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Protocol

EpiAirway™ Respiratory Toxicity Protocol

Note: This protocol is written for use with the EpiAirway™ Model (AIR-100-DAY20-TOX).

This protocol is an update to previous work described in Jackson et al. (2018). The dose-range-finding step has been eliminated, the exposure time has been extended to 4 hours to match that of in vivo testing as stipulated by OECD TG 403 (Guideline for the Testing of Chemicals, Acute Inhalation Toxicity, 2009), and a 20-hour post-incubation was added to increase sensitivity of the assay. In addition, 2 presentations utilizing the assay have been included as references.

I. Overview

This assay method requires fifteen (15) EpiAirway tissues (part #: AIR-100-DAY20) to determine the airway toxicity of one test material: 9 tissues for the test material doses (6 for MTT and 3 for histology), and 3 each for the positive (PC) and the vehicle/negative controls (NC). Testing of 2 test materials requires 24 tissues, testing of 3 test articles requires 33 tissues, etc. (Note: These numbers of tissues assume that test materials are all soluble in the same vehicle/negative control). AIR-100-DAY20 tissues are shipped every Monday from MatTek Headquarters (Ashland, MA, USA) and MatTek Europe (Bratislava, Slovakia) via overnight express delivery service.

The EpiAirway tissues are exposed to three fixed doses of the test material for 4 hours and the resultant tissue viability, relative to the vehicle/negative control exposed tissues, is determined using the MTT assay. Histology is performed to increase confidence in the MTT results. The effective dose of the test material (mg/tissue) that reduces tissue viability by 25% (ED-25) is mathematically interpolated. This protocol also presents the prediction model which allows the translation of the in vitro result into an in vivo GHS irritation category.

II. Method

Tissues required:
a. Nine (9) tissues (n=3/concentration) for each test material.
b. Three (3) or six (6) tissues for vehicle/negative control (NC) that will be treated with ultrapure water and/or corn oil (depending upon chemical solubility of the test materials).
c. Three (3) tissues for positive control (PC) that will be treated with at least one respiratory irritant (Table 1). The recommended positive control is toluene (200 mg/mL). For a valid assay, the viability for the positive control must be ≤75% of the NC.
d. If an untreated control is desired (optional), reserve n=3 tissues which will be cultured in AIR-100-ASY for the duration of the experiment.

1. Pre-equilibration:
a. In a sterile hood, unpackage the EpiAirway tissues and transfer them into 6-well plates containing 1.0 mL/well of assay media (Part #: AIR-100-ASY) according to the standard procedure outlined in the "EpiAirway™ AIR-100 Use Protocol" (Document #: MK-24-007-0027).
b. Equilibrate the tissues overnight in a 37±1°C, 5±1% CO_2, 90±10% RH incubator.
c. Following equilibration, gently rinse the apical surface of the EpiAirway tissues twice by adding 400 µL of phosphate buffered saline (PBS) containing calcium and magnesium (Part #: TEER-BUFFER) to the culture insert. Tilt the insert and aspirate to remove all liquid and mucus from the tissue surface, being careful not to damage the tissue surface.
d. Place the rinsed EpiAirway tissues into 6-well plates containing 1.0 mL of fresh AIR-100-ASY medium.

2**. Preparation of dosing solutions:** Prepare test material **dosing solutions** of 200, 100, and 20 mg/mL (corresponding to 20, 10, and 2 mg/tissue) in either ultrapure water or corn oil, depending upon the solubility of the test article. A positive displacement pipet is suggested for accurate dispensing of corn oil solutions. If a test material is not soluble in either water or corn oil, prepare a fine suspension/emulsion in either vehicle.

a. Prepare 200 mg/mL dosing solution: On an analytical balance, weigh out 200 mg of the test material and dissolve in 1.0 mL of corn oil or ultrapure water.

b. Prepare 100 mg/mL dosing solution: Dilute the 200 mg/mL solution 1:1 with the vehicle. Example: Add 300 µL of the 200 mg/mL solution from the step above to 300 µL of the vehicle and mix thoroughly.

c. Prepare 20 mg/mL dosing solution: Dilute the 100 mg/mL solution 1:4 with the vehicle. Example: Add 100 µL of the 100 mg/mL solution from the step above to 400 µL of the vehicle and mix thoroughly.

3. **Apply dose to tissues:** Apply 100 µL of each dosing solution (20, 100, and 200 mg/mL) to the apical surface of the EpiAirway tissues (n=3 tissues/dose).

4. **Insert vapor cap:** Immediately after application of the dose, insert a vapor cap (Part #: MILCEL-MTK-CAP) into the top of each tissue culture insert to prevent evaporation of the applied dose.

5. **Incubate dosed tissues:** Incubate the dosed tissues in a 37°C, 5% CO_2, 95% RH incubator for 4 hours ± 5 minutes.

6. **Completion of exposure period:** At the conclusion of the 4 hour exposure period, remove the vapor caps and decant the test material from the tissue surface onto a clean absorbent material (paper towel, gauze, etc.). Rinse off the test material by adding 400 µL of TEER buffer to the apical surface of the EpiAirway tissue. Carefully aspirate off the TEER buffer by tilting the insert to the liquid. Repeat the rinse twice (3 rinses total).

7. **Post-exposure incubation:** Transfer the tissues into 6-well plates containing 1.0 mL of fresh AIR-100-ASY (pre-warmed to 37°C) per well and incubate the tissues for 20 hours in a 37°C, CO_2, 95% RH incubator.

8. **Tissues for MTT assay**: N=2 of the tissues from each test condition are used to determine the tissue viability using the MTT viability assay, as per the procedure outlined in the EpiAirway AIR-100 Use Protocol MK-24-007-0027.

9. **Tissues for histology**: N=1 of the tissues from each test condition are used for the histology, as per the Histology Sample Preparation Procedure, Protocol OH-24-007-0001.

III. Determination of ED-25

Determine ED-25: Calculate the ED-25 using the spreadsheet available from MatTek Technical Service.

IV. Prediction model – In Vivo Categorization of Test Articles

1. **Assign an in vivo category to test material:** Based on the ED-25, a GHS category can be assigned to the test material, as per the Table 1.

Table 1. Prediction model: Conversion of ED-25 to In Vivo Category				
Dose of test material applied				
mg/tissue	mg/cm^2	mg/mL	GHS Category	Examples
≤ 5	≤ 8.3	≤ 50	Cat. 1&2	Crotonaldehyde, Chloroacetaldehyde, Allyl alcohol
5 to 20	8.3 to 33.3	50 to 200	Cat. 3&4	Methyl propyl ketone, Toluene, Xylene
≥ 20	≥ 33.3	≥ 200	Cat. 5& No Category	Ethanol, Piperonyl butoxide, Isoflurane

2. **Compare to benchmark chemicals**: Results for test materials should be compared to known respiratory irritants and non-irritants (benchmark controls) that are similar in chemical nature to the test material(s) being evaluated. Benchmark controls may include allyl alcohol and chloroacetaldehyde (Cat 1 & 2), toluene and xylene (Cat 3 & 4), or ethanol and isoflurane (Cat 5 and No Category) as listed in the Table 1.

3. **Additional (optional) endpoints**: EpiAirway culture medium may be collected for assessment of released cytokines. Trans-Epithelial Electrical Resistance (TEER) can also be measured to determine effects on tissue barrier function.

V. References

1009. Multi Species 3D Airway Tissue Models for Translational Inhalation Toxicity. Jackson GR, Durand S, Coen K, Landry T, Klausner M, Kaluzhny Y, Armento A, Ayehunie S. Presented at the Society of Toxicology meeting, March, 2023, Nashville, TN.

1001. Development and evaluation of in vitro inhalation model to predict acute respiratory toxicity of mists and volatile liquids. Kaluzhny Y, Jackson GR, Markus J, Kearney P, Letasiova S, Klausner M, Armento A. Presented at the Society of Toxicology meeting, March, 2023, Nashville, TN.

837. Prevalidation of an Acute Inhalation Toxicity Test Using the EpiAirway In Vitro Human Airway Model. Jackson, G.R., Jr., Maione, A.G., Klausner, M., Hayden, P.J., 2018. Appl In Vitro Toxicol 4, 149-158.

794. Development of an in vitro cytotoxicity model for aerosol exposure using 3d reconstructed human airway tissue: application for assessment of e-cigarette aerosol. Neilson L, Mankus C, Thorne D, Jackson G, DeBay J, Meredith C. Toxicol In Vitro. 2015 Oct: 29(7):1952-62.

VI. Materials Provided

EpiAirway® DAY20 Tissues (Part No. AIR-100-DAY20-TOX)

Quantity	Description	Part No.
24	EpiAirway Day 20 tissues	AIR-100-DAY20
4	6-well plates (sterile)	MW-15-003-0027
2	24-well plates (sterile)	MW-15-003-0028*
1	TEER buffer, bottle, 100 mL	TEER-BUFFER
1	Assay medium, 125 mL	AIR-100-ASY
1	EpiAirway (AIR-100) protocol	MK-24-007-0027
1	Toluene (2 mL bottle) – positive control	

** Shipped only if MTT-100 is ordered*

VII. Additional Materials & Services – must be ordered separately

MTT assay kit (Part No. MTT-100)

Quantity	Description	Part No.
1	MTT diluent solution, 8 mL	MTT-100-DIL
1	Extractant solution, 60 mL	MTT-100-EXT
1	MTT concentrate (5:1), 2 mL	MTT-100-CON

Note: MTT-100 kits must be ordered separately

Quantity	Description	Part No.
1	Vapor caps	MILCEL-MTK-CAP

Note: 24 vapor cap needed/ AIR-100-DAY20 kit. Minimum order: 6 vapor caps

Quantity	Description	Part No.
1	H&E histology slide	AIR-HIS
1	Photography of histology slide	AIR-PHO

Note: Minimum order: 12 histology slides.

VIII. Materials required – not included

Corn oil	Vehicle (non-polar)	CAS No: 8001-30-7
Ultrapure water	Vehicle (polar)	CAS No: 7732-18-5