

15-hour Bacterial Food Growth

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Notes

- Three days of preparation. See [bacteria growth schedule](#)
 - **Day 1** Prepare media and inoculate the 18-hour seed culture.
 - **Day 2** Inoculate 15-hour culture at OD 0.001 using the seed culture.
 - **Day 3** Wash, aliquot, and freeze the 15-hour culture.
- Expected yield = 74.00 +/- 10.53 mL ml of OD100 food per liter of culture.
 - See [Bacterial food preparations](#) spreadsheet.
- Use sterile filter tips for all pipetting steps.

Supplies

- Vortex mixer
- Stir plate
- Autoclaved 4 L flasks*
- 1X Horvitz Super Broth (see protocol below)* Use this guide to help estimate the requirements for each compound ([Horvitz Super Broth prep](#))
 - Tryptone (US Biological #T8750)
 - Yeast extract (Sigma #Y1625)
 - 100% Glycerol (Fisher Scientific #BP2291)
 - dH2O
 - KH₂PO₄ (Fisher Scientific #P285 3)
 - K₂HPO₄ (Fisher Scientific #P288500)
- 14 mL Fisherbrand culture tubes with blue snap cap (for growing bacteria overnight/storing blank) (Fisherbrand #149569C)
- Spectrophotometer (BioRad, smartspec plus)
- One autoclaved beaker with stir bar (volume of beaker should hold 1.5 times the amount of food you plan to prepare, include stir bar before autoclaving)
- Four autoclaved Thermo Scientific 500 mL capacity flat-bottomed centrifuge bottles (Thermo Scientific #75003699)
- Filter tips for p20, p200, and p1000 pipettes (USA Scientific #1121-2710 #1120-8710 #1126-7710)
- Automated transfer pipette
- Sterile serological pipets (10 mL and 50 mL) (Fisherbrand #13-678-11E #13-676-10R)
- Eppendorf or similar repeat pipette
- Repeat pipette tips (sterile, 50 mL) (Fisher Scientific #13-683-717)
- Sterile 50 mL conical tubes (for washing bacteria)* (Fisherbrand #05-539-8)
- Disposable 1.5 mL cuvettes (Fisherbrand #14955128)
- K medium (see protocol below)*
 - 5M NaCl (Fisher Scientific #BP358-10)

- 1M KCl (US Biological #C10121513)
 - 1M CaCl₂ (Amresco #97062-590)
 - 1M MgSO₄ (Fisher Scientific #BP213-1)
 - Unfiltered HTA cholesterol (1 g cholesterol (Amresco #0433-250G) in 200 mL 200 proof ethanol (Fisher Scientific #BP2818500))
 - 1.7 mL Posi-click tubes or 15 mL conicals (for freezing and storing bacteria)*
 - Tube labels for labeling frozen bacterial aliquots*
 - Freezer boxes or equivalent for storing aliquots in the -80°C freezer*
- * (amount will vary depending on how much food you want to make)

Protocol

Day 1

1. Make enough 1x Horvitz Super Broth (1x HSB) to accommodate the amount of food your experiments require (see broth protocol below). Prepare the broth directly in 4 L growth flasks. After the broth is prepared, store the flasks on your benchtop until needed (Day 2). Maintain the foil cover used for the autoclave step to prevent contamination.
2. Make sure the HB101 bacteria plate is less than one month old. Working HB101 bacteria plates are in walk-in 4°C on the far upper left shelf labeled “working bacteria cultures”.
3. Work in the sterile hood with PPE. Transfer 5 ml of 1x HSB from one of the culture flasks to five separate culture tubes (Fisherbrand, #149569C). Pick a single colony using a disposable inoculation loop (Fisherbrand, #22-363-607) into each of three separate culture tubes. This triple inoculation ensures that at least one of them will grow. The remaining two culture tubes will be used as negative controls and should contain 1x HSB only.
 - a. If the bacteria plate is older than one month, ask Robyn to streak a fresh plate from the frozen HB101 stock. Grow the streaked plate at 37°C overnight before picking single colonies. Parafilm the plate and keep it at 4°C for up to one month.
4. Incubate the cultures and the negative control tubes for 18 hours at 37°C shaking at 180 rpm in the Andersen Lab incubator (Bottom level, Infors HT - Multitron). Place the tubes in a tube rack then place the tube rack on the adhesive pad for shaking.
5. Ensure that you have all the necessary supplies to complete the rest of the procedure.

Day 2

NOTE: Before inoculating the large culture, make sure to warm the broth for 1 hour at 37°C with 180 rpm shaking in the Andersen lab incubator (Infors HT - Multitron). Place flasks on the adhesive pad for shaking.

6. 18 hours after inoculating the seed cultures, check that the HB101 cultures are turbid (cloudy) and the negative control tubes are not.
 - a. If the negative control tubes are cloudy, your broth may be contaminated. You should start over. If none of your three HB101 cultures have grown, first check

that the plate you picked from is less than one month old. If the bacteria did not grow overnight and the source plate is fresh, try another colony, remake the broth, or ask Robyn to streak out a new plate from the frozen stock.

7. Transfer the cultures and negative controls to the 4°C room (left side of room on counter space) for seven hours before inoculating 15-hour cultures. Standard practice is to inoculate cultures at the end of a workday (5 PM). The seven-hour period at 4°C aligns this protocol with normal work hours.

IMPORTANT: Before inoculating the 15-hour culture, make sure to warm broth for 1 hour at 37°C with 180 rpm shaking in the Andersen lab incubator.

8. Beginning 6.5 hours after the three 18-hour cultures were transferred to 4°C, measure the OD₆₀₀ (optical density at 600 nm) of one of your culture tubes with the spectrophotometer (BioRad, smartspec plus). If all three of your 18-hour cultures grew, choose one and discard the other two.
 - a. Prepare serial dilutions in 1.5 mL cuvettes (fisherbrand cat# 14955128) and use the negative control medium as the blank.
 - b. 10, 20, 50, and 100 (serial dilutions)
 - i. Add 180 µL of culture to 1620 µL of blank to get 1:10 dilution
 - ii. Add 800 µL of 1:10 to 800 µL of blank to get 1:20 dilution
 - iii. Add 600 µL of 1:20 to 900 µL of blank to get 1:50 dilution
 - iv. Add 500 µL of 1:50 to 500 µL of blank to get 1:100 dilution
 - c. Measure the OD of all dilutions using (BioRad, smartspec plus). Immediately before placing the cuvettes in the spec be sure to mix the sample by pipetting up and down three times using a 1 mL pipet. Avoid bubbles because they will affect OD reading.
 - d. Do not use OD measurements that fall outside the linear range of the spec (0.2 - 0.8).
 - e. Multiply the OD measurement by its dilution factor for each dilution.
 - f. Take the average of these adjusted OD measures to estimate the OD of the 18-hour starter culture.
9. Calculate how much of the 18-hour starter culture you need to inoculate the 15-hour culture at an OD of 0.001.
 - a. You can choose your own 15-hour culture volume but be sure to maintain at least a 4:1 ratio of flask volume to culture volume in the autoclaved flask (e.g. 1 L of culture in a 4 L flask). Standard practice is to use 4 L culture flasks with 1 L culture volume.
 - b. To determine volume of 18-hour starter culture needed to inoculate the 15-hour culture at OD of 0.001, use the formula $C_1V_1 = C_2V_2$. Use the average of the OD measures as C_1 , OD of 0.001 as C_2 , and the 15-hour culture volume as V_2 .
 - c. Before inoculating the 15-hour culture, make sure to warm broth for 1 hour at 37°C with 180 rpm shaking in the Andersen lab incubator.
 - d. Before inoculating the 15-hour culture, make sure to check the clarity of the 1x HSB in the culture flask(s). If the 1x HSB is cloudy, it is likely contaminated, do not inoculate a contaminated flask! Make fresh 1X HSB and start again.

10. In the sterile hood, using gloves, lab coat, mask, and sterile technique, prepare a 5 mL negative control tube (Fisherbrand, #149569C) to test the 1x HSB for contamination. If you made 1x HSB in multiple culture flasks on day 1, choose a single culture flask to test for contamination.
 - a. Place the negative control tube in a tube rack then place the rack onto the adhesive pad in the 37°C incubator with 180 rpm shaking (bottom level, Infors HT - Multitron).
11. In the sterile hood, using gloves, lab coat, mask, and sterile technique, inoculate the **pre-warmed** culture flask(s).

IMPORTANT: Before inoculating the 15-hour culture flask(s), make sure to warm the broth for 1 hour at 37°C with 180 rpm shaking in the Andersen lab incubator.

 - a. Carefully mix the 18-hour seed culture, then use a sterile filter tip to aspirate the inoculant volume calculated in step 9.
 - b. Dispense the volume directly into the culture flask. Do not add the inoculant to the side of the culture flask; dispense it directly into the broth.
12. Incubate the inoculated culture flask(s) for 15 hours at 37°C with 180 rpm shaking in the Andersen lab incubator (Infors HT - Multitron). Place flasks on the adhesive pads for shaking.

Day 3

13. After 15 hours of growth, remove all flasks from the incubator and set them in the 4°C walk-in cold room. Check that the negative control culture is not cloudy. If the negative control is cloudy/contaminated, the 15-hour culture(s) are likely contaminated too. Do not use contaminated cultures! Start the protocol over again.
14. Select up to two 4 L culture flasks to centrifuge at a time and leave the other flasks at 4°C. Working in the sterile hood, pour approximately 500 mL of the 15-hour culture from a 4 L culture flask into a 500 mL flat-bottomed centrifuge bottle (Thermo Scientific, #75003699). Repeat this step up to three more times to prepare a maximum of four 500 mL flat-bottomed centrifuge bottles. Ensure the volumes in each pair of centrifuge bottles are roughly equal. This step should be done by transferring excess culture volume in one centrifuge bottle to the other until the levels are equal by eye. The Eppendorf 5810R centrifuge can spin four centrifuge bottles at a time.
 - a. Retain at least one 4 L culture flask in the hood. This flask will serve as a waste container to hold the waste supernatant after each spin.
15. Centrifuge the bacterial cells in the flat-bottomed bottles at 3500 rpm for 10 minutes in the Eppendorf 5810R.
 - a. Check for an obvious pellet. If there is no pellet, spin again.
16. In the hood wearing gloves, lab coat, and mask, discard the supernatant, pour 10 mL K medium from conicas and vortex to resuspend the pellet. Once resuspended, add an extra 20 mL of K medium, vortex to resuspend completely, and transfer to a sterile 50 mL conical tube. You should have one 50 mL conical tube for each flat-bottomed centrifuge bottle after transferring.

- a. Pour directly from conicals instead of using pipettes when adding K medium.
 - b. Retain the flat-bottom centrifuge bottles for pelleting the other cultures.
17. Move the 50 mL conical tubes to the 4°C cold room and select another set of culture flasks.
18. Repeat steps 14 - 17 until all the culture flasks have been pelleted and transferred to 50 mL conical tubes. In the end, you should have one 50 mL conical for each 500 mL of culture you pelleted.
19. Take all the 50 mL conical tubes from the cold room and centrifuge them at 3500 rpm for 10 minutes in the Eppendorf 5810R.
20. In the hood wearing gloves, lab coat, and mask, check for a pellet, remove the supernatant, vortex to resuspend in 10 mL K medium, then once resuspended, add 20 mL more of K medium vortex to resuspend completely, and centrifuge them at 3500 rpm for 10 minutes in the Eppendorf 5810R.
21. Repeat this K medium wash one more time for a total of three washes. Remember to perform all washes in the sterile hood with gloves, lab coat, and mask.
22. After the final wash, the supernatant should be clear.
23. In the hood wearing gloves, lab coat, and mask, discard the supernatant and resuspend by adding 10 ml of K medium per 50 mL conical.
24. In the hood wearing gloves, lab coat, and mask, vortex the 50 mL conicals until the pellet is completely resuspended, then transfer each conical to a single autoclaved glass beaker using a sterile 25 mL pipette. Place the beaker with a stir bar on a stir plate in the hood to maintain a well mixed bacterial suspension. The beaker should be large enough to accommodate 1.5 times the total amount of OD100 bacteria suspension that you expect to make. Expect $\sim 75 \pm 10$ mL of OD100 bacterial suspension per liter of initial culture volume. Allow the contents of the beaker to mix well before proceeding with step 26.

IMPORTANT: Be sure to **record** the volume transferred to the beaker from each conical so you can calculate the total culture volume later.

IMPORTANT: **Adjust the speed of the stir bar very carefully to avoid splashing the culture. Never place the beaker on the stir plate with stirring turned on. Switch it off, place the beaker on the stir plate, then adjust the stirring speed up slowly from 0.**

25. Once all the 50 mL conicals are transferred to the beaker, calculate the total volume in the beaker by summing each transfer volume. The final volume in the beaker must be known to accurately prepare an OD100 food source.
26. Measure the OD of this known volume of washed bacterial suspension **in triplicate**. Triplicate serial dilutions are necessary to accurately measure the culture OD.
 - a. Use 1:20, 1:350, 1:700, and 1:1400 (serial dilutions). You should make the dilutions in the 1.5 mL cuvettes (fisherbrand cat# 14955128) with K medium as the blank.
 - i. Add 100 μ L of mixed bacterial suspension to 1,900 μ L of K medium to get 1:20 dilution
 - ii. Add 103 μ L of 1:20 dilution to 1,697 μ L of K medium to get 1:350 dilution

- iii. Add 750 μL of 1:350 to 750 μL of K medium to get 1:700 dilution
 - iv. Add 500 μL of 1:700 to 500 μL of K medium to get 1:1400 dilution
 - b. Measure OD of all dilutions using (BioRad, smartspec plus). Immediately before placing in the spec be sure to mix the sample by pipetting up and down three times using a 1 mL pipet. Avoid bubbles because this will affect OD reading.
 - c. Do not use OD measurements that fall outside the linear range of the spec (0.2 - 0.8).
 - d. Multiply the OD measurement by its dilution factor for each dilution.
 - e. Take the average of these adjusted OD measures to estimate the OD of the culture.
 - f. You should now have triplicate OD measures, one per serial dilution.
- 27. In the hood with gloves, lab coat, and mask, dilute the 15-hour bacterial suspension to an OD100 with K medium. Use the formula $C_1V_1 = C_2V_2$ to dilute the suspension and use the average of the triplicate OD measures as C_1 . Use the volume you transferred to the beaker as V_1 and OD100 as C_2 .
- 28. Label 1.7 mL Posi-click tubes or 15 mL conicals "15h HB101, OD100" the date, and the initials of the preparer(s) on the cap with printed labels.
- 29. In the sterile hood, using gloves, lab coat, mask, and sterile technique, aspirate a large volume of the stirring culture with an ethanol-sterilized repeat pipetter fitted with a sterile 50 mL tip. Then, aliquot the OD100 bacterial suspension into 1.7 mL Posi-click tubes. Alternatively, use the automated transfer pipet with sterile pipettes to aliquot into 15 mL conicals.
 - a. The volume of each aliquot will depend on your experimental design.
 - b. If aliquoting small volumes (< 2 mL) mix the bacterial suspension in the 50 mL tip after filling 10 tubes by swirling the pipette five times in each direction. Repeat this mixing every 10 tubes.
- 30. If using microfuge tubes, put them in a white cardboard freezer box with "15h HB101, OD100" the date, and the initials of the preparer(s). If using 15 ml conicals, freeze them upright in an open, metal tube rack then transfer them laying horizontally to a cardboard freezer box with "15h HB101, OD100" the date, and the initials of the preparer(s). Store the box in a -80°C freezer.
- 31. Record the culture data in the [Bacterial_food_preparations](#) spreadsheet.
- 32. Before using the food in an assay, test it for contamination.
 - a. In the sterile hood, using gloves, lab coat, mask, and aseptic technique, thaw at least 0.5 mL of the OD100 food.
 - b. Spot three 6 cm NGMA test plates with 100 μl of the culture and place the plates at 37°C overnight.
 - c. The next day, check the HB101 lawns on plates for normal growth and no contamination.
 - i. A normal HB101 lawn will appear as a light yellow/brown spot with nearly uniform thickness. It's normal for the color of the lawn to appear slightly darker along the perimeter of the spot because it grows thicker there.

- ii. A contaminated culture might be mottled in color, contain distinct non-HB101 colonies of different colors or thicknesses, contain fungal hyphae, or all of the above. **Do not use contaminated food!** Throw it away and start again.
 - d. Record the results of the contamination test on the [Bacterial food preparations](#) spreadsheet.
33. If the culture is contamination free, use as needed.

Solutions

1 L 1X Horvitz Super Broth

(for different volumes see [Horvitz Super Broth measurements](#))

1. Mix the following in a 4 L Erlenmeyer flask:
 - a. 12 g Tryptone (US Biological #T8750)
 - b. 24 g yeast extract (Sigma #Y1625)
 - c. 4 mL 100% Glycerol (Fisher Scientific #BP2291)
 - d. 900 mL dH₂O
2. Be careful to be accurate when measuring the glycerol, add glycerol after water, and pipette broth solution up and down 2-3 times to remove glycerol from the inside of the pipette.
3. Autoclave on liquid cycle for 40 minutes.
4. Once the broth is cool, add 100 mL of sterile 1X Super Broth potassium phosphate buffer (see protocol below).

1L 1X Super Broth Potassium Phosphate Buffer

(for different volumes see [Horvitz Super Broth measurements](#)). Prepared stocks of 1X Super Broth Potassium Phosphate Buffer can be found on the liquid reagents shelf. Ask the czar to make more if necessary. Make separately from Horvitz Super Broth and add only after both are cooled.

1. For a total of 1 L:
 - a. Mix the following:
 - i. 23.1 g KH₂PO₄ (Fisher Scientific #P285 3)
 - ii. 125.4 g K₂HPO₄ (Fisher Scientific #P288500)
 - iii. Up to 1 L dH₂O
 - b. Make 100 mL aliquots in 250 mL pyrex bottles.
 - c. Autoclave on liquid cycle for 30 minutes.

K medium

1. Mix the following reagents in order and add water in between each salt (the working K medium reagent stocks are stored in bottles above the HTA prep bench, Bench 8):
 - a. 5.1 mL 5M NaCl

- b. 16 mL 1M KCl
 - c. 1.5 mL 1M CaCl₂
 - d. 1.5 mL 1M MgSO₄
2. Add water up to 500 mL
3. Filter sterilize
4. Add 125 µL unfiltered HTA cholesterol 5 mg/mL in 100% ethanol. 5 mg/mL cholesterol is stored above the HTA prep bench, Bench 8..
 - a. To make fresh 5 mg/mL add 1 g cholesterol (Amresco #0433-250G) to 200 mL 200 proof ethanol (Fisher Scientific #BP2818500) in an autoclaved pyrex bottle.

Shaking notes

The orbital diameter of a shaker is linearly related to the oxygen transfer rate (OTR), which is the rate at which oxygen is transferred into the liquid from the air. The agitation is also linearly related to the OTR at roughly 1:1. According to Newton's second law of motion, force = mass x acceleration. Therefore, you can use the equation below to move from shaker to shaker with similar results.

$$\text{rpm_new} = \sqrt{\text{rpm_old}^2 * (\text{orbital_dia_old} / \text{orbital_dia_new})}$$

References:

Bates, MK, Phillips, DS, O'Bryan, J. Shaker Agitation Rate and Orbit Affect Growth of Cultured Bacteria. Thermo Fisher Scientific Inc. 2016.

Autoclave notes

1. Use Silverman 4th Floor Autoclave #1, reserve time via the CLP calendar portal (<https://www.clp.northwestern.edu/scheduling/>)
 - a. This autoclave fits eight 4L flasks
 - b. If only autoclaving 1-2 flasks, use tub, if more flasks, create a dummy flask that holds the probe.
2. Open by pressing once on the foot pedal on the bottom right, attach the metal cart to the rail so that it clicks, and pull the tray out fully. (*Caution! The metal tray may be hot! Use heat proof gloves.*)
3. Arrange media flasks on the tray, with one dummy flask towards the middle.
 - a. Dummy flask should contain 1 L of water, volume equal to that of the broth in other flasks.
 - b. Place the green temperature probe into the dummy flask so that the metal section of the probe is under the water.
4. Push the tray back into the autoclave carefully to avoid getting the temperature probe tangled, release the cart, and close the autoclave by pressing once on the foot pedal.
5. Start Liquid cycle with the following parameters once the door is fully closed.
 - a. Purge Time 1:00

- b. Sterilization Time 0:40:00
 - c. Sterilization Temp 121.0°C
 - d. Total Time: 1:15:00
6. Once the cycle is ended, open the autoclave by pressing once on the foot pedal, attach the cart to the rails, and slowly pull the tray out fully to avoid tangling the temperature probe. (*Caution! Both the metal tray and the flasks will be very hot! Use heat proof gloves.*)
 7. Remove flasks from the tray onto a heat proof surface, push the tray back into the autoclave, detach the cart, and close the autoclave by pressing once on the foot pedal.