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SOP: PP040.2
Modified: 7-13-23 AS

Growth of *Mycobacterium* Normoxic/Hypoxic Culture Pairs Using a Fermentor/Bioreactor

Materials and Reagents:

1. *Mycobacterium tuberculosis*, 1 ml frozen stock or other *Mycobacterium* culture
2. Biosafety cabinet (BSC)
3. Rubbermaid transport cart
4. Absorbent bench liner
5. Wypall
6. Pipette boat half filled with 2.5% Vesphene solution
7. Extra outer gloves
8. Biosafety bag and holder for trash, autoclave tape
9. One squeeze bottle with 2.5% Vesphene solution
10. One squeeze bottle with 70% ethanol
11. 7H11 + OADC agar plate
12. 2x 500ml E-flasks containing 250ml GAS medium (SOP M001)
13. Inoculation loop, 10µl
14. P-200 pipettor
15. P-200 tips, sterile, aerosol-resistant tips
16. Ziploc freezer bags
17. Fernbach flask (x2 empty), 2.8L with sterile cotton and cheesecloth
18. Cell scrapers
19. Serological pipettes, 10ml, 25ml and 50ml, sterile, aerosol-resistant
20. Serological pipettor
21. Falcon centrifuge tubes, 15ml 50ml, sterile
22. 0.2 µm VacuCap bottle filtration units
23. Parafilm
24. Harvard trip balance
25. Sodium azide
26. 2:1chloroform methanol
27. Centrifuge with rotors and buckets for 15 and 50ml conicals
28. BioFlo 115 Bioreactor control unit including DO probes, heat jackets, exhaust heaters, and pH probes
29. Bioreactor Vessels (2), each containing 3.75L GAS medium (SOP M001) Eppendorf North America, Inc., Single Use 5 L Vessel units
30. Incubator
31. Freezer, -80ºC
32. Autoclave
33. .8/.2 µm syringe filter or steri-flip 50mL conical
34. 60ml syringe
35. Orbital shaker (or environmental shaker incubator)
36. Bead beater
37. Bead berating tubes
38. Trizol
39. sodium azide
40. LJ slant
41. Microcentrifuge
42. peristaltic pump
43. Vacuum compressor pump with sump

Protocol:
Start Day:

1._____ Set up a BSC (SOP SP041b) in the BRB, POD3, or any BSL2 space for BSL2 organisms and thaw a 1mL frozen stock of mycobacteria. One stock can make 2-5 large 7H11 plates. Other mycobacteria besides *M. tuberculosis* may be grown with this technique but may require different media or growth timelines.

2._____ Transfer 200µL of the stock to a large 7H11 plate and streak to grow as a lawn with a sterile bent plastic loop.

3._____ Place inoculated plates in Ziploc bags, seal, and place in the warm room if in the BRB or in the incubator if elsewhere. Grow with the media on bottom (opposite from other bacteria). Depending upon the strain, a lawn could take 2 to 6 weeks to form. Most strains take 4 weeks. Make at least 2 large plates, one for a normoxic culture and one for a hypoxic culture.

4._____ Take down the BSC.

5._____ Incubate at 37°C until a thick lawn has formed. Check growth after 2 days. Inspect for contamination.

Two-six Weeks Later:

6._____ Set up a BSC in the BRB and using a sterile cell scraper, aseptically transfer cells from one plate to a Fernbach flask containing 1 L of GAS media (or 500 mL E-flasks with stir bars if in the POD). Repeat with more Fernbach flasks.

7._____ Flame the flask neck if possible.

8._____ Place the Fernbachs in the warm room for two weeks or the E-flasks into the incubator with stir plate at 37°C for one week. BSL2 strains can be grown in an environmental shaker at 37°C until good growth is observed.

9._____ Take down the BSC.

One-Two Weeks Later: Once There is Sufficient Cell Growth

10._____ Prepare 10 liters of GAS media (or other media determined by strain) in large glass flasks and allow to cool to room temp after autoclaving.

11._____ Set up the BSC. Place an empty bioreactor vessel, peristaltic pump and the GAS media into the BSC with the pump in the middle. Remove the shipping lid of the vessel to expose the tubes. Position the exhaust line so that it has no “kinks” in it that will prevent gas movement. Using sterile technique unscrew cap of any of the capped ports.

12._____ Tape VacuCap outlet over open port and run the tubing through the peristaltic pump.

13._____ Remove the plastic sinker from the end of the VacuCap and attach to the tapered end of a 10ml serological pipet that has the cotton end snapped off. Turn on the pump to move the media from the flask. It is then filtered through the VacuCap and dispensed into the sterile vessel.

14._____ Fill each vessel to 3.5-3.75L of GAS media. Once filled, tuck all tubing back under the shipping lid of vessel. Remove the vessel from the BSC and place in an incubator, warm room, or use heat jacket over night for a contamination check.

15._____ Prepare the DO probes (see manual). Autoclave DO probe at 15 min liquid cycle, this only needs to be done once if the probe is new or has not been stored and unused for a long period of time. The DO probe must be polarized and charged prior to use. Plug the probe into the control unit via the DO probe cable and turn on the control unit. Allow the probe to charge for at least 8 hours, over night is preferred. Calibrate probe: Unplug the DO line and set zero when the number stabilizes. Insert DO probe and temperature probe into the vessel. Attach exhaust heater and incubator jacket around the vessel. Attach gas

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line to the sparge filter and place the rotor motor on top of the vessel. Sparge air at 1 vvm. Run the agitator at 65. Set span at 100 for the DO calibration when the number stabilizes.

16._____ Prepare the pH probes (see manual). Autoclave pH probe at 15 min liquid cycle. Remove from bag and remove cap. Calibrate probe: Attach pH probe to the pH cable and plug into the control unit. Rinse with water, measure pH 7 standard, set Zero as pH 7 when the number stabilizes, rinse with water, measure pH 4 standard, set Span as pH 4 when the number stabilizes. Take a 30 mL sample of media from the sample port of the vessel using a syringe or reserve media from the vessel filling to confirm pH. Any discrepancy between the pH value on the control unit and the separate pH meter can be adjusted on the set span of the pH calibration on the control unit. Do not mix up probes.

17._____ Set up a BSC in the room containing the bioreactors. Place the Fernbachs or eflasks and bioreactor vessels into the BSC. Gently swirl the flask to create a whirlpool and set down to allow the cells to settle. They will settle into a nice pile in the center.

18._____ Pipette the cells from the flasks to the bioreactor vessels through an open port. Maintain sterility.

19._____ Sanitize pH probe with vesphene and then ethanol. Then wash in sterile Milli Q water. Aseptically install probe. Seal with parafilm.

20._____ Remove the bioreactor vessels from the BSC and bring the vessels back to the control units and attach the pH cables to the pH probes. Gently insert DO probes and temperature probes into the vessels. Attach the exhaust heater to the filtered exhaust tube and wrap the incubator jacket around the vessel. Attach the gas lines to the sparge filters and place the rotor motors on top of the vessels. On the Summary screen of the main control unit, set the gas option to 4-Gas, the temperature set to 37°C, the DO set to 35, and the pH set to 6.6 (for GAS media). Then switch to the Cascade screen, here set the cascade to Agitation/Gasflo. The parameters for these are to be set with a low limit of 60 and a high limit of 150 for Agitation, and a low limit of 0 and a high limit of 0.5 VVM for the Gasflo (can be set higher or lower if needed).

21._____ Set up batch with trendlines on the computer using the Biocommand software (see manual).

22._____ Take down the BSC.

Sample removal, if needed, wear a PAPR from this point on

23._____ Check cultures two days post inoculation for infection. Check all equipment and tanks. Continue monitoring cultures remotely.

24._____ Set up the BSC in the room if samples are needed. If samples are not needed skip to step 31.

25._____ Remove two 40 mL samples from each vessel using the sample port and syringe. Place samples onto vesphene soaked Wypalls on cart. Transfer the four syringes to BSC. Express contents into 50 mL conicals, centrifuge to pellet with 3.75K RPM for 10 min. decant supernatant to new 50ml conical.

26._____ For CFP (raw culture filtrate protein), attach one sample from each vessel to a steri-flip conical. Use the vacuum pump to filter CFP to the new container. Cap and wrap with parafilm. Freeze at -80°C or store at 4°C with sodium azide added. If steri-flips are unavailable, pour the supernatant into a reservoir boat. Pull up the liquid with a syringe and attach a .8/.2 or .2 µm syringe filter. Slowly press the liquid through the filter into a new 50 mL centrifuge tube. This CFP sample can now be labeled and removed from the BSL3 for analysis. Freeze or add 50 mg of sodium azide to the sample for 4C storage if not immediately analyzed.

27._____ Freeze the cells for later analysis or move the cells to a glass tube and add 2:1 chloroform methanol. The cells can be removed from the BSL3 after testing on an LJ slant. If RNA extraction is desired, this must be done immediately when sample is taken. Resuspend cell pellet in 1ml Trizol and transfer to a bead beating tube and bead beat 3 times for 30 sec on and 30 sec off. Using a microcentrifuge, spin down the beads at

max speed for 10 mins and transfer Trizol extract to a new 1.7 mL eppi tube. Be careful not to take any of the pelleted beads. Bead beating and microcentrifuging must be done inside the BSC. Bead beating tubes with bead pellet exposed to Trizol must be disposed of as Haz. Waste. Freeze the Trizol extracts at -80°C.

28._____ Label the 2x50ml centrifuge tubes or glass tubes with the appropriate information (strain, lot number, date, medium, and technician name).

29._____ Take down the BSC.

30._____ Repeat steps 18-24 as needed. Spent media with cell samples are usually taken at harvests and frozen.

Start hypoxic conditions

31._____ Initiate hypoxic conditions on one vessel 4 days after inoculation by changing the set point of the DO in the Summary screen to 1. If the DO fails to fall naturally within 1 day, change the Cascade gas flow lower limit is to 0.2. Remotely monitor transition to hypoxic conditions. Harvest hypoxic culture 10 days post hypoxic conditions initiation (14 days post inoculation). Harvest normoxic culture 10 days post inoculation.

Harvest of vessel

32._____ Set up a BSC.

33._____ Place two empty Fernbach flasks into the BSC.

34._____ Detach and turn off all lines attached to the bioreactor and remove to a BSC in the BRB or POD. Turn off all items on the display screen. See book chapter (978-1-0716-1460-0) for previous harvest method.

35._____ Once in the BSC, remove the pH probe from the vessel. Wipe/clean the probe with Vesphene and then ethanol, cap, and put in a biosafety bag to be autoclaved.

36._____ Use the pH port or unscrew any other port for harvest of cells/media. Snap off the cotton end of a 10ml serological pipet and fit the tapered end onto the end of sterile tubing to create a harvesting wand. Feed the sterile tubing through the peristaltic pump to the collection flask. Pump the bioreactor contents into the flask.

37._____ When the collection flask is full, turn off the pump and allow the cells to settle. Carefully pour the CFP into the second empty Fernbach flask as the CFP collection flask. Continue pumping the vessel culture in the cell collection flask until the vessel is empty.

38._____ Pipette out the remaining media along with the cell pellet into 50 mL or 230 mL centrifuge tube. Allow cells to settle and remove remaining media. If cells do not flocculate, pellet can be centrifuged at 2.5K for 15 minutes. May be necessary for some strains. Hypoxic/Normoxic cells are not washed with water prior to irradiation and will therefore have residual media (to reduce oxygenation, especially important for hypoxic cells). Record final cell pellet weight, triple bag for irradiation, and freeze.

39._____ If collecting CFP, place a sterile 4L glass bottle, along with a VacuCap, into the biosafety cabinet. Using autoclave tape, fasten the tubing from the vacuum pump to the BSC to prevent the tubing from causing accidental spillage.

40._____ Open the 4L glass bottle and VacuCap package. Snap off the cotton end of a 10ml pipette. Aseptically remove from plastic wrapper. Place the broken end into the flask containing CFP and insert the pointy end into the VacuCap hose. Place the VacuCap on the mouth of the bottle and attach the tubing from the vacuum pump to the VacuCap.

41._____ Carefully watch the bottle begin to fill. If more than an inch of foam forms, the bottle was not rinsed correctly or there may be a crack in the bottle. Start over with a new bottle and discard old one and

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VacuCap. Also watch for crack formation due to the pressure. Carefully tip the vessel to remove most of the CFP and avoid sucking up the cell pellet.

42._____ Once empty, add Vesphene into the vessel through the open pH port and place in a large autoclave bag for disposal.

43._____ The cell pellet is ready to be removed from the BSL-3 for γ-irradiation (PP004.1) or 2:1 chloroform methanol (PP009.2) added for lipid extraction. Label the CFP and cell pellet with the appropriate information (strain, lot number, date, medium, and technician name) and parafilm the lids. Write down the weight of the cell pellet on the label along with the other information and fill in the large scale growth worksheet. Parafilm the cap and package the 230mL Polycarbonate conical with pellet in three layers of biohazard bags with Lysol spray between layers 2 and 3. Seal each layer with tape. Label the outside of the package and freeze in a -70 until the sample can be transported to Biochemistry for irradiation. The cell pellet is ready to be removed from the BSL-3 for γ-irradiation or frozen at –80ºC for DNA extraction. To remove the bacterial cell pellet from the BSL-3, follow SOP PP004.2. Post irradiation, Alamar blue assay may be done with SPAS media. Need transport certification to transport infectious material. For DNA extraction see SOP PP009.2.

44._____ Take down the BSC.

Resources

The manuals can be found at:
http://eshop.eppendorfna.com/products/New_Brunswick_BioFlo_115_fermentor

Knabenbauer, P. 2016. Establishment and systematic characterization of Mycobacterium tuberculosis in bioreactors. Graduate thesis, Colorado State University. Publication number 10240636.

Book chapter978-1-0716-1460-0_1.pdf pg 46-58
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