



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 (NCI-H441)

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Adapted from the NanoImpactNet SOP, Cliff *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

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To all authors: Please note that all text in blue is only a guide – only provide information that is applicable to the specific SOP being written.

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 the addition of complexities to the system.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used for culturing NCI-H441 cells in submerged conditions and at an air-liquid interface (ALI). This is the beginnings of the lung model with adaptations that can then be added to this base model.

Limitations:

They are available for ENM exposures 24 hours after being switched to ALI, leaving 72 hours of stability for investigation of extended or repeat-dose particulate effects.

There are also restrictions placed on the cell line which state the cells are only permitted for use for research purposes only and proposed commercial uses must be negotiated with the National Cancer Institute.

“The line is available with the following restrictions:

1. This cell line was deposited at the ATCC by Dr. Adi F. Gazdar and is provided for research purposes only. Neither the cell line nor products derived from it may be sold or used for commercial purposes. Nor can the cells be distributed to third parties for purposes of sale, or producing for sale, cells or their products. The cells are provided as service to the research community. They are provided without warranty of merchantability or fitness for a particular purpose or any other warranty, expressed or implied.
2. Any proposed commercial use of the these cells, or their products, must first be negotiated with the National Cancer Institute (NCI). For further information, please contact NCI’s Technology Transfer Center at NCI_TTC_Contact@mail.nih.gov or by phone at (240)-276-5514.”

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)?	Y/N
Has the method been submitted for standardisation (to OECD, CEN, ISO,...) in its own right or as part of another	Y/N

standardisation project?

Is the method included in an existing standard (or ongoing standardisation work)

Y/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 *nanostuctured 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 – Americal 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 aseptic culture and cryopreservation of NCI-H441 cells in either culture flasks or transmembrane well inserts.

NCI- H441 is a hyperdiploid human cell line. It has a modal chromosome number of 52, but there is a high frequency of cells with 53 chromosomes.

They have a population doubling time of 58 hrs in medium with serum; 99 to 138 hrs in serum-free medium.

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

- Cell line utilised is a Human Papillary Adenocarcinoma derived epithelial cell line, NCI-H441 (ATCC HTB-174).
- For further information:
 - https://www.lgcstandards-atcc.org/products/all/HTB-174.aspx?geo_country=gb#generalinformation

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 (1X) (A10491-01, GIBCO®, Paisley, UK)

PBS pH 7.4 1X, MgCl₂ and CaCl₂ Free (14190-094, GIBCO®, Paisley, UK),

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

DMSO (276855, Sigma Aldrich®, UK)

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

Virkon (Rely+On™ Virkon®, DuPont, UK)

5.3 Apparatus and equipment used:

- Liquid Nitrogen
- Cell Freezing Aid
- Laminar Class II Tissue Culture Hood
- 37°C and 5% CO₂ ISO Class 5 Hepa Filter Incubator
- Water Bath (37°C)
- Autoclave Tape
- Centrifuge
- T-25 (690175) and T-75 (660175) Tissue Culture Flask
- Pipette Controller
- 5mL, 10mL and 25mL sterile pipettes
- P1000 and P200 micropipettes
- Non-Filtered, Sterile 200µl and 1000µl Pipette tips
- 50mL Centrifuge Tubes
- 50mL Skirted Falcon Tubes
- Haemocytometer
- 0.2mL and 1.0mL Eppendorf Tubes
- Light Microscope

5.4 Reporting of protected elements:

There are restrictions associated with this SOP as previously indicated (Section 1.1). They are licenced for use within research institutions and not within industry. There are no 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.

Trypan Blue solution is a known teratogen, so it is advised that any pregnant individuals take extra caution when handling the chemical and should follow additional health and safety guidelines stated by the Occupational Safety and Health Administration (OSHA) in SOP 3.21 Reproductive Toxins, Mutagens, Teratogens and Embryotoxins – Procedures for Safe Handling and Storage. However, alternative cell viability assays (e.g. Erythrocyte B) could be used instead if preferred.

5.6 Reagent preparation:

5.6.1 Cell Culture Medium (CCM)

RPMI 1640 (1X) supplemented with

- 1% Penicillin-Streptomycin (corresponds to 100 Units/mL Penicilin and 100 µg/mL Streptomycin)

- 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.

From a fresh 500 mL medium bottle;

Remove:

- 55.5 mL of medium

Add:

- 5.5 mL Penicillin-Streptomycin
- 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/cm²) under aseptic conditions and place in the incubator (5% CO₂, 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 125g 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% CO₂.
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-Culture

- NCI-H441 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 in order to label future cultures properly. It is important to know the passage number the cells are currently being grown at in case differences in experimental results are noted and could potentially be due to variation in the passages used.
- NCI-H441 cells are maintained in T25, T75 or T175 cell culture flasks, in which the cells are passaged at >80% confluency every 3-4 days with fresh medium replacement occurring every 2-3 days. Medium is poured off into a waste vessel and fresh CCM (T25=5mL, T75=15mL and T175=90mL) 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 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 >80% confluent by checking under a light microscope (see Figure 1).

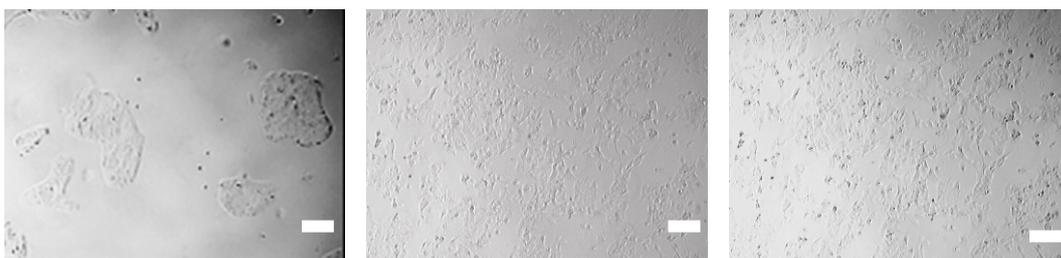


Figure 1: Phase contrast image of NCI-H441 cells a) not confluent b) at confluency >80%, c) fully confluent (overgrown). Scale bar: 50 μ m.

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 2.5 mL Trypsin-EDTA and place flask back into the incubator at 37°C and 5% CO₂ for 5 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 125g, 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 pre-warmed (37°C) CCM by pipetting up and down until all cells are suspended (no pellet or cell agglomerates can be observed).
10. 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 2) using a cell counter.

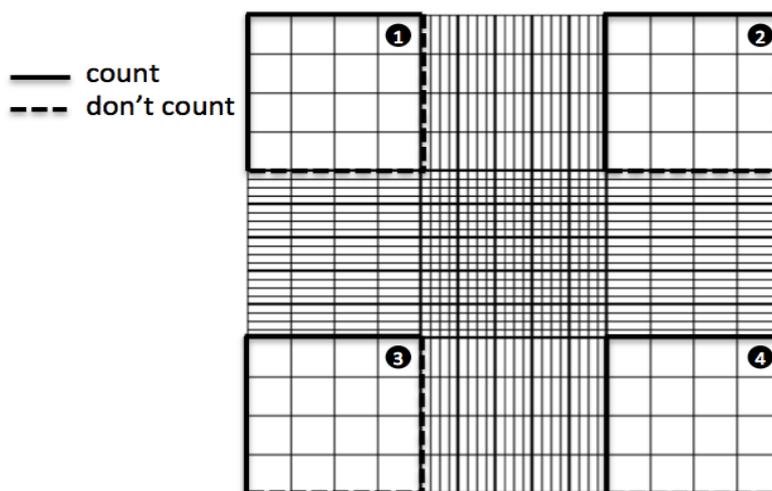


Figure 2 Counting cells using a haemocytometer
(Hemocytometer.org, 2018)

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

$$\text{Total cells/mL} = \text{counted cells} \times \frac{\text{dilution}}{\text{\# of squares counted}} \times 10^4$$

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:

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

Using the live cell number calculated above, determine the dilution required to get the correct cell number of between 2×10^3 and 1×10^4 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% CO₂).

14. Replace the medium with fresh prewarmed CCM every 2-3 days and subculture when they reach >80% confluency.

See appendix (Section 9, page 19) 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 sub-culture) 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 that of the sub-culture protocol (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 1×10^6 cells/mL in prewarmed CCM.
3. Add 5% (v/v) DMSO.
4. Pipette 1mL of the cells (diluted to 1×10^6 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. After preparation, place plate 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 2.5×10^5 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., 125'000 cells/insert, which is equivalent to 112'500 cells/cm²) 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% CO₂.
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 3 days
3. Remove and discard CCM. Medium is poured off to waste.
4. Apply 1.5 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 24 h prior exposures in the incubator at 37°C and 5% CO₂.

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/>