rinse the plate twice with 150 µl/well of pre-warmed (37 °C ± 2 °C) sterile PBS.

8.3.3 Add 100 µl of DMEM medium and incubate in 5 % CO_2, 95 % humidity at 37 °C overnight (18-24 h).

8.4 Cell viability assay (Day 4)

8.4.1 The cell viability is measured using MTS cell proliferation assay as described in ISO 19007.

8.4.2 Thaw MTS reagents at room temperature or water bath at 37 °C. Prepare MTS solution in DMEM according to the manufacturer's instructions.

NOTE Avoid air bubble formation while filling with MTS solution as it can affect the absorbance reading. Measure absorbance at λ=700 nm in addition to λ =490 nm. Discard data for the wells that show elevated absorbance at λ=700 nm.

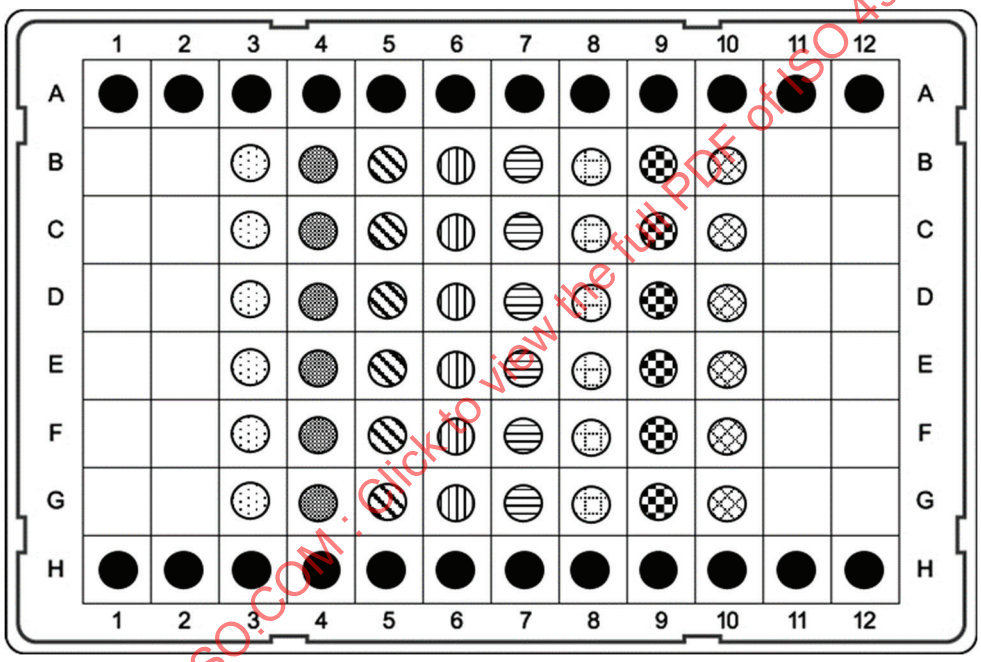
8.4.3 Discard DMEM medium from cells by suction and immediately fill with 120 µl/well of MTS mixture in each well of the plate.

8.4.4 Incubate the plate at 37 °C for 2 h in a humidified, 5 % CO_2 atmosphere.

8.4.5 Record the absorbance for both plates at 490 nm using a multiple well plate reader.

8.5 Evaluation of artefacts due to possible NP interferences with the MTS assay (in the dark)

In terms of NPs interference in MTS assay, four possibilities must be considered, as shown in Figure 4.



Key

- PBS only
- media-only control
- NPs in media
- MTS substrate control
- NPs with MTS substrate
- MTS + NADH control
- NPs with MTS + NADH
- formazan control
- NPs with formazan

Figure 4 — Schematic diagram of the interference test plate loading

8.5.1 Some NPs can directly interfere with the optical readout of MTS assay.

To assess for this interference: incubate NPs in media at the same concentration as in 8.2.1 (column 4) and compare to media-only control (column 3). Measure optical absorbance at λ=490 nm.

8.5.2 Extracellular NPs adsorbed to cells can also bind tetrazolium salt outside cells and prevent formazan product formation.

To check for this interference: incubate NPs with MTS substrate (tetrazolium salt) only (column 6) and compare to MTS substrate control (column 5). MTS reagent concentration should be the same as in 8.4.3.

8.5.3 Extracellular NPs adsorbed on cell membrane can catalyse tetrazolium salt to soluble formazan.

To check for this interference: incubate NPs with MTS substrate and NADH (co-factor) (column 8) and compare to MTS + NADH control (column 7). MTS concentration is the same in 8.4.3 and NADH concentration is 250 µmol/l.

8.5.4 Extracellular NPs can bind formazan and interfere with absorbance readings.

To check for this interference: incubate NPs with formazan only (column 10) and compare to formazan control (column 9). The same MTS + NADH control as in column 7 is loaded in columns 9 and 10, and NPs are treated in column 10 after 2 h to compare absorbance.

NOTE Assessment of potential optical path interference artefacts due to NP dosing can occur by evaluating the mean and variance of the absorbance of wells in the separate plate layout. To evaluate the interference, NP concentration should be the same as in 8.2.1. If the mean of the absorbance of wells in the NP-treated group (columns 4,6,8, and 10) is within the mean and variance of non NP-treated group (columns 3,5,7, and 9), interference by the treated NP can be ignored.

8.6 Data analysis

8.6.1 Calculate averages and standard deviations for three replicate wells before calculating cell viability.

8.6.2 Plot cell viability as a function of UVA exposure duration for “no treatment”, “positive control”, and “nanoparticles” column data with row B as a 100 % viability reference.

NOTE Some NP such as SiO_2, TiO_2 and ZnO have demonstrated certain cytotoxicity even without UVA light. If there is no difference in MTS absorbance between the NP-treated group (B8-B10) and non NP-treated group (B2-B4), toxicity by the treated NP regardless of UVA irradiation does not need to be considered. This is when the mean of B8-B10 exist within the mean and variance of B2-B4.

8.6.3 Fit all three plots to a linear function and determine the slopes (a_1, a_2, and a_3).

8.6.4 Compare the linear slopes for all three samples in columns 2 to 4, 5 to 7, and 8 to 10.

8.6.5 Confirm the intensity in column 11 and columns 1, 12 to check for the NP interference in absorbance readings.

NOTE Assessment of potential optical path interference artefacts due to NP dosing can occur by evaluating the mean and variance of the absorbance of wells in column 11. These blanks undergo the same procedure (same incubation time, discharge after UVA exposure, incubation with MTS substrate) than exposed cells in columns 8 to 10. If the mean of the absorbance of wells in column 11 is not within the mean and variance of the only MTS reagent wells (columns 1 and 12), it can suggest that the remaining NP in column 11 has not been adequately removed during PBS washing step, introducing the interference artefacts.[15] Therefore, this protocol cannot be used if repeated experiments yield the same results.

8.6.6 Adjust the "treatment" and "positive control" slopes for negative control by subtraction:

$$A_2 = a_2 - a_1 \quad (2)$$

$$A_3 = a_3 - a_1$$

8.6.7 Calculate the sample phototoxicity T relative to the positive control by taking the ratio of A_3 and A_2

$$T = A_2 / A_3 \quad (3)$$

NOTE Cell viability for "no treatment" at a maximum UVA exposure (row G) should not be lower than 80 %.

9 Report

9.1 Test report

The test report shall include the following information:

— test date;

— a reference to this International Standard, i.e. ISO 4962:2024;

— name of the testing laboratory;

— full details concerning the test sample (manufacturers name, manufacturing date, batch no., purity, particle size, intended application, MSDS);

— the manufacturer's name and lot # of cell line (mouse fibroblast);

— absorbance spectrum of stock NP suspension at test concentration in DMEM medium (phenol red free);

— the manufacturer's name and lot # of DMEM culture media;

— the manufacturer's name and purity of Chlorpromazine hydrochloride;

— the manufacturer's name and purity of FBS;

— the manufacturer's name and purity of BSA;

— the manufacturer's name and lot# of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium);

— the manufacturer's name and lot# of Trypan blue solution (4 g/l);

— the manufacturer's name and model number of the UV crosslinker;

— the manufacturer's name and model number of the UV power meter;

— the manufacturer's name and model number of the 96-well plate;

— the manufacturer's name and model number of the 96-well plate reader;

— the detailed description of the 96-well plate reader settings used for absorbance measurements;

— description of the UV light shield (see 8.3.1);

— records of observations;

— assessment of the results, including statistical methods.

9.2 Report data format

9.2.1 Light intensity of the UVA light source, measured with UV power meter, expressed in W/cm^2 (see 7.5.2).

9.2.2 Cell doubling time according to 7.4.2.9.

9.2.3 The $A_{490\ nm}$ absorbance values, for each well at 490 nm in table format (see 8.4.5).

Table 1 — Absorbance values for the 96-well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A(A,1)	A(A,2)	A(A,3)	A(A,4)	A(A,5)	A(A,6)	A(A,7)	A(A,8)	A(A,9)	A(A,10)	A(A,11)	A(A,12)
B	A(B,1)	A(B,2)	A(B,3)	A(B,4)	A(B,5)	A(B,6)	A(B,7)	A(B,8)	A(B,9)	A(B,10)	A(B,11)	A(B,12)
C	A(C,1)	A(C,2)	A(C,3)	A(C,4)	A(C,5)	A(C,6)	A(C,7)	A(C,8)	A(C,9)	A(C,10)	A(C,11)	A(C,12)
D	A(D,1)	A(D,2)	A(D,3)	A(D,4)	A(D,5)	A(D,6)	A(D,7)	A(D,8)	A(D,9)	A(D,10)	A(D,11)	A(D,12)
E	A(E,1)	A(E,2)	A(E,3)	A(E,4)	A(E,5)	A(E,6)	A(E,7)	A(E,8)	A(E,9)	A(E,10)	A(E,11)	A(E,12)
F	A(F,1)	A(F,2)	A(F,3)	A(F,4)	A(F,5)	A(F,6)	A(F,7)	A(F,8)	A(F,9)	A(F,10)	A(F,11)	A(F,12)
G	A(G,1)	A(G,2)	A(G,3)	A(G,4)	A(G,5)	A(G,6)	A(G,7)	A(G,8)	A(G,9)	A(G,10)	A(G,11)	A(G,12)
H	A(H,1)	A(H,2)	A(H,3)	A(H,4)	A(H,5)	A(H,6)	A(H,7)	A(H,8)	A(H,9)	A(H,10)	A(H,11)	A(H,12)

9.2.4 Cell viability plots as a function of UVA exposure duration for "no treatment", "positive control", and "nanoparticles" column data.

9.2.5 Slopes of linear fits to plots in 9.2.4 (a_1, a_2, and a_3).

9.2.6 Adjusted plots A_1, A_2, A_3 (see 8.6.6).

9.2.7 Value of *T*.

10 Precision

10.1 Repeatability

The absolute difference between two independent measurements carried out within a short time period, for the same sample in the same laboratory by the same operator using the same experimental and analytical measuring device, will not exceed the repeatability limit, *r*, in more than 5 % of cases.

Typical precision data for three different laboratories obtained in an inter-laboratory test are given in Annex D.

10.2 Reproducibility

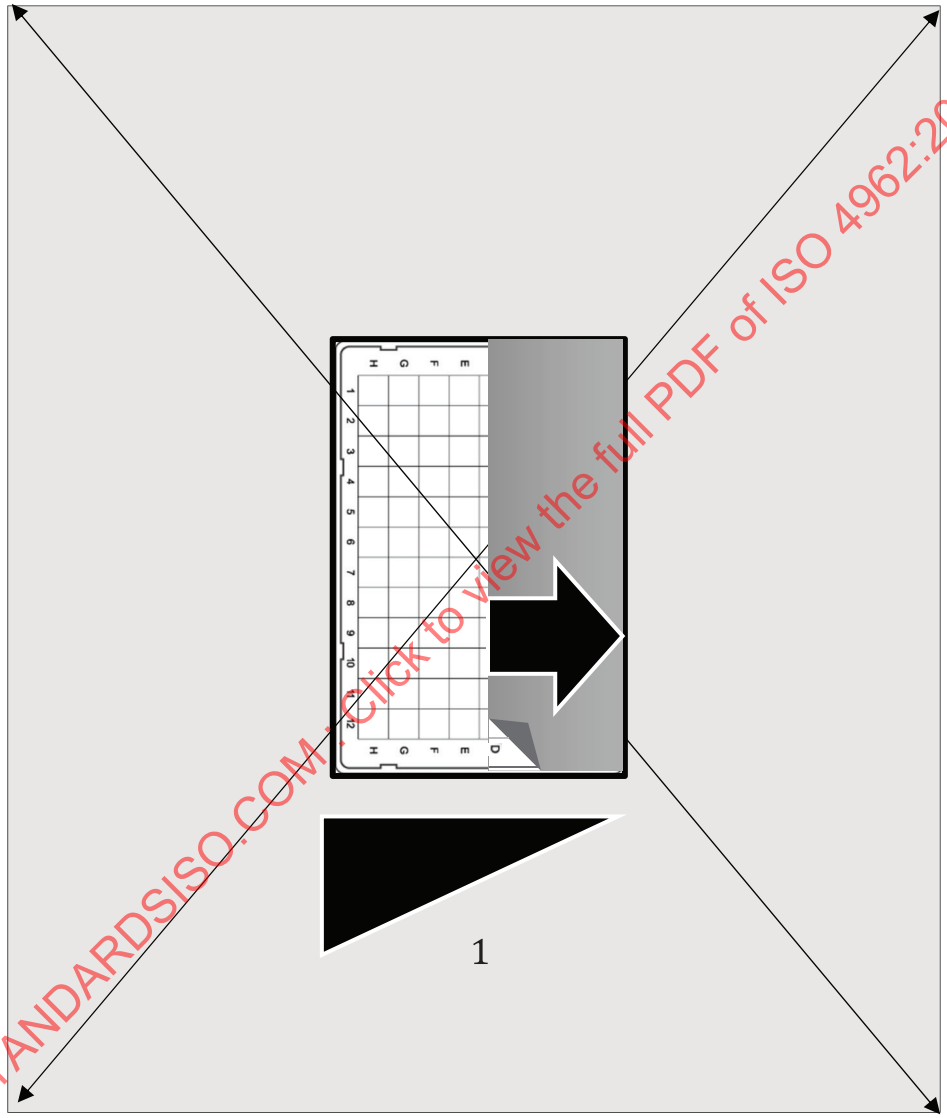
The absolute difference between two individual measurements carried out using the same test method, for the same sample in different laboratories by different operators using different experimental and analytical measuring devices, will not exceed the reproducibility limit, *R*, in more than 5 % of cases.

Typical precision data for three different laboratories obtained in an inter-laboratory test are given in Annex D.

Annex A
(informative)

Schematic diagram of 96-well plate position

Ensures the positioning of a 96-well plate in the UV crosslinker at the same central location, as shown in Figure A.1.



Key

1 UV exposure time

NOTE This figure shows the position of a 96-well plate inside the UV crosslinker and the schematic of the prescribed illumination sequence accomplished by sequential movement of the mask.

Figure A.1 — Schematic diagram of 96-well plate position

Annex B
(informative)

Verification of plate reader uniformity

Verification of plate reader uniformity involves the following steps.

a) Thaw MTS reagents at room temperature or water bath at 37 °C. After completely thawing the MTS reagents, 2,5 ml MTS reagents are mixed with 12,5 ml DMEM medium (phenol red free).

b) Fill with 120 µl/well of mixture in each well (B2-G11).

c) Incubate the plate at 37 °C for 1 h in a humidified, 5 % CO_2 atmosphere.

d) Record the absorbance at 490 nm using a multiple well plate reader.

Table B.1 shows the absorbance values of MTS/DMEM medium mixture at 490 nm.

Table B.1 — Absorbance value of MTS/DMEM medium mixture at 490 nm

	2	3	4	5	6	7	8	9	10	11
B	0,172 9	0,176 1	0,175 9	0,178 1	0,178 2	0,178	0,177 1	0,175 5	0,177	0,176 9
C	0,175 3	0,176 4	0,177	0,178 4	0,177	0,178 2	0,180 2	0,178 8	0,18	0,177 6
D	0,173 1	0,177 1	0,176 2	0,178 4	0,177 6	0,180 1	0,178	0,174 5	0,179 7	0,179 2
E	0,174 6	0,174 4	0,175 4	0,176	0,176	0,176 5	0,178 4	0,176 9	0,178 9	0,176 8
F	0,175 2	0,176 8	0,175 1	0,175 8	0,175	0,175 3	0,177 5	0,175 8	0,178 2	0,177 2
G	0,170 8	0,170 6	0,171 1	0,171 6	0,172 7	0,175 4	0,174 6	0,169 7	0,171	0,171 4

— SD = 0,002 728, Mean = 0,175 91

— CV = 0,002 728/0,175 91 x 100 = 1,550 77 %

$$CV\,(Coefficient\ of\ Variation, \%) = \frac{Standard\ Deviation}{Mean}\ x\ 100$$

Annex C
(informative)

Dispersing procedure for TiO_2 nanoparticles in DMEM

C.1 Steps to prepare aqueous TiO_2 nanoparticle dispersion

a) Using an analytical balance and an aluminum or polystyrene weighing dish, weigh 0,5 g of TiO_2 powder to achieve the desired concentration in a 50 ml DIW volume.

b) Add the weighed mass of powder to a 100 ml, ≈ 5 cm diameter cylindrical glass beaker.

c) Place the beaker inside the 125 x 65 mm glass dish and secure the beaker in the centre of the dish by use of clamps or other locking device to ensure that the beaker remains in place during sonication.

d) Fill the 125 x 65 mm glass dish with enough water and chopped ice to allow for the ice water bath level to encase the beaker to approximately the level of the water contained in the beaker.

e) Immerse the sonicator horn into the liquid in the beaker down to about 2,5 cm below the liquid level in the beaker. Centre the horn in the beaker; the horn should not touch the sides or the bottom of the beaker.

f) Select a sonicator setting that yields a delivered power of approximately 50,0 W.

g) Operate the sonicator at this delivered power level for 15 min, using an 80 % pulsed operation mode (e.g., 80 % on / 20 % off during each second of operation time), or similar on/off time sequence.

h) After sonication, transfer the aqueous dispersion to an amber borosilicate glass container and store at ambient temperature until further use. Do not refrigerate.

C.2 Steps to prepare TiO_2 nanoparticle dispersions in DMEM (phenol red free) with 10 % FBS

a) Prepare a 10 mg/ml TiO_2 aqueous dispersion dispersed in C.1.

b) Prepare 50 ml of DMEM-FBS by mixing 5 ml of FBS with 45 ml of DMEM (1x). The resulting pH should be ≈ 7,8.

c) Weigh 0,8 g of BSA powder and transfer to a 10 ml amber glass vial. Add 10 ml of DIW to the vial with the BSA, seal and gently shake to allow for complete dissolution of the BSA. Do not use the BSA solution until visible material is completely absent (allow approximately 1 h). The final product is a transparent 80 mg/ml BSA solution in DIW.

d) Add 19 µl of the 80 mg/ml aqueous BSA solution into a clean 10 ml amber glass vial.

e) Add 150 µl of the 10 mg/ml TiO_2 aqueous dispersion into the vial with 19 µl of BSA solution.

f) Using a calibrated pipette, add 14,83 ml of the DMEM-FBS prepared in b) to a 30 ml amber glass vial.

g) Transfer 169 µl of the TiO_2/BSA/DIW mixture obtained in e) into the 30 ml vial with 14,83 ml of DMEM-FBS, to yield a 100 µg/ml (TiO_2), 100 µg/ml (BSA) dispersion in DMEM-FBS. The pH of the resulting dispersion should still be comparable to that of the original medium. This procedure results in a 1,1 % dilution of the DMEM-FBS by the water added with the TiO_2 and BSA stocks.