PATROLS
Advanced Tools for NanoSafety Testing

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ANNEX 3207

PATROLS Standard Operating Procedures (SOP)

Guidance Document for the induction of the inflamed alveolar epithelial model

SOP_PATROLS 3207_Lung_Co-cultures_inflammatory model

SOP_PATROLS_ 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	09/04/2020	Initial Document	Barbara Drasler
1.1	14/04/2020	Additional detail added from AMI	Anne Bannuscher and Barbara Rothen-Rutishauser
1.2	15/04/2020	Final comments implemented	
2.0	29/05/2020	Version distributed to WP3 members	
2.1	29/05/2020	All comments from WP3 members integrated and uploaded to server	

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1 Introduction:

DOMAIN: Diseased 3D In Vitro lung model system for ENM hazard assessment

According to the World Health Organization (WHO), several hundred million people suffer from chronic lung inflammation which plays an important role in the pathogenesis of various respiratory diseases, such as acute lung injury, asthma and chronic obstructive pulmonary disease. Individuals with pre-existing chronic diseases and bacterial or viral infections are more susceptible to potential hazardous effects of engineered nanomaterials (ENM). Most of *In Vitro* human lung models used for ENM hazard assessment mimic a healthy human tissue. Therefore, diseased human lung models mimicking a general, non-specific inflammation present a powerful tool for ENM hazard assessment *In Vitro* aiming the relevance to patients with acute or chronic lung inflammation.

For the assembly of the multicellular human lung model, composed of alveolar epithelial cells (A549), human monocyte-derived macrophages (MDMs) and dendritic cells (MDDCs), cultivated at the air-liquid interface (ALI), refer to: [1]. Cultivation of epithelial cells, e.g., A549, should be followed the SOP for the cell line (i.e., SOP_PATROLS_A549). The differentiation of monocytes into MDMs and MDDCs and co-culturing with human epithelial cells is described with all experimental details in [1].

1.1 *Scope and limits of the protocol*

This SOP was established with the intention to be used for developing inflammatory 3D *In Vitro* lung co-cultures. These provide a physiologically relevant model for ENM hazard assessment associated with diseased individuals, i.e., suffering acute or chronic lung inflammation.

Limitations: (i) Detection of the cytokines IL-1β and TNF-α at both, the protein secretion and gene expression levels, is at the lower detection limit when 12-well plates are used (as opposed to 6-well plates). Please refer to the SOP specific limitations for the applied epithelial cells (i.e., SOP_PATROLS_A549). (ii) The A549

co-cultures 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.

2 Terms and Definitions:

Substance

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

3 Abbreviations:

ALI – Air-liquid interface

ATCC – American Tissue and Cell Collection

CCM – Cell Culture Medium

ENM – Engineered Nanomaterials

FBS – Foetal Bovine serum

LPS – Lipopolysaccharide

PBS – Phosphate Buffered Saline

RPMI - Roswell Park Memorial Institute-1640 Medium

4 Principle of the Method:

This method presents establishment of an inflamed model by challenging the multicellular human lung model with lipopolysaccharide (LPS). The inflammatory model may be used for exposures to ENM following the corresponding SOP (e.g., SOP_PATROLS_Cloud_Aerosolization or SOP_PATROLS_QUASI-ALI).

5 Description of the Method:

5.1 *Biological setting & test system used:*

This SOP has to be carried out under laboratory-based conditions, with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

5.2 *Chemicals and reagents used:*

FBS (10270-106, GIBCO®, Switzerland)

L-Glutamine (25030081, GIBCO®, Switzerland)

LPS (isolated from *E. coli*, strain O55:B5, L2880, Sigma-Aldrich, Switzerland)

RPMI 1640 (11879020, GIBCO®, Switzerland)

Pen/Strep (15140-122, Penicillin/Strepmyocin 100X or 10,000U/mL, GIBCO®, Switzerland)

5.3 *Apparatus and equipment used:*

- 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)
- Centrifuge
- Pipette Controller
- 5 mL and 10 mL sterile pipettes
- P1000 and P100 micropipettes
- Non-Filtered, Sterile 100 µL and 1000 µL Pipette tips
- 0.2 mL and 1.0 mL Eppendorf Tubes or 15 mL Centrifuge Tubes
- 6 or 12-well cell culture inserts (transparent, PET, 3 µm pore size) either Falcon® (Cat. No. 353091 or 353181, respectively, Falcon, Corning brand, USA) or Transwell® (preferred, Cat. No. CLS3452-24EA or CLS3462-48EA, respectively, Corning, USA)
- 6 or 12-well cell culture plates with flat bottom, not required specific, for example Falcon® Polystyrene Permeable Support Companion Plate, with Lid, sterile (Cat. No. 353502 or 353503, respectively, Falcon, Corning brand, USA)

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:*

Working with non-tested human blood samples, *i.e.* MDMs and MDDCs, involves specific care to prevent transmission of HIV and Hepatitis B and C. The use of protective measures such as gloves, gowns, masks, and eye protection as well as vaccination against the Hepatitis B virus is mandatory. These reduce the risk of exposing the skin or mucous membranes to potentially infectious fluids. Avoid using sharp, or fragile utensils to minimize potential injury. 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 *Nanomaterials used / handling procedures:*

Not applicable for this SOP.

5.7 *Reagent preparation:*

5.7.1 Cell Culture Medium (CCM)

RPMI supplemented with:

- 1% Penicillin-Streptomycin (corresponds to 100 Units/mL Penicilin 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 4-6 °C after preparation and can be kept for three 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.

5.7.2 LPS

Prepare a stock solution of LPS in sterile, endotoxin-free water at 1 mg/mL under sterile conditions. After adding water to lyophilized LPS, pipette up and down again (or vortex) in order to ensure mixing. Prepare aliquots of, e.g., 20-30 µL in 0.2 mL Eppendorf Tubes and store at -20 °C at least during a one-year period. Avoid freeze-thaw cycles and store the aliquot in use at 4°C.

5.8 *Procedure:*

Co-culture assembly including epithelial cells (A549), human monocyte derived macrophages and dendritic cells, and its cultivation at air-liquid interface (ALI) should be followed according to the protocol described in [1]. Cultivation of the epithelial cells (A549) prior to assembling the model should be done accordingly to the SOP_PATROLS_A549.

4.8.1. Challenge with LPS

At least two co-culture models, grown at ALI, are required (i.e., one serving as an untreated model and one to be LPS-challenged).

1. Warm CCM to 37°C in a water bath (~20-30 min).
2. Defreeze one LPS stock aliquot at room temperature.

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

3. Prepare LPS in the warmed CCM at 1 µg/mL in a 1.0 mL Eppendorf tube or in a 15 mL Centrifuge Tube.[1] Pipette the LPS stock solution up and down gently before diluting to the working concentration (1 µg/mL). After adding to CCM, pipette up and down again (or vortex) in order to ensure mixing.

[1] The volume depends on the number of inflammatory models to be prepared (i.e, 0.6 mL per model plus 0.1 mL for pipetting error).

4. Remove and discard basal CCM of the co-culture model grown at ALI. CCM is poured off into a waste vessel.
5. Add 0.6 mL or 1.2 mL of CCM containing LPS (at 1 µg/mL) to the basal side of untreated each model for 12- and 6-well plates, respectively. Add 0.6 mL or 1.2 mL of CCM (without LPS) to untreated models.
6. Incubate for 48 h in the incubator (37 °C, 5% CO_2) without changing CCM.
7. Upon incubation, exposure to ENM can be performed for the defined post-exposure times.

    7.1. Exposure to ENM: The inflammatory model can be used for exposures to ENM following the corresponding SOP (e.g., SOP_PATROLS_Cloud_Aerosolization or SOP_PATROLS_QUASI-ALI).

8. Following exposures and post-exposure incubation, basal CCM is collected for analysis of pro-inflammatory reactions and barrier integrity (Drasler et al. *In preparation*):

    8.1.: Pro-inflammatory reactions upon exposures are assessed using enzyme-linked immunosorbent assay (ELISA) for pro-inflammatory markers, tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-8) and their respective gene expression via real-time quantitative reverse transcription polymerase chain reaction (real-time RT-qPCR).

    8.2: A loss of alveolar barrier integrity shall be measured via barrier permeability assays, for examples a dextran-based permeability assay.

5.9 Quality control & acceptance criteria:

Visual inspection of the cell culture should ensure there are no contaminations. Cell viability should be checked via a membrane rupture assay (e.g., lactate dehydrogenase assay on basal CCM samples). Upon fixation, morphology of the fluorescently stained models can be visualized with a confocal laser scanning (Drasler et al. *In preparation*).

Data Analysis and Reporting of Data:

Not applicable for this current SOP.

6 Publications:

Drasler et al. *In preparation* (will be updated when submitted)

7 References:

Not applicable for this current SOP.

1. Barosova, H., et al., *Multicellular Human Alveolar Model Composed of Epithelial Cells and Primary Immune Cells for Hazard Assessment.* Journal of Visualized Experiments, 2020. **In press**.
2. Drasler et al. *In preparation* (will be updated when submitted)