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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

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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 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).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

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.

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.

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.

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

5 Materials

5.1 Cell line

Established cell lines are preferred and where used shall be obtained from recognized repositories. Follow the basic principles of cell culture techniques regarding expanding a frozen stock of cells so that the MTS assay for nanocytotoxicity can be performed [12].

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 −130 °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.

NOTE 1 It is important to check cells regularly [e.g. morphology, doubling time, modal chromosome number, short tandom repeat (STR) testing] because sensitivity in tests can vary with passage number.

NOTE 2 Nanoparticle can interact with cells through different mechanisms. It is useful to include both a phagocytic cell line (i.e. macrophage) and a non-phagocytic cell line (i.e. epithelial or fibroblast) in these studies. Assay results with the use of these two cell types can provide insight into the mode of action for nanoparticle toxicity.

5.2 Assay

5.2.1 MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]\PMS- phenazine methosulfate [CAS#138169-43-4].

The reagent is reduced in the presence of cellular enzymes and forms a coloured product that is soluble in the culture media. The optical density of the culture media is correlated with cell count in a culture vessel in the absence of artefacts that can occur if the culture conditions affect reductase activity within the cells and if the nanoparticle causes interference effects in the assay readout. The reagent is described in Reference [2] and the reagent materials are available from different vendors.

5.3 Controls

5.3.1 Chemical positive control material, $CdSO_4$, shall be used as positive chemical control.

NOTE 1 Cd^{+2} ions are toxic to animals and cells through an oxidative stress mechanism, see Reference [13].

NOTE 2 Cadmium containing compounds, including water soluble compounds such as $CdCl_2$ and $CdSO_4$ are assigned the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) signal word of Danger.

Cadmium (Cd) is a toxic heavy metal and its disposal and use are regulated in some countries. In the case where Cd cannot be used as a positive chemical control, an alternative chemical control shall be selected. The control compound should be soluble in aqueous media, sufficiently stable for the time course of the experiment and readily available as a purified product from commercial vendors. Non-metallic chemicals such as phenol, DMSO and detergents such as Tween 80 can be used as positive chemical controls, with the protocol undergoing additional validation for the use of these chemicals.

5.3.2 Positively charged polystyrene nanoparticles, (diameter 60 nm, dispersed in water) shall be used as a nanoparticle positive control material. The use of these nanoparticles as positive controls in A549 and Raw 264.7 cells has been validated in interlaboratory studies (see Table 1).

NOTE 1 For dispersion protocol and biological activity of the cationic polystyrene nanoparticles, see Reference [10].

NOTE 2 Positively charged polystyrene (amine terminated) induce toxic oxidative stress in many cells, see Reference [14].

NOTE 3 Nanoparticles of quartz, silica and silver are also cytotoxic to many cell types and could be used as positive controls, see Reference [15].

6 Apparatus

6.1 Incubator, 37 °C ± 1 °C, humidified, 5 % CO_2/air.

6.2 Flat bottom 96 multi-well plates.

6.3 96 multi-well plates with U bottom, for dosing plate use.

6.4 24 multi-well plates with flat bottom, for cell health and growth rate only.

6.5 96 well plate photometer microtitre plate reader.

6.6 Centrifuge, capable of at least 2 000 g acceleration.

6.7 Multichannel pipette (at least 8 position), with 200 µL volume/pipette.

6.8 Laminar flow cabinet, standard biological hazard.

6.9 Tissue culture flasks, 25 cm^2 and 75 cm^2.

6.10 Inverted phase contrast microscope.

6.11 Stereomicroscope.

6.12 Laboratory balance.

6.13 Electronic cell counter or hemocytometer

6.14 Micropipette.

6.15 Vortex mixer.

7 Nanoparticle test 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. Alternatively, nanoparticles can be dispersed with biologically compatible chemical stabilizers, coatings, such as albumin, or directly in culture medium using the appropriate serum. Specific dispersion techniques are not discussed in this document. Details for dispersion can be found in the references cited in the NOTEs and in ISO/TS 19337.

NOTE 1 Several procedures have been published that identify methods to reproducibly disperse nanoparticles[15][16][17] and characterize nanosuspensions and their stability. Dispersion protocols from the NANOGENOTOX Joint Action are publicly available on the internet.

NOTE 2 For biologically compatible chemical stabilizers see Reference [19]. For coatings such as albumin see Reference [20]. For compatible culture medium, see Reference [21].

NOTE 3 Chemical stabilizers such as albumin can introduce high background levels in cell viability assays. It is important to use control experiments (i.e. stabilizer only) to determine the effect of the stabilizer on the assay readout.

With nanoparticles dispersed in a liquid media such as H_2O, the volume fraction of the nanoparticle media in the cell culture media shall be below the fraction that is toxic to the cell culture.

The liquid media supporting the nanoparticle suspension can be toxic to cells and cause a false positive toxicity measurement. Control experiments with liquid media should be performed to determine at what volume fraction is the liquid media toxic to the cells.

NOTE 1 A 1 mg/1 ml suspension would produce a water content of ~10 % in cell culture media for a 100 µg/ml exposure. When using water as a dispersion vehicle, a guideline is to keep the final concentration of water below 10 % of the total volume to reduce significant vehicle effects. If higher concentrations of vehicle are required for nanoparticle dose preparation, it is important to validate the higher concentration of vehicle does not interfere with the assay results.

The type of suspension process used shall be carefully considered in order to rule out false positive cytotoxic effects that are not due to the nanoparticles

For nanosuspension stability evaluation, two factors shall be evaluated:

a) stability against agglomeration (reflected in the mean particle size); and

b) stability of the colloidal suspension (reflected by precipitation and sedimentation).

Nanosuspensions should be tested for the presence of endotoxins in accordance with ISO 29701.

8 Preparations

8.1 General

All solutions (except culture medium), glassware, etc., shall be sterile and all procedures should be performed under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

8.2 Culture medium

The culture medium shall be sterile.

The culture medium with or without serum shall meet the growth requirements of the selected cell line. Antibiotics may be included in the medium provided that they do not adversely affect the assays.

Storage conditions such as refrigerator temperature shall be validated.

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

8.3 Preparation of cell stock culture

Using the chosen cell line and culture medium, prepare sufficient cells to complete the test. 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. Additional cell line information is in Annex A.

Good cell culture practices should be used. See Reference [12] additional instructions if required.

8.4 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:

a) Grow the cells in 24 well plates for 24 h and 48 h:

   1) transfer 200 000 cells/ml in 500 µL of culture medium per well with eight replicates per time period;

   2) use one plate for each time period (24 h and 48 h);

   3) gently move the plates into the incubator without agitation to avoid disturbing cell attachment resulting in non-uniform deposition;

   4) verify that incubators have been recently calibrated for: temperature, humidity, CO_2 concentration. Record metrics in a laboratory notebook to establish charting metrics;

   5) at each time point (24 h and 48 h), remove one plate from the incubator;

   6) make note of the apparent health and morphology of the cells with a stereomicroscope.

b) Assess cell number and viability with trypan blue:

   1) remove culture medium from the wells with gentle pipetting;

   2) harvest the cells using the manufacturer's instructions;

   3) collect the culture medium containing cells in a centrifuge tube;

   4) spin the supernatant with the added cells in a centrifuge at 400 g for 5 min to form a pellet;

   5) discard the supernatant;

   6) add 25 µL (0,4 % trypan blue in PBS) to 100 µL culture medium;

   7) resuspend the pellet in trypan blue/culture medium with a pipette;

   8) deposit the cells on a hemacytometer;

   9) 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[22] for details;

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

8.5 Verification of plate reader uniformity

Ensure that the instrument is operating properly prior to performing the measurement.

8.6 Control preparation

8.6.1 Control description

Positive control materials shall be $CdSO_4$ and positively charged polystyrene. See Clause 5 for more detail on the selection of these positive controls.

Separate experiments shall be performed to determine whether the nanoparticles and antibiotics can potentially interfere with the assay.

Prepare stock solutions in sterile and endotoxin free ultra pure water (<1,1 µS/cm at 20 °C).

8.6.2 $CdSO_4$ stock solution preparation (10mM)

Cells shall be exposed to $CdSO_4$ at final concentrations of 1 µM, 10 µM, 25 µM, 50 µM, and 100 µM.

a) Dissolve and dilute $CdSO_4$ in ultra pure water to 10mM concentration;

b) Store the 10mM $CdSO_4$ at 4 °C. Sterile filtration is not required.

8.6.3 Nanoparticle control suspension preparation

Adjust positive polystyrene concentration to 10 mg/ml with ultra pure water.

NOTE At this concentration, the maximum dosing concentration in the test plate (100 µg/ml) will result in 1.0 % (w/v) water vehicle in the cell culture media. This stock preparation, which was used in study described in Annex B, is used as an example for all the following procedures. If a more dilute nanomaterial is used as a stock solution, the fraction of vehicle in the cell culture media will be increased. See Clause 7 for preparation of other nanoparticle suspensions.

8.7 Precision pipetting

A calibrated pipette shall be used to dispense reagents into the 96-well plate used for this assay. If possible, a calibrated multichannel pipette that is capable of simultaneously dispensing from at least 6 pipette tips during a single ejection in a 96-well plate should be used to perform this procedure. A previous study indicated that variability in cell seeding between wells using a multichannel pipette is lower than the variability from separate pipetting steps. [21]

NOTE With the small volumes of fluids, cells and nanoparticles used in the 96 well experiments, it is important that the pipette system is carefully calibrated and procedures used to dispense fluids precisely. A more detailed description of procedures vendor. As an example, Cerionix application note AN1-12 12/06 describes "Precision- and Accuracy-Based Validation of Pipette Tips Used on Automated Liquid Handling Platforms Following Multiple Cleanings with 'Cold' Atmospheric Plasma".

9 Characterization of nanoparticle impact on cell viability

9.1 General

Due to potential variability in nanosuspensions, three independent replicate assays should be conducted on different days with new suspensions. The protocol steps for each assay are summarized in the flow chart shown in Figure 1.

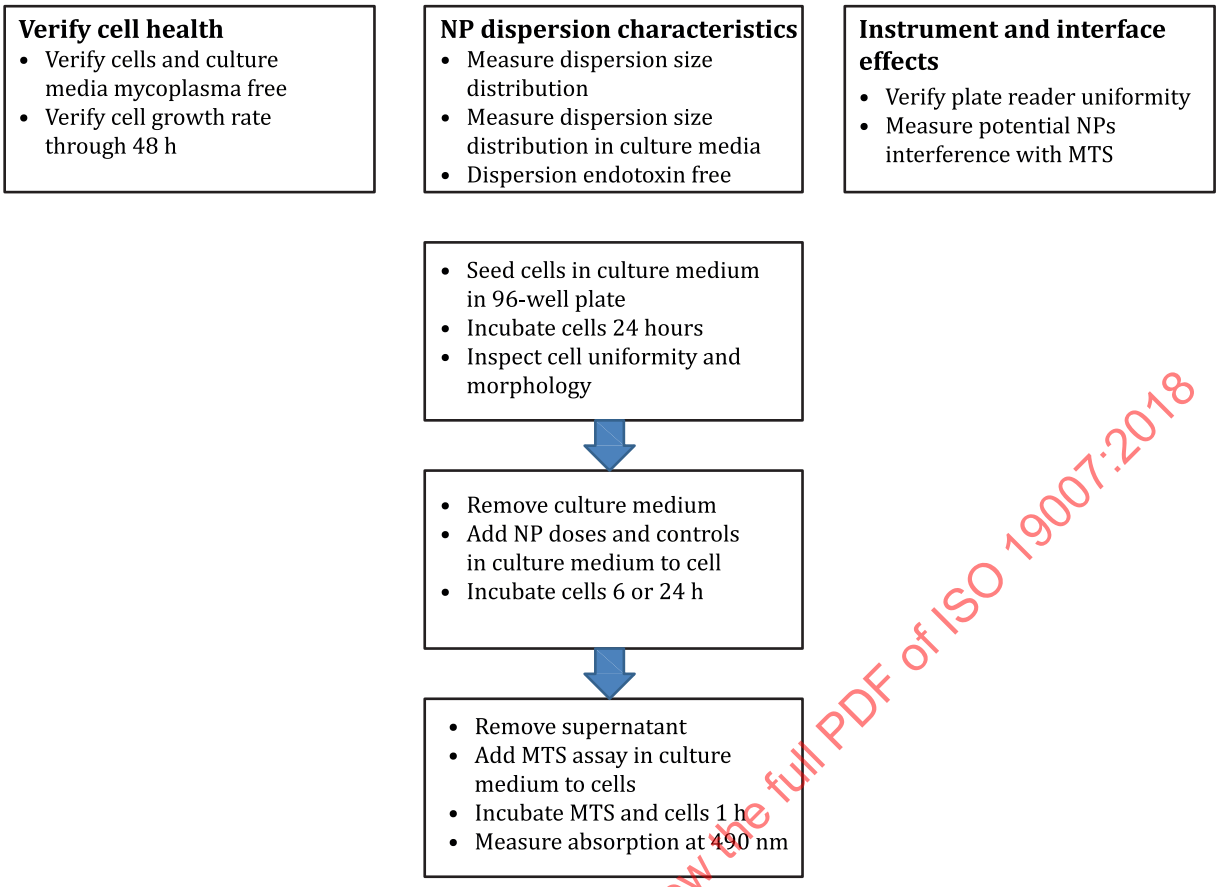


Figure 1 — A simplified process flow for characterizing the potential cytotoxicity of nanoparticle dispersions

9.2 Preparation of the cell plate

9.2.1 The cells should be collected, counted and then re-suspended in culture medium at ~7,5 × 10^4 cells/mL, as verified with a cell counter or hemacytometer and stereomicroscope.

— Cells shall be seeded in culture medium at the appropriate density so that cultures will not reach confluence by the end of the test;

— Approximately 1 000 000 cells are required for a single plate (~30 % excess).

NOTE The cell concentration described here is based on a cell seeding density of 1.5x10^4 cells/well in a 96 well plate. This is appropriate for the A549 cells described in Annex B. Depending on the cell type, doubling time, and MTS reduction activity, this cell seeding density can be validated in preliminary experiments. See NOTE 1 in 9.5.2.

9.2.2 Transfer culture with cells (200 µL) into each well in columns 3-6 and 8-10, as shown in Figure 2.

9.2.3 Transfer complete cell culture media with no cells (200 µL) into each well in columns 2, 7 and 11, as shown in Figure 2, hashed wells. Cells shall be seeded at 1,5 × 10^4 cells/well in each of the clear wells. Columns 2, 7 and 11 shall have medium alone and column 6 shall have cells alone in the culture medium.

NOTE Wells in a single column are seeded with a single multichannel pipetting step. See 8.7.

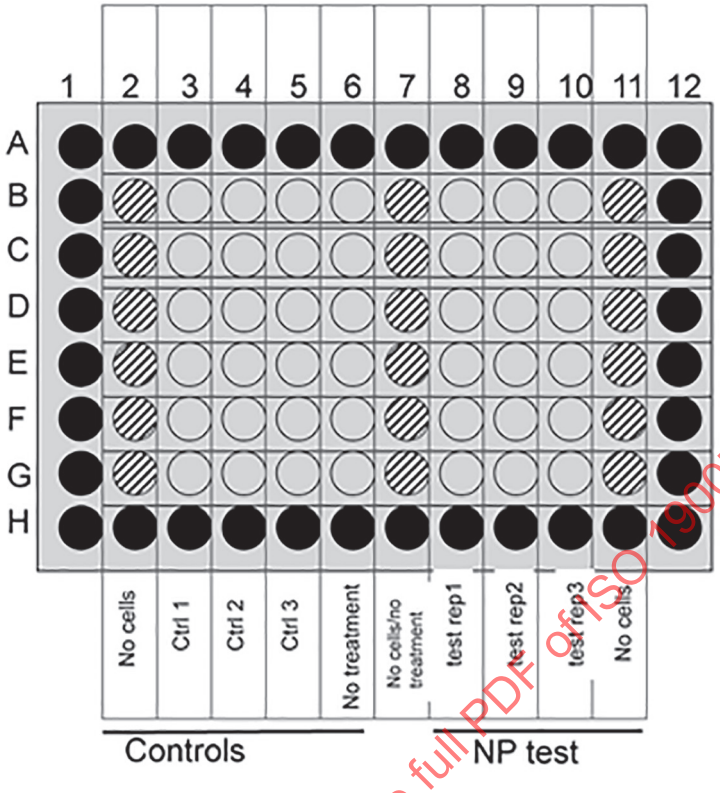


Figure 2 — Cell culture plate layout

9.2.4 Gently transfer the plate to a humidified incubator at 37 °C ± 1°C with 5 % CO_2 so cells are evenly distributed.

a) Incubate the cells for 24 h ± 2 h.

b) After the cell plate has incubated for 24 h, inspect the cells with a microscope to determine whether the cells are uniformly distributed and also make note of the apparent health and morphology of the cells.

9.3 Prepare the nanoparticle dosing plate

9.3.1 Figure 3 shows the basic layout of a dosing plate. Columns 6 and 7 wells shall contain cell culture media + 1 % H_2O. Columns 2–5 and columns 8–11 contain positive control material and test sample nanoparticle doses, respectively. Column 2 and column 11 serve as assay interference wells for both the positive chemical control and the NP test sample, respectively. The volumes shall be in accordance with

Table 2. The content of these wells shall be transferred to the cell plate with an 8-channel multichannel pipette steps for each row.

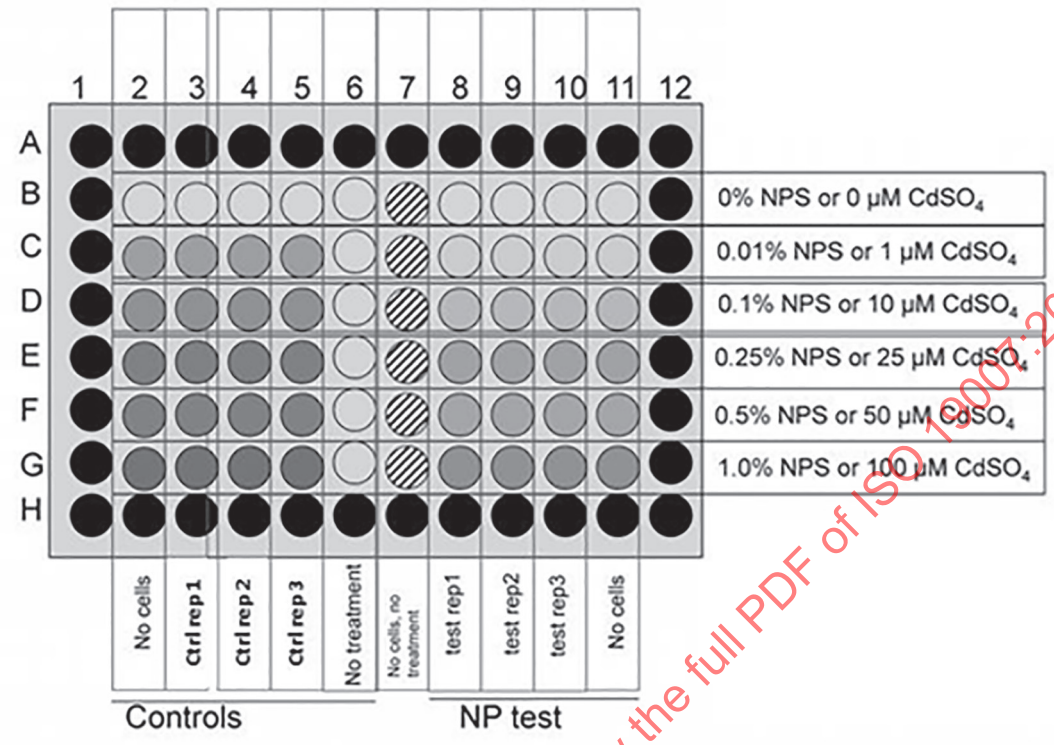


Figure 3 — Dosing plate layout

NOTE The NPS weight percentage shown in Figure 3 is for the +PS-NP suspension described in Clause 7. For other nanoparticle preparations that require higher weight percentages in the dose preparations, see guidance in Clause 7 for determining the amount of NP suspension to add to each dose and maximum vehicle percentages.

9.3.2 Dosing plate preparation.

a) Columns 2, 3, 4 and 5 shall receive doses of $CdSO_4$ in culture medium specified in Table 2.

b) Column 8, 9, 10 and 11 shall receive positively charged polystyrene nanoparticles or test sample nanoparticles in culture medium with doses specified in Table 2.

c) Column 6 and 7 receive 200 µL of culture medium with 1 % H_2O.

NOTE 1 The 1 % H_2O concentration in the cell culture media will depend on the stock nanoparticle concentration. See Clause 7 for guidance.

NOTE 2 Prepare the nanoparticle dosing plate approximately 3 h before the cell plate is removed from the incubator. This allows immediate transfer of the contents in the dosing plate to the cells after removal of media from the cells. This also reduces long term precipitation effects that can occur when the NP are suspended in the cell culture media.

Table 2 — Preparation of culture media, chemicals and nanoparticles (NP) in dosing plate

Row Letter	Complete Cell Culture Media (µL) in Columns 2–5 and 8–11	$CdSO_4$ positive control material (100 mmol/L) in complete cell culture media in columns 2 and 3 (µL)	Positive charged PS (100 µg/ml) in complete cell culture media in column 4 (µL)	Test sample NP (1 % suspension concentration) in complete cell culture media in columns 8–11 (µL)
B	200	0	0	0
C	198	2	2	2
D	180	20	20	20
E	150	50	50	50
F	100	100	100	100
G	0	200	200	200

9.4 Expose cells to nanoparticles in culture medium

9.4.1 After 24 h incubation of the cell plate, remove cell culture media from each well, with a pipette positioned at the bottom edge of the well.

9.4.2 The contents of each column in the dosing plate shall be carefully transferred to the cell plate with a pipette set to 200 µL (e.g. a multichannel pipette).

9.4.3 The plates shall then be transferred to the incubator for 24 h.

NOTE If extended incubation times including 48 h or 72 h are used for the assay, then the protocol steps in this document are validated for these incubation times.

9.4.4 After the indicated exposure time, remove the plates from the incubator.

9.4.5 Remove the dosing treatments and non-adhered unhealthy cells using a pipette and gentle aspiration.

9.5 Expose cells to MTS Assay

9.5.1 Transfer 200 µL of the MTS (317 µg/ml) reagent in culture medium into each column of the cell plate.

Do not use the expel step in the pipetting procedure to prevent the formation of air bubbles.

9.5.2 Incubate the cells with the MTS reagent in the dark for 60 min at 37 °C in a humidified, 5 % CO_2 atmosphere.

NOTE 1 The incubation time might vary depending on the cell type, cell concentration, and MTS reagent concentration. This is especially true if primary cells are used. For a new cell type, preliminary experiments are performed to determine the appropriate incubation time. For example, the time required for a culture well of non-treated cells to have an optical density between 1.0 and 2.0 (490 nm) after the addition of the MTS reagent are used as an appropriate incubation time.

NOTE 2 The MTS reagent is relatively non-toxic to cells and can be directly added to the NP exposure media without the use of the separate media removal step described in this document. Although this is a simplification of the procedure, this modification is only be useful for NP suspensions that do not introduce optical absorption artefacts. This modification is only valid for the use with a particular nanoparticle.

9.6 Measurement of formazan absorbance

9.6.1 Set the plate reader so that raw absorbance measurements are recorded for every well in the plate.

9.6.2 Absorbance shall be measured at 490 nm using a plate reader.

9.6.3 Measure the absorbance in each well. Take note of any wells that have air bubbles.

NOTE 1 An additional procedure that can be used to detect optical path issues in the culture wells (e.g. air bubbles, fingerprints, etc) is to take absorbance measurements at a reference wavelengths that does not detect the MTS reagent (e.g. 650 nm). Significant variations between the wells for this reference measurement could indicate the presence of an optical path obstruction in a well. Such a procedure is validated before it is used as part of the protocol.

NOTE 2 The protocol from the NanoGo interlaboratory comparison of nanoparticle cytotoxicity used a separate centrifugation step to remove any residual NP from the MTS cell culture media mixture before it is read on a absorbance plate reader. After incubation with the MTS reagent, the plate was centrifuged at 2000g for 10 min and the supernatants were transferred to a new plate and read on the plate reader[1]. The protocol in this document can be modified with this procedure, but the procedure undergoes validation before general use.

10 Cell viability analysis

Perform a simple spreadsheet-based analysis of the toxicity data.

a) The average background level for nanoparticles in culture medium (from column 11) shall be subtracted from each well.

b) The resulting absorbance from each dosing well in a technical replicate shall be normalized to the absorbance value in the no-treatment well in the technical replicate (row B). These background-subtracted and normalized values represent the fraction of cells that remain after dose treatment.

c) After normalization, the values from a single row in the technical replicates (i.e. columns 3-5 or 8-10) shall be averaged and standard deviations for each dosing condition shall be determined.

d) If toxicity was observed, estimate an EC_{50} value and and a 95 % confidence interval from the data set using GraphPad Prism or other statistical software applications.

If a treatment causes enhanced cell proliferation, viability will appear to increase. Additional proliferation assays such as BrdU or cell counting should be used to confirm this result.

Assement 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. If the mean absorbance and absorbance variance is significantly different than the MTS reagent wells (column 7), it may suggest that the NP are introducing interference artefacts.[23]

11 Interpertation of Assay Results

The MTS assay is a screening assay and provides a rapid method to assess potential toxic interactions with biological cells. In general, a single screening assay is not adequate to fully interpertate the toxicity of a nanoparticle. The results of this assay should be used with the results of other assays to potentially understand the mechanism of action of toxicity and how the data can be used in a risk assessment model.

Annex A
(informative)

Potential cell lines and assays

Cell lines that are applicable to use with the MTS assay include: mouse macrophage; Abelson murine leukaemia virus transformed (RAW 264.7), human lung epithelial cell (A549), human bronchial epithelial (BEAS-2B), rat alveolar type II epithelial (RLE-6TN), mouse liver epithelial hepatocyte (HEPA-1), human microvascular endothelial (HMEC), and rat aorta, thoracic/medial layer (A10) cell lines. These cell lines have been used to evaluate cell viability in the presence of nanoparticles in some experiments. Additional cell lines such as those described in ISO 10993-5 which describes cytotoxicity testing for medical devices could also be adapted for use in the protocol, but validation of the protocol steps would be required. These cell lines include mouse embryo (Balb/c 3T3), mouse fibroblast (L929) and Chinese hamster lung fibroblast (V79).

While the MTS Assay was used for this document, other assays may also be applicable for assessing cell viability including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), XTT(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide), and other water-soluble tetrazolium salts-based assays (e.g. WST-1 or WST-8). The MTS assay is an improved version of the MTT Assay[23].

Annex B
(informative)

Example: the MTS assay using the A549 cell line (EMPA-NIST protocol)

B.1 General

This protocol is an extension of the protocol from the non-peer reviewed IANH described in Annex C. This protocol includes preparation of the dosing plate and more specific details of cell handling. This protocol was used with an A549 cell line to assess the cytotoxicity effect of positively charged polystyrene and ceria nanoparticles. An interlaboratory comparison between five international measurement laboratories was performed using this protocol. The experimental design is described in Reference [22] and the international laboratory comparison data will be published elsewhere and are summarized at the end of this protocol. This protocol does not use the centrifugation step to reduce potential NP interference effects that is described in the NanoGo protocol.

B.2 Experimental procedures

B.2.1 Basic procedure

The effect of nanoparticles on A549 cell cytotoxicity is determined by using a modified version of this document.

B.2.2 Material

B.2.2.1 Cell line

A549, human epithelial; carcinoma transformed (Verified with DNA identification Cell cultures) shall be free of mycoplasma.

B.2.2.2 Assay

MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]\ PMS- phenazine methosulfate[2].

B.2.2.3 Chemical media and sera

B.2.2.3.1 Roswell Park Memorial Institute medium (RPMI-1640)

B.2.2.3.2 FBS (Fetal Bovine Serum, heat inactivated)

B.2.2.3.3 Penicillin

B.2.2.3.4 Streptomycin

B.2.2.3.5 L-glutamine

B.2.2.3.6 0,05 % Trypsin-EDTA

B.2.2.3.7 MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

B.2.2.3.8 Phosphate Buffered Saline, (PBS) [Ca^{2+} and Mg^{2+} Free]

B.2.2.3.9 DMEM Phenol red free

B.2.2.3.10 $CdSO_4$ (Positive chemical control)[13]

B.2.2.3.12 Positively charged polystyrene nanoparticles, (diameter < 100 nm) [positive nanoparticle control][10]

The NH_2-PS NP suspended in H_2O were obtained from Bangs Laboratories Inc (Fishers, Indiana, US), lot number 10351 and inventory number L120117F, 10 % (w/v). This information is presented for the convenience of the user of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

B.2.2.3.13 Distilled water or any purified water suitable for cell culture

B.2.2.3.14 Amphotericin B (antifungal agent)

B.2.3 Apparatus

See Clause 6.

B.3 Preparations

B.3.1 General

All solutions (except culture medium), glassware, etc., shall be sterile and all procedures should be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

B.3.2 Culture medium

B.3.2.1 Culture medium

B.3.2.1.1 10 % Fetal Bovine Serum (FBS)

B.3.2.1.2 90 % Roswell Park Memorial Institute medium (RPMI-1640)

B.3.2.1.3 10 µg/ml streptomycin

B.3.2.1.4 100 IU/ml penicillin

B.3.2.1.5 0,2 mg/ml L-glutamine

B.3.2.1.6 2,5 µg/ml Amphotericin B

Due to potential degradation of the L-glutamine, the culture medium should not be stored more than three weeks. Clear labelling of time that an assay is performed with respect to the reception time of the cell culture media should be recorded.

B.3.2.2 Cell culture medium without serum

B.3.2.2.1 Roswell Park Memorial Institute medium (RPMI-1640) with:

B.3.2.2.2 10 µg/ml streptomycin

B.3.2.2.3 100 IU/ml penicillin

B.3.2.2.4 0,2 mg/ml L-glutamine

B.3.3 Preparation of cell stock culture

B.3.3.1 Cell thawing

Upon thawing, cells should be passaged at least two times before use in experiments. The cells should not be kept longer than three months in culture.

Immediately after thawing and within the first passage, cell growth may be slow.

B.3.3.2 Growth of cells[16]

Grow cells in a 75 cm^2 cell culture flask with 20 ml culture media in an incubator under standard growth conditions (37 °C, 5 % CO_2 in humidified air).

NOTE 1 A549 cells are cultured in complete cell culture medium (see reagent preparation in C.3.2).

NOTE 2 A549 cells grow rapidly with a typical population doubling time of 24 h. When the cells are grown in phenol red-containing medium, a change in colour from red to yellow could indicate culture overgrowth (i.e. depletion of nutrients).

B.3.3.3 Cell harvesting

B.3.3.3.1 Remove culture medium from the flask and wash the cells twice with 2 ml PBS.

B.3.3.3.2 Apply 2 ml 0,05 % trypsin-EDTA to the PBS in the flask and incubate for 3 min at 37 °C, 5 % CO_2.

B.3.3.3.3 Release the cells by vigorously shaking the flash and transfer the cells.

B.3.3.3.4 Transfer the cells by adding 20 ml of tissue culture medium with serum to inactivate the trypsin and rinsing the bottom of the flask three to five times.

B.3.3.4 Counting cells to determine cell health

B.3.3.4.1 Sample approximately 20 ml (2 ml Trypsin-EDTA + 18 ml of the complete cell culture media) of cell suspension into 50 ml conical tube using a pipette.

B.3.3.4.2 Centrifuge cell suspension to precipitate cells as pellet for 5 min at 200 g.

B.3.3.4.3 Remove the supernatant and add approximately 1 ml media solution and re-suspend the pellet into the media solution.

B.3.3.4.4 Take 30 µL of cell suspension and add into 30 µL of 0,4 % (w/v) trypan blue/PBS in a 2 ml microcentrifuge tube. Mix the solution by pipetting with 200 µL micropipette.

NOTE As live cells can also be stained by trypan blue after extended periods of time, perform cell counting in 30 min.

B.3.3.4.5 With a cover slip in place, use a 200 µL micropipette to transfer 10 µL of trypan blue-cell suspension at each end of the hemocytometer. Try to avoid bubbles within the hemocytometer and cover slip.

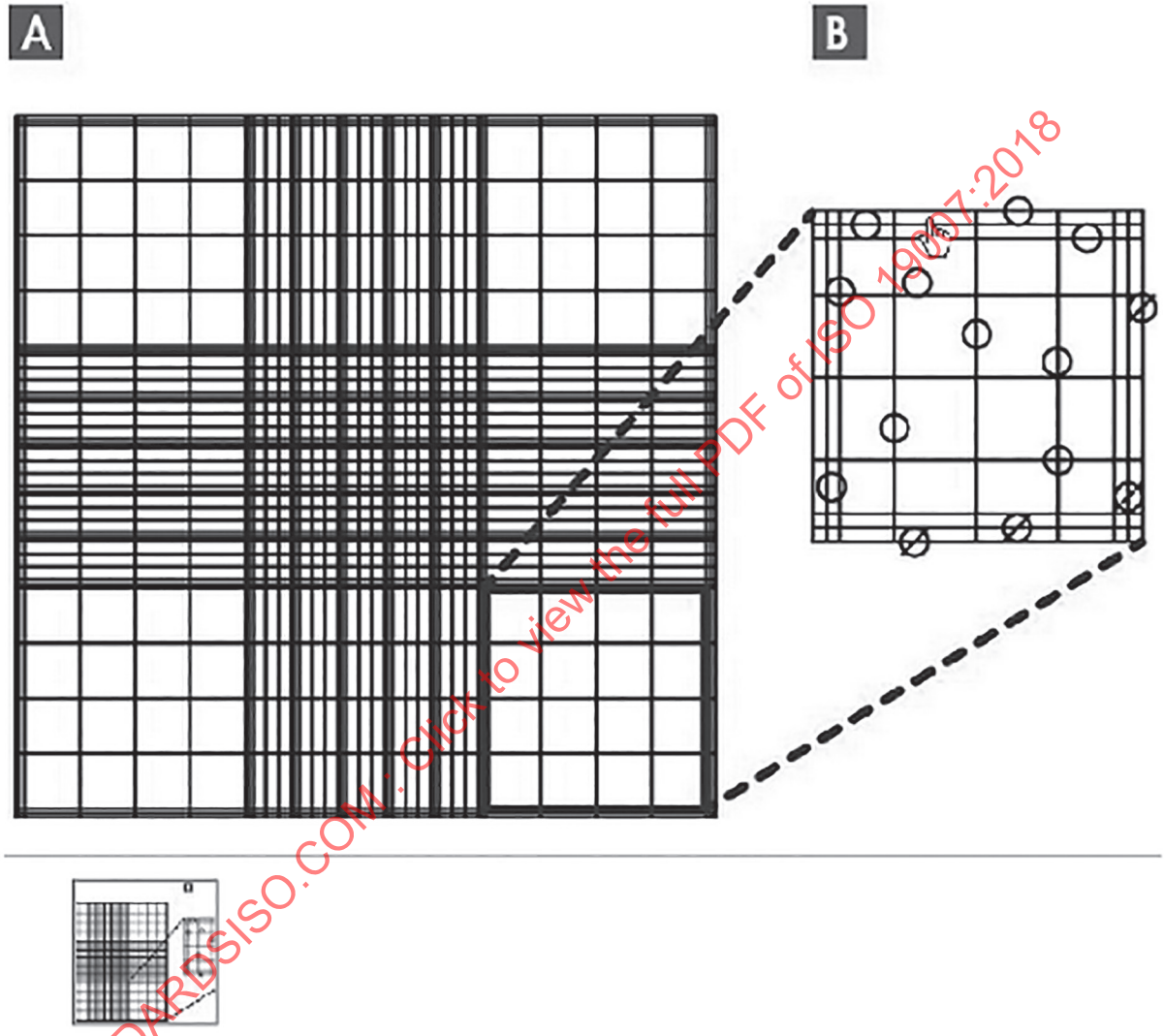


Figure B.1 — Schematic diagram of a hemocytome (cell counting chamber)

B.3.3.4.6 Count all the cells (non-viable cells stain blue, viable cells will remain opaque) in the 4 1 mm^2 squares at the corners (A1, A3, A7, A9, B1, B3, B7 and B9 in Figure B.1), which gives a total of eight squares for counting. Do not count cells on the right and lower border (see inset). If greater than 25 % of cells are non-viable, the culture is not being maintained in the appropriate amount of media. The current cell suspension should be abandoned and cells have to be re-cultured.

NOTE If there are less than 50 or more than 200 cells per large square, repeat the procedure adjusting to an appropriate dilution factor.

B.3.3.4.7 Calculate the number of cells per unit volume (cells/mL) for this protocol using the following Formula: the concentration of cells = Average cell count × the dilution factor × 10^4/ml.

NOTE The complete equation for using a hemocytometer is available with the manufacturers instructions.

B.4 Procedure

B.4.1 Cell seeding

B.4.1.1 $7{,}5 \times 10^5$ cells ($7{,}5 \times 10^4$ cells/ml) are suspended in 10 ml of complete cell culture medium for 1 96-well plate.

B.4.1.2 Cells are seeded at $1{,}5 \times 10^4$ cells/well (200 µL) in each of the blue wells (see Figure B.2) columns 3-6 and 8-10).

NOTE 1 It is important that cells in a single column are seeded with a single multichannel pipetting step.

NOTE 2 Stripped wells contain complete cell culture media only.

NOTE 3 Black wells are whenever possible filled with complete cell culture medium only. If operationally necessary they can be kept empty.

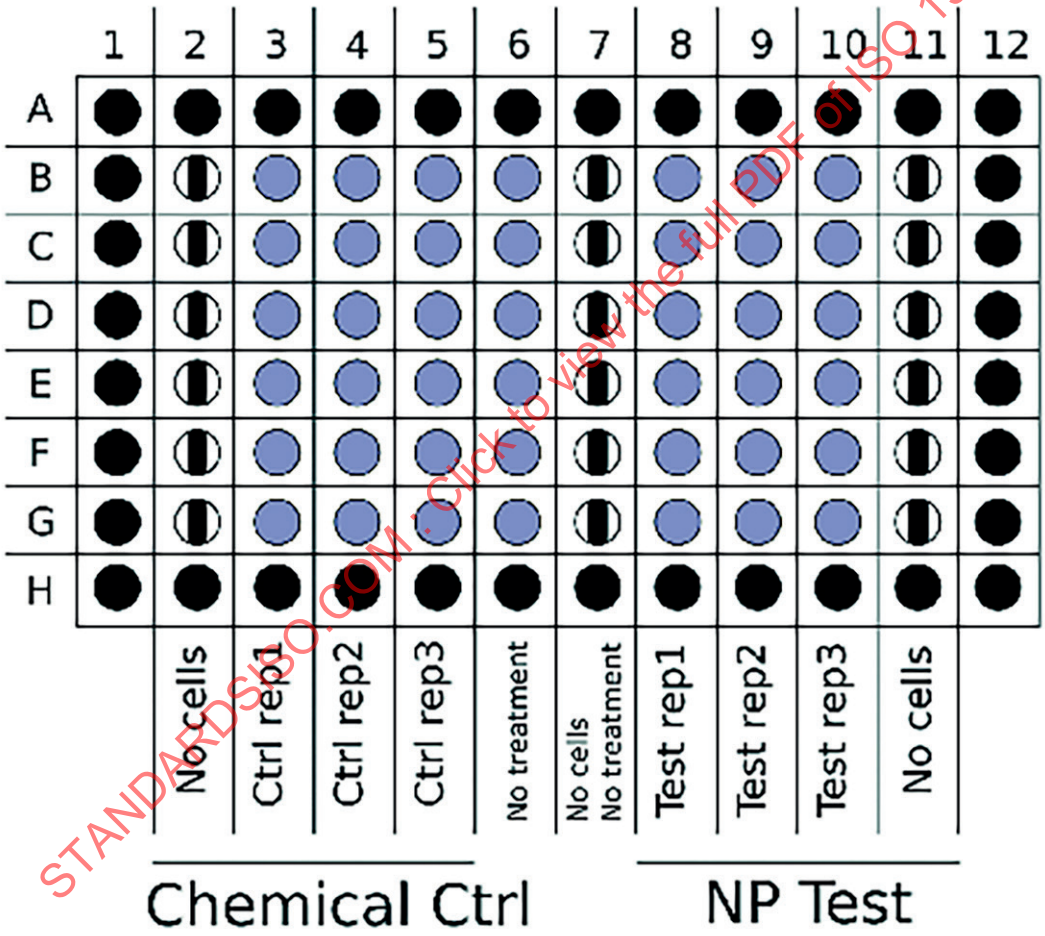


Figure B.2 — Seeding of the cells in a 96 well plate

Cells are seeded at 1,5 × 104 c/w with 200 µL complete cell culture medium in each of the blue wells. Stripped wells contain complete cell culture medium only, whereas black wells could either be filled with complete cell culture medium (better) or stay empty. Cells in a single column should be seeded with a single multichannel pipetting step.

B.4.1.3 The 96 well plate with the seeded cells is cultured for 24 h in a humidified incubator at 37 °C with 5 % CO_2.

B.4.2 Preparation of the working concentration of chemical positive control materials

240 µ L sterile H_2O (bi distilled water) are mixed with 2 376 ml cell culture medium without serum. This mixture #1 is used in all the following steps for the preparation of the working concentrations of the chemical controls:

1) 40 µ L of 10 mmol/L $CdSO_4$ are mixed with 3 960 µ L of mixture #1 = > 100 µ mol/L $CdSO_4$

2) 2 000 µ L of 100 µ mol/L $CdSO_4$ are mixed with 2 000 µ L of mixture #1 = > 50 µ mol/L $CdSO_4$

3) 2 000 µ L of 50 µ mol/L $CdSO_4$ are mixed with 2 000 µ L of mixture #1 = > 25 µ mol/L $CdSO_4$

4) 2 000 µ L of 25 µ mol/L $CdSO_4$ are mixed with 3 000 µ L of mixture #1 = > 10 µ mol/L $CdSO_4$

5) 200 µ L of 10 µ mol/L $CdSO_4$ are mixed with 1 800 µ L of mixture #1 = > 1 µ mol/L $CdSO_4$

6) Mixture #1 is used as 0 µ mol/L $CdSO_4$

NOTE 1 The resulting mixtures are vortexed before use for further dilution steps.

NOTE 2 The previous preparation of working concentrations of the chemical controls are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same.

For example: two 96 well plates:

— 2 × 40 µ L of 10 mmol/L $CdSO_4$ are mixed with 2 × 3 960 µ L of mixture #1 = > 100 µ mol/L $CdSO_4$

— 80 µ L of 10 mmol/L $CdSO_4$ are mixed with 7 920 µ L of mixture #1 = > 100 µ mol/L $CdSO_4$)

B.4.3 Preparing the working concentrations of nanoparticle test samples

Cell culture medium without serum is directly used without any addition of water. This medium #2 is used in all the following steps for the preparation of the working concentrations of the nanoparticles:

a) 4 µ L of 10 % PS-NH_2 are mixed with 3 996 μ L of medium #2 = > 100 μ g/ml PS-NH_2

b) 2 000 µ L of 100 µ g/ml PS-NH_2 are mixed with 2 000 µ L of medium #2 = > 50 µ g/ml PS-NH_2

c) 2 000 µ L of 50 µ g/ml PS-NH_2 are mixed with 2 000 µ L of medium #2 = > 25 µ g/ml PS-NH_2

d) 2 000 µ L of 25 µ g/ml PS-NH_2 are mixed with 3 000 µ L of medium #2 = > 10 µ g/ml PS-NH_2

e) 200 µ L of 10 µ g/ml PS-NH_2 are mixed with 1 800 µ L of medium #2 = > 1 µ g/ml PS-NH_2

f) Medium #2 is used as 0 µ g/ml PS-NH_2

B.4.3.1 The initial 10 % PS-NH_2 stock solution tube with the solvent shall be placed onto the vortex and before adding distributing the nanoparticle containing solution.

B.4.3.2 The resulting PS-NH_2 nanoparticle-medium mixtures shall be vortexed before used for further dilution steps and again vortexed before they are used for treatment.

NOTE 1 The previous preparation of working concentrations of the nanoparticle suspensions are sufficient for the treatment of one 96 well plate. If more than one 96 well plates are treated at the same time then increase the corresponding amounts with the factor of the number of the treated plates, but keep otherwise the ratios the same. For example, 2 96-well plates:

— 2 × 4 µ L of 10 % PS-NH_2 are mixed with 2 × 3 996 µ L of medium #2 = > 100 µ g/ml PS-NH_2 or

— 8 µ L of 10 % PS-NH_2 are mixed with 7 992 µ L of medium #2 = > 100 µ g/ml PS-NH_2

B.4.4 Treatment of a A549 cells with chemical positive control materials and nanoparticles

B.4.4.1 Wells of columns 2-5 and of columns 8-11 are emptied completely by pumping out the supernatant cell culture medium.

B.4.4.2 Washing with PBS: Wells of columns 2-5 and of columns 8-11 are filled with 200 µL PBS per well and then emptied completely. This washing step is repeated three times.

B.4.4.3 Columns 8-11 are immediately treated with the working concentrations of the nanoparticles according to the plate layout shown in Figure B.3.

B.4.4.4 Columns 2-5 are immediately treated with the working concentration of the chemical control according to the plate layout shown in Figure B.3.

B.4.4.5 The 96 well plate is cultured for 24 h after the treatment in a humidified incubator at 37 °C with 5 % CO_2.

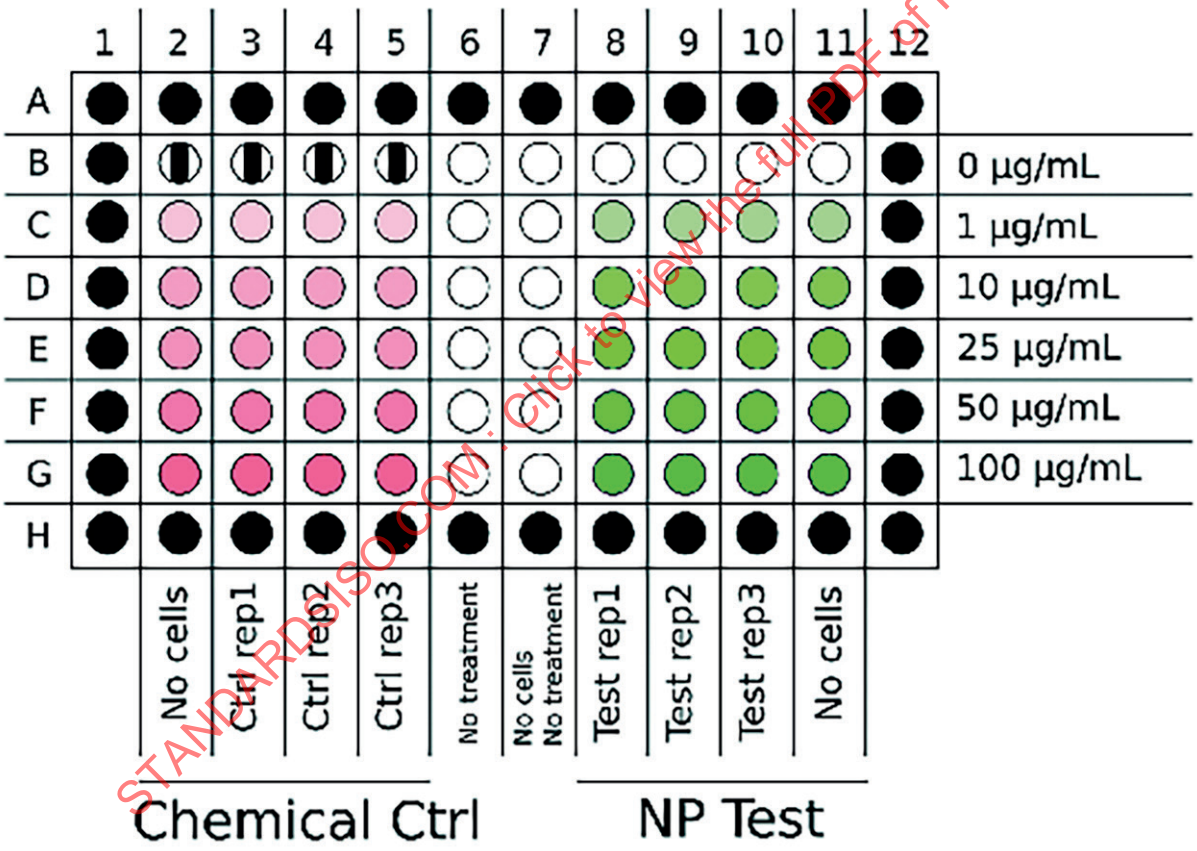


Figure B.3 — Dosing plate layout

White wells contain complete cell culture medium, whereas black wells can either contain complete cell culture medium (better) or stay empty. Stripped wells contain mixture (1). Columns 8-11 and 2-5 are treated column by column with a 10-channel multichannel pipette (200 µL).

B.4.5 Measurement of cytotoxicity

Cellular viability is determined by the MTS assay, which measures the reduction of {3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium}

(MTS) in the presence of phenazine methosulfate (PMS). It produces a formazan product that has an absorbance maximum at 490 nm to 500 nm. After incubation with the indicated doses of NP for 24 h at 37 °C, formazan absorbance is measured at 490 nm.

a) 2,5 ml MTS reagents are mixed with 12,5 ml RPMI-1640 medium (phenol red free). This mixture is called (3). There is no addition of serum, L-glutamine, Penicillin and Streptomycin to the (phenol red free) RPMI-1640 medium.

b) All 96 wells of the plate are emptied completely from the supernatant cell culture medium by pumping with a pipette.

c) All 96 wells of the plate are immediately filled with 120 µ L/well of mixture (3).

d) The 96 well plate is incubated for 60 min in an humidified incubator at 37 °C with 5 % CO_2.

e) The absorption measurements are performed with a plate reader at a wavelength of 490 nm.

f) These raw absorption results are used for the detailed evaluation of the assay test.

Meeting these control specifications (see Table B.1) is critical for achieving measurement assurance in the MTS nano-cytotoxicity assay. Additional information about the controls can be found in Reference [22].

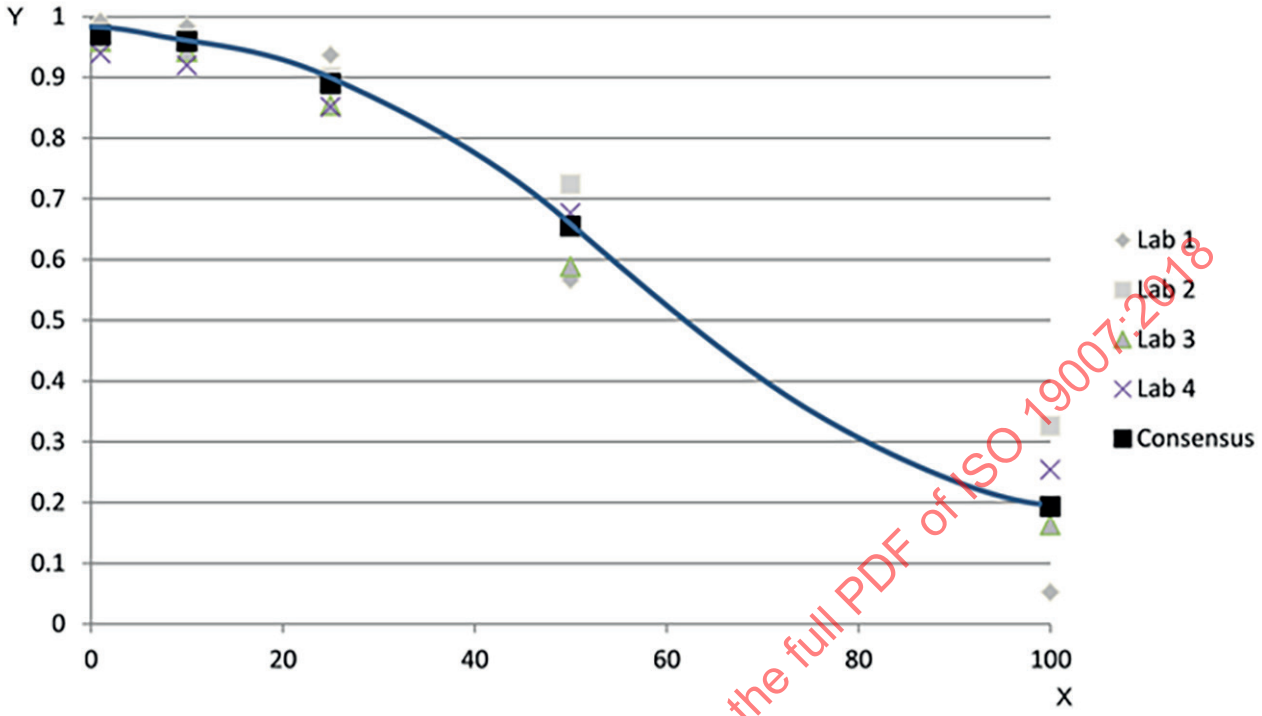
Table B.1 — System Specifications for the MTS assay as defined by the interlaboratory comparison for A549 cells

Control	Serum-containing media		
	Target value	Range	Variability
Background chemical control [1]	0,06 OD	0,05–0,09 OD	< 6 %
$CdSO_4$ control (EC50 value, well B3-G5)	77,2 umol/L	54,3–99,4 umol/L	
Within pipette variability (wells B6-G6)	2,0 OD	1,8–2,3 OD	< 7 %
No cell and no treatment control (wells B7-G7)	0,06 OD	0,05–0,09 OD	< 6 %
Background NP [2] (wells B11-G11)	ND		
Between pipette variability (wells B3-B5, B8-B10)	1,5 OD	1,3–1,8 OD	< 12 %
1) If no additional background from the chemical reaction control is observed.			
2) No values given, because some of the laboratories observed a background signal while others did not.			

B.4.6 Representative Cytotoxicity Dose-Response Curves

B.4.6.1 Figure B.4 shows representative cytotoxicity dose-response curve from 5 different laboratories.

B.4.6.2 Each data point shall be an average of absorbance values from several replicates in each laboratory. The consensus dose-response curve can be generated through Monte Carlo modelling techniques. Figure is based on Figure 3c from Reference [2] and was generated using Plot Digitizer v2.6.8



key

X +PS-NP dose concentration (µg/ml)

Y relative absorbance at 490 nm

Figure B.4 — Dose-response curves from 5 different laboratories for +PS-NP treatment of A549 cells in serum-containing media

Annex C
(informative)

Example: MTS assay using the RAW 264.7 cell line (IANH protocol)

C.1 General

This MTS assay protocol was developed through the International Alliance for NanoEHS Harmonization (IANH) in 2008 and used in the initial rounds of interlaboratory comparisons between the IANH members. This protocol was applied to the RAW 264.7 cell line to verify cell growth rate and viability and then to assess the cytotoxicity effect of positively charged polystyrene and ceria nanoparticles. The data from this study was not published, but is presented as Figures in this annex.

C.2 Experimental procedure

C.2.1 Basic procedure

C.2.1.1 General

The health and growth rate of the Raw 264.7 cells are verified by using 8.4 of this document. The effect of nanoparticles on Raw 264.7 cell cytotoxicity is determined by using Clauses 9 and 10 of this document.

C.2.1.2 Materials

C.2.1.2.1 Cell line

RAW 264.7, mouse macrophage; Abelson murine leukaemia virus transformed (Verified with DNA identification), American Type Culture Collection [ATCC], Manassas, VA, USA; ECACC No. 88102702, European Collection of Cell Cultures, Salisbury, Wiltshire SP4 0JG, UK). Cell cultures shall be free of mycoplasma.

C.2.1.2.2 Assay

MTS[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]\
PMS- phenazine methosulfate[2]

C.2.1.2.3 Chemicals media and sera

C.2.1.2.3.1 Dulbecco's Modified Eagle Medium (DMEM)

C.2.1.2.3.2 FBS (Fetal Bovine Serum)(heat inactivated)

C.2.1.2.3.3 Penicillin

C.2.1.2.3.4 Streptomycin

C.2.1.2.3.5 L-glutamine

C.2.1.2.3.6 0,05 % Trypsin-EDTA

C.2.1.2.3.7 MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]

C.2.1.2.3.8 Phosphate Buffered Saline, (PBS) [Ca^{2+} and Mg^{2+} Free]

C.2.1.2.3.9 DMEM Phenol red free

C.2.1.2.3.10 $CdSO_4$ (Positive chemical control) [13]

C.2.1.2.3.11 Cerium dioxide (ceria) nanoparticles (diameter ~100 nm) [Negative particle control]

C.2.1.2.3.13 Positively charged polystyrene nanoparticles, (diameter < 100 nm) [positive nanoparticle control][10]

The NH_2-PS NP suspended in H_2O were obtained from Bangs Laboratories Inc (Fishers, Indiana, US), lot number 10351 and inventory number L120117F, 10 % (w/v). This information is presented for the convenience of the user of this document and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

C.2.1.2.3.14 Distilled water or any purified water suitable for cell culture

C.2.1.2.3.15 Amphotericin B (antifungal agent)

C.2.1.3 Apparatus

See Clause 6.

C.3 Preparations

C.3.1 General

All solutions (except culture medium), glassware, etc., shall be sterile and all procedures shall be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (biological hazard standard).

C.3.2 Culture medium

For routine culture:

— 10 % Fetal Bovine Serum (FBS) (heat inactivated)

— 90 % DMEM

— 100 µg/ml streptomycin

— 60 µg/ml penicillin (100 IU/ml)

— 292 µg/ml L-glutamine

— 2,5 µg/ml Amphotericin B

Due to potential degradation of the L-glutamine, the culture medium should not be stored more than three weeks. Clear labelling of time that an assay is performed with respect to the reception time of the cell culture media should be recorded.

C.3.3 Preparation of cell stock culture

C.3.3.1 Cell thawing method

C.3.3.1.1 Transfer culture media (10 % FBS) into a centrifuge tube, place on ice and allow to cool for 5 min.

C.3.3.1.2 Thaw frozen cells in 37 °C water bath until there is a small amount of ice left. (It will continue to melt after you take it out of the water bath.)

C.3.3.1.3 Remove the cells with gentle scraping and aspiration with a pipette.

C.3.3.1.4 Add cells drop wise to a 15 ml centrifuge tube containing culture medium.

C.3.3.1.5 Spin the cells and culture medium in a centrifuge at 400 g for 5 min and remove the culture medium.

C.3.3.1.6 Re-suspend the cells in 10 ml of culture medium in a 15 ml centrifuge tube (10 % FBS).

C.3.3.1.7 Spin the cells and culture medium in a centrifuge at 400 g for 5 min and remove the culture medium.

NOTE Steps C.3.3.1.4-C.3.3.1.6 significantly dilutes (i.e. removes) the DMSO preservation media from the cell culture.

C.3.3.1.8 Resuspend the cells in 5 ml culture medium (10 % FBS) and transfer to a 25 cm^2 tissue culture flask.

C.3.3.2 Growth of cells[17]

C.3.3.2.1 Wash cells once gently with culture media and change culture media every 2–3 d.

Use 15 ml media (10 % FBS) for 75 cm^2 tissue culture flask or 5 ml for 25 cm^2 tissue culture flask.

C.3.3.2.2 Grow cells 2–3 d at 37 °C, 5 % CO_2 before passaging.

The Raw 264.7 cells are to be grown adherent to the flask.

Ensure that the cells don't exceed 80 % confluence with a stereomicroscope.

C.3.3.3 Passaging or splitting cells

C.3.3.3.1 Wash cells with 5 ml warm or room temperature media.

C.3.3.3.2 Extract the cells and culture medium from the flask with a pipette and gentle scraping.

C.3.3.3.3 Transfer the cells and culture medium to a centrifuge tube.

C.3.3.3.4 Wash the flask with culture media and add to the centrifuge tube.

C.3.3.3.5 Spin the cells and culture medium in a centrifuge at 400 g for 5 min and remove the culture medium.

C.3.3.3.6 Re-suspend the cells in culture medium.

C.3.3.3.7 Split 1:4 or 1:5 to ensure optimal growth.

C.3.3.4 Preparing culture for experiments

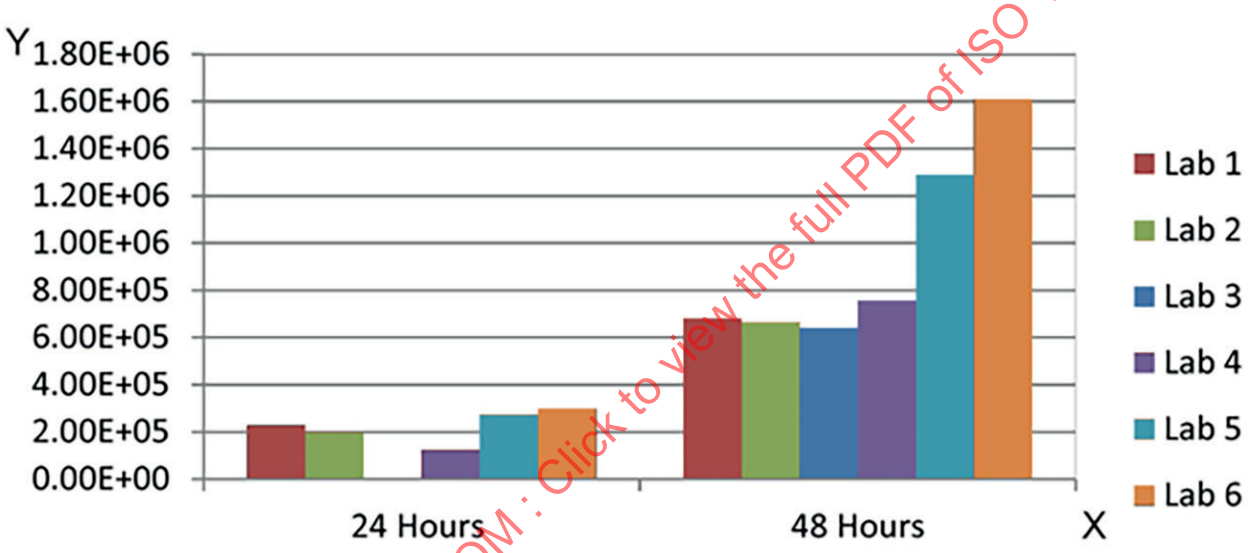
Suspend the cells in culture medium (10 % FBS) without phenol red to a concentration of 2×10^5 cells/mL.

C.3.3.5 Verify cell growth and viability

Verify Raw 264.7 cell growth rate and viability using 8.4 of this document.

C.3.4 Results of the IANH growth rate and cell viability assessment

Two important factors to assess in the health of cells are the growth rate and viability through the time of the experiments. Six groups in the IANH performed these experiments and determined that the cells multiplied and doubled within 24 h and continued to multiply through 48 h as is shown in Figure C.1. Furthermore, most groups found the cells to have viability greater than 90 % through the 48 h experiments is shown in Figure C.2.



Key

X growth time

Y cell count

Figure C.1 — Counts of RAW 264.7 cells for six different laboratories at 24 h and 48 h

Lab 3 did not measure growth rate at 24 h.