PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for a quasi-air-liquid interface exposure of nanoparticles to cells grown at an air-liquid interface

This is a a) SOP used by members of PATROLS only or (b) 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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Reviewed by:
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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	04/07/2019	Initial Document	Kirsty Meldrum

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1 Introduction:

DOMAIN: Exposure of various nanomaterials at an air-liquid interface (ALI) for engineered nanomaterial hazard assessment

Due to the constant increase in their production, exposure to engineered nanomaterials (ENM) poses an inevitable health risk to both humans and the environment through long-term, repetitive, low-dose exposures. The majority of the literature however, focuses on short-term, high-dose exposures. Hazard assessment of ENM, when applying alternative testing strategies to *in vivo* research, has previously engaged 2D test systems. Such standard model systems have their limitations, and it is widely accepted that they do not adequately represent the biological matrix *in vivo.* Advanced, 3D models in this sense have received heightened attention and pose a potential valid alternative to invasive *in vivo* approaches.

As a step in this process, exposures to these ENM can be implimeted through the use of a quasi-ALI exposure regieme.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for exposure of ENM to cells grown at an ALI. This method allows ENM exposures over both an acute and chronic, repeated dose regime.

Limitations:

A specific volume of ENM needs to be added to the apical surface of the culture, and it is not possible to determine which cell has been exposed to a certain concentration of ENM. This leads to the possibility that some cells within the culture are exposed to a higher concentration of ENM complared to others. An argument could be made that this mimics physiological exposure situations and is therefore acceptable.

1.2 Validation state of protocol

Level of advancement towards standardization	Level reached (please mark only one with "X")
Stage 1: Internal laboratory method under development	
Stage 2: Validated internal laboratory method	x
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N
If yes, specify	[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Agglomerate

Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate

Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanoscale

Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanotechnology

Application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material.

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.3]

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]

Nano-object

Discrete piece of material with one, two or three external dimensions in the *nanoscale*.

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.5]

Nanostructure

Composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* region.

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.6]

Nanostructured material

Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

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

Manufactured nanomaterial

Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Incidental nanomaterial

Nanomaterial generated as an unintentional by-product of a process.

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See “ultrafine particle” in ISO/TR 27628:2007, 2.21

Particle

Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

Substance

Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

3 Abbreviations:

ALI – Air-liquid interface

ENM – engineered nanomaterials

4 Principle of the Method:

This method aims to standardise the method for exposing cell cultures grown at an ALI to ENM using a quasi-ALI method (Endes *et al*., 2014).

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and at a minimum of Biological Safety Level 1 conditions.

Various cell lines can be used as required, however, they must be at an ALI before exposure.

5.2 Chemicals and reagents used:

ENM of interet

5.3 Apparatus and equipment used:

All tissue culture equipment was sourced from Greiner Bi-One, UK unless stated otherwise.

- Laminar Class II Tissue Culture Hood
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- Water Bath (37˚C)
- Pipette Controller
- 5 mL, 10 mL and 25 mL sterile pipettes

- P1000 and P200 micropipettes
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips
- 50 mL Centrifuge Tubes
- 50 mL Skirted Falcon Tubes
- Light Microscope

5.4 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.

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

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.

An alternative to trypan blue staining (as this is a teratogen) includes the use of Erythrosin B. It can be diluted to 0.1% in PBS and used in the same way as trypan blue solution.

5.1 Nanomaterials used / handling procedures:

It is important to ensure that all nanomaterial handled are done so in an appropriate maner. Standard health and safety precautions associated with working with nanomaterials as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/legislation/guidelines/guidance-protection-health-and-safety-workers-potential-risks-related), should be adopted when conducting this SOP.

5.2 Reagent preparation:

5.2.1 CCM

Medium appropriate to your specific cell line should be made and warmed to 37°C in a water bath before use.

5.2.2 Nanpmaterial suspension

The nanomaterial of interest should be weighed out, suspended and sonicated as outlined in the NANOGENOTOX dispersion protocol (http://safenano.re.kr/download.do?SEQ=175).

5.3 Procedure:

5.3.1 Quasi-ALI exposure

1. Warm medium required reagents to 37°C in a water bath (~20-30 min).
2. Determine the range of doses required for the specific exposure protocol before continuing.

From here on, all steps need to be completed under aseptic conditions.

3. Before adding the exposures, wash the apical side of the membrane with 1ml of CCM and remove the washing to waste. Ensure the culture has no liquid on the apical surface before adding the samples.

4. Using the warmed CCM, the correct dillutions should be make of the nanomaterials. It is suggested to make a minimum of 1ml of the exposure concentrations. Pipette the stock nanomaterial solution up and down gently before diluting in the CCM. After adding to CCM, pipette up and down again in order to ensure complete mixing. Use immediately.
5. For a 6 well transwell insert with a surface area of 4.2cm^2, 100µl of the sample is added to the apical surface of the culture. For smaller sized inserts, the volume/cm^2 should be calculated to ensure comparisons can be made.
6. Return cultures back to the incubator at 37°C and 5% CO_2 for the length of exposure time required.

5.4 Quality control & acceptance criteria:

Visual inspection of the culture should ensure there are no contaminations.

Nanomaterials, once suspended, should be diluted within 30mins of sonication and then used immediately.

6 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

7 Publications:

Not applicable for this current SOP.

8 References

Endes, C.; Schmid, O.; Kinnear, C.; Mueller, S.; Camarero-Espinosa, S.; Vanhecke, D.; Foster, E.J.; Petri-Fink, A.; Rothen-Rutishauser, B.; Weder, C.; et al. An in vitro testing strategy towards mimicking the inhalation of high aspect ratio nanoparticles. *Part. Fibre Toxicol.* **2014**, *11*, 40.

Jacobsen NR, Pojano G, Wallin H and Jensen KA (2010). Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA report. March 2010. National Research Centre for the Working Environment. 8 pp.

http://safenano.re.kr/download.do?SEQ=175