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 and Materials

- Drugs
  - Cortisone acetate, (Sigma catalog #C3130, supplied as a 25 gram vial)
  - Cyclophosphamide, (Cytoxan, Mead Johnson supplied as a 500 gram vial)
  - Ceftazidime (Tazicef, Glaxo Smithkline, supplied as a 1 gram vial)
- Inhalation Chambers
  - Acrylic chamber (2ft.2in x 1ft.2in x1ft 6 in.) (Scott Filler, MD, Harbor-UCLA, Figure 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, Figure 2)
    - This is a self contained, HEPA filtered unit.
- Mice – Male; ICR mice, 18-20 g, Harlan
  - Acceptable equivalent: Male or Female; BALB/c mice, 18-20 g, Charles River or National Cancer Institute.
- 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 (Fisher)
- BD Unopette System for leukocyte enumeration (Fisher Biomedical Cat # 02-687-40).
- 23, 25, 27, 30 gauge needles
- 1.0 ml syringes
- Potato Dextrose Agar plates (PDA)

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 male ICR 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 – **Neutropenic model**
     - Immunosuppressive drugs are made and used at the following concentrations:
       - Cortisone acetate [25 mg/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 15 minutes before using. (Note: This drug should be prepared the same day of use).
       - Cyclophosphamide [25 mg/ml] should be dissolved by the addition of sterile water at a 25 mg/ml concentration in the vial. (Note: the concentration of this drug will change in the second round of immunosuppression of the animals to 20 mg/ml, thereby changing the amount of sterile water added to the vial). Store at 4^{o}C.
       - Antibiotic Ceftazidime [50 mg/ml] dissolve by addition of sterile saline (20 ml) to 1 g 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) 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. The vial should be vortexed multiple times during injection.
- In addition, mice will begin receiving a daily dose of the antibiotic ceftazidime [50 mg/kg] subcutaneously (0.2 ml/mouse) to prevent bacterial infections due to immunosuppression 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 20 mg/ml stock to aid in calculating doses).

- Immunosuppression – **Steroid model**
  - Cortisone acetate [10 mg per mouse]: Weigh out the necessary amount of cortisone acetate and add sterile PBS containing 0.05% Tween 80. Vortex this suspension vigorously and sonicate for 15 minutes before using. (Note: This drug should be prepared the same day of use).
  - Antibiotic Ceftazidime [50 mg/ml] dissolve by addition of sterile saline (20 ml) to 1 g vial. Store at 4^{o}C.
  - At days -4, –2, 0, +2 and +4 with respect to inoculation, administer cortisone acetate at 10 mg per mouse in 0.2 ml volume subcutaneously Cortisone acetate may require a 23 or 25 gauge needle. NOTE: Cortisone acetate will also settle rapidly. The vial should be vortexed multiple times during injection.
  - In addition, mice will begin receiving a daily dose of the antibiotic ceftazidime [50 mg/kg] subcutaneously (0.2 ml/mouse) to prevent bacterial infections due to immunosuppression that is induced for the duration of the study.

- Inoculation of mice
  - Optional: On the morning of inoculation verify that the mice are leukopenic by tail vein phlebotomizing 10% of the control mice (10 µl 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 chamber in the laminar flow hood and place all mice (maximum of 70 per run) to be infected in the inhalation chamber. Tape along the edge of the door facing out, and the top to avoid

directing exiting conidia towards the hood opening. Also, plug 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 6 ml of suspension. While the apparatus is disconnected, gently agitate the chamber to redistribute the mice. (The mice will tend to huddle in 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.
- 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*). Prepare 1:10 and 1:100 dilutions of the

homogenate and streak 100 µl of each onto PDA plates in duplicate. Incubate overnight at 37°C and count the colonies the next day.

- **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.
  - 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*). Prepare 1:10 and 1:100 dilutions of the homogenate and streak 100 µl of each onto PDA plates in duplicate. Incubate overnight at 37°C and count the colonies the next day.

- <u>Disinfection of the chamber</u>
  - **Acrylic Chamber**
    - Add 6 ml of Amphyl to the Micro Mist® nebulizer, turn air on (as done previously) and run the nebulizer for 12 - 15 minutes.
    - 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 needed. 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 6 ml 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 (Appendix 1).

- 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, whichever is shorter.

7. Attachments

   Appendix 1. Madison Chamber Decontamination

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 post-infection	Calculated Range 95% CI	Mean % DOD ± S.E	Calculated Range 95% CI	Mean % 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.

Sheppard DC, Graybill JR, Najvar LK, Chiang LY, Doedt T, Kirkpatrick WR, Bocanegra R, Vallor AC, Patterson TF, Filler SG. Standardization of an experimental murine model of invasive pulmonary aspergillosis. Antimicrob Agents Chemother. 2006 Oct;50(10):3501-3.

10. History

Version 1.00. Original

Version 1.10. Revisions made to text for purposes of clarification and uniformity. Figures and Appendix 1 added.

11. Examples of Deliverables

N/A

Figure 1. Acrylic Inhalation Chamber Apparatus for Aspergillosis

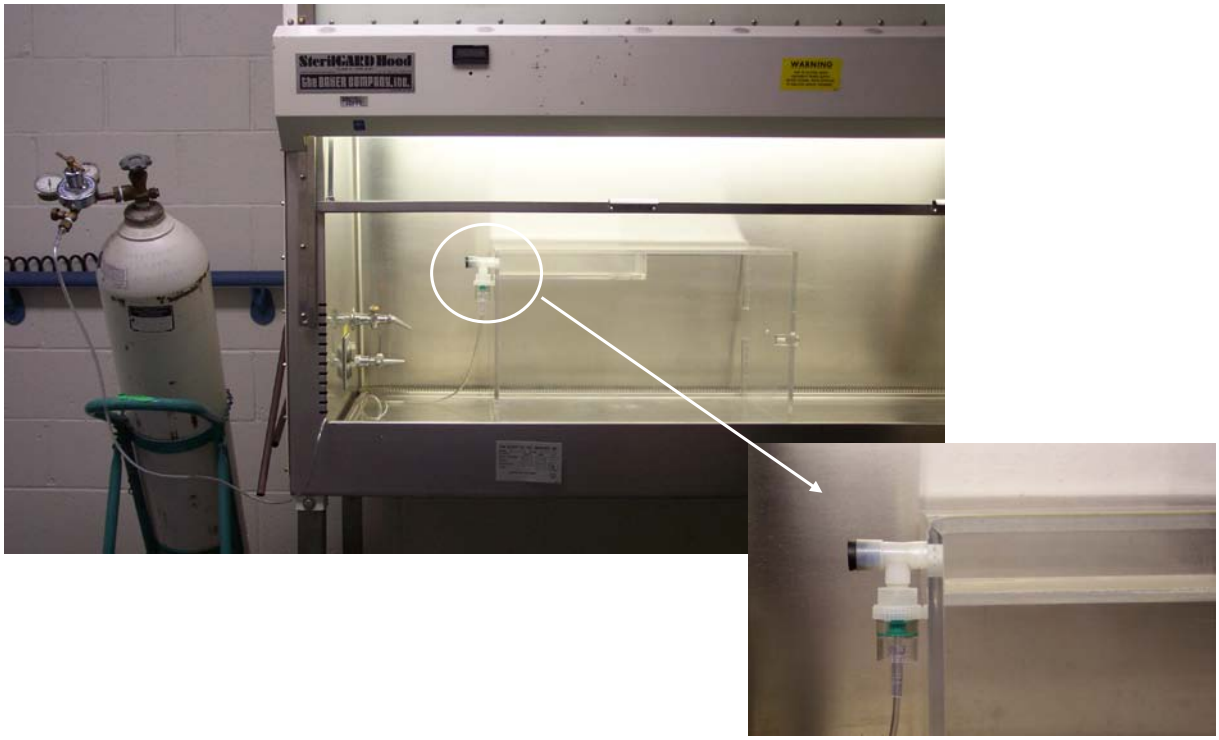
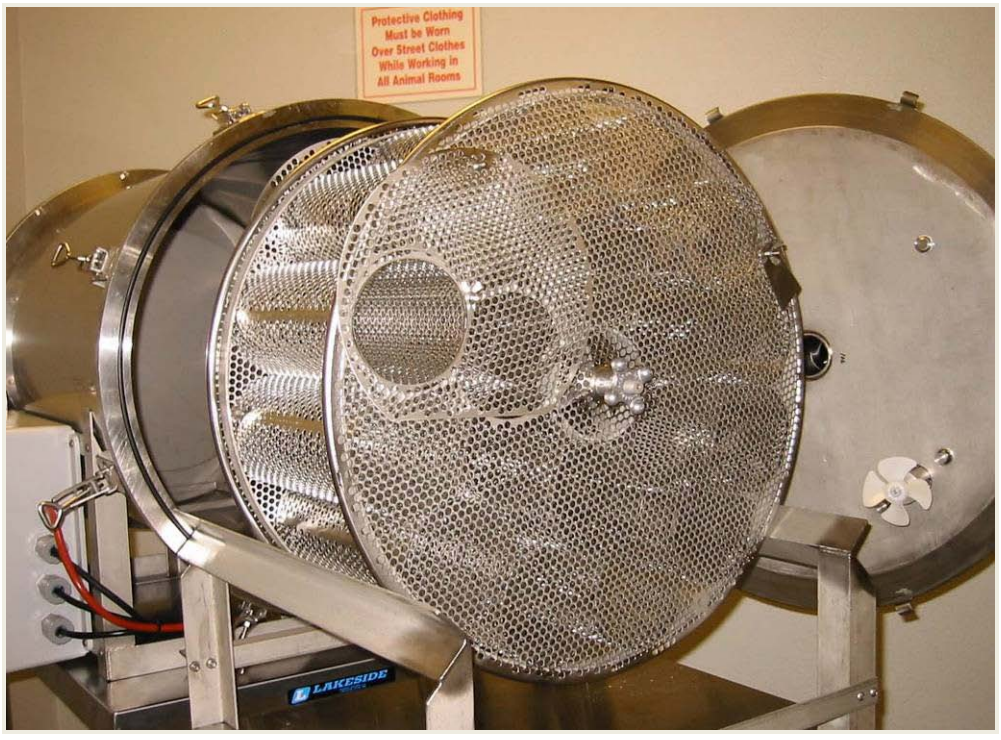


Figure 2. Madison Inhalation Chamber Apparatus for Aspergillosis



APPENDIX 1. Madison Chamber Decontamination

Madison Chamber Decontamination

- **Refer to Figure A**
  - Attach the Certek model #1414RH as shown in Fig 1.
  - Add 10 – 11 g paraformaldehyde to the metal vessel labeled "FORM"
  - Add 10 – 11 g Ammonium Carbonate to the metal vessel labeled "NEUT"
  - Add 15 – 20 ml H_20 to the vessel so labeled
- **Refer to Figure B**
- Set Timer to 4 hours (lower left of panel)
- Set Toggle switch to Humidity (lower right of panel)
- Turn unit "ON" (upper left of panel)
- Press reset – then press START (top center of panel)
- Certek unit will run automatically until finished (Sequence Complete light) OR power fails (Power Loss light) both on center top of panel.
  - If Power loss light is illuminated, press RESET and then START to complete the cycle
  - If Sequence Complete light is illuminated, turn unit off and reattach hoses (as indicated by dashed lines)

Certek model #1414RH

For full information, visit the company website:
http://www.certekinc.com/generators.html

- **General Operating Protocol - All Models**
- **The space to be decontaminated is measured and the volume calculated in cubic feet. The space is then sealed. The relative humidity inside the space is measured or sensed, depending upon the model. The CERTEK Generator/Neutralizer generator is then connected to the space with the appropriately sized connections. The humidifier, formaldehyde, and neutralizer canisters are then loaded with the proper amount of material as calculated using the formulas in the operating manual or the amount determined by other evaluations. The generators are then programmed with the operating sequence choices as determined by the operator and then started by pushing the "Start" button. The space is then conditioned to the proper relative humidity. After this is accomplished, the generator starts formaldehyde insertion. The amount of air required to insert the formaldehyde/neutralizer is circulated through the generator when the insert sequences are active. After the formaldehyde insertion cycle, the generator holds for a preselected contact time (infinitely selectable). After the contact time, the generator automatically begins the neutralizer insert cycle, inserting the neutralizing gas into the space. At the conclusion of this cycle, a preset one hour neutralizer contact cycle begins. When this is complete, the space may be reopened. The product of the reaction between the formaldehyde and the neutralizer is a white powder, which has a slight "fishy" odor. For safety, the space should be ventilated and air samples taken prior to occupancy. Built-in safety features in all of the CERTEK generators permit completely automatic operation without the necessity of a technician's constant attention.**

Figure A

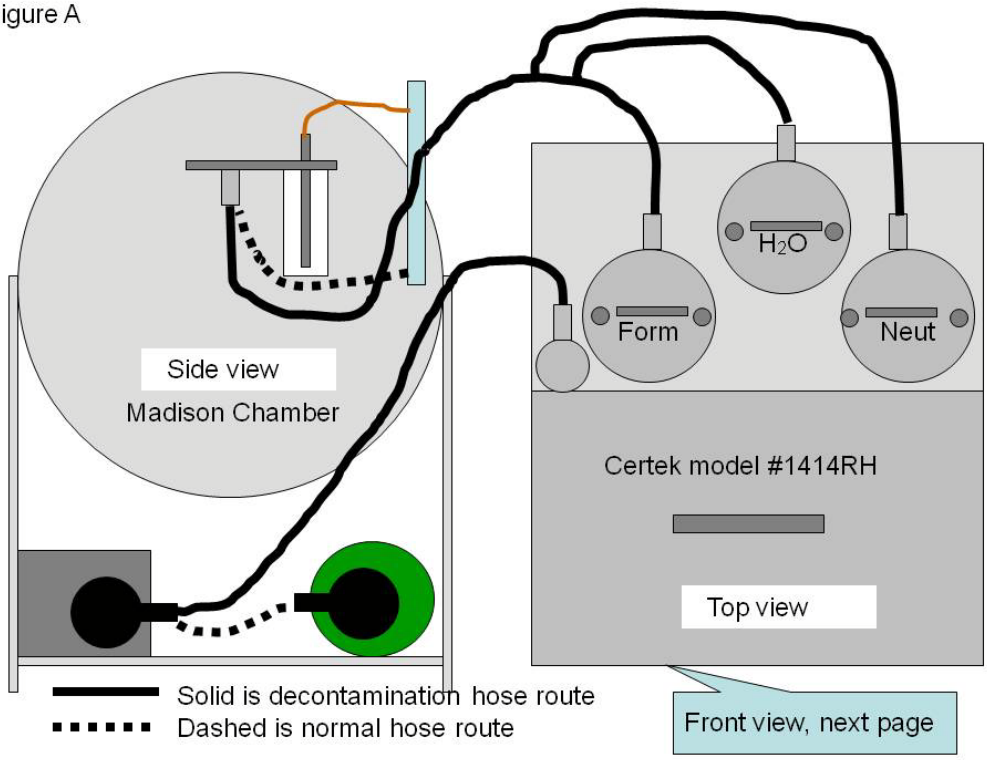


Figure B