PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for cell culture of lung epithelial cell-line (A549)

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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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	13/09/2018	Initial Document	Kirsty Meldrum
1.1	01/10/2018	Additional detail added from AMI	Hana Barosova
1.2	17/12/2018	Final comments implemented	Hana Barosova
2.0	18/12/2018	Version distributed to WP3 members	Kirsty Meldrum
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2.3	11/06/2019	Updated and uploaded to server	Kirsty Meldrum
2.3.1	13/06/2019	Addition of identifier code	Kirsty Meldrum

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1 Introduction:

DOMAIN: Advanced 3D *in Vitro* Model Systems for ENM 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 first step in this process, cells must first be cultured in submerged conditions, before being switched to more physiologically relevant conditions and adding complexities to the model.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for developing advanced 3D lung cultures *in vitro* which can provide a physiologically relevant assessment of the hazards associated with ENM exposures over both an acute and chronic, repeated dose regime. This is the beginnings of the lung model with adaptations that can then be added to this base model.

Limitations:

The cells cultured at an air-liquid interface will only remain stable for ENM exposures until day 4 after being switched to an air-liquid interface (ALI). They are available for ENM exposures 24 hours after being switched to ALI.

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	
Stage 3: Interlaboratory tested method	x
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

ATCC – American Tissue and Cell Collection

RPMI - Roswell Park Memorial Institute-1640 Medium

FBS - Fetal bovine serum

DMSO - Dimethyl sulfoxide

CCM – Cell Culture Medium

PBS – Phosphate Buffered Saline

4 Principle of the Method:

This method aims to standardise the method for growing A549 cells in both culture flasks and transwell inserts. As well as the cryopreservation of the cells.

This protocol will be broken into key stages:

1. Culturing cryopreserved cells
2. Sub-culturing cells
3. Freezing cells
4. Culturing cells an at air-liquid interface

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.

Cell line utilised is a Human Caucasian lung Carcinoma derived epithelial cell line, A549 (ATCC CCL-185).

- For further information:

- https://www.lgcstandards-atcc.org/products/all/CCL-185.aspx?geo_country=gb

5.2 Chemicals and reagents used:

0.05% Trypsin-EDTA (5300-054, GIBCO®, Paisley, UK) (an alternative to this could be Accutase (00-4555-56, Thermo Fisher Scientific)

FBS (10270-106, GIBCO®, Paisley, UK)

RPMI 1640 (11879020, GIBCO®, Paisley, UK)

PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Paisley, UK),

Pen/Strep (15140-122, Penicillin/Streptomycin 100X or 10,000U/mL, GIBCO®, Paisley, UK).

Trypan Blue Solution (CAS# 72-57-1) (T8154-100 mL, Sigma Aldrich®, UK)

Virkon (Rely+OnTM Virkon®, DuPont, UK)

5.3 Apparatus and equipment used:

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

- Liquid Nitrogen
- Cell Freezing Aid
- Laminar Class II Tissue Culture Hood
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- Water Bath (37˚C)
- Autoclave Tape
- Centrifuge
- T-25 (690175) and T-75 (660175) Tissue Culture Flask
- Cell culture insert (353091, Corning)

- Cell culture companion plate (353502, Corning)
- 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
- Haemocytometer
- 0.2 mL and 1.0 mL Eppendorf 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.

5.6 Reagent preparation:

5.6.1 CCM

RPMI supplemented with

- 1% Penicillin-Streptomycin (corresponds to 100 Units/mL Penicillin and 100 µg/mL Streptomycin)
- 1% L-glutamine (corresponds to 2 mM L-Glutamine)
- 10% FBS (heat inactivated, please follow the ATCC protocol for heat inactivation (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx, page 19))

Culture medium and supplements do not require pre-heating before supplementation but should be stored at between 4-6°C after preparation. Can be kept for 3 months at this temperature.

Antibiotics are added to the medium to reduce bacterial contamination.

In a 500 mL medium bottle add:

- 5.5 mL Penicillin-Streptomycin
- 5.5 mL L-glutamine
- 50 mL FBS

Order of addition does not need to be specific, but medium needs to be mixed after each addition.

5.6.2 Freezing Medium

Cell culture medium (as prepared in section 5.7.1) with

- 5% (v/v) DMSO

For example – 1 confluent T75 flask will require around 10 mL of freezing medium to be prepared (500 µL of DMSO in 9500 µL of CCM).

- DMSO should not be added to the CCM in advance but should be added once the final concentration of cells has been achieved and freezing is imminent. *i.e.* resuspend 10^7 cells in 9500 µL of CCM, pipette up and down several times to mix properly and then slowly add 500 µL of DMSO. Mix properly (again by pipetting up and down), aliquot and place immediately to the cell freezing aid.

Freezing medium is freshly prepared before each use and must be used immediately and not stored.

5.7 Procedure:

5.7.1 Thawing Cells

1. Warm all required reagents to 37°C in a water bath (~20-30 min).
2. Prepare a T75 flask (size of the flask and amount of the medium should be adjusted based on cell number in cryo vial) with 15 mL of CCM (*i.e.* 0.2 mL/cm^2) under aseptic conditions and place in the incubator (5% CO_2, 37°C) for a minimum of 15 min to allow the pH to settle in preparation for new cells. A specific pH level is not required, instead the equilibration period is to ensure there is not a significant change in pH after the initial seeding that might lead to cell shock. Smaller or larger flasks can be used after the 1st passage.
3. Remove one vial of cells from liquid nitrogen storage and gently swirl in a 37°C water bath (to ensure uniform thawing of the cell suspension) ensuring the O-ring and lid is not submerged (to reduce the potential for contamination) until the contents are almost thawed (between 1 and 2 min).
4. Remove the vial from the water bath and decontaminate with 70% ethanol, ensuring again that the decontaminant does not interact with the lid and the O-ring.

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

5. Pipette slowly (drop by drop) the contents of the vial into a centrifuge tube with 9 mL of prewarmed CCM and centrifuge at 125 g for 5 min.
6. Discard the supernatant into waste (this is laboratory specific so follow laboratory specific protocols) and re-suspend (via pipetting up and down) the cell pellet in 1 mL of prewarmed CCM.
7. Transfer the re-suspended cells into the pre-prepared medium in the T75 flask (from step 2) and incubate the culture at 37°C and 5% CO_2.
8. Change the medium every 2-3 days. Medium is poured off into a waste vessel and 15 mL of fresh cell culture medium is pipetted into the flask and the flask replaced back into the incubator.

5.7.2 Sub-Culturing

- A549 cells are provided by ATCC in cryovials frozen previously in liquid nitrogen with the number of passages identified. Please, keep the information about the original cell passage available when preparing aliquots for your experiment.
- A549 cells are maintained in T25, T75 or T150 cell culture flasks, in which the cells are passaged at >80% confluency every 3-4 days (on Monday/Tuesday and Thursday/Friday) with medium renewal every 2-3 days. Medium is poured off into a waste vessel and fresh CCM (T25=5 mL, T75=15 mL and T150=30 mL) is pipetted into the flask and the flask placed back into the incubator
- Cells should be passaged at least 2 times after thawing, before use in experiments or before freezing, and they should be passaged no more than 20 times in total (recovery of cells using trypan takes longer after this passage has been reached and therefore it is suggested to discard cells at this point).

Protocol:

1. Confirm if flask is >80% confluent by checking under a light microscope (see Figure 1.

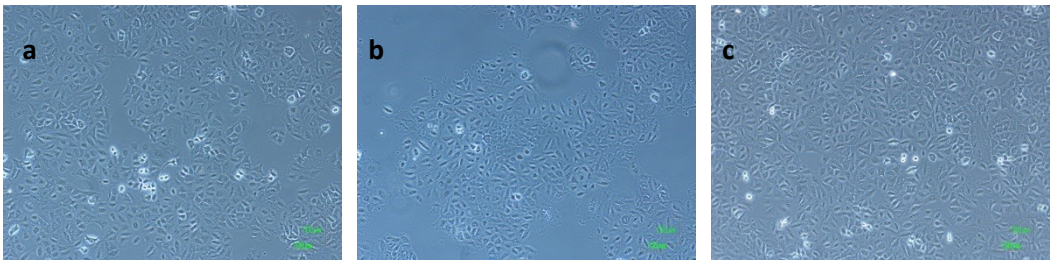

Figure 1: Phase contrast image of A549 cells a) at confluency >80%, b) not confluent, c) fully confluent (overgrown). Scale bar: 100 µm.

The following steps are completed under aseptic conditions

2. Remove and discard culture medium. Medium is poured off into a waste vessel.
3. Wash the cells with 10 mL of 1xPBS three times, discarding the PBS to waste after each wash. PBS removes serum which inhibits Trypsin.
4. Add 2 - 3 mL Trypsin-EDTA and place flask back into the incubator at 37°C and 5% CO_2 for 5-10 min ensuring cells have become detached from the flask surface.
5. Add 6 mL (double the Trypsin-EDTA volume originally added) of CCM to the flask and gently rock the flasks to ensure proper mixing. This is to ensure the trypsin has been neutralised by the FBS in the CCM and its actions on the cells are halted. If trypsin is allowed to remain in contact with the cells for too long they will not re-attach when put into a new cell culture flask.
6. Remove the complete contents of the flask into a 15 mL centrifuge tube.
7. Centrifuge the cells for 5 min at 125 g, ensure that the centrifuge is correctly balanced.
8. Return the vial containing the cells back to aseptic conditions and remove the supernatant gently, without disturbing the pellet. The supernatant can be poured off and the remainder pipetted off ensuring the pellet is not disturbed.
9. Resuspend the cell pellet in 1 mL of CCM by pipetting up and down until all cells are suspended (no pellet or cell agglomerates can be observed).
10. Count the cells (dead and alive) in the 1 mL of CCM using a haemocytometer. To achieve this;

a. Take the haemocytometer out of the box and place the cover slip on using breath vapour.

b. Determine cell viability using 0.4% trypan blue solution. Add one volume of trypan blue to one volume of cell suspension (for example 10 µL of trypan blue mixed with 10 µL of re-suspended cells).

c. Remove 10µL of the sample and disperse it underneath the cover slip.

d. Count the cells in the 4 large squares (Nr. 1-4) (see Figure 2) using a cell counter.

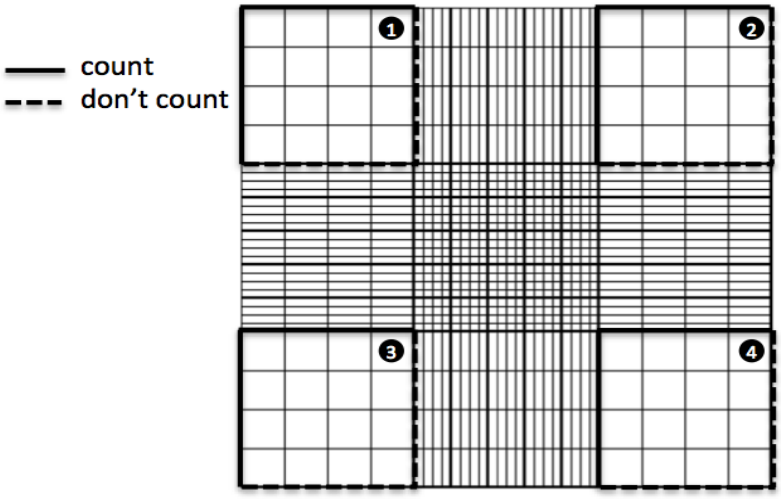

Figure 2 Counting cells using a haemocytometer (Hemocytometer.org, 2018)

11. Using the following calculation, count the number of living cells (not labelled with trypan blue) that are in your sample

$$\text{Total cells/mL} = \text{counted cells} \times \frac{dilution}{\#of\ squares\ counted} \times 10'000$$

The term 'dilution' refers to how many times the stock solution was diluted in trypan blue (in this case 2x) and the '# of squares counted' refers to the number of larger haemocytometer sections on which cells were counted (i.e. the outside 4 big squares). Using the following calculation, count the percentage of viable cells:

$$\frac{Live\ cell\ count\ (white\ cells)}{Total\ cell\ count\ (blue+white\ cells)} = \%\ viable\ cells$$

Using the live cell number calculated above, determine the dilution required to get the correct cell number of between $2x10^3$ and $1x10^4$ cells per cm^2. The seeding density depends on number of flasks required and when the flasks are required. For example a higher seeding density is used if confluent flasks are required sooner.

$C_1V_1=C_2V_2$

Where:

C_1=the concentration you currently have

V_1 = the volume you have of your current concentration

C_2 =the concentration you want

V_2 =the volume you want

12. Suspend the cells into the correct volume required and add cell suspension into each flask (as previously stated section 5.8.2).
13. Gently rock the flask and then place back into the incubator (37°C and 5% CO_2).
14. Replace the medium with fresh prewarmed CCM every 2-3 days and subculture when they reach >80% confluency.

See appendix for worked example.

5.7.3 Freezing Cells

Notes:

- Aliquots of early-passage cells (minimal 2, maximum 4 passages) should be frozen. Expand the cells so that at least 10 vials can be frozen.
- Record the total number of passages (at the time of subculture) and other data when freezing the cells. A log of the historical passage number is also important.

- Store the cryovials in different liquid nitrogen tanks if available to create a back-up of cells in case a liquid nitrogen tank fails.

Protocol:

- Cryopreservation follows the same steps as sub-culturing (section 3.2 until step 12).

1. Count cells to determine current cell density (as outlined in previous section point 10).
2. Dilute cells to $1x10^6$ cells/mL in prewarmed CCM.
3. Add 5% (v/v) DMSO.
4. Pipette 1 mL of the cells (diluted to $1x10^6$ cells/mL) in the freezing medium+cell solution into a cryovial.
5. Place the cryovial into a suitable cryopreservation chamber and leave overnight at -80°C before removing and putting into liquid nitrogen for long-term storage.

5.7.4 Seeding Cells onto Culture Inserts

Notes:

- The following procedure is adjusted for 12-well inserts. It can however be applied to different insert sizes, by recalculating the cell number and amount of medium based on the well insert surface area.
- If using cell culture inserts from other provider, note that the growth area of the insert can also differ.

Protocol:

The following are completed under aseptic conditions

1. Pipette 1.5 mL of pre-warmed CCM to each well of 12-well cell culture plate. As prepared plate place into the incubator at 37°C and 5% CO_2. Prepare cell suspension with known concentration following steps 1 – 11 from sub-culturing protocol (section 5.8.2).

2. Dilute cells to a concentration of $5x10^5$ cells/mL in prewarmed CCM.
3. Take the pre-prepared cell culture plate from incubator and place under aseptic conditions.
4. Carefully uncover the cell culture plate, unpack one by one the cell culture inserts and place them into pre-prepared wells in cell culture plate.
5. By pipetting up and down carefully mix the cell suspension. Pipette 0.5 mL of cell suspension (i.e., 250 000 cells/insert, which is equivalent to 277 777 cells/cm^2) on the top of the membrane in the cell culture insert.
6. Cover the cell culture plate and place back into the incubator at 37°C and 5% CO_2.
7. Change the medium every 3 – 4 days.

5.7.5 Culturing Cells at Air-Liquid Interface

The following steps are completed under aseptic conditions

1. Cells are growing in the cell culture inserts as prepared in “Seeding Cells on Cell Culture Inserts”.
2. Let the cells become confluent for 4 days in case of assembling cell co-cultures, or 5 days for monocultures (in order to keep the same ‘age’ of the cells).
3. Remove and discard CCM. Medium is poured off to waste.
4. Apply 0.6 µL of pre-warmed CCM to the bottom part of the well (under the cell culture insert). The CCM should be touching the membrane from the bottom, but not leaking onto the top of the insert.
5. At this point cells are apically exposed to air, which is referred as ALI.
6. Expose cells to the air for 24 h prior exposures in the incubator at 37°C and 5% CO_2. During this period A549 are able to release surfactant proteins which then cover the aqueous lining layer (Blank et al., 2006).

5.8 Quality control & acceptance criteria:

Not applicable for this current SOP.

6 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

7 Publications:

Not applicable for this current SOP.

8 References

https://www.hemocytometer.org/hemocytometer-protocol/

BLANK, F., ROTHEN-RUTISHAUSER, B. M., SCHURCH, S. & GEHR, P. 2006. An optimized in vitro model of the respiratory tract wall to study particle cell interactions. *J Aerosol Med,* 19**,** 392-405.