Standard Operating Procedure for Animal Tissue Homogenization

1. Purpose

   This Standard Operating Procedure (SOP) will provide information necessary for the uniform completion of tissue homogenization of organs harvested from laboratory animals infected with experimental pulmonary aspergillosis and will provide sufficient information to allow determination of colony forming units of *Aspergillus* within these tissues.

2. Scope

   This SOP will encompass homogenization of organs from mice and guinea pigs and will provide two functionally equivalent methods for homogenization of the tissues derived from these model animals.

3. Definitions.

   "Homogenization" means to prepare uniform dilution of a known quantity of tissue suspended within a known quantity of a suitable diluent and uniformly macerate or crush the tissue in such a manner as to disperse minute fragments of tissue evenly throughout the mixture.

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

   - **Materials and Reagents: Method A. Tissue Grinder**
     - Weigh paper
     - Petri dish
     - Wheaton® Potter-Elvehjem Tissue Grinders [Grinding Chambers #358039 10 ml (for mice) and #358054 30 ml (for guinea pigs) with Teflon® pestles ¼" diameter stainless steel rod] (Millville, NJ)
     - Amphyl (Revco) [Acceptable equivalent: Vesphene (Steris) or Decon (Decon Labs)]
     - Tissue Grinder: RW16 Basic S1 Overhead stirrer, 15 V 50/60 Hz (IKA® works Inc., Wilmington NC)
     - Snap top tubes (5ml, 12 x 75 mm) (BD Falcon #352054)
     - Petri dish
     - Homogenization buffer (sterile saline, gentamicin [0.8 µg/ml] chloramphenicol [0.05 mg/ml] (Sigma-Aldrich).
     - Parafilm
     - Potato Dextrose Agar plates (PDA)
   - **Materials and Reagents: Method B. Whirl Pak Bag**
     - Whirl Pak Bags® (Fisher Scientific, Pittsburgh, PA)
     - Sabouraud dextrose plates (SAB)
     - PDA plates
     - Sabouraud dextrose agar

- Petri dish
- Sterile saline
- chloramphenicol (Sigma-Aldrich)
- Sharpie style pen

6. Procedure
   - ***NOTE: Due to the nature of working with infected animal tissues, all procedures described in this SOP should be performed in a biohazard laminar flow hood with proper caution. Hood bottom should be lined with absorbent pads to soak up any animal tissue spillage or aerosol induced by any of the procedures described (i.e. Whirl Pak Bag seepage or breakage). Disinfectant (70% alcohol or Amphyl®) should be accessible inside the hood at all times.***
   - **Method A. Tissue Grinder homogenization**
     - Using sterile technique, freshly harvested organs (placed on Petri dishes) are individually weighed, weights recorded and processed as described:
       - **Guinea pig:** One gram of each organ is extracted (spleen will be less than one gram) and placed in a 30 ml Wheaton® glass homogenization tube containing 9 ml of Homogenization buffer.
         - ***Note****: Due to the difficulty of homogenizing the lung tissue, roughly chop the tissue before placing into the tube with the buffer prior to homogenization.*
       - **Mice:** Entire organ is placed in a 10 ml Wheaton® glass homogenization tube and bring up entire volume (tissue + buffer) to a total of 2 ml (add 2 ml of buffer to an empty 10 ml Wheaton tube and use this volume as your guide) with homogenization buffer.
     - Any tissue left over is sterilely placed in a Whirl Pak bag® labeled with study number, animal identification, date of extraction, and name of organ. Bags are frozen and stored at -70°C.
     - An appropriate sized pestle is placed in the homogenization tube containing the organ and buffer. Organ is homogenized using the tissue grinder: RW16 Basic S1 Overhead stirrer, (IKA® works Inc., Wilmington NC)] set at a speed of approximately 1300 rpm or a setting of 8 on the instrument scale. Homogenize the tissue by going up and down the tube with the pestle twice only so as to keep the state of homogenization of each organ uniform from sample to sample.
     - Based on the volume, pour the homogenate into a 5 ml snap top tube for mouse homogenates, or a 15 ml conical tube for guinea pig homogenates.
     - Label PDA plates and snap top tubes with animal number, organ, and dilution (i.e. GP 1, lung, 0 or -1 or -2).
     - Make appropriate dilutions (1:10, 1:100, 1:1000) in 900 µl of homogenization buffer and plate in duplicate (100 µl of the same dilution on each ½ of a single PDA plate).

- Place parafilm sealed plates at 37°C for 24-36 hours and count colonies from the different dilutions. Calculate CFU/gram of organ.

- **Method B. Whirl Pak Bag technique**
  - Murine tissues
    - In sterile fashion, dissect tissues (for lung, do not include heart) and weigh them in Petri dish. Place tissue in Whirl Pak Bag and add 5 ml of sterile homogenization buffer.
    - Close bag expressing as much air out as possible (leaving approximately 4 cm of length from the point at which the bag is secured shut to the tissue to allow for "crushing" of the tissue.
    - Utilizing a large sharpie pen, roll it toward the bottom of the bag over the tissue being careful not to rupture the bag. Crush the tissue in this fashion with approximately 25 crush strokes. Attempt to take the parenchymal tissue off of the connective tissue as you crush the specimen.
    - Pour homogenates into snap top tubes and make dilutions.
    - Plate 100 µl of concentrated specimen in duplicate on PDA plates
    - ***NOTE#1: If the bag should rupture, then transfer the contents to a new bag rinsing the original with an extra 5 ml of saline. Plating should be done with usual volume 100 µl, but calculations revised to reflect the change in volume.***
    - ***NOTE#2: The number of rolling crush strokes needed to homogenize the tissues will vary by tissue type. Lungs, for instance will require more strokes than will kidneys or brain. Uniformity of the homogenate is the goal.***
  - **Alternative Plating Method: Overlay Technique**
    - Dilute specimen 1:10 (1 ml specimen to 9 ml of Sterile saline) and plate 1ml of this dilution on a Sabouraud dextrose plate.
    - Overlay plate with SAB agar supplemented with 0.05mg/ml chloramphenicol and swirl 10-12x clockwise and 10-12x counter clockwise. Allow agar to solidify.
    - Incubate overnight at 37°C and count colonies early in the morning (due to possible overgrowth of fungus on the plates).
    - After counting, leave the plates an additional 24 hours at room temperature to finalize counts.
    - Calculate the CFU per mouse/per gram of tissue.

7. Attachments

   N/A

8. Deliverables

   Two types of deliverables are possible. First, an accounting of colony forming units per gram of tissue examined is expected. Second, aliquots of these homogenates should be prepared and frozen (-20°C) for reference / experimental purposes.

9. References

Graybill JR, Kaster SR. Experimental murine aspergillosis. Comparison of amphotericin B and a new polyene antifungal drug, SCH 28191.Am Rev Respir Dis. 1984 Feb;129(2):292-5.

Patterson TF, George D, Ingersoll R, Miniter P, Andriole VT. Efficacy of SCH 39304 in treatment of experimental invasive aspergillosis. Antimicrob Agents Chemother. 1991 Oct;35(10):1985-8

10. History

Version 1.00. Original

Version 1.1. Revisions made to text for purposes of clarification and uniformity.

11. Examples of Deliverables

N/A