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Cellular viability - WST-1 assay in NR8383 macrophages

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1. Scope

This Standard Operating Procedure (SOP) describes the analysis of the cellular toxicity of nanomaterials via WST-1 assay as part of Work Package 5 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).

3. Materials and Instruments

3.1. Materials

- Sterile pipette tips
- 96-well microplates, flat bottom, sterile (BD Falcon #353072)
- Culture media with and without phenol red (see Table 1)
- 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
- Triton X-100, CAS 9002-93-1 (Sigma #T8787)
- Hydrogen peroxide (H_2O_2) 30% (Roth #8070.2)
- Sterile PBS (Sigma #D8537)
- Cell line in culture
- Polystyrene Conical Centrifuge Tube 15 ml (BD Falcon #352095)
- Polystyrene Conical Centrifuge Tube 50 ml (BD Falcon #352070)
- Disperser 5 ml (Band PD-Tips #702390) und 0.5 ml (Brand PD-Tips #702384)
- Rotisolv HPLC gradient water (Roth #A511.2)

3.2. Instruments

- Microscope
- Hemacytometer
- Multistep pipette
- Single channel pipette
- 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
- Vortexer
- Water bath 37°C

4. Experimental procedure

4.1. Cell culture

- Seed cells in an appropriate cell density in a 96-well plate (Table 1)

Table 1: Cultivation conditions for NR8383 cells

Cell line	Cell density	Cultivation time	Culture medium	Assay medium	WST-1 Incubation
NR8383	40.000 cells/well	48 h	Ham's F-12K (+ phenol red) +15% FCS +1% HEPES +1% GlutaMAX	DMEM F-12 (wo phenol red) +1% HEPES	2h

- Culture cells in 96-well plate in 200 µl medium per well
- Run samples as six replicates
- Avoid all marginal wells (Figure 1, green panels) as cells grow differently
- Use the outer wells of the plate as blank controls (Figure 1, row A and row H)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0 µg/cm^2	1 µg/cm^2	2 µg/cm^2	5 µg/cm^2	10 µg/cm^2	20 µg/cm^2	40 µg/cm^2	80 µg/cm^2	1% Triton-X	85 µM H2O2	Blank
B	Blank	0 µg/cm^2	1 µg/cm^2	2 µg/cm^2	5 µg/cm^2	10 µg/cm^2	20 µg/cm^2	40 µg/cm^2	80 µg/cm^2	1% Triton-X	85 µM H2O2	Blank
C	Blank	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	Blank
D	Blank	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	Blank
E	Blank	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	Blank
F	Blank	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	Blank
G	Blank	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	Blank
H	Blank	0 µg/cm^2	1 µg/cm^2	2 µg/cm^2	5 µg/cm^2	10 µg/cm^2	20 µg/cm^2	40 µg/cm^2	80 µg/cm^2	1% Triton-X	85 µM H2O2	Blank

w/o cells + 0,5% Triton-X

Figure 1. Plate design for a WST-1 assay in the course of nanOxiMet

4.2. Preparations

- Weight 20 mg (+/- 0.5 mg) particles in a 50 ml Falcon Tube
- Disperse the particles in 20 ml HPLC gradient water → **Stock solution: 1 mg/ml**
- Sonicate particles with the Cuphorn and with following settings:
    - Duty cycle: 20%
    - Time: 10 min
    - Power: 5.71 (~ 200 W)

4.3. Treatment

- Prepare nanoparticles test suspensions or positive controls (Table 2)
- As the dispersion of nanoparticles results in a dilution of the media by water, this water amount has to be compensated by adding HPLC gradient water to lower concentrated nanoparticle dispersions and controls

NP dilution	in µg/ml	Volume of NP solution (µl)	Cell culture media (µl)	HPLC gradient water (µl)	Final volume (µl)
80 µg/cm²	256 µg/ml	512	1488	0	2000
40 µg/cm²	128 µg/ml	256	1488	256	2000
20 µg/cm²	64 µg/ml	128	1488	384	2000
10 µg/cm²	32 µg/ml	64	1488	448	2000
5 µg/cm²	16 µg/ml	32	1488	480	2000
2 µg/cm²	6.4 µg/ml	12.8	1488	499.2	2000
1 µg/cm²	3.2 µg/ml	6.4	1488	505.6	2000
0 µg/cm²	0 µg/ml	0	1488	512	2000

	Concentration	Volume of 10% stock solution	Cell culture media (µl)	HPLC grade water (µl)	Final volume (µl)
Triton-X	1%	200	1488	312	2000

	Concentration	Volume of 1 M H_2O_2 stock solution*	Cell culture media (µl)	HPLC grade water (µl)	Final volume (µl)
H_2O_2	85 mM	170	1318	512	2000

*Preparation of a **1 M H_2O_2 solution:** 101 µl (H_2O_2 30%) + 899 µl medium

Table 2. Pipette scheme for different nanoparticle suspensions and positive controls.

- Rinse wells with 100 µl/well with serum-free medium without phenol red
- **NR8383 cells have to be centrifuged before that rinsing step (1200 rpm, 10 min)**
- Incubate cells with test materials in 100 µl/well in serum-free media without phenol red at 37°C, 5% CO_2 for 24 hours

4.4. Incubation with WST-1

- Expose two of the six replicates to 0.5% Triton-X-100 in serum-free medium without phenol red (Figure 1, red frames)
- Incubate plates for 15 minutes at 37°C and 5% CO_2
- Add 10 µl of WST-1 solution/well
- Incubate cells at 37°C and 5% CO_2 for 2 hours

4.5. Plate reading

- Shake plate for one minute and measure absorbance of the samples using a measure wavelength at 450 nm and a reference wavelength at 630 nm

4.6. Data analysis

- The average absorbance of the blanks (WST-1 without cells) is subtracted from sample absorbance at 450 nm and absorbance are corrected by their respective reference
- Corrected absorbance of dead cells is subtracted from live cells corrected absorbance

((Sample A_{450nm} - blanks A_{450nm})-(sample A_{630nm} - blanks A_{630nm})) without Triton-X-100

MINUS

((Sample A_{450nm} - blanks A_{450nm})-(sample A_{630nm} - blanks A_{630nm})) with Triton-X-100

- Results are expressed relative to medium control
- Three independent experiences using 6 replicates should be reported

4.7. Test acceptance criteria

Absorbance at 450 nm - absorbance at 630nm of:

- $A_{450\ nm}$- $A_{630\ nm}$ of medium controls (cells incubated in culture media without phenol red) should be between 0.5 and 2, standard deviation should be <0.3.
- Positive controls (cells exposed to H_2O_2 or Triton-X-100) should be lower than the controls

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 bio hazardous waste.