

Standard Operating Procedure for Guinea Pig Inhalational Pulmonary Aspergillosis

1. Purpose

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

2. Scope

This SOP and will provide sufficient information to infect guinea pigs 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 guinea pigs.

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, (Cytosan, Mead Johnson, supplied as a 500 milligram vial)
 - Ceftazidime (Tazicef, Glaxo Smithkline, supplied as a 1 gram vial)
- Inhalation Chambers
 - Acrylic chamber (2ft.2in x 1ft.2in x 1ft 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.
- Guinea Pigs – Male Hartley guinea pigs 450 – 550 g, Charles River
- 10% Bleach
- Sterile water [Acceptable equivalent: Sterile normal saline]
- 70% ethanol [for cleaning Madison chamber]

New Animal Models for Invasive Aspergillosis (IA)

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Version 1.10

- Certek Formaldehyde Generator/Neutralizer (Certek) [for decontaminating Madison Chamber]
- paraformaldehyde prills (JT Baker Cat # S898-07) [for decontaminating Madison chamber]
- ammonium carbonate (Sigma-Aldrich Cat # A9516) [for decontaminating 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).
- Needles 23, 25 gauge needles
- Syringes, 10 ml, 3ml, 1ml
- 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.*
- Guinea Pigs
 - Use male Hartley guinea pigs 450 – 550 g. Each experiment should include 4 to 8 guinea pigs per treatment or control group and a comparable group of uninfected controls. An additional 1-2 guinea pigs will be sacrificed 1 hour post infection from each individual run of the chamber to confirm the delivered inoculum.
- Immunosuppression Regimen
 - 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°C.
 - Antibiotic Ceftazidime [50 mg/ml] dissolve by addition of sterile saline (20 ml) to 1 g vial. Store at 4°C.
 - At day –2 prior to inoculation, administer cortisone acetate [250 mg/kg] subcutaneously (approximately 5 ml / guinea pig) and cyclophosphamide [250 mg/kg] intraperitoneally (approximately 5 ml/guinea pig) to all the guinea pigs. A 25 gauge needle will work

for the cyclophosphamide, but cortisone may require a 23 gauge needle. Cortisone acetate will also settle rapidly, and it should be vortexed multiple times during injection.

- In addition, guinea pigs will begin receiving a daily dose of the antibiotic ceftazidime [50 mg/kg] subcutaneously (0.5 ml/guinea pig) to prevent bacterial infections due to immunosuppression that is induced for the duration of the study. Injection sites should be alternated with subsequent injections.
- On day +3 post infection, the immunosuppression regimen should be repeated using the same concentration of cortisone acetate [250 mg/kg]. However, the concentration of cyclophosphamide is 200 mg/kg (prepare a 20 mg/ml stock to aid in calculating doses).
- Inoculation of Guinea Pigs
 - Optional: On the morning of inoculation verify that the guinea pigs are leukopenic by saphenous vein phlebotomizing control animals (0.7 ml per guinea pig, one half of micropipette capillary tube) and counting neutrophils using the Unopette® system. Do not bleed guinea pigs to be infected – this increases mortality. The leukocyte count should be <1000.
 - **Acrylic Chamber** (optional inhalational infection chamber)
 - Place the inhalational chamber in the laminar flow hood and the compressed air cylinder to the Micro Mist® nebulizer which in turn is connected to the inhalation chamber by tygon tubing. Make sure to seal all connections with several layers of parafilm so as to avoid any leaks. Place up to 8-9 guinea pigs into the chamber per run. Seal the chamber with tape along the edge of the door facing out and the top to avoid directing exiting conidia towards the hood opening. Plug the hole in the 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 reservoir (or acceptable equivalent) close and begin to run air

- through the nebulizer at 100 kPa until the nebulizer begins to splutter, usually about 13-15 minutes.
- Turn off the compressed air and refill the nebulizer reservoir with an additional 6 ml of the conidial suspension. At this time gently rock chamber to redistribute the guinea pigs. (The Guinea pigs will tend to huddle in the chamber).
 - Reconnect the nebulizer and run at 100 kPa until it splutters (approximately 30-35 min.) and stops delivering aerosol. Turn off compressed air at this point and leave the guinea pigs in the chamber for a total exposure time of 1 hour from the beginning of the run.
 - After 1 hour, open chamber and transfer guinea pigs to their cages with 1 guinea pig placed in a temporary cage.
 - One hour later, sacrifice one guinea pig per run to confirm the conidial delivery for that particular run.
 - Utilizing sterile technique, extract lungs (or other organs if needed) and homogenize 1 gram of tissue in 9 ml of sterile saline (refer to *Standard Operating Procedure 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 one guinea pig in each individual housing cage, within the cage rack, then place the rack into the chamber. The Madison Chamber will hold a maximum of 18 guinea pigs. Seal 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 guinea pigs from chamber to their housing cages.
 - Within 1 hour, sacrifice 1-2 guinea pigs to confirm the conidial delivery.
 - Using sterile technique, extract lungs (or other organs if needed), weigh the tissue and homogenize 1 gram in 9 ml of sterile saline
 - Assess CFU (refer to *Standard Operating Procedure 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.
 - Disinfection of the chambers
 - **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 with 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 6ml of 10% bleach should be nebulized to disinfect the channel which is not accessible for cleaning directly. The cage should then be extensively rinsed out with 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, Madison Chamber Decontamination).
- Monitoring of Guinea Pigs
 - Monitor guinea pigs daily for signs of distress
 - 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.
 - Guinea Pigs that are moribund should be euthanized humanely using approved methods such as pentobarbital overdose or CO₂ asphyxiation.

The goal should be to have virtually all guinea pigs die by euthanasia rather than by infection.

- The experiment should be continued for at least 12 days after inoculation or until all the guinea pigs 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 counts <1000.
- This model consistently produces 100% mortality utilizing either the acrylic or Madison chamber aerosol challenge. For validity, the length of survival of control infected guinea pigs utilizing this protocol should fall within the indicated ranges:

6.76 ± .18 (n=17) Acrylic chamber (Mean Day of Death ± SE)

8.09 ± .23 (n=23) Madison chamber (Mean Day of Death ± SE)

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.

Vallor AC, Kirkpatrick WR, Najvar LK, Bocanegra RC, Kinney MC, Fothergill AW, Herrera ML, Wickes BL, Graybill JR, Patterson TF. Assessment of Pulmonary *Aspergillus fumigatus* Tissue Burden by Quantitative PCR, Galactomannan EIA and Quantitative Culture in Guinea Pigs. *Antimicrob Agents Chemother.* 2008 Jul;52(7):2593-8.

10. History

Version 1.00. Original

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

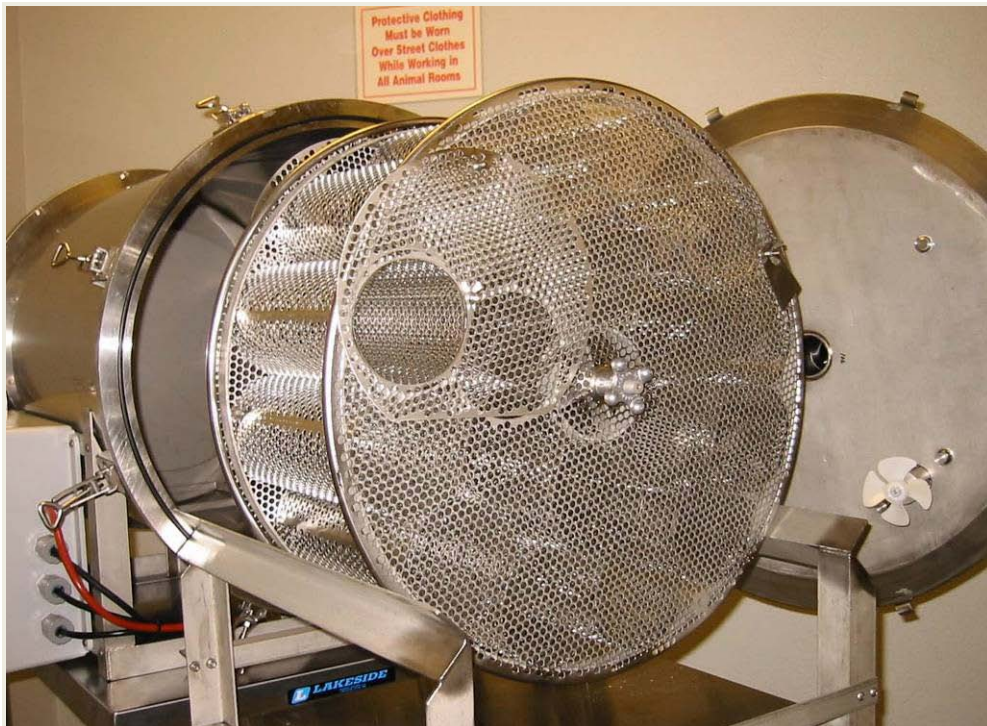
11. Examples of Deliverables

N/A

Figure 1. Acrylic Inhalation Chamber Apparatus for Aspergillosis



Figure 2. Madison Inhalation Chamber Apparatus for Aspergillosis



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 H₂O 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.**

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Figure A

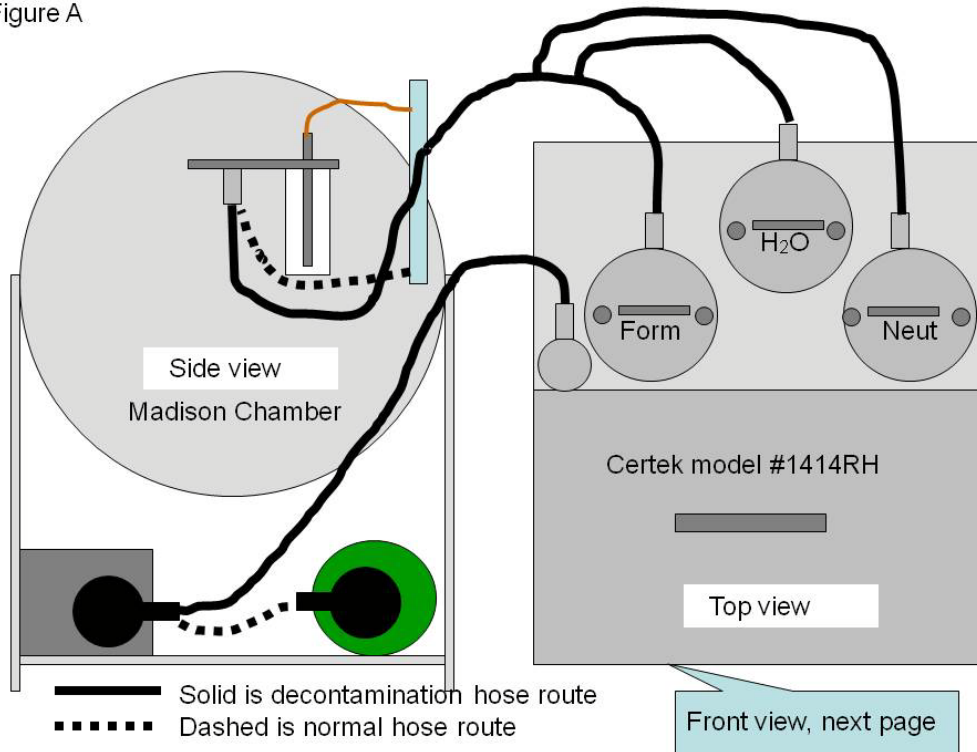


Figure B

