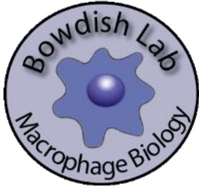

MACROPHAGE KILLING ASSAY

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BACKGROUND

This protocol is used to determine a macrophage population's ability to kill bacteria. It works by incubating macrophages and bacteria together so the macrophages can phagocytose the bacteria. The macrophages are then placed in sterile water in order to lyse them. Bacteria inside the macrophages will then be in solution, which can be plated and counted. This assay can help to quantify the macrophage population's ability to kill bacteria. *Note: assay requires a minimum of 120 minutes to be completed.

MATERIALS

- Tryptic Soy Agar, 5% Sheep's Blood, 10ug/mL Neomycin plates (must be at room temperature for the assay)
- Tryptic Soy Broth (TSB)
- PBS
- Sterile water
- Spectrophotometer
- Vortex
- Lidocaine solution (4mg/mL lidocaine-HCl, with 10mM EDTA in PBS)
- Cell lifter
- Trypan blue and hemocytometer

PROTOCOL

PART I: Preparation of *S. pneumoniae*

1. Thaw 1mL frozen aliquot of *S. pneumoniae.*
2. Add aliquot to 4mL tryptic soy broth in a falcon polystyrene tube. Take care not to shake tube excessively as increased oxygen slows the growth.
3. Incubate tube until OD_{600} is 0. 5. Use tryptic soy broth as a blank. Do not overgrow the culture.
4. Remove 1mL into a 1.5mL tube and spin at 15,000rpm for 1 minute.
   *Additional 4mL of culture can be frozen as stock at a 800uL culture:200uL 80% glycerol ratio and stored at -80°C.
5. Remove supernatant and resuspend pellet in 1mL PBS and keep on ice. 100uL of this sample will be used per macrophage sample.

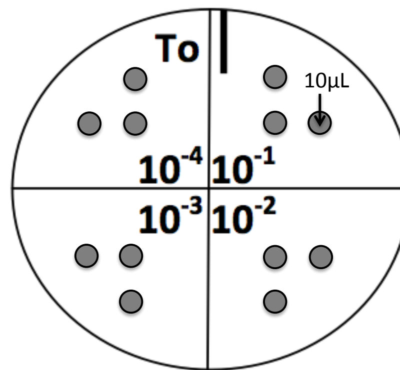
*Note: 1mL is assumed to be 1×10^8 CFU, so 100uL used later once resuspended in PBS is 1×10^7CFU

PART II: Harvesting Bone-marrow Derived Macrophages from Plates

1. Remove media and wash plate with PBS.
2. Add 10mL lidocaine solution. Place the plate on ice for 25-30 minutes.
3. Scrape the plate using a cell lifter. Check that all macrophages are lifted under the microscope.
4. Remove the solution into a 50mL conical tube and spin at 1500rpm for 5 minutes at 4°C.
5. Remove the supernatant and resuspend the pellet in 5mL PBS.
6. Add 10uL of this solution to 10uL of trypan blue and use hemocytometer to determine the number of live cells/mL.
7. Prepare solution to get 1×10^6 cells in 900uL PBS.

PART III: Starting the Killing Assay

1. Titre the *S.pneumoniae* from Part I: prepare serial dilutions of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} and on a blood agar plate divided into quarters (one for each dilution), plate 3 x 10uL per dilution.
2. Mix 1×10^6 macrophages and 1×10^7 *S. pneumoniae* (900uL and 100uL, respectively) into a 1.5mL tube resulting in an MOI of ~10.
2. Incubate the solution for 1 hour at 37°C with rotation to allow for phagocytosis of bacteria by macrophages.
3. During the incubation, label and prepare 4 tubes (for each serial dilution: 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}) for each time point (Time zero (To), 30m, 60m, 90m, 120m) with 900uL of sterile water. Prepare one blood plate per time point by dividing into quadrants (one for each serial dilution) as illustrated below.



PART IV: Time Zero Killing Numbers & Plating

1. Spin the macrophage-bacteria mix at 1500rpm for 5 minutes.
2. Carefully remove all of the supernatant to remove extracellular bacteria and resuspend in 1mL PBS and vortex.
3. Remove 100uL of the macrophage/bacteria mix and add to the 10^{-1} Time zero (T_o) tube with 900uL water in it.

4. Put the macrophage/bacteria mix back into the incubator with rotation and start the timer for 30 minutes again.
5. Complete serial dilutions removing 100uL of the 10^{-1} dilution and adding it to the 900uL sterile water for the 10^{-2} dilution, repeating this process until the 10^{-4} dilution. Vortex well and change pipette tips at each step.
6. Work backwards from the 10^{-4} to the 10^{-1} dilution, plating three 10uL drops of each dilution into its appropriate quadrant. Since working from lowest to highest concentration, there is no need to change pipette tips.
10. Leave the plate uncovered for the drops to dry. Once dry, cover and place plate upside down in the incubator (37°C with 5% CO_2) and leave overnight.
11. Repeat steps 3-10 for remaining time points (30m, 60m, 90m, 120m).

PART V: Reading Plates & Determining Killing Numbers

1. Count number of colonies resulting from each drop plated. Average the CFU's from the three drops per dilution and multiply by 10^2 and the dilution factor to get CFU/mL.
   Sample calculation: At 30m time point the 10-^{4} dilution had 28, 30, and 32 colonies. Average of 30 colonies x 10^2 x 10^4 = 3 x 10^6 CFU/mL
2. A properly performed killing assay should result in curves similar to the graph below: