PATROLS
Advanced Tools for NanoSafety Testing

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Annex 3109

PATROLS Standard Operating Procedures (SOP)

Guidance Document for cell exposure at the air-liquid interface using VITROCELL® automated exposure station

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	19/08/2019	Initial Document	Hedwig Braakhuis
1.1		Additional details added by RIVM	
1.2		Final comments implemented	
2.0		Version distributed to WP3 members	
2.1		All comments from WP3 members integrated and uploaded to server	

Table of Contents

1 Introduction:

DOMAIN: Human Toxicology

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 exposures under submerged conditions. Such approaches poorly mimic realistic human exposures, and possess several limitations, such as issue with measuring a delivered dose, and interaction with exposure medium. Therefore, air-liquid interface (ALI) exposure chambers allowing for deposition of ENM aerosols onto cell surface were developed. VITROCELL® automated exposure station equipped with a Quartz crystal microbalance (QCM) for measuring online deposition of ENMs, is referred in this SOP.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for developing an exposure protocol for a lung cell culture model which can provide a physiologically relevant assessment of the hazards associated with ENM exposures over both an acute and chronic, repeated dose regime. This SOP provides only instructions on how to handle the cell model; for handling the VITROCELL® automated exposure station in general please refer to the instructions provided by VITROCELL®.

Limitations

ENM aerosols can be generated via different methods, including nebulizing an ENM suspension and aerosol generation from dry powder using for example a Vilnius Aerosol Generator (VAG). For nebulization, ENMs have to be suspendable in liquid, to be able to provide the stable ENM suspension (to achieve this, refer to NanoReg protocol: 'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media'). There are no models available yet that are able to predict the deposited dose on the cells, therefore, first preliminary experiments are needed to fine-tune the aerosolization and concentration.

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	x
Stage 2: Validated internal laboratory method	
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

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

CCM- cell culture medium

ENM – engineered nanomaterials

QCM – quartz crystal microbalance

TEM – transmission electron microscopy

TEER- Transepithelial electrical resistance

4 Principle of the Method:

This method aims to standardise the exposure of cells to ENM aerosols using VITROCELL® automated exposure station.

This protocol will be broken into key stages:

1. Preparing the device
2. Cell handling
3. Cleaning the device

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under controlled laboratory conditions, with all work following safe handling of ENMs.

- VITROCELL® automated exposure station (VITROCELL®, Germany) equipped with
  - QCM
  - Option to enhance deposition by applying a current (max – or + 1000 V)
  - Different sizes of cell culture inserts can be used, their use depends on the available trumpets in the system. This protocol refers to the use of 6-well and 12-well inserts.
- For further information refer to: https://www.vitrocell.com/inhalation-toxicology/exposure-systems/automated-exposure-station

5.2 Chemicals and reagents used:

- PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Switzerland)
- Ultrapure water
- 70% Ethanol
- CCM specific for cells used (please refer to culturing protocol of specific cell line)
- Isotonic sterile 0.9% NaCl solution

5.3 Apparatus and equipment used:

- P1000, P200, P2.5 micropipettes
- Sterile 10 µL, 200 µL and 1000 µL pipette tips
- Sterile 50 mL centrifuge tubes
- Sonicator
- Water bath (37°C)
- VITROCELL® automated exposure station

- Nebulizers (different for different materials/ different mesh sizes available, choose based on the particle size)
- Cell cultures growing on hanging cell culture inserts (for example Falcon® Cat. No. 353181, Falcon, Corning brand, USA, or Transwell®, Cat. No. CLS3462-48EA, Corning, USA)
- TEM grids
- Ultrasonic Bath Sonifier (no need to be specific, for example Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)
- Evom2 Voltohmmeter (World Precision Instruments Inc., USA) for TEER measurement

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:

Standard health and safety precautions associated with working within a laboratory environment and handling ENMS, 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.

5.6 Applicability:

The SOP has been demonstrated for Dörntruper quartz 12 (DQ12) and is applicable to all ENM that can be stably generated over time and of which the aerosol characteristics can be sufficiently monitored.

5.7 Reagent preparation:

Please, follow specific protocols for ENM suspension preparation

Tier 1 ENMs are usually suspended in ultrapure water, or following NanoReg protocol: ‘Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media’.

5.8 Procedure:

5.8.1 Preparing the VITROCELL® automated exposure station

Figure 1 shows the VITROCELL® automated exposure station with its all parts.

1. Turn on the VITROCELL® automated exposure station and wait until the temperature reaches 37°C and the humidity reaches 85% (~ ? min).
2. Warm the cell culture media to 37°C in a water bath (~20 – 30 min).

3. Prepare aerosol generation.

Figure 1: The VITROCELL® automated exposure station.

5.8.2 Cell handling

1. Culture cells as described in corresponding SOP, i.e. SOP_PATROLS_A549, SOP_PATROLS_hAELVI, SOP_PATROLS_Calu-3, etc. on permeable cell culture inserts and let them grow for several days (based on the corresponding SOP) to become confluent and differentiate into an epithelial barrier. For a typical ALI exposure using the VITROCELL® automated exposure station, 15-20 inserts with a confluent cell layer are needed. These consist of 3 clean air controls, 3 incubator controls that will be handled similar to the other inserts without exposure in the VITROCELL® system, 6 to 8 inserts for aerosol exposure (depending on the use of 0, 1 or 2 microbalances), 1 to 3 inserts for measurement of the maximum LDH release, and 3 spare inserts in case the TEER of some of the inserts is not sufficient.
2. Remove apical medium and grow cells at the ALI according to the description in the SOP.
3. At the day of exposure, wash cells once with culture medium, check cell morphology and optionally measure the TEER of the cell model using an EVOM2 device.
4. Put 1.5 mL/1 mL of HEPES buffered CCM to the basolateral side of 6-wells/12-wells plates and transfer the culture inserts with cells to the plates. NOTE During

exposure, no CO2 is present in the VITROCELL® system. Therefore, HEPES buffered culture medium (25mM HEPES) is used during transport and exposure. This medium is used for both the exposed cells in the VITROCELL® system as the incubator control cells.

5. Put the cells in a portable incubator of 37 °C during transport.
6. At the VITROCELL® system, fill the exposure modules with HEPES buffered CCM. The amount of CCM depends of the used unit, and type of cell culture insert. Keep in mind, that medium should reach bottom of the membrane, but should not leak on the top of the membrane to keep cells at the ALI. When using 6-wells inserts, add 6.0 mL of HEPES buffered CCM to the exposure modules.
7. Transfer the inserts with cells from the plates to the exposure modules using tweezers. Check that no air bubbles are below the cell surface and remove any CCM on the apical side of the inserts. Keep the plates containing CCM in an incubator for after exposure.
8. Close the exposure modules and close the door of the VITROCELL® system.
9. Use the touch-screen display to choose exposure duration, air-flow rate and electrostatic deposition enhancement. The display can also be used to check humidity and temperature. Usually, an exposure duration of 4 hours is chosen, with a flow-rate of 50 ml/min at 37 °C and 85% humidity. The modules in the first layer (Figure 1) are used for clean air exposure, inserts in this layer are used as clean air exposure controls. The other modules in the second and third layer can be used for aerosol exposure, including 2 modules for microbalances to measure online deposition.
10. The leak test should be conducted before starting exposure, the leakage needs to be smaller than 5 ml/min. When the leak test finished, the exposure can be started.
11. At the end of exposure, open the VITROCELL® system, open the exposure modules and place the cell culture inserts back to the cell culture plates and transfer to the portable incubator. Collect the media from the modules (exposed samples) and from the plates (incubator controls) for later analysis.
12. Back at the cell culture lab. Fill culture plates with standard CCM according to the SOP of the cells and transfer the cell culture inserts to the plates. Put in the incubator until next exposure or until analysis.
13. Proceed to cleaning.

5.8.3 Cleaning

1. Aspirate the cell culture media from the VITROCELL® exposure modules, and thoroughly rinse with ultrapure water. Rinse the inlets with ultrapure water.
2. Fill the exposure modules with 70% ethanol and rinse the inlets using 70% ethanol. Let it dry.

5.8.4 Testing for nanomaterial interference:

N/A

5.9 Quality control & acceptance criteria:

The deposited dose is continuously monitored using QCM and can be checked regularly using TEM and/or ICP-MS (i.e., every repetition of the experiment, or every week during long-term repeated exposures).

6 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

7 Publications:

Not applicable for this current SOP.

8 References