PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

PATROLS Standard Operating Procedures (SOP)

Guidance Document for the *in situ* detection of ENM elemental distribution within different compartments of 2D epithelial tissue *in vitro* model

SOP_PATROLS_ This is a SOP recommended for external use by PATROLS

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	18.04.2021	Initial Document	Barbara Rothen-Rutishauser
1.1		Additional detail added from AMI	Ilaria Zanoni, Anna Costa
1.2		Final comments implemented	
2.0		Version distributed to WP1 members	
2.1		All comments from WP1 members integrated and uploaded to server	

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1 Introduction:

DOMAIN: Biodistribution of nanomaterials at the human epithelial lung tissue after single and repeated exposures

The potential hazards of ENMs are not only determined by the physico-chemical properties of the particle per se but also on the interactions of the particles with immediate surrounding environments. Once inside cells, ENMs distribution in different compartments also provides indications as to their potential biological impact, as well as how to specifically design ENMs for effective cell targeting and drug delivery. There is a need to apply mechanistic based testing strategies enabling to understand interactions of ENMs at a cellular level. It is accepted that in vitro results can be useful for ranking ENMs either by mechanistic studies enabling a deeper insight into mechanisms of ENM-induced (potentially even nano-specific) effects or serving as a basis for follow-up in vivo studies.

Cultivation of epithelial cells, e.g., A549, should be followed the SOP for the cell line (i.e., SOP_PATROLS_3101). For the preparation of ENM dispersions the NANoREG dispersion protocol should be followed.

1.1 *Scope and limits of the protocol*

This SOP was established with the intention to be used to assess the cellular uptake and translocation, of nanomaterials (ENMs) across an A549 lung epithelial cell monolayer by measuring elemental distribution in different cell compartments, e.g. apical, apical wash, intracellular and basal, using inductively coupled plasma optical emission spectrometry (ICP-OES). This protocol can also be used for any other epithelial tissue.

Limitations: (i) The A549 cells can be kept as stable monolayer only under submerged conditions, therefore the submerged conditions is recommended for the long-term repeated exposures (over 3 days). (ii) ENMs have to be suspendable in liquid, to be able to provide the stable ENM suspension (to achieve this, refer to NANoREG dispersion protocol).

2 Terms and Definitions:

Nanomaterial

Material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of nano-object and nanostructured material. [SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Engineered nanomaterial (ENM)

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

3 Abbreviations:

ENM – Engineered Nanomaterials

FBS – Foetal Bovine serum

PET - Polyethylene terephthalate

RPMI - Roswell Park Memorial Institute-1640 Medium

ICP-OES - Inductively Coupled Plasma Emission Spectroscopy

4 Principle of the Method:

This method presents the experimental approach to expose ENM to A549 cell monocultures, grown on permeable transwell membrane inserts in 6 well wells. and track ENM distribution through different cell compartments.

5 Description of the Method:

5.1 *Biological setting & test system used:*

This SOP has to be carried out under laboratory-based conditions, with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

The human adenocarcinomic alveolar basal epithelial type II cell line A549 was grown as described in the SOP_PATROLS_3101. Herein, A549 cells were cultured at a density of 2.5 × 10^5 cells per insert on BD Falcon cell culture inserts (high pore density PET membranes, 3.0 µm pore size) for 5 days prior to exposure.

5.2 *Reporting of protected elements:*

To the best of our knowledge, this SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described.

5.3 *Health and safety precautions:*

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

5.4 *Nanomaterials used / handling procedures:*

ENM samples were prepared starting from stocks at 2.56 mg mL-1 in MilliQ water + BSA, prepared accordingly to the NANoREG dispersion protocol. The stocks were then diluted in RPMI +10 % FBS medium reaching the desired concentration in the concentration range of 12.5 – 100 µg/mL.

5.5 *Procedure:*

5.5.1 A549 cell exposures to ENM, single and repeated exposure scenario

Cultivation of the epithelial cells (A549) was done accordingly to the SOP_PATROLS_3101. After 5 days under submerged condition, single short-term exposure of ENMs was done with a concentration of 100 µg/mL and post-incubation for 24h and 80h. In the long-term repeated exposures on A549 cells, the cells were exposed repeatedly every day up to 5 days, using 25 µg/mL ENMs concentration, resulting in a total exposure of 100 µg/mL and 5 days incubation time in total.

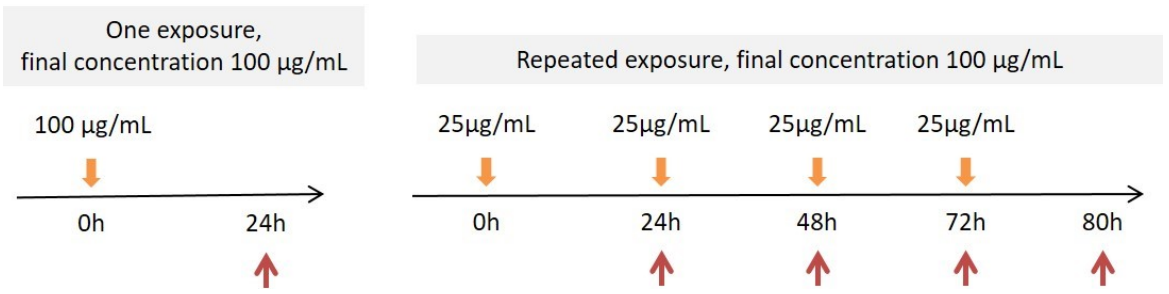

Figure 1: Exposure scenarios and sample collection indicated by red arrow.

Apical, apical cell Wash and Basal samples were collected after 24 h, 48 h, 72 h and 80 h of exposure, while the Cells fraction was collected at the end of the experiment at 80 h. To analyse the cellular uptake of the material, whole membranes with the cells attached were cut out.

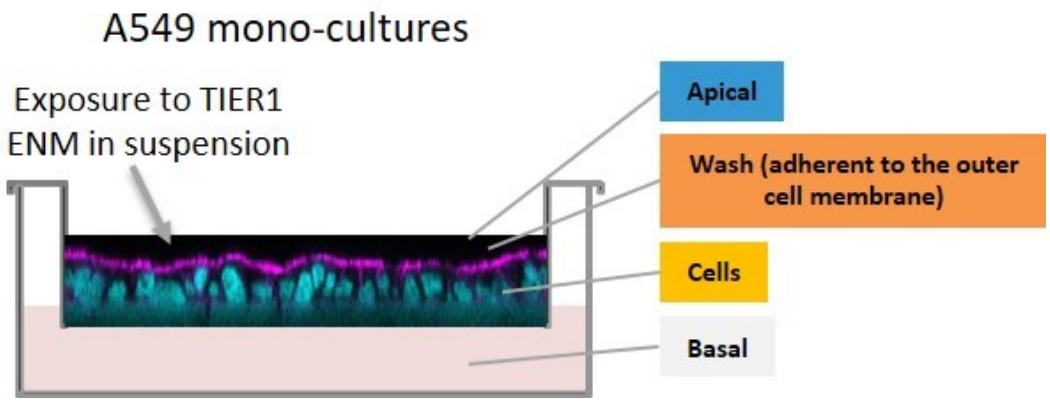

Figure 2: Schematic indicating the sample collection after ENM exposure to A549 cell cultures.

5.5.2 Inductively Coupled Plasma optical emission spectrometry (ICP-OES)

Acid digestion was performed to obtain the real concentration of ENMs in medium by inductively coupled plasma optical emission spectrometry using an ICP-OES 5100 – vertical dual view apparatus coupled with OneNeb nebulizer (Agilent Technologies, Santa Clara, CA, USA). Digestive procedure was performed adding 0.2 mL of hydrogen peroxide (H_2O_2 30wt% in water), 0.2 mL of sulfuric acid (H_2SO_4 96%), 0.2 mL of phosphoric acid (H_3PO_4 85%) and 0.2 mL of nitric acid (HNO_3 65%) in to 0.5 mL sample and adding 1.5 mL of MilliQ water. The treated samples were ultrasonicated for 10 min in an ultrasonic bath. Calibration curves were obtained with

0.01, 0.05, 0.1, 1.0, 10.0 and 50.0 µg/mL standards prepared in RPMI, using the same digestive procedure applied to samples. The low detection limit was 0.01 µg/mL.

5.6 Quality control & acceptance criteria:

Visual inspection of the cell culture should ensure there are no contaminations. ENM dispersions should be tested regularly for endotoxin contaminations.

Data Analysis and Reporting of Data:

Not applicable for this current SOP.

6 Publications:

Lehner et al. *In preparation.*

7 References:

Not applicable for this current SOP.