MY LABORATORY METHOD

EVERY MONDAY

Soil Wash

1. Under safety hood put 15g soil from the big tube into a 50ml falcon tube and add 15ml of pbs.
2. Vortex the sample for 5 seconds.
3. Tape horizontally onto the orbital shaker and run at 225rpm for 10 minutes.
4. Allow wash to settle for 10 minutes.
5. Under the safety hood, transfer the wash to a 5ml tube.

Cytometer Wash

1. Check that the cytometer has enough chemicals and the waste bottle isn’t full. Replace/empty where necessary.
2. Are there chemicals in the little tubes? CD(isinfectant)W(ater)?
3. Turn the cytometer on.
4. Open BD Sampler on desktop.
5. Click Autocollect.
6. Plate type 24 tube.
7. Tick A1, A2, A3.
8. Fluidics > click Fast.
9. Run limits > untick 1000, enter 120ul.
10. Set threshold FSC-H 8000.
11. Wash settings > None.
12. Apply settings.
13. Select WASH file, Save.
14. Eject plate and load with lids off. Press Load Plate.
15. Open Run Display.
16. AutoRun
17. When the wash is completed click Close Run Display.
18. Eject Plate. Close lids.

Loading the clear 96 flat bottom plate

1. Put 180ul of sterile pbs in wells B, C, D, E.
2. Put 200ul of sterile pbs in well F.
3. Put 200ul of soil wash in well A.
4. Mix well A.
5. Put 20ul of wash in well A into well B. Change ends.
6. Mix well B.
7. Put 20ul of wash in well B into well C. Change ends.
8. Mix well C.
9. Put 20ul of wash in well C into well D. Change ends.
10. Mix well D.
11. Put 20ul of wash in well D into well E. Change ends.
12. Mix well E.

Run cytometer for cell count

1. Open BD Sampler on desktop.
2. Click Autocollect.
3. Plate type 96 flat bottom.
4. Tick C, D, E, F (only include B if it is fairly clear)
5. Fluidics > click Fast.
6. Run limits > untick 1000, enter 10ul.
7. Set threshold FSC-H 8000.
8. Wash settings > 1 Cycle.
9. Agitate Plate > None.
10. Apply settings.
11. Save file name AS\_ SoilWash\_CellCount\_1(2,3,4…)
12. Eject plate and load with lid off. Press Load Plate.
13. Click Manual Collect
14. Click Plot Spec.
15. X-axis > Height > FSC-H.
16. Tick Log.
17. Y-axis > Height > SSC-H.
18. Tick Log.
19. Apply, Okay.
20. Click by to Autocollect.
21. Open Run Display.
22. AutoRun
23. When the cell count is completed click Close Run Display.
24. Eject Plate. Close lid.
25. Manual Collect.
26. Select the shape area we want to be counted = P1 (we will minus this number from the remaining cells).
27. Tick Statistics.
28. Tick P1 Count.
29. Tick All Samples.
30. Tick Volume (P1).
31. Tick Events.
32. Note down the values here and save the file.
33. Close.
34. Run Cytometer Wash again.

Dilution

1. What is the cell count for tubes C (1/100), D (1/1000) and E (1/10000)? E.g. 6.9 x10^5, 7x10^4, 7x10^3.
2. Find the average of these. Multiply up to estimate cell count in 1/1 soil wash. E.g. 7 x10^7.
3. We want to standardise the cell count to 10^7 cells per ml. So we take the 1/1 cell count we have (C1), the volume we want to find (V1), the cell count we want (C2) and the volume we want to add (V2). C1 x V1 = C2 x V2. So V1 = C2 x V2 / C1.

The volume we need of the 1/1 soil wash = 10^7 x 100ul / cell count of our 1/1 soil wash.

The answer will be the number of ul of soil wash that needs to be added to pbs to make up 100ul of 10^7 cells per ml cells!

1. Next we make 2000ul of each dilution. Start by adding the right amount of pbs to each tube.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 1st 5ml tube | Pure Soil Wash |  |  | Dilution |
| 2nd 5ml tube | + 20x V1 | + remaining pbs to make up 2000ul of dilution | Add. Change ends. Mix. | 10^7 |
| 3rd 5ml tube | + 200ul from 2nd tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^6 |
| 4th 5ml tube | + 200ul from 3rd tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^5 |
| 5th 5ml tube | + 200ul from 4th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^4 |
| 6th 5ml tube | + 200ul from 5th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^3 |
| 7th 5ml tube | + 200ul from 6th tube. | + 1800ul pbs | Add. Change ends. Mix. | 10^2 |
| 8th 5ml tube | + 200ul from 7th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^1 |
| 9th 5ml tube | + 200ul from 8th tube. | + 1800 pbs | Add. Change ends. Mix. | 10^0 |
| 10th 5ml tube |  | 2000ul pbs |  | PBS ONLY |

1. Inoculate each microcosm with 100ul of the appropriate dilution. Add. Change ends. Mix a little. Change ends.

EVERY THURSDAY

Main Soil Wash (Do at the same time as prep atp soil samples)

1. Under safety hood put 15g soil from the big tube into a 50ml falcon tube and add 15ml of pbs.
2. Vortex the sample for 5 seconds.
3. Tape horizontally onto the orbital shaker and run at 225rpm for 10 minutes.
4. Allow wash to settle for 10 minutes.
5. Under the safety hood, transfer the wash to a 5ml tube.

Prepare soil samples for cell count and ATP assay

1. Use sterile spatula to take 1g soil sample from each specially marked tube and put into sterile Eppendorf tube. Mark with dilution. Repeat for each tube, changing spatula every time (27 spatulas).
2. Add 1000ul sterile pbs to each Eppendorf tube.
3. Vortex each tube for 5 seconds.
4. Tape horizontally onto the orbital shaker and run at 225rpm for 10 minutes.
5. Allow the wash to settle for 10 minutes.
6. Take three 96 clear, lidded, flat-bottomed well plates and prepare as directed.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 10^3 (tube 1) | 10^3 (tube 2) | 10^3 (tube 3) | 10^2 (tube 1) | 10^2 (tube 2) | 10^2 (tube 3) | 10^1 (tube 1) | 10^1 (tube 2) | 10^1 (tube 3) | 10^0 (tube 1) | 10^0 (tube 2) | 10^0 (tube 3) |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash |
| B | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| C | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| D | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| E | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| F | 200ul PBS |  |  |  |  |  |  |  |  |  |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | 10^7 (tube 1) | 10^7 (tube 2) | 10^7 (tube 3 | 10^6 (tube 1) | 10^6 (tube 2) | 10^6 (tube 3) | 10^5 (tube 1) | 10^5 (tube 2) | 10^5 (tube 3) | 10^4 (tube 1) | 10^4 (tube 2) | 10^4 (tube 3) |
|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash |
| B | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| C | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| D | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| E | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| F | 200ul PBS |  |  |  |  |  |  |  |  |  |  |  |

1. Put 180ul of sterile pbs in wells B, C, D, E.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | PBS | PBS | PBS | MAIN SOIL WASH |
|  | 1 | 2 | 3 | 4 |
| A | 200ul soil wash | 200ul soil wash | 200ul soil wash | 200ul soil wash |
| B | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| C | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| D | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| E | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs | 20ul sw + 180 pbs |
| F | 200ul PBS |  |  |  |

1. Put 200ul of sterile pbs in wells F 1-12.
2. Put 200ul of soil wash in wells A 1-12.
3. Mix well A.
4. Put 20ul of wash in well A into well B. Change ends.
5. Mix well B.
6. Put 20ul of wash in well B into well C. Change ends.
7. Mix well C.
8. Put 20ul of wash in well C into well D. Change ends.
9. Mix well D.
10. Put 20ul of wash in well D into well E. Change ends.
11. Mix well E.

Cytometer Wash

1. Check that the cytometer has enough chemicals and the waste bottle isn’t full. Replace/empty where necessary.
2. Are there chemicals in the little tubes? CD(isinfectant)W(ater)?
3. Turn the cytometer on.
4. Open BD Sampler on desktop.
5. Click Autocollect.
6. Plate type 24 tube.
7. Tick A1, A2, A3.
8. Fluidics > click Fast.
9. Run limits > untick 1000, enter 120ul.
10. Set threshold FSC-H 8000.
11. Wash settings > None.
12. Apply settings.
13. Select WASH file, Save.
14. Eject plate and load with lids off. Press Load Plate.
15. Open Run Display.
16. AutoRun
17. When the wash is completed click Close Run Display.
18. Eject Plate. Close lids.

Run cytometer for cell count

1. Open BD Sampler on desktop.
2. Click Autocollect.
3. Plate type 96 flat bottom.
4. Tick C, D, E, F (only include B if it is fairly clear)
5. Fluidics > click Fast.
6. Run limits > untick 1000, enter 10ul.
7. Set threshold FSC-H 8000.
8. Wash settings > 1 Cycle.
9. Agitate Plate > None.
10. Apply settings.
11. Save file name AS\_ SoilWash\_CellCount\_1(2,3,4…)
12. Eject plate and load with lid off. Press Load Plate.
13. Click Manual Collect
14. Click Plot Spec.
15. X-axis > Height > FSC-H.
16. Tick Log.
17. Y-axis > Height > SSC-H.
18. Tick Log.
19. Apply, Okay.
20. Click by to Autocollect.
21. Open Run Display.
22. AutoRun
23. When the cell count is completed click Close Run Display.
24. Eject Plate. Close lid.
25. Manual Collect.
26. Select the shape area we want to be counted = P1 (we will minus this number from the remaining cells).
27. Tick Statistics.
28. Tick P1 Count.
29. Tick All Samples.
30. Tick Volume (P1).
31. Tick Events.
32. Note down the values here and save the file.
33. Close.
34. Run Cytometer Wash again.

ATP Assay

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | A | B | C | D | E | F | G | H | I |
| 1 | 10^7 | 10^7 | 10^7 | 10^6 | 10^6 | 10^6 | 10^5 | 10^5 | 10^5 |
| 2 | 10^4 | 10^4 | 10^4 | 10^3 | 10^3 | 10^3 | 10^2 | 10^2 | 10^2 |
| 3 | 10^1 | 10^1 | 10^1 | 10^0 | 10^0 | 10^0 | PBS | PBS | PBS |

* 1. Take a white 96 well plate with no lid
  2. Take a bottle of green top Bactiter-Glo (substrate) and white top Bactiter Glo (buffer) and leave to defrost at room temp
  3. Fill well plate with 98ul pbs A-I, 1-3.
  4. Take 2ul soil wash from each of the 27 samples, add and mix.
  5. Press the ON button on the Synergy 2 machine.
  6. Gen 5.
  7. Create using existing protocol.
  8. Documents.
  9. Amy folder.
  10. ATP\_assay, double click.
  11. Take off the cover. Protective tube, pin with rubber.
  12. Clean tubes 1st.
  13. System.
  14. Instrumental control.
  15. Synergy 2.
  16. Prime.
  17. Take off green and white lids. Pour buffer into substrate.
  18. Lid back on to substrate, gentle tip shake 5 times.
  19. Pour into new 15ml tube (should be about 10ml).
  20. Remove protective tube and replace with full tube.
  21. Lid back on.
  22. Prime.
  23. Priming plate okay (if there is a problem turn it off and on again).
  24. Close door
  25. Make sure when it is done there is liquid in the tray.
  26. Insert plate A, left hand side.
  27. Click play.
  28. Load plate manually.
  29. Save run. Okay.
  30. Close lid.
  31. Take plate out.
  32. Put safety plate back.
  33. Take out green tube > put in yellow bin.
  34. Other tube back in with some water.
  35. Now to clean!
  36. System.
  37. Instrument control.
  38. Synergy 2.
  39. Prime.
  40. Prime.
  41. Close lid.
  42. Once finished, empty water.
  43. Replace tube. Purge.
  44. Empty tube again.
  45. My white plate into yellow bin.
  46. Rinse and empty other plate.
  47. Export button. Save and record data.

Dilution

1. What is the cell count for tubes C (1/100), D (1/1000) and E (1/10000)? E.g. 6.9 x10^5, 7x10^4, 7x10^3.

2Find the average of these. Multiply up to estimate cell count in 1/1 soil wash. E.g. 7 x10^7.

3We want to standardise the cell count to 10^7 cells per ml. So we take the 1/1 cell count we have (C1), the volume we want to find (V1), the cell count we want (C2) and the volume we want to add (V2). C1 x V1 = C2 x V2. So V1 = C2 x V2 / C1.

The volume we need of the 1/1 soil wash = 10^7 x 100ul / cell count of our 1/1 soil wash.

The answer will be the number of ul of soil wash that needs to be added to pbs to make up 100ul of 10^7 cells per ml cells!

4Next we make 2000ul of each dilution. Start by adding the right amount of pbs to each tube.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| 1st 5ml tube | Pure Soil Wash |  |  | Dilution |
| 2nd 5ml tube | + 20x V1 | + remaining pbs to make up 2000ul of dilution | Add. Change ends. Mix. | 10^7 |
| 3rd 5ml tube | + 200ul from 2nd tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^6 |
| 4th 5ml tube | + 200ul from 3rd tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^5 |
| 5th 5ml tube | + 200ul from 4th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^4 |
| 6th 5ml tube | + 200ul from 5th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^3 |
| 7th 5ml tube | + 200ul from 6th tube. | + 1800ul pbs | Add. Change ends. Mix. | 10^2 |
| 8th 5ml tube | + 200ul from 7th tube. | + 1800ul pbs. | Add. Change ends. Mix. | 10^1 |
| 9th 5ml tube | + 200ul from 8th tube. | + 1800 pbs | Add. Change ends. Mix. | 10^0 |
| 10th 5ml tube |  | 2000ul pbs |  | PBS ONLY |

5. Inoculate each microcosm with 100ul of the appropriate dilution. Add. Change ends. Mix a little. Change ends.