Standard Operating Procedure for Murine Inhalational Pulmonary Aspergillosis

1. Purpose

   This Standard Operating Procedure (SOP) will provide information necessary for the uniform pulmonary infection of mice by *Aspergillus fumigatus* or related fungal spore inoculum preparations.

2. Scope

   This SOP and will provide sufficient information to infect mice in either the Madison or Acrylic inhalation chambers. These chambers are utilized for the induction of inhalational pulmonary aspergillosis. This SOP introduces the process of infection and follows it from immunosuppression, through actual infection within either of the two chambers, through disinfection of the apparatus and, ultimately, monitoring the infected mice.

3. Definitions.

   For the purposes of this SOP, "infect" will mean to introduce into the animal a precise, quantified concentration of viable *Aspergillus fumigatus* conidia in a diluent suitable for suspending and stabilizing the same.

4. Responsibilities

   This SOP shall be utilized by employees of Research assistant status or higher without additional training. Research technicians may perform this work upon receipt of training.

5. Equipment

- Drugs
  - Cortisone acetate, Sigma cat #C3130
  - Cyclophosphamide, (Cytoxan, Mead Johnson) supplied as 500 gram vials
  - Ceftazidime (Tazicef, Glaxo Smithkline, supplied as1 gram vial)
- Inhalation Chambers
  - Acrylic chamber (2ft.2in x 1ft.2in x1ft 6 in.) (Scott Filler, MD, Harbor-UCLA, Attachment 1**)**
    - Inhalation chamber in laminar flow hood
    - Nebulizer – Hudson Micromist (Hudson RCI, Cat #1883)
      - Acceptable equivalent: Hudson Micromist, # HU41892, Southern Syringe Services Ltd Enfield UK (European Union)
    - Compressed air cylinder – medical grade air is not required
  - Madison chamber (University of Wisconsin at Madison, Attachment 2a)
    - This is a self contained, HEPA filtered unit.
- Mice – female BALB/c mice 18-20 grams, National Cancer Institute Strain #01B05
  - Acceptable equivalent: Male; BALB/c mice, 18-20 g, Charles River
- Microisolators
- Bleach, 10%
- Sterile water [Acceptable equivalent: Sterile normal saline]

- 70% ethanol
- Certek Formaldehyde Generator/Neutralizer (Certek) (for decontamination of the Madison chamber)
- paraformaldehyde prills (JT Baker Cat # S898-07) (for decontamination of the Madison chamber)
- ammonium carbonate (Sigma-Aldrich Cat # A9516) (for decontamination of the Madison chamber)
- Amphyl (Revco) [Acceptable equivalent: Vesphene (Steris) or Decon (Decon Labs)]
- Micropipette EDTA Tubes (Infolab)
- BD Unopette System for leukocyte enumeration (Fisher Biomedical Cat # 02-687-40).
- 23, 25, 27, 30 gauge needles
- 1.0 ml syringes

6. Procedure
    - Preparation of Inoculum
        - *Refer to Standard Operating Procedure for Preparation of Aspergillus fumigatus Test Strains for Inhalational Aspergillus Animal Pulmonary Aspergillosis Studies.*
    - Mice
        - Use female BALB/c mice (or acceptable equivalent) between 18 and 20 grams. Mice smaller than this do not tolerate the immunosuppression well. There will be a total of 10 mice per infected group plus an additional group of 10 which will remain uninfected. To confirm the inoculum of test strain delivered to the mice, 3-5 mice from each run of the chamber will be sacrificed 1 hour post infection.
    - Immunosuppression
        - Immunosuppressive drugs are made and used at the following concentrations:
            - Cortisone acetate [25mg/ml]: Weigh out the necessary amount of cortisone acetate and add sterile PBS containing 0.05% Tween 80. Vortex this suspension vigorously and sonicate for 10 seconds before using. (Note: This drug should be prepared the same day of use).
            - Cyclophosphamide [25mg/ml] should be dissolved by the addition of sterile water at a 25mg/ml concentration in the vial. (Note: the concentration of this drug will change in the second round of Immunosuppression of the animals to 20mg/ml, thereby changing the amount of sterile water added to the vial). Store at 4^{o}C.
            - Antibiotic Ceftazidime [50mg/ml] dissolve by addition of sterile saline (20ml) to 1g vial. Store at 4^{o}C.

- At day –2 prior to inoculation, administer cortisone acetate [250 mg/kg] subcutaneously (approximately 0.2 ml / mouse) and cyclophosphamide [250 mg/kg] intraperitoneally (approximately 0.2 ml/ mouse) in to all the mice. A 30 or 27 gauge needle will work for the cyclophosphamide, but cortisone may require a 23 or 25 gauge needle. **NOTE: Cortisone acetate will also settle rapidly, and it should be vortexed multiple times during injection.**
- In addition, mice will begin receiving a **daily dose** of the antibiotic ceftazidime [50 mg/kg] subcutaneously (0.2ml/mouse) to prevent bacterial infections due to neutropenia that is induced for the duration of the study.
- On day +3 post infection, the immunosuppression regimen should be repeated using the same concentration of cortisone acetate [250mg/kg]. However, the concentration of cyclophosphamide is 200mg/kg (prepare a 20mg/ml stock to aid in calculating doses).

- <u>Inoculation of mice</u>
  - Optional: On the morning of inoculation verify that the mice are leukopenic by tail vein phlebotomizing 10% of the control mice (10µl volume per mouse, one half of capillary tube) and counting neutrophils using the Unopette® system. Do not bleed mice to be infected – this increases mortality. The leukocyte count should be <1000.
  - Acrylic Chamber (optional inhalational infection chamber)
    - Place all mice (maximum of 70 per run) to be infected in the inhalation chamber and place chamber in the laminar flow hood. Tape along the edge of the door facing out, and the top to avoid directing exiting conidia towards the hood opening. Also, plug in hole in the center of door with parafilm
    - The Micro Mist® nebulizer package comes with 5 parts: the tee, tubing, mouthpiece, jar with jet and cap, and reservoir. The mouthpiece and reservoir are simply discarded and not used. The tubing is connected to the bottom of the jar and then to the air tank. The tee (it is shaped in a "T") has 3 openings. The bottom of the tee connects to the cap of the jar. The smaller opening of the tee is the one which is connected to the chamber. This opening is smaller than the hole on the side of the chamber so it must be wrapped with parafilm to ensure a tight fit into the chamber. Do not cover the opening of the tee just the outer part of the opening so that the mist is expressed into the chamber and not being released outside of the chamber. The 3rd opening of the tee, which is the larger opening, should be completely sealed off. This may be done with a rubber stopper or it may be wrapped in parafilm. (This opening is sealed off so that the mist is directed to go into the chamber and not allowed to escape through this larger opening).

- Add 6 ml of the conidial suspension to the Micro Mist® nebulizer (or acceptable equivalent) and connect the nebulizer to the inhalation chamber. Connect the nebulizer to a tank of compressed air (medical air is not necessary).
- Run air through nebulizer at 100 kPa until the nebulizer begins to splutter, usually about 13-15 minutes.
- Turn off the compressed air and refill the nebulizer with another 6ml of suspension. While the apparatus is disconnected, gently agitate the chamber to redistribute the mice. (The mice will tend to huddle due to the noise of the chamber).
- Reconnect the nebulizer and run at 100 kPa until it splutters (usually about minute 30-35) and stops delivering aerosol. Turn off compressed air at this point and leave the mice for a total exposure time of 1 hour.
- After 1 hour, open cage and transfer mice from chamber to their cages inside the hood, placing microisolators on each cage.
- Within 1 hour, sacrifice the 3-5 mice being used to confirm the conidial delivery.
- Utilizing sterile technique, extract lungs from mouse, weigh the organ and homogenize in 2 ml of sterile saline.
- Assess CFU (*Refer to Standard Operating Protocol for Animal Tissue homogenization Method A.*). Briefly, prepare a 1:10 and 1:100 dilution of the organ homogenate and spread 0.1ml of each dilution on ½ of a Potato Dextrose Agar plate (spread 0.1ml of the same dilution on the other ½ of the plate) and incubate at 37°C overnight. Count the colonies the next day.

o **Madison Chamber** (optional inhalational infection chamber)

- Place 5-7 mice to be infected in each individual housing cage, within the cage rack, then place the rack into the chamber. The Madison Chamber is designed to hold 18 individual cages, thus, the maximum number of mice per run is 126. Seal the chamber door using the attached latching system.
- Add 13-15 ml of the conidial suspension to the air-glass impinger
- Run air through impinger at 40 l/min for 1 h. This shall be followed by a 10 minute air wash, with NO input of conidia from the impinger.
- After 70 minutes (from beginning of run), open chamber door and transfer mice from chamber to their housing cages, placing microisolators on each cage.

- Within 1 hour, sacrifice the 3-5 mice being used to confirm the conidial delivery.
- Harvest lungs, homogenize with 2 ml of sterile saline, and assess CFU as described in Standard Operating Protocol Animal Tissue homogenization Method A. Briefly, prepare a 1:10 and 1:100 dilution of the organ homogenate and spread 0.1ml of each dilution on ½ of a Potato Dextrose Agar plate (spread 0.1ml of the same dilution on the other ½ of the plate) and incubate at 37°C overnight. Count the colonies the next day.

- Disinfection of the chamber
  - **Acrylic Chamber**
    - Thoroughly clean the inside of the chamber with Amphyl® (or acceptable equivalent), then de-ionized water. If more experiments are planned in the next 48 hours with the same inocula then the chamber can remain in the hood until then (leave hood on). **WARNING: Do not turn on the UV light as this will damage the chamber.**
    - If another strain is to be used, or if the chamber is to be stored, then the chamber should be disinfected with 10% bleach, and 6ml of 10% bleach should be nebulized to disinfect the channel which is not accessible for cleaning directly. The chamber (and channel) should then be extensively rinsed out with de-ionized water to remove bleach residue and dead conidia. **WARNING: Do not use alcohol to clean as this will damage the chamber.**
  - **Madison Chamber**
    - Thoroughly clean the inside of the air-glass impinger with 70% ethanol, followed by a similar cleaning with sterile water.
    - Place 15 ml of 70 % ethanol into the air-glass impinger and run air through impinger at 40 l/min for 10 min.
    - Discard and repeat step 2 using sterile water
    - Spray external surfaces of the cage rack and internal housing cages and the inside of the Madison chamber with Amphyl and soak for 10 minutes. Wipe dry and replace cage rack (and internal cages) into Madison chamber.
    - Seal Madison chamber using the attached latch system
    - Begin final paraformaldehyde disinfection (Attachment 2b).
- Monitoring of mice
  - Monitor mice daily for signs of distress, such as:
    - Rapid breathing
    - Breathing very slow, shallow and labored (preceded by rapid breathing)
    - Rapid weight loss due to dehydration

- Ruffled fur
- Hunched posture
- Body temperature less than 30°C.
- Impaired ambulation (unable to reach food or water easily)
- Evidence of muscle atrophy or other signs of emaciation (body weight is not always appropriate).
- Extensive ulcerative dermatitis and infected tumors.
- Any obvious illness such as signs of lethargy (drowsiness, aversion to activity, physical or mental alertness, anorexia (loss of appetite, especially when prolonged), bleeding, difficulty breathing, CNS disturbance and chronic diarrhea

- Mice that are moribund should be euthanized humanely using approved methods such as pentobarbital overdose or CO_2 asphyxiation. The goal should be to have virtually all mice die by euthanasia rather than by infection.
- The experiment should be continued for at least 14 days after inoculation or until all mice are dead, which ever is shorter.

7. Attachments

   "Appendix 1and 2a (source documents) and 2b, Madison Chamber disinfection".

8. Deliverables

   Analysis and interpretation of results

   - Use the log-rank test for the statistical comparisons of survival between animal groups
   - P values < 0.05 will be considered significant with adjustment for multiple comparisons.
   - Conidial delivery should be between 1000 and 10000 per animal (usually 2000-4000), although results can vary depending on homogenization technique.
   - Leukocyte count <1000
   - For validity, the percentage survival of control infected mice (strain Balb/C or ICR) at different time-points utilizing this protocol should fall within the acceptable ranges as shown in the following table.

	Balb/C (n=158)†		**ICR (n=57)‡**	
Day	Calculated	Mean	Calculated	Mean

post-infection	Range 95% CI	% DOD ± S.E	Range 95% CI	% DOD ± S.E
D7	66.53 - 80.30	73.41 ± 6.89	48.76-74.04	61.40 ± 12.64
D9	41.57 - 57.16	49.37 ± 7.80	17.95-41.70	29.82 ± 11.88
D11	31.01 - 46.20	38.61 ± 7.59	9.05-29.55	19.30 ± 10.25
D14	29.61 - 44.74	37.18 ± 7.57	7.22-27.09	17.15 ± 9.93

9. References

Sheppard DC, Rieg G, Chiang LY, Filler SG, Edwards JE Jr, Ibrahim AS. Novel inhalational murine model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother. 2004 May;48(5):1908-11.

10. History

Version 1.00.

11. Examples of Deliverables

N/A