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SOP: PP040.1
Modified: 02/27/2017 PK

Growth of *M.tuberculosis* Normoxic/Hypoxic Culture Pairs Using a Fermentor/Bioreactor

Materials and Reagents:
1. *M. tuberculosis*, 1 ml frozen stock or growing 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 eflasks containing 250ml GAS medium (SOP M001)
13. Inoculation loop, 10 µl
14. P-200 pipettor
15. P-200 tips, sterile, aerosol-resistant tips
16. Ziploc bag
17. Fernbach flask (x2 empty), 2.8L with sterile cotton and cheesecloth
18. Cell scrapers
19. Serological pippets, 10ml, 25ml and 50ml, sterile, aerosol-resistant
20. Serological pipettor
21. Falcon centrifuge tubes, 15ml 50ml, sterile
22. 0.2 um 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 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 with components for fermentor
30. Incubator
31. Freezer, -80ºC
32. Autoclave
33. .8/.2 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. Microcentrifuge
40. Watson Marlow pump
41. Custom harvest line, Silicone tubing (ID 4.8mm, OD 7.9mm)
42. Vacuum compressor pump with sump

Protocol:
Start Day:
1._____ Set up a BSC in the BRB or the POD (SOP SP041b) and thaw a 1mL frozen stock of *M. tuberculosis*. One stock can make 2-5 large 7H11 plates.

2._____ Transfer 200 µL 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 in the POD. 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 (SOP SP041b).

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

Three-Weeks Later:

6._____ Set up a BSC in the BRB and using a sterile cell scraper, aseptically transfer an entire plate to a Fernbach flask containing 1 L of GAS media (or 500 mL eflasks if in the POD). One to two Fernbach flasks in the BRB seems to be best.

7._____ Flame the flask neck if possible.

8._____ Place the Fernbachs in the warm room for two weeks or the eflasks into the incubator at 37°C for one week.

9._____ Take down the BSC.

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

10._____ Prepare 10 liters of GAS media in large 4 liter glass flasks and allow to cool to room temp after autoclaving.

11._____ Set up the BSC by wiping down with 70% ethanol and laying down a lab mat. Place an empty reactor vessel, Watson-Marlow peristaltic pump and the GAS media into the BSC with the pump in the middle. Remove the 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. Locate and unwind the one of the large liquid addition (LA) ports.

12._____ Clamp the LA line closed and cut off the tip of the line, removing the tube attachment adaptor. Fit the open end of the LA tube to the outlet port of a vacu-cap located under the vacu-cap bottle fitting. Use a clamp to secure the tube tightly. Attach the feeder line of the vacu-cap through the peristaltic pump.

13._____ Remove the buoy from the end of the feeder line. Using a 10ml serological pipet, snap off the cotton end of the pipet that attaches onto a pipettor. With the tapered nozzle of the pipet, fit this end into the open ond of the feeder line to create a media harvesting wand. Insert the wand into on the media flasks and turn on the pump at max speed. The media will be pulled from the flask, filtered through the vacu-cap and dispensed into the still sterile vessel.

14._____ Fill each vessel to 3.75-4.0L of GAS media. Once filled, tuck all tubing back under the lid of vessel. Remove the vessel from the BSC and place in the large incubator 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 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.

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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. Expel the media into a 50 mL falcon tube, measure pH with probe. Using a separate pH meter measure the pH of the media. 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 POD. 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 the pH probe port.

19._____ Sanitize pH probe with vesphene and then ethanol. Aseptically install probe. Seal with parafilm.

20._____ Remove the fermentor 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 to 40, and the pH set to 6.6. 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 45 and a high limit of 105 for Agitation, and a low limit of 0 and a high limit of 1VVM for the Gasflo.

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 POD.

25._____ Remove two 40 mL samples from each vessel using the sample port and syringe. Place samples onto vesphene soaked Wypalls on cart.

26._____ 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.

27._____ 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 syringe filter. Slowly press the liquid through the filter into a new 50 mL centrifuge tube.

28._____ This CFP sample can now be labeled and taken to Micro for analysis. Freeze or add 50 mg of sodium azide to the sample if it won't be immediately analyzed.

29._____ Freeze the cells for later analysis or move the cells to a glass tube and add 2:1 chloroform methanol. The cells can now be taken to Micro for analysis. 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 carful 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.

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

31._____ Take down the BSC.

32._____ Repeat steps 18-24 as needed. Samples are currently taken at harvests.

Start hypoxic conditions

33._____ 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

34._____ Set up a BSC in the POD.

35._____ Place two empty furnbach flasks into the BSC.

36._____ Turn off the gas flow from the BioFlo 115 control unit into the vessel and remove the heating jacket. Attach the vacuum compressor pump exhaust port to the filter of the Gas overlay port of the reactor vessel. Clamp shut all other exhaust and gas ports so the vessel is completely sealed.

37._____ Turn off the DO, pH and temperature probes, leaving the agitation on at 105 RPM.

38._____ Attach the custom harvesting line to the harvest port of the vessel using the appropriate adaptor piece. Wrap parafilm around the connection. Run the other end of the harvest line into the BSC and tape the end to the empty furnbach flask so the vessel culture in pumped into the flask. Unscrew the exhaust regulator of the pump all the way and then turn on the pump. VERY slowly increase the pressure until the culture begins to flow in the BSC collection flask.

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

40._____ Pipette out the remaining media along with the cell pellet into 50 mL or 230 mL centrifuge tube (record empty weight).

41._____ Place a sterile four liter 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.

42._____ 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.

43._____ 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 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.

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44._____ When all of the cells have been transferred, remove as much media supernatant as possible. Dispose of the pipette into the pipette boat containing a 2.5% Vesphene solution.

45._____ Turn on the benchtop centrifuge.

46._____ Remove the buckets from the rotor and place on the Harvard trip balance.

47._____ Place the 50 mL/230 mL centrifuge tube containing the bacterial pellet in one of the rotor buckets and make a water balance.

48._____ Spin at 3K for 15 minutes.

49._____ Unhook the empty vessel from the control unit and place in the BSC. Remove the pH probe from the vessel. Wipe/clean the probe with ethanol, cap, and put in a biosafety bag to be autoclaved. Add a copious amount of Vesphene into the vessel through the open pH port and place in a large autoclave bag for disposal.

50._____ Once the cell pellet is done spinning, remove the media supernatant.

51._____ Write down the wet weight on the 50 mL centrifuge tube label and freeze. Pellet is not washed with sterile water prior to γ-irradiation.

52._____ The cell pellet is ready to be removed from the BSL-3 for γ-irradiation or 2:1 chloroform methanol added for lipid extraction.

53._____ 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.