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 (Calu-3)

This is a SOP recommended for external use by PATROLS

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	24/04/2019	Initial Document	Hedwig Braakhuis
2.0	03/06/2019	Internal review round RIVM	Eric Gremmer, Rob Vandebriel, Ruiwen He, Hedwig Braakhuis

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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 limitations 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 SOP describes the base model, future adaptations such as adding other types of cells can be added to this base model if required.

2 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

HBSS – Hank's Balanced Salt Solution

3 Principle of the Method:

This method aims to standardise the method for growing Calu-3 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

4 Description of the Method:

4.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and in laboratory implementing biosafety level 1.

- Cell line utilised is a human lung adenocarcinoma derived epithelial cell line, derived from metastatic site, Calu-3 (ATCC® HTB-55™)
- For further information: http://www.lgcstandards-atcc.org/Products/All/HTB-55?geo_country=nl

4.2 Chemicals and reagents used:

- 0.05% Trypsin-EDTA (1x) (25300, GIBCO®);
- FBS (758093, Greiner bio-one);
- MEM (1x) + GlutaMAX (41090, GIBCO®);
- HBSS (1x) (14175, GIBCO®);
- Penicillin/Streptomycin (100X) (15140, GIBCO®);
- Non-Essential Amino Acids (NEAA) solution (100x) (11140, GIBCO®);
- Amphotericin B (15290, GIBCO®);
- Trypan Blue Solution (CAS# 72-57-1) (T8154-100mL, Sigma Aldrich®, UK);

4.3 Apparatus and equipment used:

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

- Liquid Nitrogen or Ultra low freezer (-130°C);
- Cell Freezing Aid (Mr Frosty, 5100-0001, Thermo Fisher Scientific, UK);
- Biohazard downflow cabinet (e.g. Clean Air 4082 SHR4);

- EVOM2™ Epithelial Voltohmmeter supplemented with STX2 Chopstick Electrode Set (World Precision Instruments Inc., FL, USA)
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator (Binder);
- Water Bath (37˚C);
- Centrifuge (Eppendorf);
- T-25 (690175), T-75 (658175) and T-175 (660175) Tissue Culture Flask ;
- Pipette Controller;
- 2 mL, 5 mL, 10 mL and 25 mL Pipettes;
- P20, P200 and P1000 micropipettes (Biohit);
- Filtered, Sterile 20 µl, 200 µl and 1000 µl Pipette tips;
- 50mL and 15mL Centrifuge Tubes;
- Haemocytometer (Bürker-Turk);
- Sterile 2.0 mL Cryo vials;
- Light Microscope (Olympus, CKX41);
- Transwell Inserts (12 mm 0.4 µm pore size, polyester)(CLS 3460, Corning);
- Transwell Inserts (12 mm, 3 µm pore size, polyester)(CLS 3462, Corning);

4.4 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.

4.5 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) 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.

4.6 Nanomaterials used / handling procedures

Not applicable for this SOP.

4.7 Reagent preparation:

4.7.1 Cell culture medium (CCM)

Minimum essential medium (MEM) + GlutaMAX (Catalog number: 410900-36) supplemented with

- 1% Penicillin-Streptomycin (corresponds to 100 U/ml Penicillin and 100 µg/ml Streptomycin);
- 1% Non-Essential Amino Acids (NEAA) solution;
- 2% Amphotericin B; (optional; see below)
- 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. Amphotericin B can be added to prevent the growth of moulds and yeasts.

To a 500 mL medium bottle add:

- 5 mL Penicillin-Streptomycin;
- 5 mL NEAA;
- 10 mL Amphotericin B;
- 50 mL FBS;

The order of adding the ingredients is not important, except that FBS should be added the last. The medium needs to be mixed after each addition.

4.7.2 Freezing Medium

CCM (as prepared in section 4.7.1) 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. Resuspend 10^7 cells in 5000 µL of CCM, pipette up and down several times (carefully to avoid sheer stress) to mix properly. Meanwhile prepare 10% DMSO in CCM in another tube with same volume (500 µL DMSO + 4500 µL CCM), then transfer the 10% DMSO to prepared cell suspension (1:1) mix these two tubes. After properly mixing (again by pipetting up and down), aliquot and place immediately to the cell freezing aid (e.g. Mr Frosty).

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

4.8 Procedure:

4.8.1 Thawing Cells

1. Warm all required reagents to 37°C in a water bath (~20-30 min).
2. Remove one vial of cells from (-130°C) storage and gently swirl in a 37°C water bath (to ensure uniform thawing of the cell suspension) ensuring the O-ring and lid are not submerged (to reduce the potential for contamination) until the contents are almost thawed (between 1 and 2 min).
3. 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.

4. Pipette slowly (drop by drop) the contents of the vial into a centrifuge tube with 9 mL of CCM and centrifuge at 130g for 5 min at RT.
5. Discard the supernatant (this is laboratory specific so follow laboratory specific protocols) and re-suspend (via carefully pipetting up and down) the cell pellet in 1 mL of prewarmed CCM.
6. Transfer the re-suspended cells into the pre-prepared CCM in the T75 flask and incubate the culture at 37°C and 5% CO_2.
7. Change the CCM every 2-3 days. CCM is poured off and 15 mL of fresh CCM is pipetted into the flask and the flask placed back into the incubator.

4.8.2 Sub-Culturing

- Calu-3 cells are provided by ATCC in cryovials frozen previously in liquid nitrogen with the number of passages identified. Keep a record of the passage number in the database of the -130°C freezer.
- Calu-3 cells are maintained in T25, T75 or T175 cell culture flasks, in which the cells are passaged at 60%-80% confluency every 7 days with CCM renewal every 2-3 days. CCM is poured off and fresh CCM (T25=5 mL,

T75=15 mL and T175=25 mL) is pipetted into the flask and the flask is placed back into the incubator.

- Cells should be passaged at least 2 times after thawing, before using in experiments or before freezing, and they should be passaged no more than 20 times in total (recovery of cells using trypsin 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 60%-80% confluent by checking under a light microscope.

The following steps are completed under aseptic conditions

2. Pour off the CCM.
3. Wash the cells with 5 mL of 1xHBSS two times, discard the HBSS after each wash. HBSS removes serum which inhibits trypsin.
4. Add 3 mL Trypsin-EDTA and place flask back into the incubator at 37°C and 5% CO_2 for 10-15min (checking after 10 min) ensuring the 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. Pour the complete contents of the flask into a 50 mL centrifuge tube.
7. Centrifuge the cells for 5 min at 130g, 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). Additional CCM can be added to dilute the cell suspension.

10. Count the cells (dead and alive) in 1 mL of CCM using a haemocytometer. To achieve this;

a. Take the haemocytometer out of the box and place the cover slip 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 (in red) (see Figure 2) using a cell counter.

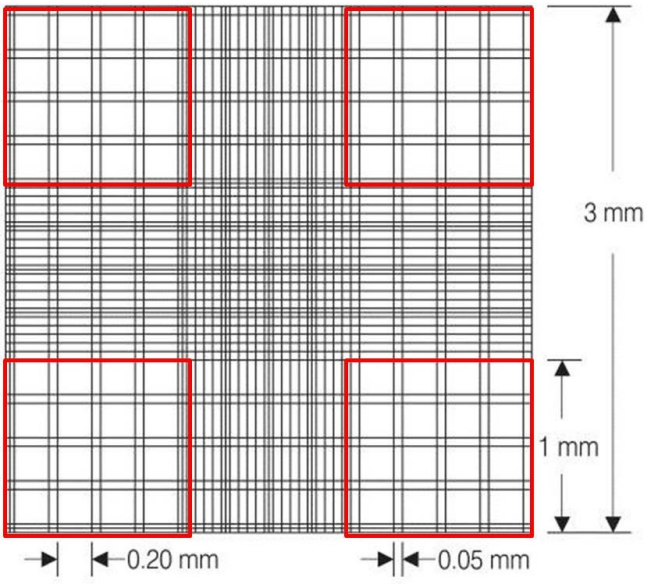

Figure 2. Counting cells using a haemocytometer

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

Total cells/mL= counted cells (4 squares) × dilution factor ÷16 × 25 × 10'000

The term “dilution” refers to how many times the stock solution was diluted in trypan blue (in this case 2x) and “counted cells” here refers to the number of cells in the 4 big squares. Using the following calculation, count the percentage of viable cells:

Viable cells % = Living cells number (white)/Total cells number (white+blue)

The seeding density for cell passage not only depends on the size of flask and number of flasks required, but also on the cell properties. For example, due to the low growth rate, it is better to passage Calu-3 cells once a week with 60%-80% confluency. So the seeding number should be around 6.0×10^6 per T175 flask and 2.0×10^6 per T75 flask. Using the number of living cells calculated above, determine the dilution required with below calculation to get the correct number in the new culture flask.

$C_1V_1 = C_2V_2$

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 CCM volume required and add the cell suspension into each flask (as previously stated in section 5.8.2).
13. Gently rock the flask and then place it back into the incubator (37°C and 5% CO_2).
14. Replace with fresh CCM every 2-3 days and subculture when they reach 60%-80% confluency.

Tips: Passage number of Calu-3 cells should keep below P25 for experiments especially under ALI testing.

See appendix for example.

4.8.3 Freezing Cells

Notes

- Aliquots of early-passage cells (minimal 2, maximum 4 passages) should be frozen. Expand the cells in such a way 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. Keep a record of the passage number in the database of the -130°C freezer.
- 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 $2x10^6$ cells/mL in prewarmed CCM.
3. Meanwhile prepare the same volume of 10% DMSO in CCM in another tube, then mix these two tubes (1:1).
4. Pipette 1 mL of the cell suspension into each cryovial.
5. Place the cryovial into a suitable cryopreservation chamber and leave overnight at -80°C before removing and putting into liquid nitrogen or -130°C for long-term storage.

4.8.4 Seeding Cells onto Culture Inserts

Notes

- The following procedure is adjusted for 6-well inserts. It can however be applied to different insert sizes by recalculating the cell number and amount of media based on the insert surface area.

- If using cell culture inserts from other providers, note that the surface area of the insert can also differ.
- Inserts are available with different pore sizes. Small pore sizes (e.g. 0.4 µm) have the advantage that the cells grow more easily and can achieve a good barrier, as measured by Trans Epithelial Electrical Resistance (TEER). However, when interested in particle translocation, these pores are too small and will trap the particles. Therefore, larger pore sizes (e.g. 3 µm) are usually chosen to test particles. The cells need a longer period of time to achieve a good TEER (see 4.8.5).

Protocol

The following is performed under aseptic conditions

1. Prepare cell suspension with known concentration following steps 1 – 11 from sub-culturing protocol (see section 4.8.2).
2. Dilute cells to a concentration of $5x10^5$ cells/ml in prewarmed CCM.
3. Take a cell culture plate with inserts and place under aseptic conditions.
4. Fill the basolateral side with 2 mL pre-warmed CCM.
5. By pipetting up and down carefully mix the cell suspension. Pipette 1.0 ml of cell suspension (i.e., 467,000 cells/insert which is equivalent to 100,000 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 2-3days.

4.8.5 Culturing Cells at Air-Liquid Interface

The following steps are performed 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 under submerged conditions.
3. Pour off the CCM.
4. Apply 1.5 mL of pre-warmed CCM to the basolateral side of the well (under the cell culture insert). The CCM should touch the membrane from the bottom, but not leak onto the top of the insert.
5. At this point cells are apically exposed to air, which is referred as culturing at the air-liquid interface (ALI).
6. Culture cells ALI for 7 days prior to exposures in the incubator at 37°C and 5% CO_2. This time is needed to achieve a TEER of >1000 Ωxcm^2.
7. Change the basolateral CCM every 2-3 days.
8. The cells can be used at the ALI for 6 weeks.

4.9 Quality control & acceptance criteria:

Not applicable for this current SOP.

5 Data Analysis and Reporting of Data:

Not applicable for this current SOP.

6 Publications:

Not applicable for this current SOP.

7 References

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