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 co-culture of an lung epithelial cell-line (A549), and macrophages derived from a monocytoic cell line (dTHP-1)

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).

Authored by:
Kirsty Meldrum[1], Harriet Risby[1], Shareen H. Doak[1], and Martin J.D. Clift[1]

[1]Swansea University Medical School (SUMS), Swansea University, UK

Reviewed by:
WP3 members

Document History:

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

Table of Contents

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 second step in this process, cells are cultured at an air-liquid interface and as part of a co-culture.

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 a progression from the base models that have also been outlined as SOPs within the PATROLS project.

Limitations:

The cells (A549s) 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	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

ATCC – American Tissue and Cell Collection

RPMI - Roswell Park Memorial Institute-1640 Medium

FBS - Fetal bovine serum

CCM – Cell Culture Medium

PBS – Phosphate Buffered Saline

PMA – phorbol 12-myristate-13-acetate

4 Principle of the Method:

This method aims to standardise the method for co-culturing A549 cells and dTHP-1 cells in transwell inserts at an ALI.

This protocol will be broken into key stages:

1. Culturing A549 and dTHP-1 cells on transwell inserts

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) and a differentiated blood derived monocyte cell line, THP-1 (ATCC TIB-202).

- For further information:
    - https://www.lgcstandards-atcc.org/products/all/CCL-185.aspx?geo_country=gb
    - https://www.lgcstandards-atcc.org/products/all/TIB-202.aspx?geo_country=gb

5.2 Chemicals and reagents used:

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 (10,000U/mL and 10000µg/ml), GIBCO®, Paisley, UK).

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

5.3 Apparatus and equipment used:

- Laminar Class II Tissue Culture Hood
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator
- Water Bath (37˚C)
- 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.

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.

5.7 Procedure:

5.7.1 Growing the co-culture

All steps need to be completed under aseptic conditions.

Studies have been completed to determine the optimum time for the addition of dTHP-1s after they have been exposued to PMA and are in their recovery period.

This protocol is completed on the day 4 of submerged growth of the A549 cells grown in 6 well transwell inserts.

1. A549 cells are grown on transwell inserts as outline in SOP_PATROLS_A549, while THP-1 cells are differentiated as outlined in SOP_PATROLS_THP-1.
2. THP-1 cells are allowed to differentiate in PMA for 48 hours, before being allowed to recover in CCM (as outline in the SOP_PATROLS_THP-1 SOP). 24 hours after the switch to CCM, they are removed from the flask they are in with accutase.
3. Remove and discard CCM. CCMis poured off into a waste vessel.
4. Wash the cells with 10 mL of 1xPBS three times, discarding the PBS to waste after each wash. PBS removes serum which inhibits Trypsin.
5. Add 2.5 mL Accutase (for a T25 cell culture flask) and place flask back into the incubator at 37°C and 5% CO_2 for 5 min ensuring cells have become detached from the flask surface.
6. Add 6 mL (double the Accutase volume originally added) of CCM to the flask and gently rock the flasks to ensure proper mixing. This is to ensure there is a large enough volume to collect all the cells within the flask.
7. Remove the complete contents of the flask into a 15 mL centrifuge tube.

8. Centrifuge the cells for 5 min at 125g, ensure that the centrifuge is correctly balanced.
9. 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.
10. Resuspend the cell pellet in 1 mL of pre-warmed (37°C) CCM by pipetting up and down until all cells are suspended (no pellet or cell agglomerates can be observed).
11. Count and check cell viability using the Trypan Blue Exclusion Assay (see section 5.5 for health and safety precaution and alternative assays); 1:1 ratio of cell suspension to pre-filtered 0.4% trypan blue solution. Prior to cell counting, using a 1mL syringe take 1mL of trypan blue solution and filter with a 0.45µm filter unit into a sterile, 1mL Eppendorf tube.

To achieve this;

a. Take the haemocytometer out of the box and place the cover slip on using breath vapour.
b. 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). Remaining filtered trypan blue solution can be stored at room temperature up to 3 months for future use.
c. Remove 10µL of the sample and disperse it underneath the cover slip.Ensure the solution is dispersed by capillary action underneath the cover slip and covers the entire grid without any bubbles.
d. Count the cells in the 4 large squares (Nr. 1-4) (see Figure 1) using a cell counter.

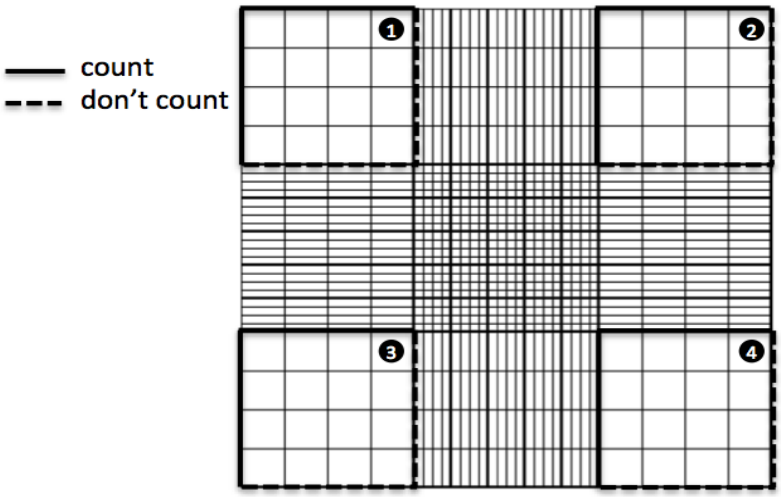

Figure 1 Counting cells using a haemocytometer (Hemocytometer.org, 2018)

12. 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:

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

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

13. 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.
14. Count the cells and allow for $1x10^5$ cells/ml
15. Remove the media from the apical side of the A549 cell culture
16. Apply 500µl of the dTHP-1 cell suspension to the apical side of the transwell and allow 2 hours for the cells to adhere (Rothen-Rutishauser *et al*., 2005).
17. After 2 hours remove all the CCM from the apical side of the membrane and wash the insert by applying 1ml of CCM and removing again to waste.
18. By removing all the apical CCM the cells are apically exposed to air, which is referred as ALI.
1. Allow 24hours for habituation before completing any futher experiments in the incubator at 37°C and 5% CO_2.

5.8 Quality control & acceptance criteria:

Visual inspection of the culture should ensure it is healthy and free from contaminations.

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/

Rothen-Rutishauser BM, Kiama SG, Gehr P. A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol. 2005;32.