Determination of the Efficacy of Grignard Pure Using a Controlled Time Release Application in an Occupied and Unoccupied Spaces

Test Microorganism(s)

Bacteriophages

MS2 Bacteriophage ATCC 15597-B1
Phi 6 bacteriophage ATCC 21781-B1

Gram Positive Bacteria

Staphylococcus epidermidis ATCC 12228*Listeria innocua* ATCC 33090

Gram Negative Bacteria

Pseudomonas fluorescens ATCC 13525
Salmonella typhimurium ATCC 53648
Klebsiella aerogenes ATCC 51697

Mycobacteria

Mycobacterium smegmatis ATCC 607

Mold Spore

Aspergillus brasiliensis ATCC 16404

Protocol Number

ARE Lab Protocol # ARELPRO10984.10

SPONSOR

Grignard Pure LLC
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PERFORMING LABORATORY

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DATE

June 5, 2023

Protocol: Determination of the Efficacy of Grignard Pure Using a Controlled Time Release in an Occupied Space

1.0 Purpose

1.1. The purpose of this study is to evaluate the reduction of Grignard Pure™ solution against a variety of airborne pathogens in an aerosol chamber to support air sanitization for occupied space label claims.

2.0 Good Laboratory Practice

2.1. This study will be conducted in accordance with Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) regulation 40 CFR Part 160, Subpart F (160.05). Any deviations from the EPA GLP regulations will be noted in the final study report. The quality assurance team will review vital steps throughout the testing process and will sign off as they are reviewing.

2.2. If necessary, an external Quality Assurance Unit may be provided for this study. The following exceptions to EPA Good Laboratory Practice may be noted in the final study report.

2.3. A datalogger placed in the chamber monitors the air temperature and relative humidity throughout testing. The captured data from each trial will be exported and archived in the study file.

2.4. All calibration certificates for the equipment used throughout the study will be copied and then archived into the study file.

3.0 Scope

3.1. This protocol outlines the stepwise procedure for assessing the bioaerosol reduction efficacy of Grignard Pure when being used in a controlled time release manner at reducing viable microorganism concentrations in an indoor environment.

3.2. This protocol will be strictly adhered to during testing. Any deviations to the protocol will be justified and recorded in the final report for review.

4.0 Test Substance Characterization and Handling

4.1. According to 40 CFR, Part 160, Subpart F (160.105): Each batch of test substance shall be characterized as to identity, strength, purity, composition, and solubility (as applicable), and shall be documented prior to use in this assay. The stability of the test substance shall be determined prior to or concurrently with this study.

4.2. The test substance is handled as follows:

4.2.1.The test substance is stored at ambient temperature.

4.2.2. The test substance is well mixed prior to use (if applicable).

4.2.3. The test substance is handled safely in accordance with the Chemical risks stated in the SDS or by the Study Sponsor.

5.0 Lab Ware and Consumables

5.1. 200µl Micropipette + tips for dispensing 20-200µl volumes
5.2. 1000µl Micropipette + tips for dispensing 100-1000µl volumes
5.3. Sterile 1.7mL microcentrifuge tubes for sample processing and serial dilutions
5.4. Sterile 50mL conical tubes for sample processing
5.5. Sterile 15mL conical tubes for sample processing
5.6. 250mL suspension culture flask with vent cap for growth of bacterial and viral cultures & stocks
5.7. Serological Pipettor and pipettes (25mL) for dispersing PBS into the AGI-30 impingers

6.0 Lab Equipment

6.1. Test substance (Grignard Pure™) being assessed for performance.
6.2. Dispersal device used to disperse the test substance during testing
6.3. A closed, 16 cubic meter bioaerosol test chamber with negative pressure capabilities
6.4. Magnehelic differential pressure gauge to monitor the pressure within the test chamber
6.5. Calibrated mass flow meters to monitor the flow of air or vacuum air flow during sampling
6.6. 24-jet Collison Nebulizer to aerosolize the selected test microorganism
6.7. Dry powder eductor used for dispersal of mold spores
6.8. Ace Glass Inc. Impingers (AGI-30) to capture the bioaerosol samples during testing
6.9. Calibrated Scale to measure out reagents or samples as appropriate during the experiment.
6.10. SKC Biostage Single Stage Impactor used for low concentration sampling of bacteria and spores
6.11. Incubator to facilitate growth of plated microorganisms at specified temperatures
6.12. A closed, 16 cubic meter bioaerosol test chamber with negative pressure capabilities
6.13. Vortex laboratory mixer for equilibration of biological samples
6.14. Centrifuge for spinning down cultures to be used in testing
6.15. 1/3 HP Gast Rotary Vane Vacuum Pump for sampling to be done at a proper air flow
6.16. Biological Safety Cabinet
6.17. TSI AM520 for monitoring of particle concentration within the test chamber during testing
6.18. -20+/-2°C Freezer for storage of certain biological stocks, samples, and reagents
6.19. -80+/-2°C Freezer for storage of certain biological stocks, samples, and reagents
6.20. 6+/-2°C Refrigerator for storage of certain biological stocks, samples, and reagents
6.21. Steam Sterilizer for sterilization of biological buffers
6.22. Temperature and Humidity Sensor for monitoring temperature and humidity throughout the trials

7.0 Solutions and Reagents

7.1. Deionized water for reparation of PBS and other stock solutions
7.2. Tryptic Soy Broth (TSB) for growth of microorganism stocks
7.3. Tryptic Soy Agar (TSA) for plating of test microorganisms
7.4. Brain infusion broth for growth of microorganism stocks
7.5. Brain infusion agar for plating of test microorganisms
7.6. Potato dextrose agar for plating of the mold test samples
7.7. Antifoam A concentrate for reducing the foaming that occurs upon nebulization with solutions containing surfactants or other foam-inducing excipients
7.8. Phosphate buffered saline solution (PBS) growth and maintenance of stocks as well as capture of test microorganisms during sampling
7.9. Polysorbate 80 (Tween 80) as an additive to PBS to enhance the survival of microorganisms
7.10. Polystyrene Latex Microspheres for PSL device characterization

8.0 Personal Protective Equipment

8.1. Lab coats for protection of skin from contact with various solutions and reagents being used
8.2. Lab gloves for personal protection as well as reducing risk of contamination
8.3. Safety glasses for personal protection from contact with various solutions and reagents being used
8.4. Personal respirator for personal protection from contact with various aerosolized particles

9.0 Experimental Design

9.1 A general sequence of the main aerosolization procedure of the experiment is given below

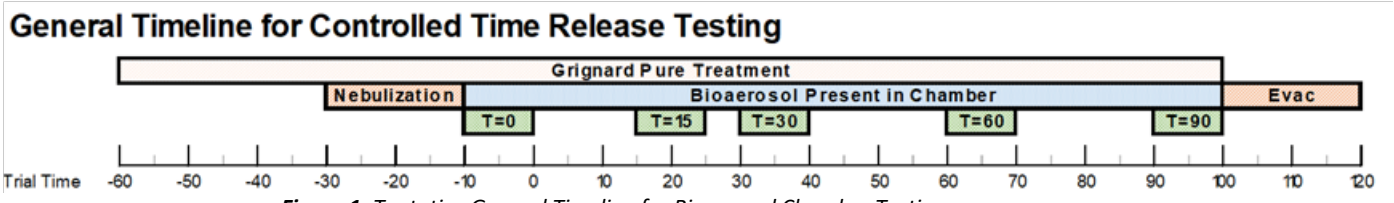

Figure 1: *Tentative General Timeline for Bioaerosol Chamber Testing*

10.0 Test System Identification

10.1. When applicable, all test and control tube racks will be labeled with the microorganism, test substance, lot identifier, and project number prior to initiation of the study and during incubation. Petri dishes will be labeled prior to initiation of the study with microorganism and project number during incubation. The test substance and usage will be traced according to SOP's extant in the laboratory.

11.0 Test Microorganisms

Test Organsims	ATCC Reference Number	Growth Medium	Incubation Parameters
MS2 Bacteriophage	15597-B1	Tryptic Soy Broth	37+/- 2°C
Escherichia coli (MS2 host)	15597	Tryptic Soy Broth	37+/- 2°C
Phi 6 Bacteriophage	21781-B1	Tryptic Soy Broth	28 +/- 2°C
Pseudomonas syringae (Phi 6 host)	21781	Tryptic Soy Broth	28 +/- 2°C
Staphylococcus epidermidis	12228	Tryptic Soy Broth	37+/- 2°C
Pseudomonas fluorescens	13525	Tryptic Soy Broth	28 +/- 2°C
Salmonella typhimurium	53648	Tryptic Soy Broth	37+/- 2°C
Klebsiella aerogenes	51697	Tryptic Soy Broth	37+/- 2°C
Listeria innocua	33090	Brain heart Infusion Broth	37+/- 2°C
Aspergillus brasiliensis	16404	Potato Dextrose Agar	23 +/- 2°C
Mycobacterium smegmatis	607	Brain heart Infusion Broth	37+/- 2°C

12.0 Maintenance, passage, and storage of test species

12.1. Obtain strains of each organism to be used in testing from a reputable source such as the American Type Culture Collection (ATCC). Every 12 months (or sooner if the quality of the culture stock is compromised or if the culture stock runs out) prepare new stock cultures of the test species from the original source. If the original stock is unavailable order a new batch of the beginning stock from the same source for replacement.

13.0 Culture Initiation

13.1. To start any culture wipe the outside of all ampules/vials of the freeze-dried cultures with a towelette prewetted with 91% isopropyl alcohol and open inside a class 2 biosafety cabinet.

For bacterial species

13.2. Open vial according to enclosed instructions that comes with the species.
13.3. Rehydrate the entire pellet with approximately 0.5 mL of the recommended broth
13.4. Aseptically transfer the entire contents to a 5-6 mL tube of the recommended broth
13.5. Additional test tubes can be inoculated by transferring 0.5 mL of the primary broth tube to these secondary tubes.
13.6. Streak a loopful of the suspension onto two recommended agar plates to test for purity of the culture.
13.7. Incubate the plates at 37°C for 24-48 hours and inspect the colonies for typical colony morphology.
13.8. If any secondary species are present on the streak plate a new stock must be ordered and prepared.

For mycobacteria species

13.9. Open the vial according to enclosed instructions

13.10. Rehydrate the entire pellet with approximately 0.5 mL of broth. Aseptically transfer the entire contents to a 5-6 mL tube of broth. Additional test tubes can be inoculated by transferring 0.5 mL of the primary broth tube to these secondary tubes.

13.11. Use several drops of the primary broth tube to inoculate an agar plate and/or agar slant.

13.12. Incubate at 37°C for 2-7 days.

For viral species

13.13. Before opening the phage vial, prepare an actively growing culture of the recommended host strain using the instructions provided in the for bacterial species section above. The host should be 16-18 hours old.

13.14. Pick one colony from the isolation plate and homogenize in 5 mL of the appropriate broth. Incubate at 37°C while shaking (160-180 rpm) until the growth reaches OD_{600} of 0.1 to 0.4.

13.15. Add approximately 1.0 mL of the recommended broth to a freeze-dried bacteriophage vial or 0.5 mL to a liquid cryovial. Infect each 5 mL culture with 100 µL of the bacteriophage. Shake at 160-180 rpm in 37°C overnight.

13.16. After 16 to 18 hours, centrifuge phage culture at 4000 g for 10 minutes. Filter the lysate with a 0.2 µm or 0.45 µm PES sterile filter. The filtrate can be stored at 4°C.

For mold species

13.17. Purchase the mold species for testing in the form of spore powder.

13.18. Suspend 100mg of spore stock into 10ml of PBS, serially dilute the sample, plate, and enumerate to determine the concentration of the spore powder stock in cfu/g.

13.19. Store the spore powder in distinctly marked containers in the 6+/-2°C refrigerator until ready for use.

14.0 Cryopreservation of cultures

14.1. Prepare a culture of the desired species with the instructions provided in the culture initiation section of the protocol.

14.2. Add 1.0ml of sterilized glycerol to the culture, shake well and incubate for 2 hours.

14.3. Mix well and aliquot into labelled vials displaying the source, name, date, passage number and lot number. Store Species for no longer than 12 months.

15.0 Test Chamber Specifications

15.1. The test chamber is constructed of 304 stainless steel with three viewing windows and an air-tight lockable chamber door

15.2. The internal chamber dimensions are 9.1 ft x 9.1 ft x 7 ft, with a displacement volume of 579 cubic feet or 16,000 liters.

15.3. The chambers internal environment is monitored through internal sensors for relative humidity and temperature. The internal sensors log all data collected to be exported at any time.

15.4. For air sampling, the chamber is equipped with four 3/8-inch diameter stainless steel probes that are positioned 18" from the nearest sidewall.

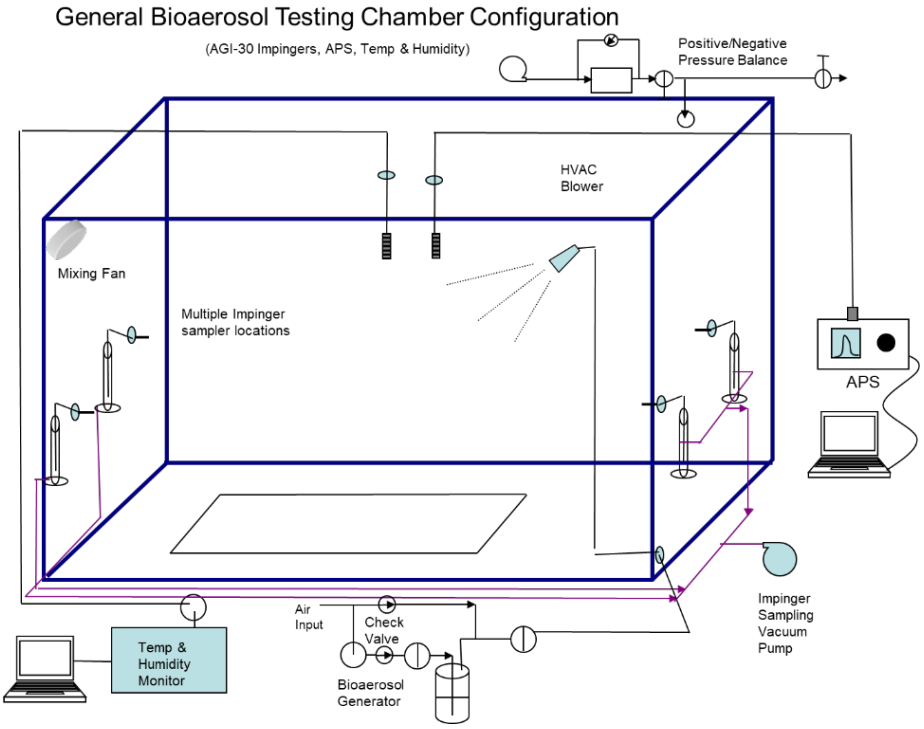

Figure 2: *General bioaerosol testing chamber configuration of the 16m³ chamber used in the testing.*

16.0 Test Requirements & Bioaerosol Chamber Set-Up

16.1. Bioaerosol testing will be conducted using a dynamically balanced bioaerosol chamber with integrated decontamination systems.

16.2. For air sampling, the chamber is equipped with four 3/8-inch diameter stainless steel probes that are positioned 18" from the nearest sidewall. (Figure 1).

16.3. Temperature and relative humidity will be monitored throughout each trial by a remote sensor to ensure the proper levels are maintained. The levels will be recorded in the lab notebook at the beginning of each bioaerosol sample.

16.3.1. Temperature range: 25°C +/- 3°C

16.3.2. Relative humidity range: 30-40%

16.4. The reservoir of the device will be filled with Grignard solution to the fill line on the reservoir before each trial

16.5. The device will be turned on to the proper settings using the work instructions provided by Grignard Pure, LLC.

16.6. The device will be primed by allowing the device to run on the selected controlled time release settings for 20 minutes to allow for lines to fill with Grignard Pure solution.

16.7. SOP will be provided by Grignard for operation of the dispersal device

16.8. After priming the device, it will be placed in the center of the chamber, 3 ft. off of the ground on a stainless-steel table.

16.9. A Collison 24-jet nebulizer will be filled with approximately 40 mL of prepared biological stock and operated at 40 psi for a period of 20 minutes.

16.10. The nebulization stock for each trial will be prepared according to the Preparation of Test Organisms section of this protocol. Once the culture is resuspended in PBS 100µL of antifoam A will be added to the stock to reduce the shearing of the microorganism observed during nebulization

16.11. The AGI-30 impingers will be filled with 20 mL of sterilized PBS with an addition of 0.005% v/v Tween 80 for bioaerosol collection.

16.11.1. The addition of Tween 80 will be used in order to increase the impinger collection efficiency and de-agglomeration of all microorganisms.

16.12. The chamber mixing fan, located in the corner of the chamber under the nebulizer port pointing towards the far corner ceiling, will be turned on for the duration of the trial to ensure a homogenous mixture of bioaerosol is present throughout the chamber

16.13. Blank settling glass slides will be placed in the chamber prior to the initiation of the trial to assess the amount of settling yielded by the microorganisms in the control trial when compared to the challenge trial.

16.14. The 1” x 2” settling glass slides will be placed on a table located 4 ft. away from the dispersion device and remain in the chamber for the duration of the trial.

17.0 Soil Load

17.1. The soils used in the study will be based off the guidelines outlined in ASTM OECD 2013

17.2. The soils to be used in the aerosolization study will consist of:

17.2.1. 0.5g of yeast extract in 10ml of PBS

17.2.2. 0.5g of bovine serum albumin in 10ml of PBS

17.2.3. 0.04g of mucin extract in 10ml of PBS

17.3. All soil additives will be filtered through a 0.22um pore size syringe filter to ensure sterility.

17.4. All soil batches will be made fresh for each round of testing to avoid any effects of aging on the mixtures.

18.0 Stock Preparation Procedure

Bacterial Stock Preparation

18.1. Thaw the freezer stock for use in the trial by removing it from the freezer and running it under 40°C water until the stock is completely thawed.

18.2. Incubate 250 ml of tryptic soy broth (30g/l) containing the thawed freezer stock of the desired organism overnight at the appropriate temperature listed in the species table.

18.3. If necessary, centrifuge stock cultures for 10 minutes at 3000rpm in an LD-3 centrifuge in sterile 15 mL conical tubes, remove growth media, and re-suspended the cells in sterile PBS buffer for aerosolization.

18.4. Extract 100ul from the organism stock and serially dilute it in sterile PBS in 1.5mL microcentrifuge tubes, and then plate it on the recommended agar plates and place it in the incubator for stock concentration determination.

18.5. Streak the culture on tryptic soy agar plates to ensure no contamination was present prior to starting the trials.

18.6. Add in required prepared soil solution prior to use in testing.

Note: Test working stocks will be grown in sufficient volume to satisfy use quantities for all tests conducted using the same culture stock material.

Viral Stock Preparation

18.7. Grow the host bacterium in a similar fashion to the bacterial vegetative cells in an appropriate liquid media.

18.8. For viral cultures infect the liquid bacterial media during the logarithmic growth cycle of the bacteria with the specific bacteriophage.

18.9. Add the viral stock to the host media in an appropriate MOI to culture a viral stock of >1.0E9 pfu/mL

18.10. Centrifuge the viral stock/ host bacterium culture for 10 minutes at 4000 rpm in sterile 50 mL conical tubes, discard the spent cell pellet at the bottom of the conical tube.

18.11. On the day of use the stocks will be removed from the freezer, thawed, and resuspended in PBS and broth for nebulization.

Mold Stock Preparation

18.12. Obtain mold spores in purified bulk powder form at a concentration of 1 x 10^9 cfu/g.

18.13. Verify the bulk powder spore concentration by taking an aliquot of weighed dry powder prepared in suspension of PBS + 0.005% Tween 80 at a mass:volume ratio to obtain a concentration of 1 x 10^9 cfu/ml.

18.14. Plate this aliquoted spore suspension, prior to testing, to verify the concentration.

18.15. Streak plate the spore suspension to verify purity prior to testing.

18.16. Store the purified bulk powder in the 6+/-2°C refrigerator until ready to use.

18.17. Weigh out the appropriate amount of spores using a calibrated scale to be dispersed prior to each trial.

19.0 Neutralization Testing

19.1. Prior to the bioaerosol testing, the Grignard Pure formulation will be tested in liquid form to ensure that all reduction yielded in the trial is due to the performance in the chamber and not because of kill occurring in the impinger sample fluid.

19.2. If the test substance fails with any of the species to be tested, then an additional neutralization step must occur during sampling of the bioaerosol.

19.3. The neutralization challenge will occur at a range of 10x dilutions designed to provide a broad range of possible Grignard concentrations that could occur in an impinger sample during testing. The Grignard solution will be diluted in sterile PBS

19.3.1. Grignard chamber concentrations tested will be: 0.04mg/m^3, 0.4mg/m^3, and 4.0mg/m^3

19.4. The desired organism will be grown overnight according to the organism growth procedure prior to testing.

19.5. Prior to neutralization testing the organism will be diluted to >1.0 x10^3 pfu/cfu per ml to allow for susceptibility of the organism to be at the highest.

19.6. A 100µl sample of the organism will be placed in DI water and allowed to sit for 15 minutes at room temperature in the biosafety cabinet. After 15 minutes the sample will be serially diluted and plated on a tryptic soy agar plate and placed in the incubator.

19.7. 100ul samples of the same organism will be placed in PBS containing all of the Grignard concentrations listed above as well as normal unaltered PBS for comparison to the DI water dilution. After a 15-minute contact time in the biosafety cabinet the sample will be diluted in PBS and plated on tryptic soy agar plates and placed in the incubator.

19.8. The plates will be counted and recorded in a lab notebook for analysis

19.9. The organism will undergo testing with this procedure for each concentration of Grignard solution listed above.

19.10. Each organism that will be tested in the bioaerosol chamber will undergo this testing to ensure that no additional reduction is occurring in the liquid after microorganism capture

19.11. If the results are within 50% of each other, then the Grignard Pure formulation will be confirmed to not affect the microorganisms when in the neutralization PBS.

20.0 Grignard Pure Concentration Determination

20.1. The devices reservoir will be filled to the fill line of the reservoir with Grignard Pure prior to every run

20.2. The device will be turned on to the proper settings using the work instructions provided by the Grignard Company

20.3. The device will be primed outside of the chamber by actuating the dispersal button on the device for a minimum of two minutes.

20.4. The device will then be placed in the chamber on a stainless-steel table in the middle of the chamber 3ft. above ground level.

20.5. The TSI AM520 will be placed in the chamber 3 ft. away from the dispersal device at the same height.

20.6. The device will be shut in the chamber and dispersed on a set schedule of time determined by the study sponsor

20.7. A mixing fan will be running in the chamber to assure a homogenous mixture is achieved

20.8. After the dispersal the chamber air will be monitored by the TSI AM520 for the duration of the trial.

20.9. At the end of the trial the data will be extracted from the TSI AM520 using the work instructions provided by TSI to check for the Grignard Pure air concentration throughout the trial.

20.10. If the concentration determined by the TSI AM520 does not match the desired concentration set forth by the study sponsor the settings of the device will be adjusted and the trial will be re-run.

21.0 Bioaerosol Sampling

21.1. Either AGI-30 impingers or SKC biostage single stage impactor are used for bioaerosol collection to determine the chamber concentration during testing.

21.2. These bioaerosols are connected to the bioaerosol chamber via sample ports located at opposite corners of the chamber.

21.3. The aerosol samplers vacuum source is maintained at a minimum negative pressure of 17 inches of Hg during all sampling to assure critical flow conditions of the samplers.

21.4. All samplers are flow characterized using a calibrated TSI model 4040 mass flow meters

22.0 Bioaerosol Testing Timeline

22.1. During Grignard Pure challenge trials, prior to the introduction of bioaerosol, the Grignard Pure dispersal device will be allowed to disperse the Grignard Pure solution into the chamber air on a predetermined setting for 30 minutes to simulate a room being continuously treated with Grignard Pure.

22.2. After 30 minutes of pre-treatment with Grignard Pure the bioaerosol will be introduced into the chamber through nebulization using a 24-jet Collison nebulizer.

22.3. Following bioaerosol generation, baseline concentrations will be established for each pilot challenge test by sampling simultaneously with two AGI-30 impingers located at opposite corners of the chamber. This sample will be known as T-0. For this study T-0 will be when the first sample is taken after the nebulization of the bioaerosol.

22.4. Control trials will begin with the nebulization of the bioaerosol and will not involve pre-treatment to the room.

22.5. AGI samples will be collected for a set amount of time at intervals predetermined throughout the entire test period.

22.6. Collected impinger chamber samples will be pooled and mixed at each sample interval for each test and placed in 15mL conical tubes.

22.7. The conical tubes containing the samples will immediately be taken to a biosafety cabinet to be serially diluted in PBS and plated on tryptic soy agar plates

22.8. In between samples the impingers will be taken to the lab sink to be rinsed 6x with DI water and then dried prior to being used for the next sample

22.9. Subsequent impinger samples will be taken at various time points throughout the trial.

22.10. These samples will be enumerated for viable concentration to measure the effective viable bioaerosol reduction achieved during operation of the device dispersing the Grignard solution over time.

22.11. The settling slides will be removed from the chamber after the evacuation of the chamber after each trial. These sample will be enumerated for viable concentration to determine the change in settling between the Grignard Pure trial and the control trial

22.12. Each slide will be placed in separate individually labeled 50mL conical tubes containing 10mL of sterile phosphate buffered saline solution + tween 80 for sample extraction.

22.13. The 50ml conical tubes will be vortexed and shaken vigorously for 2 minutes prior to sample extraction

22.14. All samples will be plated in triplicate on the appropriate media over a minimum 3 log dilution range.

22.15. Plates will be incubated for the recommended amount of time at the recommended temperature and enumerated for viable colony forming units or plaque forming units (cfu or pfu) to calculate aerosol challenge concentrations in the chamber and reduction of viable microorganism

23.0 Statistical Analysis

23.1. The data will be subjected to appropriate statistical analyses for the preparation of the final report.

23.2. Analysis of the test data will include, at minimum, the average of the data sets as well as the standard deviation of the data sets.

24.0 Data Analysis/ Calculations

Calculation of % Reduction and LOG Reduction

24.1. The starting bioaerosol concentration of the challenge trial will be compared to the starting concentration of the control trial to calculate the effect of the Grignard Pure™ chamber pre-treatment. The method for nebulization and nebulization stock will remain the same from the control to the challenge trial so the concentration of the challenge trial would be

expected to be the same as the control. The change in the starting concentration seen in the challenge trial will therefore be attributed to the pretreatment of the chamber.

24.2. To determine the log reduction of the trial at each time point, the percent of viable microorganism remaining airborne in the chamber will be compared to the initial bioaerosol concentration of the uninhibited control trial. This percent remaining will then be multiplied by the log10 function to yield log reduction.

24.3. For net reduction, the natural losses from the control trial will be subtracted from the test trial to account for natural losses not caused by the test solution.

24.4. AGI – 30 impinger collection calculation:

24.4.1. Viable aerosol concentration collection (C_a) = cfu or pfu/L of chamber air.

24.4.2. Viable Impinger concentration collection (C_{Imp}) = cfu or pfu/ml

24.4.3. Sampler collection volume (I_{vol}) = X ml collection fluid (impinger), sampler dependent

24.4.4. Sample flow rate (Q_{imp}) = Y L/min, sampler dependent

24.4.5. Sample time (t) = Z minutes, test dependent.

24.4.6. For viable impinger aerosol concentration collection (C_a) = cfu or pfu/L of chamber air:

$$C_a = \frac{C_{Imp} \cdot I_{vol}}{Q_{imp}} t$$

24.5. To evaluate the viable aerosol delivery efficiency and define operation parameters of the system, calculations based on (theoretical) 100% efficacy of aerosol dissemination will be derived using the following steps:

24.5.1. Concentration of the stock suspension (C_s) pfu/ml

24.5.2. Collison 24 jet nebulizer use rate (R_{neb}) volume of biological stock dispersed at X psi air supply pressure.

24.5.3. Aerosol Generation time (t)

24.5.4. Chamber volume (V_c) = 15,993 Liters

24.5.5. Assuming 100% efficiency, the quantity of aerosolized viable particles (V_P) per liter of air in the chamber for a given biological stock concentration (Cs) is calculated:

$$V_P = \frac{C_s \cdot R_{neb}}{V_c} t$$

24.6. The aerosol system viable delivery efficiency (expressed as %) is:

$$Efficiency = \frac{C_a}{V_p} \cdot 100$$

24.7. All triplicate runs will be averaged to obtain a mean and standard deviation for each trial.

24.8. Total viable biological concentrations along with reductions over time will be Log reductions of aerosolized biologicals will be calculated and graphed for each trial.

25.0 Quality Control

25.1. Sterility Control: One plate of growth medium will be incubated alongside the test plates. All reagents will be evaluated by plating 1.0 ml of growth media and incubated alongside the test. The acceptance criterion for this control is lack of growth.

25.2. Viability Control: The growth/neutralizing media will be challenged in duplicate with <100 CFU the test bacterium, mycobacterium, mold and <100 PFU bacteriophage/ virus and incubated alongside the test to confirm the media can support the growth its low numbers.

25.3. Purity Control: A streak plate will be prepared for each test culture or host culture and incubated alongside the test to confirm the purity of the test culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism

26.0 Control Acceptance and Criteria

26.1. All sterility controls must be free of any visible growth.

26.2. Viability Control must demonstrate growth in all media with <100 PFU/plate or <100 CFU/plate.

26.3. Purity Control must demonstrate a pure culture.

26.4. Neutralization Validation: The mean number of CFU on the plate unexposed to the test substance and those on the plate exposed to the test substance must be within 50%.

26.5. Temperature and RH readings must stay within range required for the test.

27.0 Study Acceptance and Criteria

27.1. Test Substance Performance Criteria: After correction for bacterial settling and natural biological decay, the test substance must demonstrate ≥99.9% (≥3.0 $\log_{10}$) reduction in the viability of the bacterial, mold, mycobacterium, and bacteriophages over the parallel untreated controls.

27.2. If cytotoxicity is present, the virus/bacteriophage titer should be increased if necessary to demonstrate ≥ $\log_{10}$ reduction in PFU/m^3 beyond the cytotoxic level.

27.3. Baseline Acceptance criteria: The control recovery must demonstrate a minimum of 4.2 Log_{10} to a maximum of 5.0 $\log_{10}$ CFU/m^3 or PFU/m^3 at the start of the treatment for a valid test.

28.0 Retesting

28.1. If any controls or results fail to meet the criteria outlined in this study the failure will be noted and a retest will occur.

28.2. If the retest of the same species results in a failure the test will be aborted to launch an investigation to find the root cause.

28.3. Upon the results of the investigation a CAPA will be established to ensure the failure does not happen again.

29.0 Report

29.1. Results are reported and accurately and fully, in accordance with Environmental Protection Agency GLP 40 CFR Part 160.

29.2. Report will include but not limited to: Purpose, scope, background device and device pictures, organisms tested, test condition, all methods, calculations, statistical analysis, results and conclusions.

29.3. Appendices with additional data, figures and raw plates counts will be provided

30.0 Protocol Amendments and Deviations

30.1. All amendments to and/or deviations from this protocol and the reasons for such shall be documented, approved by management (as may be required), signed by the Study Director and at the study sponsor, dated and maintained with the protocol.

30.2. Any amendments to and/or deviations from this protocol will be recorded in the raw data and documented in the final report.

31.0 Record Retention

31.1. Data and records to be maintained by ARE Labs include, but are not limited to, the study protocol, amendments and deviations, test article receipt and handling, chamber temperature and humidity records, laboratory notebooks, study correspondence, and study final report.

31.2. All data, records, documents, notes, or other materials generated by the study will be collected and securely stored on site at the ARE facility.

31.3. All recording of data will be made directly, concurrently, and legibly in indelible ink. All data entries will be dated on the date of entry and signed or initialed by the person responsible for the entry. Any changes to data entries will be made to not obscure the original entries. The changes will be justified and then checked and signed by the study director and the QA representative.

32.0 References

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STUDY INFORMATION

Testing parameters to be completed by the Study Sponsor/ Representative

Test Substance Name	
Test Substance Batch Numbers	
Manufacture Date(s)	
Expiration Date(s)	
Test substance active Ingredient(s)	□ Alcohol □ Peroxide □ Quaternary Ammonia □ Sodium Hypochlorite
Test Substance Active Concentration as submitted (for Neutralization information)	
Active Ingredient Level	□ At or below Lower Certified Limit (LCL) □ At or below nominal
Test Substance Storage Conditions	□ Room Temperature □ 2-8˚C □ Other:
Test Substance Hazards	□ None Known: Use Standard Precautions □ Material Safety Data Sheet, attached □ Other:
Test Substance Dilution	□ Ready to Use (RTU) □ Dilution ratio: (e.g., 1 oz per gallon, 1:255)
Dilution to be made	□ N/A, RTU □ Dilution(s) to be tested: _____ defined as _____ + _____ (e.g. :1 oz/gallon) (amount of test substance) (amount of diluent)
Test Substance Diluent	Not applicable for RTU Product

Organic Soil Load	□ None □ 5% fetal bovine serum □ Other:
Contact time (s)	
Exposure Temperature	□ Room Temperature □ Other:
No Number of Test Carriers / Replicates	
Spray Instructions	
Neutralization / Subculture Broth	ARE Lab to determine. Sponsor authorizes pre-test neutralization confirmation assay to be conducted to determine appropriate neutralizer if need. □ Use:
EPA 40 CFR Part 160.31(d) requires testing facility management to assure that the test, control, and reference substances have been appropriately tested for identity, strength, purity, stability, and uniformity, as applicable	Identity, strength, purity, and uniformity, as applicable, of the test lots has been or will be completed prior to efficacy testing □ YES □ No ▪ Performed following 40 CFR Part 160 GLP regulations: □ Yes □ No Stability testing has been or will be completed prior to efficacy testing or concomitantly with efficacy testing: ▪ Performed following 40 CFR Part 160 GLP regulations: □ Yes □ No
Certificate of Analysis (CoA)	□ CoA for each batch provided. CoA will be appended in the final report.
Test Substance Shipment Status	□ Use test substance already present at AER LAB. □ Test substance will be shipped. Estimated arrival date, if known:
Protocol Modification	□ Testing to be performed as outlined in the protocol. □ The following protocol modifications are to be performed:
Regulatory Agency(s) that report may be submitted to	□ EPA □ Health Canada

PROTOCOLS APPROVAL

ARE Labs Author: Title: Research Scientist	Signature: Date:
ARE Labs Reviewed By: Title: Director of Operations	Signature: Date:
ARE Labs Reviewed By: Title: QA	Signature: Date:
Client Reviewed By: Title:	Signature: Date:
Client Approved By: Title:	Signature: Date: