SP034.2: Alamar Blue Assay
Modified: 7-31-23 AS
Using the plate reader in 102E

Materials and Reagents:

1. *M. tuberculosis* cells, inactivated by γ-irradiation (stored in the skinny -80 third shelf after transport from irradiation services on main campus), samples and negative control
2. *M. tuberculosis* cells, actively growing (from cryovials stocks in the skinny -80 in 101D)
3. Biosafety cabinet
4. Rubbermaid transport cart or container (don't tip or jar the 96-well plate)
5. Absorbent bench liner
6. Wypall
7. Pipette boat half filled with 2.5% Vesphene solution
8. Extra outer
9. Biosafety bag and holder for trash, autoclave tape
10. Squeeze bottle with 2.5% Vesphene solution
11. Bunsen burner and striker (recommended on bottles/lids)
12. Centrifuge and Sorvall benchtop centrifuge rotor, containing 15 ml centrifuge tubes hanging buckets
13. Microplate spectrophotometer (102E)
14. Computer (102E) with Microplate reader software installed and an excel spreadsheet for data and calculations (templates in raw data folder in Alamar Blue folder in Anne's folder in the TB contract on the T drive)
15. Stir plate in warm room
16. Warm room 102B BRB (37°C) storage during the incubation time
17. 7H9 media + OADC + 0.05% tween 80 (SOP M006, give enough lead time for media prep to make the media, 200ml is plenty for 2 plates)
18. Cell scraper or loop, 1/sample
19. P-200 8 channel pipetman
20. P-200 LTS pipette filter tips, sterile, ~2 packs/sample
21. P-200 pipetman
23. Reagent reservoirs, 50ml capacity, ~3 total
24. Serological pipettor
25. Serological pipettes
26. 15ml plastic conical tubes containing sterile stir bars (1/sample + controls) and plastic rack to hold them (individual autoclaved stir bars in glass tubes made ready by media prep)
27. 96-well plate, sterile, one plate per 2 samples
28. Alamar Blue reagent (on shelf in walk-in 4°C in BRB hallway)
29. Aluminum Foil (to keep the reaction dark)
30. Ziploc bag
31. Freezer, -80°C
32. Autoclave

Protocol:
Day 1-4
1. ___Set up the BSC (SOP SP041b).
2. ___ Remove about 100µg (enough to cover the bottom of the 15 ml conical) of irradiated whole cell slurry from a thawed pellet with a cell scraper or loop. Dig deep through the center of the pellet to get the best representative sample. Take a new sample if assay needs to be redone. Place sample in a 15ml conical tube containing 3-5ml of 7H9 media + OADC + 0.05% tween 80 and a sterile stir bar (flea). A minimum volume would be enough cells to be visibly cloudy in 1200µL of media. Also works if cells were irradiated in media (remove sample, spin down, and replace media for best results).
3. ___ Place samples and controls in the warm room on a stir plate over the weekend or for 3 days at ~300rpm. This disperses the cells evenly through the media and is a good check if any cells are active. It also revives the positive control.

4. ___Clean up BSC.

Day 5

5. ___After incubation, set up the BSC.
6. ___ Place 200µL sample/well in wells 1A-E and 7A-E (if there is a second sample). This gives 5 replicates, a minimum of 3 replicates are needed.
7. ___ Place 200µL of a negative control (dead Mtb) in well 1F. Place 200µL positive control (live Mtb)/well in wells 1G-H and 7F-H. Place 200µl/well 7H9 media + OADC + 0.05% tween 80 in all wells of columns 6 and 12 as blanks (use the multichannel).
8. ___ Move the plate reader into the 102E Biosafety Cabinet, turn on, click back arrow until diagnostic preformed, log on: 00000.
9. ___ Place the plate in the plate reader and remove the lid.
10. ___ Set up new endpoint protocol using the single wavelength setting of 600nm, 96 well plate setting, click read plate, and then copy and paste into a raw data template. Save the raw O.D.s.
11. ___ Based on the O.D. of each sample (5 replicates), make 200µL of a 1:10 dilution of the sample in columns 2 and 8 (if there is a second sample) of the sterile 96-well plate. The raw data template has the volumes calculated to save time.
12. ___ Add 180µl 7H9 media + OADC + 0.05% tween 80 to wells A-H 3-5 and 9-11 (if second sample) and make serial dilutions of 1:10 columns using the multichannel pipettor (20µL from column 2 to 3 and so forth). Discard 20µL from columns 5 and 11 to keep the correct volume. Mix thoroughly (important!) before pipetting and discard tips after transfer (standard serial dilution protocol).
13. ___Using the multichannel, remove 20µL from the blanks (columns 6 and 12).
14. ___Using the multichannel, add 20µL of Alamar Blue reagent to each well, except for columns 1 and 7 (if second sample). Wells of columns 1 and 7 will turn false-positive due to the number of bacterial cells present, even if they are inactivated. Thus, there is no benefit to adding Alamar Blue to these sample wells. Alamar blue is added last so that all wells will start reacting at the same time and so give accurate results.
15. ___Place the plate in the plate reader (in the BSC). Change the settings of the plate reader program to dual wavelengths of 600 nm (reference filter) and 570 nm (measurement filter) and use the ratio setting. Click read plate. Save the raw O.D.s and copy and paste to excel template. (Alternatively, 540nm and 630nm measurements can be used depending on available filters. i.e. using the plate reader in POD3)
16. ___ Wrap the plate in foil and place in a Ziploc bag, turn off and put the plate reader away.
17. ___Place in the warm room on the shelf.
18. ___Clean up BSC.

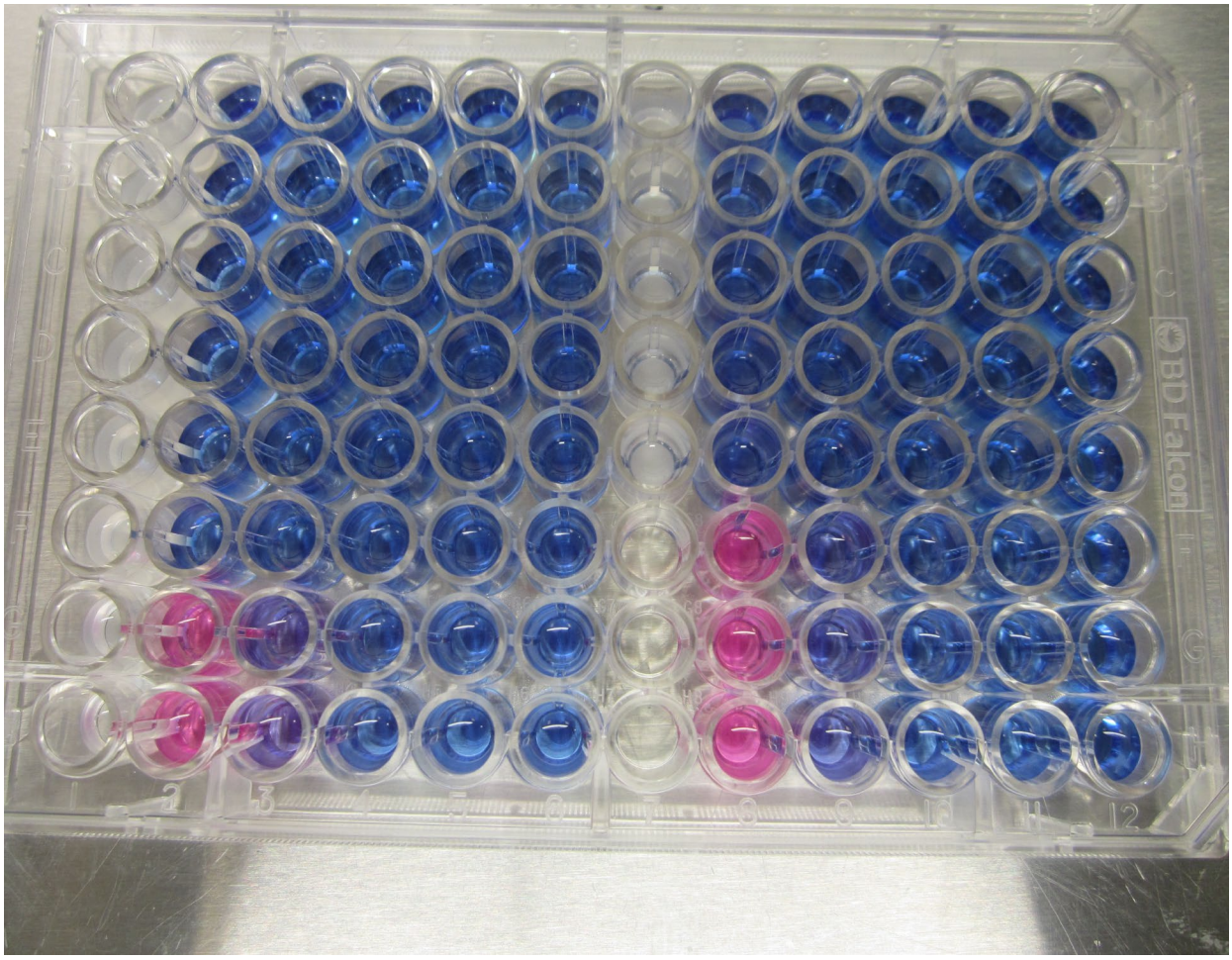
Day 6

18. ___ Repeat steps 15 to 17 after 24 hours and put back into the warm room.

Day 7

19. ___ Repeat steps 15 to 16 after 48 hours, then place the plate in a biosafety bag and autoclave as trash.
20. ___ Insert data from microplate reading into QC excel spreadsheet template. Save the excel sheet with the lot # for the irradiated cells as soon as the data from the OD @ 0, 24, and 48hr is entered into the excel template. Compare the change of slope for the positive control (0.1; the trendline is a black solid line with triangles) with the change of slope for the sample (0.1; the trendline is a blue diamond dot line with blue diamonds). The assay is valid when the sample slope change is 10 fold less than the positive slope change. Compare positive 0.01 to sample 0.01 ONLY if the OD of any values @ 0.1 are > 4.0.

    Example of plate @48h



Notes:

To determine the amount of suspension needed to make the initial dilution of 1:10, use the calculation $C_1V_1 = C_2V_2$ where C1 is the O.D. reading of the suspended cells, V_1 is the variable being solved for, C_2 is 1.0 and V_2 is 200µl. Just use the raw data excel spreadsheet to auto-calculate.

References: Alamar Blue Instruction Book
SOP: SP034