SOP: SP059

Infection of macrophages

Materials and Reagents:

1. 37°C incubator with 5% CO_2
2. THP-1 cells (See SOPs: SP058)
3. cRPMI (see SOP: M023)
4. Phorbol Myristate Acetate (PMA) (Cat # P1585, stored at -20°C)
5. *M. tuberculosis* infectivity stock (SOP: SP060)
6. Tissue culture/biosafety hood
7. PBS (1X)
8. Serological pipettes – 5 mL, 10 mL, 25 mL
9. Table top centrifuge
10. 20 µL, 200 µL and 1 mL pipet tips
11. P20, P200 and P1000 pipetmen
12. Hemocytometer
13. Trypan blue
14. Microscope
15. Cell counter
16. Camera
17. Falcon tubes – 15 mL & 50 mL
18. 12-well plates, tissue culture grade (Company, cat #)
19. 7H9 media
20. 2.0 mL screw-capped microcentrifuge tubes
21. 1.7 mL microcentrifuge tubes
22. 7H11 quad plates (2 plates per infected well)
23. Sterile loops
24. 0.05% SDS
25. Cell Scrapers – 18 cm (BD Falcon cat#: 353085)

Protocol:

Day 1

1. _____ In a clean hood, transfer actively growing THP-1 cells to a falcon tube, centrifuge and enumerate cells (Notes 1 & 2).
2. _____ Seed each well with 5.0 x 10^5 THP-1 cells in 3 mL of fresh cRPMI media with 200 nM (final concentration) PMA added to each well of a 12-well plate (Note 3 & 4).
3. _____ Incubate 72 hrs in the 37 °C incubator with 5% CO_2.

Day 4 – Part 1

5._____Look at cells under microscope to make sure they look healthy and are not contaminated, cells will be larger in appearance and spread out (take a picture!).
6._____Remove media, wash gently with PBS, twice. Add 2 mL of fresh media to each well.
7._____Prepare bacteria for the infection: Thaw infectivity stock in a 37 °C water bath. Transfer bacteria to a microcentrifuge tube and spin at 1200 rpm for 10 min.
8._____ Gently remove supernatant and resuspend bacteria in 1 mL of cRPMI media.
9._____ Sonicate bacteria in a water bath for 10 – 20 seconds.
10._____ To further break up clumps, pipet up and down slowly using a 200 µL pipet tip.
11._____ Add cRPMI to the bacteria so that the concentration is 5 x 10^5/mL of media (cRPMI), add 1 mL of this to infect macrophages at an MOI of 5:1 to cells.
12._____ Incubate the bacteria + macrophages for 4 hrs in the 37 °C incubator with 5% CO_2.

Day 4 – Part 2

13._____ Remove media and save in a 15 mL Falcon tube (Note 5 & 6). Wash infected cells 3 times with PBS (1 wash with 3 mL, and 2 washes with 1mL). Discard the wash.
14._____ For wells included in the 4hr timepoint: Add 1 mL of PBS with warmed 0.05% SDS and scrape cells (Note 7 & 8). Transfer cells to a 2 mL screw-capped microcentrifuge tube.
15._____ Centrifuge lysed cells @ at 1000-1200 rpm at room temperature for 10 minutes (Note 9).
16._____ Resuspend bacterial pellet (may not be visible) in 500 µL of 7H9 media. Prepare dilutions and plate on 7H11 quad plates for enumeration (Note 10). Incubate 7H11 plates in the 37 °C warm room for 4 weeks and then perform colony counts.
15._____ To the remaining, unlysed wells, add 3 mL of fresh media and incubate cells in the 37 °C incubator with 5% CO_2.

Day 5 – *24 hour infection time point*

16._____ Remove media and save in a 15 mL Falcon tube (Note 5).
17._____ Wash infected cells 3 times with PBS (1 wash with 3 mL, and 2 washes with 1mL). Discard the wash.
18._____Add 3 Add 1 mL of PBS with warmed 0.05% SDS and scrape cells (Note 7 & 8). Transfer cells to a 2 mL screw-capped microcentrifuge tube.
19._____ Centrifuge lysed cells @ at 1000-1200 rpm at room temperature for 10 minutes (Note 9).
20._____ Resuspend the pellet in 500 µL of PBS. Make serial dilutions and plate on 7H11 media (Note 10).

Day 7 – *72 hour infection time point*

21._____ Repeat steps 16 – 20 on 72 hour infected wells.

Day 8 – *96 hour infection time point*

22._____ Repeat steps 16 – 20 on 96 hour infected wells.

Notes:

1. See SOP: SP067 for cell counting procedure
2. Use trypan blue to determine the number of viable/dead cells
3. See SOP: SP068 for differentiation of THP1 procedure
4. Be sure to set up a minimum of three wells per infection time point and if infection is being done with a mutant *Mtb*, be sure to include a WT *Mtb* control in triplicate for each time point.
5. This media can be used for LDH assay for the calculation of host cell lysis – 100 µL needed for duplicate assay.
6. If exosome purification is to be performed, filter media with a .22 um syringe filter and bring back to microbiology for exosomes isolation protocol.
7. Reference: Srivastava et al 2007.
8. For the lysis of 10 wells, prepare 10 mL of 0.05% SDS by adding 50 µL of 10% SDS to 10 mL of PBS.
9. If centrifugation is performed at 4 °C, the SDS will start to crystalize, if this happens, warm back to room temperature and re-centrifuge.
10. To make sure that the concentration of the infectivity stock is correct, plate serial dilutions of the working stock. Start with 1 mL of neat, then 10-fold dilutions (ex: if stock is 10^6, add 100 ul of stock to 900 ul fresh media for the 1:10 dilution (10^5), take 100 ul of 10^5 stock and add it to 900 ul of fresh media for the 1:100 dilution (10^4)….continue in this manner until 10^1 dilution is ready. Plate 10 ul of each dilution onto 7H11 media.