

MTS assay in A549 cells

Detection of cell viability/activity

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1 Introduction

The viability of a cell culture system serves as a measure of acute cytotoxicity. To assess the number of viable cells in culture several methods are available. Here we describe the usage of a tetrazolium compound which is soluble in water and cell culture medium: MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-cyboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt).

2 Principle of the Method

The CellTiter 96[®] AQ_{ueous} One Solution (later on simply called MTS) contains the MTS reagent itself and an electron coupling reagent (phenazine ethosulfate; PES) in a stable solution. MTS is added directly to the cells. PES is membrane permeable, enters the cell and is reduced by mitochondrial enzymes (dehydrogenases involving NADPH or NADH), active only in viable cells. The reduced PES is then able to transform the MTS reagent to its formazan product. The resulting color is quantified by an absorbance measurement at 490 nm.

3 Applicability and Limitations

The assay has been used to assess proliferation as well as cytotoxicity. In general the colorimetric readout correlates to the number of viable cells in a cell culture system. Whether an increase in OD is due to an increase in cell number or an increase in enzymatic activity cannot be distinguished with this assay alone.

NM-related consideration: The large (most often reactive) surface area of NMs may be able to process the MTS molecule to its formazan product without cellular contribution. Furthermore the mere presence of NMs might influence the OD measurement. These issues are addressed in the related SOP “*NM interference in the MTS assay*”. Both cell free controls cannot be calculated against values from cellular measurements. They serve as qualitative estimations of NM only reactions that do not involve cellular contribution.

4 Related Documents

Table 1: Documents needed to proceed according to this SOP and additional NM-related interference control protocols.

| Document ID | Document Title |
|------------------------------|--|
| V_MTS_interference | <i>NM interference in the MTS assay</i> |
| cell culture_A549 | <i>Culturing A549 cells</i> |
| M_NM suspension_metal oxides | <i>Suspending and diluting Nanomaterials – Metal oxides and NM purchased as monodisperse suspensions</i> |
| M_NM suspension_carbon based | <i>Suspending and diluting Nanomaterials – Carbon based nanomaterials</i> |

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5 Equipment and Reagents

5.1 Equipment

- Absorbance reader for multi-well plates (to measure optical density (OD) at a wavelength of $\lambda=490$ nm)
- Centrifuge (for cell pelleting; able to run 15 ml as well as 50 ml tubes at 200 x g)
- Conical tubes (15 ml and 50 ml; polypropylene or polystyrene; e.g. from Falcon)
- Flat bottom 96-well cell culture plates
- Hemocytometer
- Laminar flow cabinet (biological hazard standard)
- Light microscope (for cell counting and cell observation)
- Microreaction tubes (1.5 ml; e.g. from Eppendorf)
- Multichannel pipette (with at least 8 positions; volume range per pipetting step at least from 50 μ l to 200 μ l)
- Vortex®

5.2 Reagents

For cell culturing and differentiation:

- Fetal Calf Serum (FCS)
- L-glutamine
- Neomycin¹⁾
- Penicillin¹⁾
- Phosphate buffered saline (PBS)
- Roswell Park Memorial Institute medium (RPMI-1640)
- Streptomycin¹⁾
- Trypsin-EDTA (0.05%)

¹⁾ bought as a 100x concentrated mixture of Penicillin, Streptomycin and Neomycin (PSN) e.g. from Gibco.

Additionally necessary to dilute carbon based NM:

- 10x concentrated RPMI-1640
- Sodium bicarbonate solution, 7.5% (NaHCO₃) [CAS-number: 144-55-8]

Buffers, solvents and detection dye itself:

- CellTiter96® AQ_{ueous} One Solution [Promega; Cat. No. G3580-G3582]
- Cadmium sulfate 8/3-hydrate (3 CdSO₄·8H₂O) [CAS number: 7790-84-3]
Note: Toxic! Handle with special care!
- RPMI-1640 WITHOUT phenol red
- Pluronic F-127 [CAS number: 9003-11-6]

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5.3 Reagent Preparation

5.3.1 Complete cell culture medium

Basic medium:

- RPMI-1640

supplemented with:

- 10% FCS
- 1x PSN, which results in final concentrations of:
 - 50 µg/ml Penicillin
 - 50 µg/ml Streptomycin
 - 100 µg/ml Neomycin
- 2 mM L-glutamine

5.3.2 Pluronic F-127

Stock:

- 160 ppm in ddH₂O: 160 µg/ml (=16 mg/100 ml)

5.3.3 Cadmium sulfate

Prepare a 1 M stock solution in ddH₂O. Can be stored at 4°C for several months.

- Dissolve 2.57 g CdSO₄·8/3 H₂O in 10 ml ddH₂O.

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6 Procedure

6.1 General remarks

This SOP includes an optimized plate setup that allows assessing several sources of variability as well as the toxicity of the chemical positive control (CdSO_4) on one 96-well plate. Only the number of NM concentrations is limited.

6.2 Flow chart

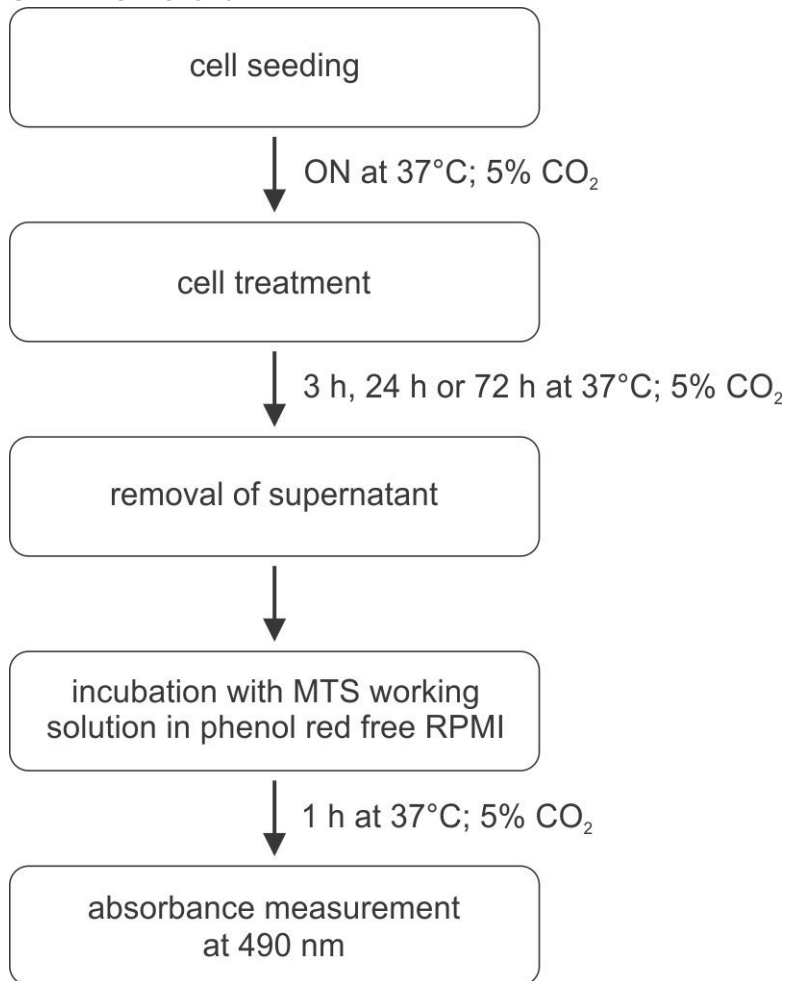


Figure 1: Brief outline of the workflow.

6.3 Cell seeding

6.3.1 Cell culture

A549 cells are grown in T75 cell culture flasks in a total volume of 20 ml of complete cell culture medium. They are kept at 37°C, 5% CO_2 in humidified air in an incubator (standard growth conditions according to SOP “Culturing A549 cells”).

6.3.2 Cell seeding into 96-well plate

- One days prior to experimental start harvest and count cells as described in SOP “Culturing A549 cells”.

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- Seed 8000 cells in 200 µl complete cell culture medium per well into a 96-well cell culture plate. Stick to the pipetting scheme given in Figure 2.
Note: The cell number of **8000 cells per well** is optimized to suit all three time points chosen (up to 72 h). If different incubation periods are analyzed, cell densities should be adapted accordingly. E.g. for a maximum of **48 h of incubation 10000 cells per well** should be seeded.
- For one 96-well plate (see Figure 2) 4×10^5 cells are suspended in 10 ml complete cell culture medium (4×10^4 cells/ml). To assess three time points of NM incubation (3 h, 24 h and 72 h) prepare three identical plates using 1.2×10^6 cells suspended in 30 ml complete cell culture medium.
- Using a multichannel pipette (6 channels) 200 µl of this cell suspension are distributed into each of the green wells (B3 to G6 and B8 to G10, Figure 2).
Note: It is important that cells in a single column are seeded with a single multichannel pipetting step!

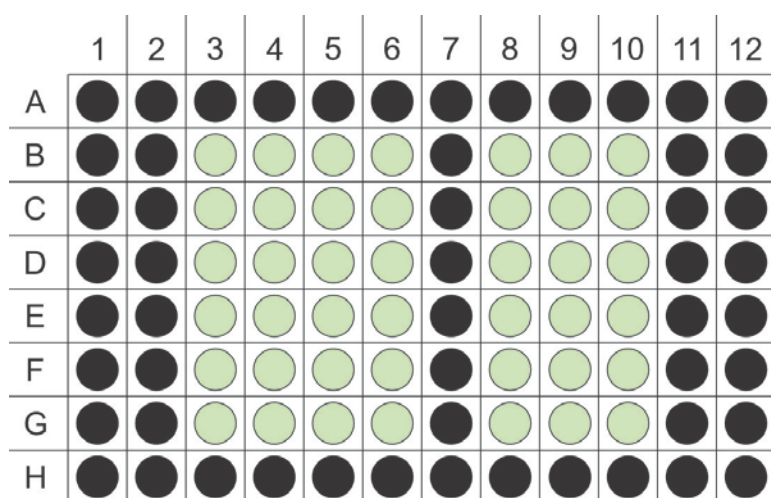


Figure 2: Cell seeding into a 96-well plate.

Cells are seeded at a density of 8000 cells per well in 200 µl complete cell culture medium into each of the green wells. Black wells receive 200 µl complete cell culture medium each.

- Black wells (Figure 2) receive 200 µl complete cell culture medium only.
- Incubate cells overnight (ON) in a humidified incubator at standard growth conditions.

6.4 Dilution of CdSO₄ (chemical positive control)

Prepare serial dilutions of the stock solution (1 M) in ddH₂O. Volumes given are enough for all three time points (3 96-well plates) as described above.

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
 - Add 50 µl of the 1 M stock solution to tube 1.
 - Add 45 µl ddH₂O to tubes 2 to 6.
- 50 µl CdSO₄ stock solution in ddH₂O → 1 M (1)
 - 5 µl of 1 M CdSO₄ stock solution (1) are mixed with 45 µl ddH₂O → 100 mM (2)
 - 5 µl of 100 mM CdSO₄ (2) are mixed with 45 µl ddH₂O → 10 mM (3)
 - 5 µl of 10 mM CdSO₄ (3) are mixed with 45 µl ddH₂O → 1 mM (4)
 - 5 µl of 1 mM CdSO₄ (4) are mixed with 45 µl ddH₂O → 0.1 mM (5)
 - 45 µl ddH₂O → solvent control (6).

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Preparation of final dilutions:

- Label six conical tubes (15 ml total volume) as follows:
 1. 10000 μM CdSO_4
 2. 1000 μM CdSO_4
 3. 100 μM CdSO_4
 4. 10 μM CdSO_4
 5. 1 μM CdSO_4
 6. Solvent control: ddH₂O
- Add 4 ml complete cell culture medium to each tube.
- Add 40 μl of the respective CdSO_4 sub-dilution or the solvent (ddH₂O):
 1. 40 μl of the stock solution (1 M) are mixed with 4 ml medium
→ 10000 μM CdSO_4 (1)
 2. 40 μl of the 100 mM sub-dilution are mixed with 4 ml medium
→ 1000 μM CdSO_4 (2)
 3. 40 μl of the 10 mM sub-dilution are mixed with 4 ml medium → 100 μM CdSO_4 (3)
 4. 40 μl of the 1 mM sub-dilution are mixed with 4 ml medium → 10 μM CdSO_4 (4)
 5. 40 μl of the 0.1 mM sub-dilution are mixed with 4 ml medium → 1 μM CdSO_4 (5)
 6. 40 μl ddH₂O are mixed with 4 ml medium → solvent control (6)

6.5 Dilution of nanomaterials

For this SOP we distinguish two types of nanomaterials (NM) according to their solvent, suspension properties and highest concentrations used in the assay. See also respective related documents (3).

- (1) Metal oxide NM, Polystyrene beads and all NM delivered as monodisperse suspensions by the supplier: solvent either determined by the supplier or ddH₂O; sub-diluted in ddH₂O; highest concentration in assay 100 $\mu\text{g}/\text{ml}$
- (2) Carbon based NM: suspended and sub-diluted in 160 ppm Pluronic F-127; highest concentration in assay 80 $\mu\text{g}/\text{ml}$

Volumes given in the following dilution schemes are enough for all three 96-well plates.

Note: "Mixing" in the context of diluting NMs means, the solvent containing tube is put on a continuously shaking Vortex® and the previous sub-dilution (or stock suspension, respectively) is put dropwise into the shaking solvent. The resulting suspension stays on the Vortex® for additional 3 seconds before proceeding with the next sub-dilution.

(1) Metal oxide NM:

Prepare serial sub-dilutions of the stock suspension (1 mg/ml) in ddH₂O:

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
- Add 1 ml of the 1 mg/ml stock suspension to tube 1.
- Add 500 μl ddH₂O to tubes no. 2, 3, 5 and 6.
- Add 600 μl ddH₂O to tube 4.

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1. 1 ml NM stock suspension in ddH₂O → 1 mg/ml (1)
2. 500 µl of 1 mg/ml stock suspension are mixed with 500 µl of ddH₂O → 500 µg/ml (2)
3. 500 µl of 500 µg/ml (2) are mixed with 500 µl ddH₂O → 250 µg/ml (3)
4. 400 µl of 250 µg/ml (3) are mixed with 600 µl ddH₂O → 100 µg/ml (4)
5. 500 µl of 100 µg/ml (4) are mixed with 500 µl ddH₂O → 50 µg/ml (5)
6. 500 µl ddH₂O → solvent control (6)

Preparation of final dilutions:

- Label six conical tubes (15 ml total volume) as follows:
 1. 100 µg/ml
 2. 50 µg/ml
 3. 25 µg/ml
 4. 10 µg/ml
 5. 5 µg/ml
 6. Solvent control: ddH₂O
- Add 3.6 ml complete cell culture medium to each tube.
- Mix on the Vortex with 400 µl of the respective NM sub-dilution or solvent (ddH₂O):
 1. 400 µl of the stock suspension (1 mg/ml) are mixed with 3.6 ml medium → 100 µg/ml (1)
 2. 400 µl of 500 µg/ml sub-dilution are mixed with 3.6 ml medium → 50 µg/ml (2)
 3. 400 µl of 250 µg/ml sub-dilution are mixed with 3.6 ml medium → 25 µg/ml (3)
 4. 400 µl of 100 µg/ml sub-dilution are mixed with 3.6 ml medium → 10 µg/ml (4)
 5. 400 µl of 50 µg/ml sub-dilution are mixed with 3.6 ml medium → 5 µg/ml (5)
 6. 400 µl ddH₂O are mixed with 3.6 ml medium → solvent control (6)

(2) Carbon based NM:

Prepare serial sub-dilutions of the stock suspension (500 µg/ml) in 160 ppm Pluronic F-127:

- Label six microreaction tubes (1.5 ml total volume) with 1 to 6 (relates to steps 1-6 below).
 - Add 2 ml of the stock suspension to tube 1.
 - Add 800 µl 160 ppm Pluronic F-127 to tubes 2 to 6.
1. 2 ml NM stock suspension in Pluronic F-127 → 500 µg/ml (1)
 2. 800 µl of 500 µg/ml stock suspension (1) are mixed with 800 µl Pluronic F-127 → 250 µg/ml (2)
 3. 800 µl of 250 µg/ml (2) are mixed with 800 µl Pluronic F-127 → 125 µg/ml (3)
 4. 800 µl of 125 µg/ml (3) are mixed with 800 µl Pluronic F-127 → 62.5 µg/ml (4)
 5. 800 µl of 62.5 µg/ml (4) are mixed with 800 µl Pluronic F-127 → 31.3 µg/ml (5)
 6. 800 µl 160 ppm Pluronic F-127 → solvent control (6)

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Preparation of final dilutions:

- Prepare the appropriate dilution of a 10x concentrated medium stock as follows. This mixture (A) is used in all following steps for the preparation of the final NM concentrations. Mixing NM sub-dilutions with (A) will result in 1x concentrated medium containing the correct concentrations of all supplements and the respective NM concentrations.

| Reagent | Volume |
|-------------------------|--------|
| 10x RPMI | 3 ml |
| 100x PSN | 300 µl |
| 100x L-Glutamine | 300 µl |
| 7.5% NaHCO ₃ | 0.8 ml |
| 100% FCS | 3 ml |
| ddH ₂ O | 18 ml |

- Label six conical tubes (15 ml total volume) as follows:
 1. 80 µg/ml
 2. 40 µg/ml
 3. 20 µg/ml
 4. 10 µg/ml
 5. 5 µg/ml
 6. Pluronic F-127: Solvent control
- Add 3.36 ml (A) to each tube. Then mix on the Vortex® with 640 µl of the respective NM sub-dilutions or the solvent (160 ppm Pluronic F-127):
 1. 640 µl of the stock suspension (500 µg/ml) are mixed with 3.36 ml medium (A)
→ 80 µg/ml (1)
 2. 640 µl of the 250 µg/ml sub-dilution are mixed with 3.36 ml medium (A)
→ 40 µg/ml (2)
 3. 640 µl of the 125 µg/ml sub-dilution are mixed with 3.36 ml medium (A)
→ 20 µg/ml (3)
 4. 640 µl of the 62.5 µg/ml sub-dilution are mixed with 3.36 ml medium (A)
→ 10 µg/ml (4)
 5. 640 µl of the 31.3 µg/ml sub-dilution are mixed with 3.36 ml medium (A)
→ 5 µg/ml (5)
 6. 640 µl of 160 ppm Pluronic F-127 (solvent) are mixed with 3.36 ml medium (A)
→ solvent control (8)

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6.6 Application of stimuli

Make sure to have final dilutions of NMs as well as CdSO₄ in complete cell culture medium ready. Apply stimuli to all three 96-well plates. All three time points are stimulated at the same time but harvested separately.

Note: All NM dilutions have to be vortexed directly before application to the cells.

- Remove medium from wells B2 to G11 (illustrated in Figure 3).
- Add 200 µl of the respective CdSO₄ dilutions to wells B2 to G5.
- Add 200 µl of the respective NM dilutions to wells B8 to G11.
- Black wells in Figure 3 (B6 to G7) receive 200 µl complete cell culture medium each.
- Incubate plates in a humidified incubator at standard growth conditions for 3 h, 24 h, and 72 h, respectively.

| CdSO ₄ (µM) | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | NM (µg/ml) ¹⁾ | |
|---------------------------|---|---------------------------|---|---|-------------------------|---|---|--------------|----------|--------------|-------------------------|----|----|-----------------------------|--|
| | A | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | |
| 0 | B | ● | ◐ | ◐ | ◐ | ◐ | ● | ● | ○ | ○ | ○ | ○ | ● | 0 | |
| 1 | C | ● | ◑ | ◑ | ◑ | ◑ | ● | ● | ◒ | ◒ | ◒ | ◒ | ● | 5 | |
| 10 | D | ● | ◒ | ◒ | ◒ | ◒ | ● | ● | ◓ | ◓ | ◓ | ◓ | ● | 10 | |
| 100 | E | ● | ◓ | ◓ | ◓ | ◓ | ● | ● | ◔ | ◔ | ◔ | ◔ | ● | 25 | |
| 1000 | F | ● | ◔ | ◔ | ◔ | ◔ | ● | ● | ◕ | ◕ | ◕ | ◕ | ● | 50 | |
| 10000 | G | ● | ◕ | ◕ | ◕ | ◕ | ● | ● | ◖ | ◖ | ◖ | ◖ | ● | 100 | |
| | H | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | ● | | |
| | | No cells | | | triplicate measurements | | | No treatment | No cells | No treatment | triplicate measurements | | | No cells | |
| | | chemical positive control | | | | | | NM | | | | | | | |

Figure 3: Application of stimuli.

Remove medium from wells B2 to G11 carefully and apply stimuli according to the scheme illustrated here. Different red colors indicate increasing CdSO₄ concentrations. Different green colors indicate increasing NM concentrations. 0 always resembles the solvent control treatment (which is different for NM and CdSO₄ therefore once depicted in white once as stripped wells).

¹⁾ NM concentrations given here refer to metal oxide NM. Carbon based NM concentrations are detailed in the text.

6.7 MTS application and activity measurement

After appropriate time points (3 h, 24 h and 72 h, respectively) the cellular activity is measured using MTS. Volumes given in the following are enough for one 96-well plate as the MTS working solution has to be prepared freshly before usage.

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Note: MTS is diluted in RPMI-1640 without phenol red and without any other additives (such as FCS or antibiotics).

- 2.5 ml MTS are mixed with 12.5 ml **phenol red free** RPMI-1640 (referred to as **MTS working solution**).
- Remove medium from all wells of the 96-well plate.
- Add 120 µl of the MTS working solution to each well using a multichannel pipet.
- Incubate the 96-well plate for 60 minutes under standard growth conditions in a humidified incubator.
- Measure the absorbance at 490 nm in a plate reader.

6.8 Data evaluation

Calculate the mean of the three technical replicates of each concentration (NM as well as CdSO₄ treatment). Subtract the corresponding blank value (“no cells” in Figure 3). These blank corrected mean absorbance values can be used to calculate an effective concentration 50 (EC₅₀) value (this is not part of this SOP) or plotted in a bar chart to compare treated and untreated samples directly. Furthermore, the effect can be expressed in percent of the untreated/solvent treated control.

7 Quality Control, Quality Assurance, Acceptance Criteria

8 Health and Safety Warnings, Cautions and Waste Treatment

Cell seeding has to be carried out under sterile conditions in a laminar flow cabinet (biological hazard standard). For this only sterile equipment must be used and operators should wear laboratory coat and gloves (according to laboratory internal standards). Special care has to be taken during PMA handling (carcinogenic potential of the substance!).

Discard all materials used to handle cells (including remaining cells themselves) according to the appropriate procedure for special biological waste (i.e. by autoclaving).

Handle all the following chemicals with special care. **Wear suitable protective clothing** (gloves, lab coat, respiratory protection, eye protection):

- CdSO₄ is **irritant**.
It is very hazardous in case of ingestion and slightly hazardous in case of skin contact, of eye contact and of inhalation. Severe overexposure can result in death. Therefore avoid ingestion, inhalation as well as contact with skin and eyes and avoid exposure in general.

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9 Abbreviations

| | |
|--------------------|--|
| ddH ₂ O | double-distilled water |
| EDTA | Ethylenediaminetetraacetic acid |
| FCS | fetal calf serum |
| g | constant of gravitation |
| MTS | 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| NM | nanomaterial |
| OD | optical density |
| ON | overnight |
| PBS | phosphate buffered saline |
| PES | Phenazine ethosulfate |
| ppm | parts per million |
| PSN | Penicillin, Streptomycin, Neomycin |
| RPMI | Roswell Park Memorial Institute medium |

10 References

11 Annex A:

Controls on the 96-well plate – additional information that can be drawn from them

| “name” | wells on the plate (Figure 3) | description |
|-----------------------------|--|--|
| “edge effects” | outermost wells (A1-12; H1-12; A1-H1; A12-H12) | Outermost wells are not included in the analysis, no cells are seeded there. However, they are filled with cell culture medium. Outermost wells are the ones most prone to drying up during longer incubation times. Cell growth (from one well to the other) varies most in these outermost wells. Using only the inner 60 wells avoids these issues. |
| “no treatment” | B6-G6 | Cells are seeded into these six wells with one single pipetting step of the multichannel pipet. Well to well variations indicate an error of the multichannel pipet, not distributing the same volume from each tip. |
| “untreated/solvent control” | B2-B5 and B8 to B11 | Cells in these wells receive the solvent of the chemical control or the NM, respectively. Variations here might indicate another error of the multichannel pipet: not distributing the same volume with each pipetting step. |

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| “no cells – no treatment” | B7-G7 | These six wells are not treated at all but receive also the MTS working solution in the last step. The values measured here: a) should all yield the same value b) resemble the blank value of empty wells. Variations here might indicate well to well variations of the plate plastic itself or problems with the MTS reagent. |
| “no cells, CdSO ₄ ” | B2-G2 | Background corrections for the chemical control treatment. If CdSO ₄ would interfere with MTS itself (e.g. enhancing its reduction) absorbance values would change in correlation with the CdSO ₄ concentration. |
| “no cells, NM” | B11-G11 | Background corrections for the NM treatment. NM can either interfere with MTS itself (e.g. enhancing its reduction) or stick to the cell culture plastic. Both facts result would result in an increase of absorbance that can be detected here. |

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