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 (Cl-hAELVi)

SOP_PATROLS_hAELVi

This is a SOP recommended for external use by PATROLS

Adapted from the PATROLS SOP, Guidance Document for cell culture of lung epithelial cell-line (A549), Meldrum *et al*.

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	4.6.2019	Initial Document	Hana Barosova
1.1	12.6.2019	Additional detail added from AMI	Hana Barosova
2.0	12.6.2019	Version distributed to WP3 members	Barbara Rothen
2.1	18.7.2019	All commets from WP3 members intergrated and uploaded to server	Hana Barosova

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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 increased attention and pose a potential valid alternative to invasive *in vivo* approaches.

As a first step in this process, cells have to be cultured under submerged conditions before being switched to an air-liquid interface condition, which is physiologically more relevant.

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 assesement of the hazards associated with ENM exposures over both an acute and chronic, repeated dose regime. It presents the trial basic set up for culturing hAELVi cell line, which might be further adapted.

Limitations:

After submerged culture conditions for 7 days, the cells can be cultured at an air-liquid interface (ALI). They need additional 7 days at ALI to develop a tight epithelial barrier and from then, they will remain stable, i.e. keeping a tight monolayer structure, until day 28, afterwards they start to detach. It is recommended to use the cells from day 14 to day 28 for ENM aerosol exposures.

2 Terms and Definitions:

Substance

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

3 Abbreviations:

ALI – Air-liquid interface

CCM – Cell Culture Medium

PBS – Phosphate Buffered Saline

4 Principle of the Method:

This method aims to standardise the method for growing hAELVi 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 in laboratories implementing biosafety level 1.

- Cell line utilised is Cl-hAELVi, an immortalized (transfected via lentiviral vectors) type I cell line from human alveolar epithelium (commercially available by Inscreenex, Germany)
- For further information:
    - https://www.inscreenex.de/products/human-immortalized-cell-lines/alveolar-epithelial-cells-hu.html

- Kuehn A. et al. *Human Alveolar Epithelial Cells Expressing Tight Junctions to Model the Air-Blood Barrier*, ALTEX. 2016; 33(3):251-60

5.2 Chemicals and reagents used:

- 0.05% Trypsin-EDTA (5300-054, GIBCO®, Switzerland) (an alternative to this could be Accutase (00-4555-56, Thermo Fisher Scientific)
- hUAEC medium (INS-ME-1013-500ml, Inscreenex, Germany)
- hUAEC coating solution (INS-SU-1018-20ml or INS-SU-1018-100ml, Inscreenex, Germany)
- PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Switzerland)
- Pen/Strep (15140-122, Penicillin/Streptomycin 10 000 U/mL Penicillin/10 000 µg/mL Streptomycin, GIBCO®, Switzerland)
- Trypan Blue Solution (CAS# 72-57-1) (T8154-100 mL, Sigma Aldrich®, Switzerland), or alternative Erythrosin B (see 5.5 Health and Safety precautions)
- Freezing medium (INS-SU-1004, Inscreenex, Germany)

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 (Mr Frosty, 5100-0001, Thermo Fisher Scientific, Switzerland)
- Laminar Class II Tissue Culture Hood (Scanlaf Mars)
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator (NUAIRE™ DHD Autoflow)
- Water Bath (37˚C)

- Autoclave Tape
- Centrifuge
- T-25 (690175) and T-75 (660175) Tissue Culture Flask
- 12-well cell culture inserts (transparent, PET, 3 µm pore size) either Falcon® (Cat. No. 353181, Falcon, Corning brand, USA) or Transwell® (preferred, Cat. No. CLS3462-48EA, Corning, USA)
- 12-well cell culture plates with flat bottom, not required specific, for example Falcon® Polystyrene Permeable Support Companion Plate, with Lid, sterile (Cat. No. 353503, Falcon, Corning brand, USA)
- Pipette Controller
- 5 mL, 10 mL and 25 mL sterile pipettes
- P1000 and P200 micropipettes
- Sterile 200 µL and 1000 µL Pipette tips with filter
- 50 mL Centrifuge Tubes
- Haemocytometer
- 1.0 mL Eppendorf Tubes
- Light Microscope
- Cryovials (not required specific)

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 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 that can be toxic) 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 Nanomaterials used / handling procedures:

Not applicable for this current SOP.

5.7 Reagent and flask preparation:

5.7.1 Cell culture medium (CCM)

hUAEC medium (500 mL) supplemented with:

- Basal medium supplement (provided with hUAEC medium, Inscreenex, Germany)
- 0.5% Penicillin-Streptomycin (corresponds to 50 Units/mL Penicilin and 50 µg/mL Streptomycin)

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 1 month at this temperature.

Antibiotics are added to the medium to reduce bacterial contamination.

In a 500 mL medium bottle add:

- 2.5 mL Penicillin-Streptomycin
- 30 mL of basal medium supplement (provided with hUAEC medium)

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

5.7.2 Cell culture flasks, inserts or plate coating

The surface of cell culture flasks, inserts or plates have to be coated prior cell cultivation using coating solution (INS-SU-1018-100ml, Inscreenex, Germany).

Procedure:

- Cover the cell culture dish with the coating solution (see table 1 below for the required volume).
- Incubate the cell culture dish for at least 120 min (up to overnight) at 37°C in the incubator.
- Aspirate coating solution.
- Wash the coated cell culture dish once with PBS.
- Coated ware can be used immediately after aspiration, or stored at 4°C for up to 1 week.

Table 1: Recommended volumes of coating solution

Plastic ware / Insert	Area in cm^2	Volume of coating solution (mL)
T75	75	2.5
T25	25	1.4
6 well	9	0.7
12 well	4	0.25
24 well	2	0.1
96 well	0.32	0.05

5.8 Procedures

5.8.1 Thawing cells - this procedure is recommended by provider (Inscreenex, Germany)

- Pipette 4 mL CCM in a 15 mL plastic tube.
- Quickly thaw vial in preheated water bath (@37°C).
- Transfer thawed cell suspension to 15 mL plastic tube containing 4 mL medium.
- Spin-down cells at 200 g for 5 min.

- Aspirate supernatant.
- Resuspend cell pellet in CCM (7 mL is recommended for T25 flask).
- Transfer cells in desired cultivation device (recommended is T25 flask)

5.8.2 Sub-Culturing

1. Confirm if flask is >80% confluent using a light microscope (see Figure 1)

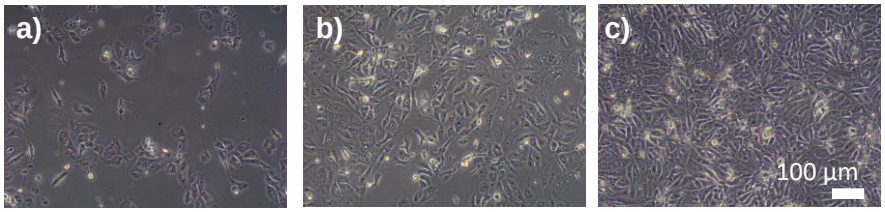

Figure 1: Phase contrast image of hAELVi cells a) not confluent, b) at confluency >80%, c) fully confluent (overgrown).

The following steps are completed under aseptic conditions

2. Remove and discard CCM medium. Medium is poured off into a waste vessel.
3. Wash the cells with 10 mL of 1x PBS three times, discarding the PBS to waste after each wash. PBS removes serum which inhibits Trypsin activity.
4. Add 0.5 - 2 mL Trypsin-EDTA and place flask back into the incubator at 37°C and 5% CO_2 for app. 5 min, ensuring cells have become detached from the flask surface.
5. Add 4 mL (triple 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 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 200 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.

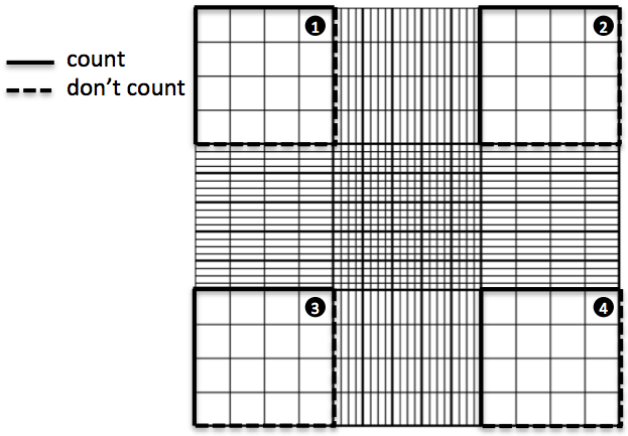

Figure 2: Counting cells using a haemocytometer (Hemocytometer.org, 2018).

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:

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

Using the live cell number calculated above, determine the dilution required to get dilution between 1:4 – 1:6. 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. Do not exceed 1:6 dilution.

12. Suspend the cells into the correct volume required and add cell suspension into each flask.
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.

5.8.3 Freezing Cells – this procedure is recommended by provider (Inscreenex, Germany)

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

- Grow the cells to 90 % confluence in the desired cell culture flask (i.e. T25, T75 or T150).
- Wash the cells with PBS.
- Trypsinize the cells with Trypsin-EDTA.
- Resuspend the cells with 5 mL of PBS, containing 2% FBS.
- Transfer cell suspension in 15 mL plastic tube.
- Spin-down cells at 200g for 5 min.
- Aspirate supernatant.
- Resuspend cell pellet with freezing medium (INS-SU-1004, Inscreenex, Germany; cell concentration $1x10^6$ cells per mL).
- Transfer cell suspension in cryovial.
- Place vials into Mr. Frosty or comparable devices (to slowly cool down vial).
- Place Mr. Frosty in -70°C overnight.
- After 24 h transfer vial from -70°C to liquid nitrogen tank for long term storage.
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5.8.4 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 $6x10^5$ 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 the pre-prepared cell culture plate.
5. By pipetting up and down carefully mix the cell suspension. Pipette 0.5 mL of cell suspension (i.e., 300'000 cells/insert, which is equivalent to 333'333 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 CCM every 2 – 3 days, by pouring off CCM from apical part, and slow pipetting of fresh CCM on the walls of inserts, and subsequently lifting the insert, sucking off the old CCM and adding fresh CCM.

5.8.5 Culturing Cells at Air-Liquid Interface (ALI)

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 7 days.
3. Remove and discard CCM. Medium is poured off to waste.
4. Apply 0.65 mL 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 another 7 days prior exposures in the incubator at 37°C and 5% CO_2. During this period hAELVi will develop tight junctions.
7. Change the CCM every 2 – 3 days.

6 Quality control & acceptance criteria:

Cells should be regularly checked under the light microscope, if they keep the morphology as presented at Figure 1, and cell viability should not drop below 70 %.

7 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

8 Publications:

Not applicable for this current SOP.

9 References

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

Kuehn A. et al. *Human Alveolar Epithelial Cells Expressing Tight Junctions to Model the Air-Blood Barrier*, ALTEX. 2016; 33(3):251-60

10 Appendix

Example of calculation of cell dilution for splitting into new flasks:

- Cells have been resuspended in 1 mL
- 10 µL of cell suspension has been added to 10 µL of trypan blue.

White cell count = 714

Blue cell count = 96

Total cell count =(714+96)= 810

Viable cells:

$$\frac{Live\ cell\ count\ (white\ cells)}{Total\ cell\ count\ (blue+white\ cells)} \text{ x100 = \% viable cells}$$

$$\frac{714}{810} \text{ x100 = \% viable cells}$$

$$\text{=88\% viable cells}$$

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

$$= 714 \text{ x} \frac{2}{4} \text{ x 10'000}$$

$$= 3570000 \text{ cells/mL}$$

$$= 3.57x10^6 \text{ cells/mL}$$

Seeding density required = $2x10^3$ cells per cm^2

Into a T75 flask = $1.5x10^5$ cells (i.e. $1x10^4$ cells/mL if 15 mL applied)

15mL is required for a T75 (5mL per $25cm^2$)

$$C_1V_1=C_2V_2$$

$$3.57x10^6xV_1=1x10^4x15000 \text{ µL}$$

$$V_1=\frac{1x10\text{^}4x15x10\text{^}3}{3.57x10\text{^}6}$$

$$V_1=\frac{150x10\text{^}6}{3.57x10\text{^}6}$$

V_1= 42 µL

42 µL of the suspension into 15mL of new cell medium.

23 new tissue culture flasks can be seeded from this suspension (42µl x 23 = ~1 mL)