

Standard Operating Procedure for Preparation of *Aspergillus fumigatus* Test Strains for Inhalational Pulmonary Aspergillosis Animal Studies

1. Purpose

This Standard Operating Procedure (SOP) will provide information necessary for the uniform completion of *Aspergillus fumigatus* inoculum preparations for use in infecting laboratory animals with experimental pulmonary aspergillosis.

2. Scope

This SOP will provide sufficient information to allow preparation of a sufficient volume of concentrated *Aspergillus fumigatus* conidia (around 10^9 to 10^{10} CFU/ml) for use in either the Madison or Acrylic inhalation chambers..

3. Definitions.

“Inoculum preparation” will mean to create 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

- Method A. (preferred)
 - Sterile phosphate buffered saline supplemented with 0.2% v/v Tween 80
 - RC5B High Speed Centrifuge (Sorvall)
 - Sorvall SS-34 rotor
 - Sterile Oakridge tubes
 - 50 ml sterile polypropylene centrifuge tubes with flat top rim seal caps
 - Parafilm
 - Sabouraud dextrose agar plates
 - Hemacytometer
- Method B. (alternative)
 - Sterile normal saline supplement with 0.2% Tween 80
 - Beckman Model TJ-6 Table-top Centrifuge or equivalent
 - Sterile disposable plastic inoculation loops (Fisher)
 - Potato dextrose agar plates
 - Parafilm
 - 50 ml sterile polypropylene centrifuge tubes with flat top rim seal caps
 - 5 ml sterile snap top polycarbonate tubes
 - Glass wool (2 x 2 piece) inserted into a 30 ml sterile glass syringe and sterilized as a unit (2)
 - Hemacytometer
 - Amphyl (Revco) [Acceptable equivalent: Vesphene (Steris) or Decon (Decon Labs)]

6. Procedure

• **Preparation of inoculum**

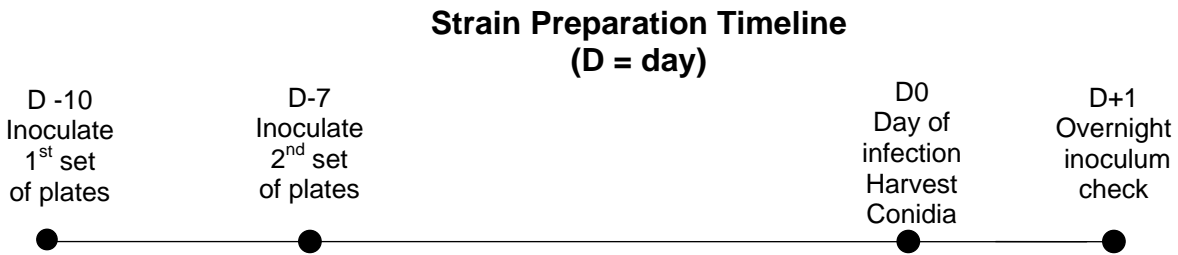
○ Method A. (preferred)

- All manipulations should be performed at room temperature in a laminar flow hood or biological safety cabinet, with disposable hospital pads lining the work area unless otherwise indicated.
- Ten days before the planned day of infection inoculate 10 Sabouraud dextrose plates for each planned run (or acceptable equivalent medium) with 10 µl of test strain (i.e. *Aspergillus fumigatus* 293) conidial stock suspension pipetted to the center of the plate. Spread the inoculum all over the surface of the plate with a sterile loop. Seal inoculated SAB plates with parafilm, invert and grow at 37°C until the day of infection.
- Note: Seven days prior to the planned day of infection, inoculate an additional 5 Sabouraud dextrose plates with the test strain to ensure contaminant-free plates of the test strain are available for harvesting conidia in the event the first 10 are contaminated or there are problems with the growth of the strain.
- On the day of infection harvest conidia with PBS + 0.2% Tween 80 (PBST) (or acceptable equivalent) by pipetting and aspirating multiple times across the surface of the plate. Use multiple washes of 8ml PBST per plate, repeatedly washing the culture surface until conidia become suspended and the liquid is dark green. This takes time (approximately 8-10 min.) as the conidia tend to float on the plate. Avoid touching the culture with the pipette as this increases the yield of hyphal fragments. Use a total of 80 ml PBST to harvest from four well-conidiated plates.
- Collect the PBST plus conidia in two high speed Oakridge centrifuge tubes, and concentrate by high speed centrifugation (we use 15,000 rpm [^ravg 17,000 X g] for 10 minutes, at 4°C no brake in a Sorvall SS-34 rotor)
- After the spin, decant the supernatant from both tubes into a sterile 100ml flask, leaving approximately 5ml volume in one tube. Reserve the supernatant for re-spinning in case insufficient numbers of conidia were isolated. Re-suspend the pellet in the 5ml volume by vortexing.
- Pipet out the suspended conidia and add it to the second centrifuge tube containing only the conidial pellet and no supernatant. Rinse the first tube with 5ml of the reserved supernatant, add to the second tube (total volume 10ml) and resuspend the second pellet. Vortex vigorously but do not sonicate. Count a 1:1000 or 1:10,000 dilution in PBST with a hemacytometer for a target inoculum of 1-2 x 10⁹ conidia/ml.

- Confirm inoculum viability by serially diluting an aliquot of the inoculum in PBST. Prepare, three serial 1:100 dilutions of the stock, followed by plating 0.05 ml on Sabouraud dextrose agar in triplicate. This should yield approximately 50 colonies per plate for a target inoculum of $1-2 \times 10^9$ conidia/ml. Incubate plates sealed with parafilm at 37°C overnight and count the colonies the next day.
- Conidial stock should be stored at 4°C until use.
- Method B. (alternative):
 - All manipulations should be performed at room temperature in a laminar flow hood or biological safety cabinet, with disposable hospital pads lining the work area unless otherwise indicated.
 - Ten days before the planned day of infection inoculate 40 potato dextrose agar plates (PDA) per planned run with 10 µl of test strain (i.e. *Aspergillus fumigatus* 293) conidial stock suspension pipetted to the center of the plate. Seal inoculated PDA plates with parafilm, invert, and grow at 37°C until the day of infection.
 - Note: Seven days prior to the planned day of infection, inoculate an additional 10 PDA plates with the test strain to ensure contaminant-free plates of the test strain are available for harvesting conidia in the event the first 40 are contaminated or there are problems with the growth of the strain.
 - On the day of infection harvest conidia with sterile saline + 0.2% Tween 80 (NaCl-T) by pipetting 10 µl to the surface of a well-conidiated plate. Scrape the entire plate with a sterile disposable plastic loop ensuring that you only scrape the surface and not dig into the agar or scrape too vigorously as to spill buffer outside of the plate and reduce hyphal contamination.
 - Aspirate the 10ml of NaCl-T + conidia from the plate and decant into a 50 ml conical tube. Add an additional 10 ml of NaCl-T of wash to the surface of the plate and combine the washes in the same conical tube.
 - Repeat as necessary for the number of plates at hand. A total of 20-25 plates will normally be needed to yield 10^9 - 10^{10} CFU/ml per one run of either the Madison or the acrylic chamber.
 - Collect and concentrate conidia in 50 ml conical tubes by centrifugation (in Beckman Model TJ-6 Table-top Centrifuge with a Beckman TH-4 swing bucket rotor) at 2500 rpm [$\text{avg } 1092 \times g$] for 10 minutes at room temperature.
 - Discard supernatant into a large beaker (containing amphyl disinfectant) taking care not to disrupt pellet (the pellet will not be very compact). Leave about 3 ml of supernatant in one tube and vortex to re-suspend pellet. Repeat with the second tube and

combine the re-suspended conidia with the contents of the initial tube. Wash tube 2 with 3ml of NaCl-T and add this to tube 1 and discard tube 2. Repeat with subsequent tubes until tube 1 reaches a volume of 40ml and start with a new tube. Repeat until the number of tubes are less than ½ the number of what you started. (You may also use a large sterile flask to collect all the conidia and washes in one container at this time only).

- Place the autoclaved glass wool/syringe unit in a 50 ml conical tube. Pour the entire conidial solution through until the tube is filled (approximately 40ml). Replace tube with a new one and repeat. (This is done to filter out any hyphal fragments that may be present). If necessary, replace the glass wool/glass syringe unit with another if the flow through begins to slow or is blocked.
 - Concentrate conidia by centrifugation at conditions previously described and combine all pellets into one tube for one last centrifugation.
 - Re-suspend the pellet in 30 ml of NaCl-T. Vortex vigorously.
 - Make 1:10,000 and 1:100,000 dilutions of the conidial stock in NaCl-T.
 - Count the dilutions on a hemacytometer to determine CFU/ml
 - Adjust the concentration of the stock with NaCl-T to yield 10^9 - 10^{10} CFU/ml, based on the needs of the specific inhalational chamber being used. See preparation notes, below.
 - Conidial stock should be stored at 4°C until use. [Conidial stock may be used up to 24 hours after it was initially prepared. However, conidia should be recounted prior to infection to re-confirm concentration.]
 - Confirm inoculum numbers by plating 100µl the following dilutions: 10^6 , 10^7 and 10^8 for an acrylic chamber run **OR** 10^7 , 10^8 and 10^9 for a Madison Chamber run on PDA plates overnight at 37°C.
- **Preparation of final inoculum for particular chambers:**
 - Acrylic chamber:
 - Prepare a final inoculum consisting of at least 13 ml of 1×10^9 conidia per ml in PBST (sterile saline + Tween 80). Note that each run will require 12 ml for infection for a single 1 hour run of the chamber.
 - Madison chamber:
 - Prepare a final inoculum consisting of 20 ml of 1×10^{10} conidia per ml in PBST (sterile saline + Tween 80). Note that each run will require approximately 12-15 ml for infection for a single 1 hour run of the chamber



7. Attachments

N/A

8. Deliverables

For the purposes of this SOP, two similar deliverables are possible. First, for the Acrylic chamber, is the preparation of a final inoculum consisting of at least 13 ml of 1×10^9 *Aspergillus fumigatus* conidia per ml in PBST or sterile saline + Tween 80. Each run will require 12 ml for infection for a single 1 hour run of the acrylic chamber

The second choice, specific to the Madison chamber will be a final inoculum consisting of 20 ml of 1×10^{10} conidia per ml in PBST or sterile saline + Tween 80. Each run will require approximately 12-15 ml for infection for a single 1 hour run of the Madison chamber

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

New Animal Models for Invasive Aspergillosis (IA)

NIH-NIAID-N01-AI-30041

Version 1.00

