Cellular viability - WST-1 assay

Protocol for adherent cells

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1. Scope

This Standard Operating Procedure (SOP) describes the analysis of the cellular toxicity of nanomaterials by Water Soluble Tetrazolium (WST-1) assay as part of Work Package 3 of the nanOxiMet project.

2. Basics

The cell proliferation reagent WST-1 is designed to be used for the non-radioactive, spectrophotometric quantification of cell proliferation, growth, viability, and chemosensitivity in cell populations using the 96-well-plate format. The assay is based on the cleavage of tetrazolium salts to formazan by cellular enzymes. An expansion in the number of viable cells results in an increase in the overall activity of mitochondrial dehydrogenases, and the formation of formazan is directly proportional to the number of metabolic active cells in the culture. The aim of this SOP is to assess nanoparticles' cytotoxicity using the WST-1 assay. In order to eliminate possible interferences from nanoparticles with the absorbance readings and/or with the WST-1 substrate, the standard procedure from the reagent provider has been adapted by Vietti et al. 2013 (doi:10.1186/1743-8977-10-52) and modified here.

3. Materials and Instrumentes

3.1. Materials

- Sterile pipette tips
- 96-well microplates, flat bottom, sterile (Costar #3599)
- Culture media with and without phenol red
- WST-1 Stock Solution (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-teterazolium, Roche Diagnostics, #11 644 807 001) - stored in 1mL aliquots at -20°C in the dark
- Triton X-100, CAS 9002-93-1 (Euromedex, cat. n. 2000-B)
- Sterile PBS
- Cell line in culture
- 15 ml plastic tubes
- Multichannel reagent reservoir

3.2. Instruments

- Microscope
- Hemacytometer
- Multichannel pipettor (10, 100, 200 µl)
- Incubator (37°C, 5% CO_2, humidified atmosphere)
- Centrifuge
- Scanning multiwell spectrophotometer with filters for 450 nm (at least between 420 to 480 nm) and a reference filter for 630nm (at least above 600).
- Biological safety cabinet, class 2
- Vortex
- Water bath 37°C

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4. Experimental procedure

4.1. Cell culture

Adapt the density of cells according to the treatment setup. For most experimental setups, a cell concentration between 0.1 and 5 x 104/well and an incubation time of 24 to 96 hours is appropriate. Cell treatments should be done at 70% confluence.

- Culture cells in 96-well plate in 200 µl of media (EXCEPT for wells A1 and B1, which must be left empty – blank controls) at 37°C, 5% CO_2 for 24 or 48 hours.
- NCI-H292 cells should be seeded at 20000 cells/cm^2 (32000 cells/ml) if treated 48 hours later or at 40000 cells/cm^2 (64000 cells/ml) if treated 24 hours later.
- THP-1 cells are seeded at 225000/cm^2 after induction of differentiation with PMA

4.2. Treatment

Prepare treatment solutions in serum-free media without phenol red. Samples are run in 6 replicates, avoid all marginal wells because cells grow differently. In order to account for NP interference with absorbance reading of WST-1, ***the same treatment is done in two plates that run in parallel***. Cells are treated with 100 µl of solution.

As the first dilution of nanoparticles results in dilution of the media (12.8% water), this has to be compensated by using media with 12.8% of HPLC grade water for the other dilutions and controls.

- Negative control (12.8% water media):
  19.2 ml HPLC grade water + 130.8 ml serum-free RPMI media without phenol red.
- Nanoparticle dilutions: 0.3 to 40 µg/cm^2 (1 to 128 µg/ml).
  Nanoparticle stock is 1 g/l.

For 2 96-well plates:

NP dilution	Volume of NP solution	Serum-free media (µl)	12.8% water media (µl)
40 µg/cm^2 (128 µg/ml)	768 µl of NP 1 g/l	5232	---
20 µg/cm^2 (64 µg/ml)	3 ml of NP 40 µg/cm^2	---	3000
10 µg/cm^2 (32 µg/ml)	3 ml of NP 20 µg/cm^2	---	3000
5 µg/cm^2 (16 µg/ml)	3 ml of NP 10 µg/cm^2	---	3000

For 4 96-well plates:

NP dilution	Volume of NP solution	Serum-free media (µl)	12.8% water media (µl)
40 µg/cm^2 (128 µg/ml)	1024 µl of NP 1 g/l	6976	---
20 µg/cm^2 (64 µg/ml)	4 ml of NP 40 µg/cm^2	---	4000
10 µg/cm^2 (32 µg/ml)	4 ml of NP 20 µg/cm^2	---	4000
5 µg/cm^2 (16 µg/ml)	4 ml of NP 10 µg/cm^2	---	4000

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If starting at a lower concentration:

For 2 96-well plates:

NP dilution	Volume of NP solution	Serum-free media (µl)	12.8% water media (µl)
5 µg/cm^2 (16 µg/ml)	96 µl of NP 1 g/l	5232	672
2.5 µg/cm^2 (8 µg/ml)	3 ml of NP 5 µg/cm^2	---	3000
1.25 µg/cm^2 (4 µg/ml)	3 ml of NP 2.5 µg/cm^2	---	3000
0.6 µg/cm^2 (2 µg/ml)	3 ml of NP 1.25 µg/cm^2	---	3000
0.3 µg/cm^2 (1 µg/ml)	3 ml of NP 0.6 µg/cm^2	---	3000

Positive control (H_2O_2): 30% H_2O_2 w/w stock is 9.88 M.

Make intermediary dilutions of H_2O_2 in cell media to bring it to 10 mM.

1 M = 10.1 µl 9.88 M H_2O_2 + 89.9 µl 12.8% water media
10 mM = 10 µl 1 M H_2O_2 + 990 µl 12.8% water media

- NCI-H292 (85 µM H_2O_2):

For 2 96-well plates: 25.5 µl of 10 mM H_2O_2 + 2974.5 µl of 12.8% water media.
For 4 96-well plates: 51 µl of 10 mM H_2O_2 + 5949 µl of 12.8% water media.

- THP-1 D (500 µM H_2O_2):

For 2 96-well plates: 150 µl of 10 mM H_2O_2 + 2850 µl of 12.8% water media.
For 4 96-well plates: 300 µl of 10 mM H_2O_2 + 5700 µl of 12.8% water media.

- Rinse wells with 100 µl/well with serum-free media without phenol red.
- Incubate cells with solution to be tested in 100 µl/well in serum-free media without phenol red, at 37°C, 5% CO_2 for 4 or 24 hours.

4.3. Incubation with WST-1

- Rinse wells with 100 µl/well with serum-free media without phenol red.
- In one set of plates add 100 µl/well of 0.2% Triton X-100 in serum-free media without phenol red (including blank controls).
- In the second set of plates 100 µl/well serum-free media without phenol red is added (including blank controls).
- Plates are incubated for 15 minutes at 37°C, 5% CO_2.
- Add 50 µl of WST-1 solution diluted 1:15 in serum-free media without phenol red to all wells and incubate at 37°C, 5% CO_2.

The appropriate incubation time after the addition of WST-1 depends on the individual experimental setup (e.g. cell type, cell density). Therefore, it is recommended to measure the absorption repeatedly at different points in time after addition of WST-1 (e.g. 0.5, 1, 2, 3, 4 hours) in a preliminary experiment. This will allow the determination of the optimal incubation period for the particular experimental setup to be used.

- NCI-H292 cells incubation time is 3.5– 4h.
- THP-1 macrophages incubation time is 30–45 min.

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4.4. Plate reading

- Measure absorbance of the samples against blank background controls using a microplate (ELISA) reader at 420–480 nm. The reference wavelength should be greater than 600 nm.

4.5. Data analysis

- The average absorbance of blanks (WST-1 without cells) is subtracted from sample absorbances and absorbances are corrected by their respective reference ((sample A_{450} - average of blanks A_{450})-(sample A630 - average of blanks A_{630})).
- Corrected absorbance of dead cells is subtracted from live cells corrected absorbance ((sample corrected $Abs_{No\ Triton}$)-(sample corrected $Abs_{With\ Triton}$)).
- Results are expressed relative to control.
- Three independent experiences using 6 replicates should be reported.

4.6. Test acceptance criteria

Absorbance at 430 nm - absorbance at 630nm of:

- controls (cells incubated in culture media without phenol red) should be between 0.5 and 2 OD and std deviation should be <0.3 as a check for homogeneous cell seeding.
- positive controls (cells exposed to H_2O_2) should be lower than control.

5. Safety precautions

Follow the safety information and regulations of the working laboratory and of materials providers. Biosafety level 1 precautions should be followed when handling cells.

6. Waste disposal

Follow the disposal advice from materials providers, if available. Any material containing cells should be discarded as biohazardous waste.