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. The numbers determined from counting the plates can help to quantify the macrophage population's ability to kill bacteria.

NOTES

- This experiment should be started around 9AM in order to leave by around 5PM
- Prior to starting this experiment, tryptic soy agar & sheep's blood plates should be made and tryptic soy broth should be made. See other protocols for those details.
- See PBMC/Nasal/Sputum/Etc Macrophage Isolation for how to culture macrophages.
- A minimum of 120 minutes is needed from time zero to ending the assay, but 180 minutes is optimal.

EQUIPMENT

- Tryptic soy agar/Sheep's blood plates, minimum of 5 for a 2 hour killing assay. 1 additional plate per additional half hour of assay time.
- Tryptic soy broth
- HBSS
- Sterile water
- Trypan blue and countess slides
- 1000uL, 100uL and 10uL pipettes and tips
- Polystyrene falcon tubes
- Spectrophotometer
- Vortex
- 1.5mL Tubes
- Licocaine solution (4mg/ml lidocaine-hcl, with 10mM EDTA in PBS)
- Microcentrifuge
- 50mL conical tubes
- Serological pipettes (1mL, 5mL, 10mL)

PROTOCOL

PART I: Preparation of S. Pneumo:

1. Thaw 1mL aliquot from -80°C freezer by warming in hand.
2. Add aliquot to **4mL** tryptic soy broth in a falcon polystyrene tube.
3. Incubate tube until OD 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. Label tubes and store at -80°C.
5. Remove supernatant and resuspend pellet in **1mL HBSS**. 100uL of this sample will be used later. Keep the 1mL on ice.

Note: 1mL is assumed to be 1x10^8, so 100uL used later once resuspended in HBSS is 1x10^7

PART II: Harvesting Macrophages from Plates

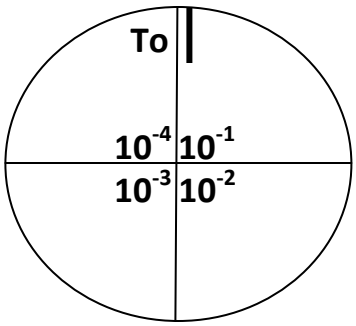
1. Remove media by tilting plate and using a serological pipette.
2. Add ~10mL lidocaine. Place the plate on ice for ~25-30 minutes.
3. Scrape the plate with a cell scraper. Be sure to cover the entire surface of the plate. Check under the microscope.
4. Remove the solution into a 50mL conical tube and spin at 1500rpm for 5 minutes at 4°C.
5. Remove the supernatant and resuspend the pellet in **5mL HBSS.**
6. Add 10uL of the solution to 10uL of trypan blue and use a countess cell counter to determine the number of live cells/mL.
7. Use the cell counter to determine how to get $1x10^6$ cells into 900uL HBSS.
   -If fewer than 1 million macrophages are available, spin the sample and resuspend in 900uL and use the whole sample.

PART III: Starting the Killing Assay

1. Plate 10uL of 10^{-5}, 10^{-6} and 10^{-7} serial dilutions of the S. Pneumo population to determine the titre.
2. Mix the $1x10^6$ macrophage and $1x10^7$ S. Pneumo solutions (900uL and 100uL, respectively) into a 1.5mL tube.
2. Incubate the solution for 1 hour at 37°C on the rotator.
3. While incubating, perform the following labeling of tubes and plates to save time:

- 5 Sets of 4 tubes should be labeled as "To, 30m, 60m, 90m, 120m" and "10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}"
  -I.e. (To -1, To -2, To -3, To -4, 30m -1, etc)

- 5 Plates should be labeled following the diagram below. Each plate also requires name, bacteria on plate, date.



- **IMPORTANT:** Be sure to continue quadrant divider onto the side of the plate and make the top line is a double line so if the lid is rotated, the samples will not be mixed up.

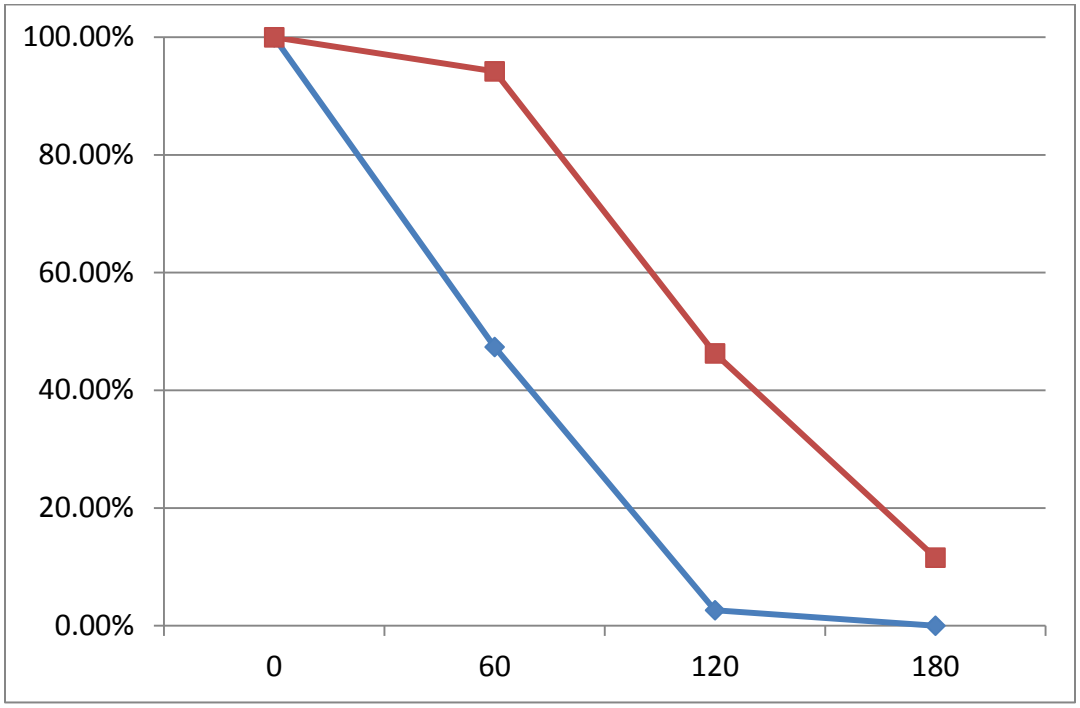
PART IV: Time Zero Killing Numbers & Plating

1. Spin the macrophage/bacteria mix at 1500rpm for 5 minutes.
2. Carefully remove supernatant and resuspend in 1mL HBSS.
3. Vortex the sample.
4. Add 100uL of the macrophage/bacteria mix to the 10^{-1} Time zero tube with 900uL water in it.
5. Vortex the tube. Remove the pipette tip.
6. With a new pipette tip, take 100uL of the 10^{-1} solution and add it to the 10^{-2} solution.
7. Put the macrophage/bacteria mix back into the incubator and start the timer for 30 minutes again.
8. Vortex the 10^{-2} tube. Remove the pipette tip. Repeat for samples 10^{-3} and 10^{-4}.
9. Work backwards by plating three 10uL drops of the 10^{-4} solution into its appropriate quadrant. NO NEED TO CHANGE THE TIP.
10. Repeat for the 10^{-3}, 10^{-2} and 10^{-1} solutions.
11. Leave the plate uncovered to dry. Place in the incubator upside down (overnight) once returning to plate the next samples.
12. Repeat for 30m, 60m, 90m, 120m, etc samples.

PART V: Reading Plates & Determining Killing Numbers

1. Count number of CFU on each drop. Usually 10^{-1}, 10^{-2} and 10^{-3} dilutions are TMTC. Average the three drops per dilution and multiply by 10^{4} and 100 to get # CFU/mL.

2. A properly performed killing assay should result in curves similar to the graph below:



LINKS AND REFERENCES

- www.bowdish.ca/lab