

HACKMANN LAB

MANUAL *for* RUMEN MICROBIOLOGY

v. 5.0



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v. 5.0

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ABOUT THE COVER

An *Isotrichid* sp. is shown from a sample of mixed protozoa (see *Preparing and culturing mixed protozoa from rumen fluid*). Image was taken using differential interference contrast microscopy at 40x.

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SECTION I: ANAEROBIC TECHNIQUE AND CULTURING

Rumen microbes are challenging to work with because they are sensitive to O₂; most are strictly anaerobic. Special equipment must be built for gassing cultures with O₂-free gas, to exclude O₂. This equipment not only dispenses O₂-free gas, but it also scrubs commercial gas of trace impurities of O₂. Once this equipment is built, many microbes can be cultured. This manual focuses on culture of pure cultures of bacteria, single-species of protozoa, and mixed rumen microbes.

Constructing and using gassing station

After (1, 2)

Construction

- 1.) See **Fig. 1** for overview.
- 2.) Assemble and mount Swagelok parts on plywood board after cutting holes for parts.
 - a. Drill ¼” holes in paint-grade plywood board (48” L x 12” H x ¾” D actual dimensions) for bolts (20-¼”) (**Fig. 2**).
 - b. Drill 3/8” holes to accommodate Swagelok parts that pass between front and rear panels (**Fig. 3**).
 - c. Paint board with several coats of black paint.
 - d. Assemble Swagelok parts and mount on front and rear panels of board using 1/2” rubber insulated clamps and bolts (**Fig. 4**). Some parts pass through front and rear panels; see **Fig. 5** for connections.
 - e. Mount board on a frame made of support stands and rods (1/2” diameter x 48” length) (see **Fig. 4B** and **6**).
- 3.) Construct O₂-scrubbing copper column.
 - a. Obtain a quartz glass tube to make the column (**Fig. 7**). Quartz glass as it is heat-stable whereas Pyrex glass will expand with heating. Ours was made to the appropriate length by special order from Chemglass.
 - b. Pack the column with thin copper turnings (**Fig. 8**). Density of copper should be 20 g/ft of column (i.e., 51-2/3 g in total). Do not pack the upper 2” and bottom 3” of the column. Insert a small amount of glass wool in the bottom of the column to hold the copper in place (**Fig. 7**).
 - c. Insert butyl-rubber stopper into each end of main tubing (**Fig. 7**). Before inserting, bore a hole in the stopper with a 3/32” drill bit (for later insertion of 1/8” copper tubing). Use butyl rubber stoppers as these are gas-impermeable.
 - d. Wrap heat tape around column, then wrap fiberglass insulating tape around heat tape (**Fig. 7**). Secure fiberglass tape at top and bottom of column using loops of 24-gauge wire.
 - e. Mount column onto tripod base using 2-prong extension clamp covered with fiberglass sleeves (**Fig. 7**).
- 4.) Set up CO₂ (99.99%), N₂ (99.999%), and H₂ (99.999%) tanks near gassing station and install 50 psi regulators (**Fig. 1**). Install a flash arrestor on the H₂ regulator (**Fig. 9**) and compression fittings on all regulators (**Fig. 10**).
- 5.) Connect various parts of station together using 1/8” copper tubing. (**Fig. 1** and **5B**). When connecting T-fittings (B-200-3) from one nozzle assembly to the next, use tubing that is 6.875” length exactly. For other connections, length of tubing is not critical. When inserting copper tubing into butyl stoppers of copper column, use soap as a lubricant to ease movement.

Once connections are complete, verify that they are gas-tight by turning on CO₂, opening 3-way valve (B-41S2) to until pressure on gauge (PGI-63C-PG30-LAQX) reaches 1 bar, then closing

valve again. If connections are indeed gas-tight, reading regulator will not visibly decrease for days.

6.) Construct gassing probes (**Fig. 11**). Bend needle by heating just above hub until red hot (**Fig. 12A**). Bend by placing red-hot section on rod of metal stand, then pulling needle around pipe (**Fig. 12B**). Use pliers to hold needle throughout. Insert bent needle into 5-mL syringe and push glass wool into syringe using another needle.

7.) Construct air-stone bubblers (**Fig. 13**).

8.) Connect gassing probes and air-stone bubblers to gassing station using butyl rubber tubing (1/4" ID, 1/2" OD; Fisher 14-168B).

9.) Plug heat tape into rheostat (**Fig. 14**) and adjust knob until column temperature reaches 350°C (measured by inserting thermocouple between heating tape and column). For our rheostat, adjusting knob to 72.5 V is usually sufficient.

Use

The column is used to scrub all O₂ from CO₂ and N₂ gas used in culturing. Commercial CO₂ and N₂ gas contains trace O₂ that will kill or inhibit rumen microbes if O₂ is not removed.

1.) If needed, reduce copper turnings in column. Reduced copper is red and is effective in removing O₂. Copper turns pale red, colorless, and then black as it is oxidized. It is ineffective in removing O₂ when oxidized.

To reduce, first turn on CO₂ or N₂ and exhaust through a gassing probe for a few minutes (to flush system of most O₂). Turn on H₂ to 0.5 bar. Combust H₂ by holding a lighter up to the gassing probe until a flame is lit. Allow H₂ to circulate until copper is red and reduced (may require several minutes).

2.) Turn on CO₂ or N₂. Wait a few minutes to flush system before use. Use flow rate of 5 mL/s (achieved by pressurizing system to 1 bar and turning needle valve [B-SS2] 3.75 rotations).

Fig 1. Overview of gassing station.



Fig 2. Placement of bolts. $A = 7.6''$, $B = 1.5''$, $C = 3.75''$, $D = 4.75''$, $E = 2.75''$, $F = 5.625$, $G = 3.75''$, $H = 6.325''$.

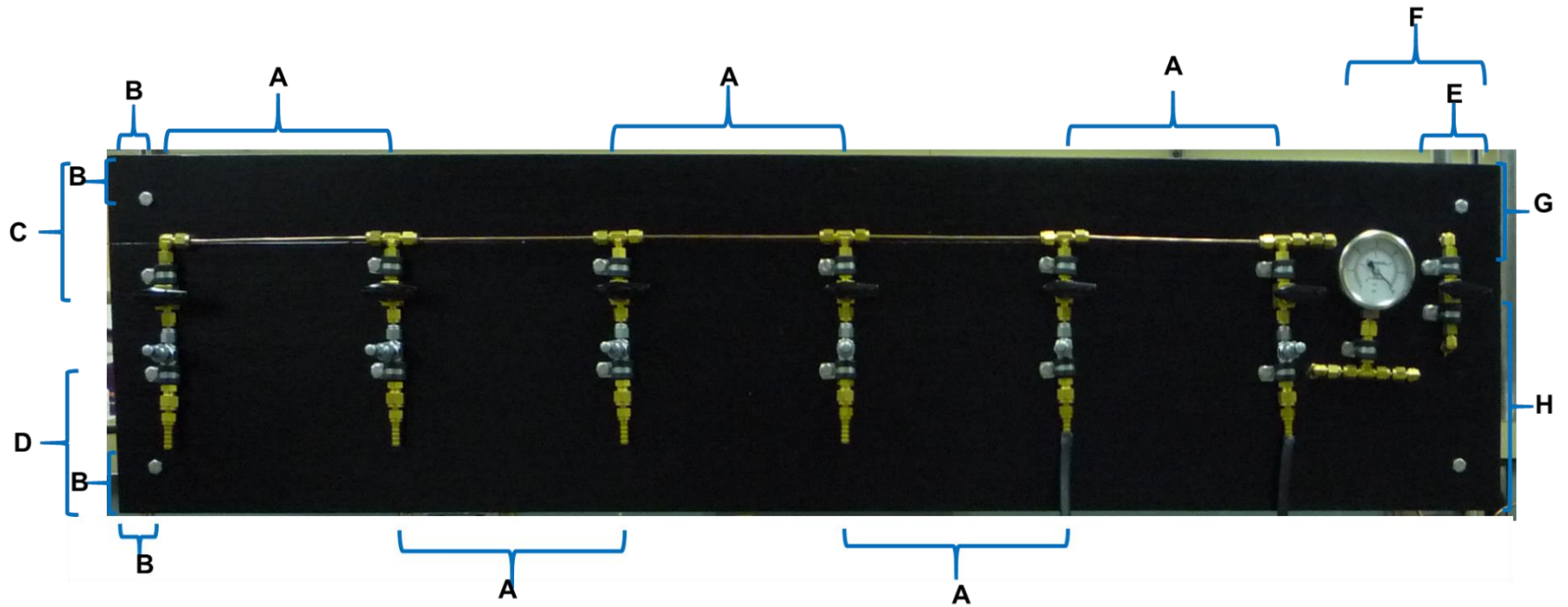


Fig 3. Placement of holes for Swagelok parts. A = 2", B = 2.75", C = 6.5", D = 4.75", E = 3.25", F = 6.75".

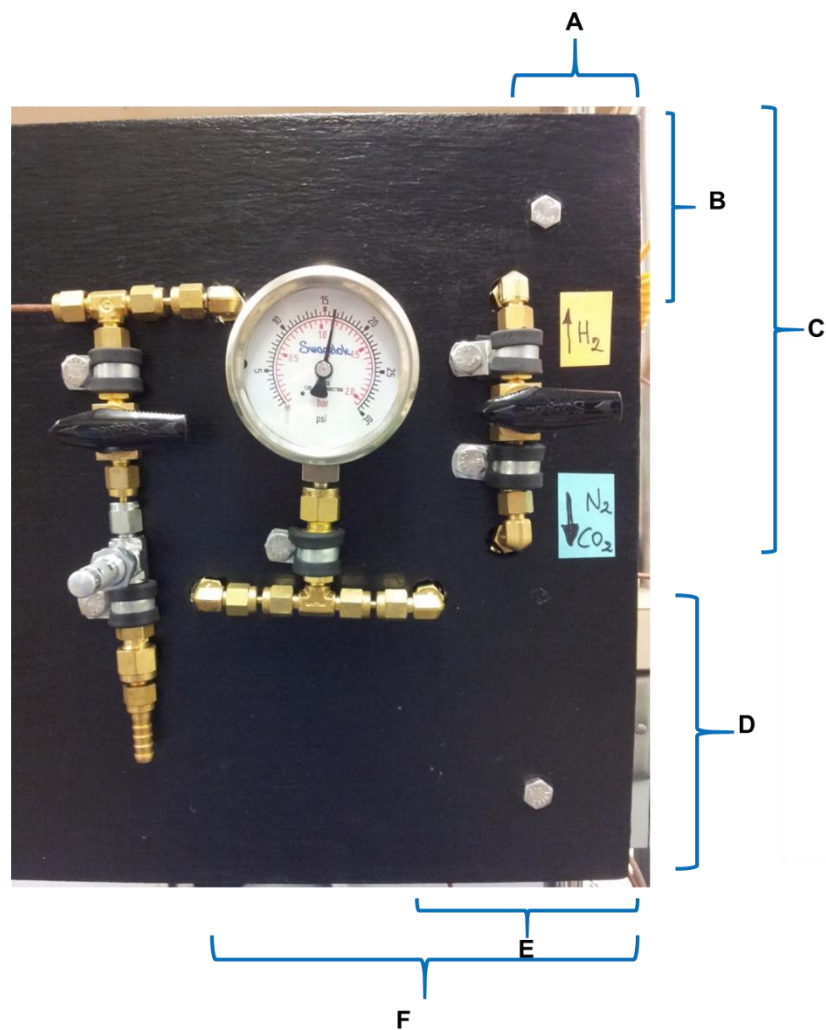
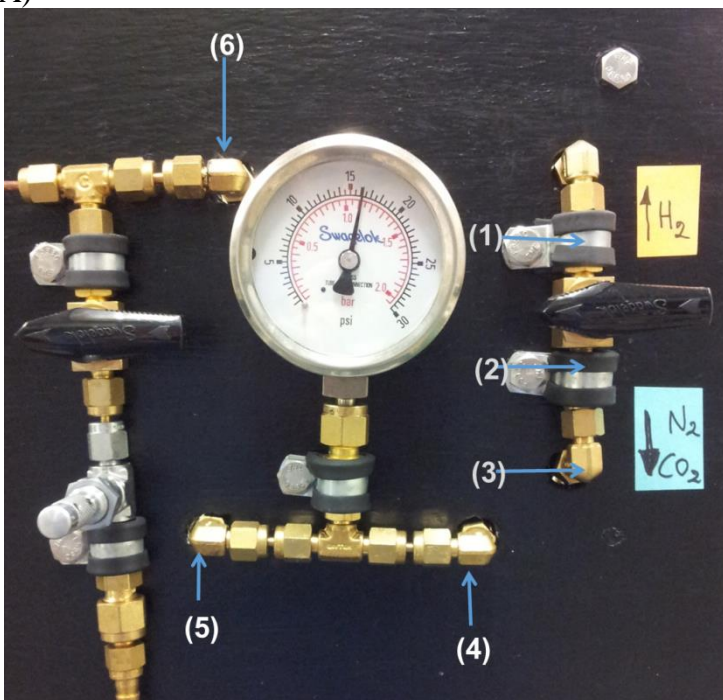


Fig 5. Connections between (A) front and (B) rear panels.

(A)



(B)

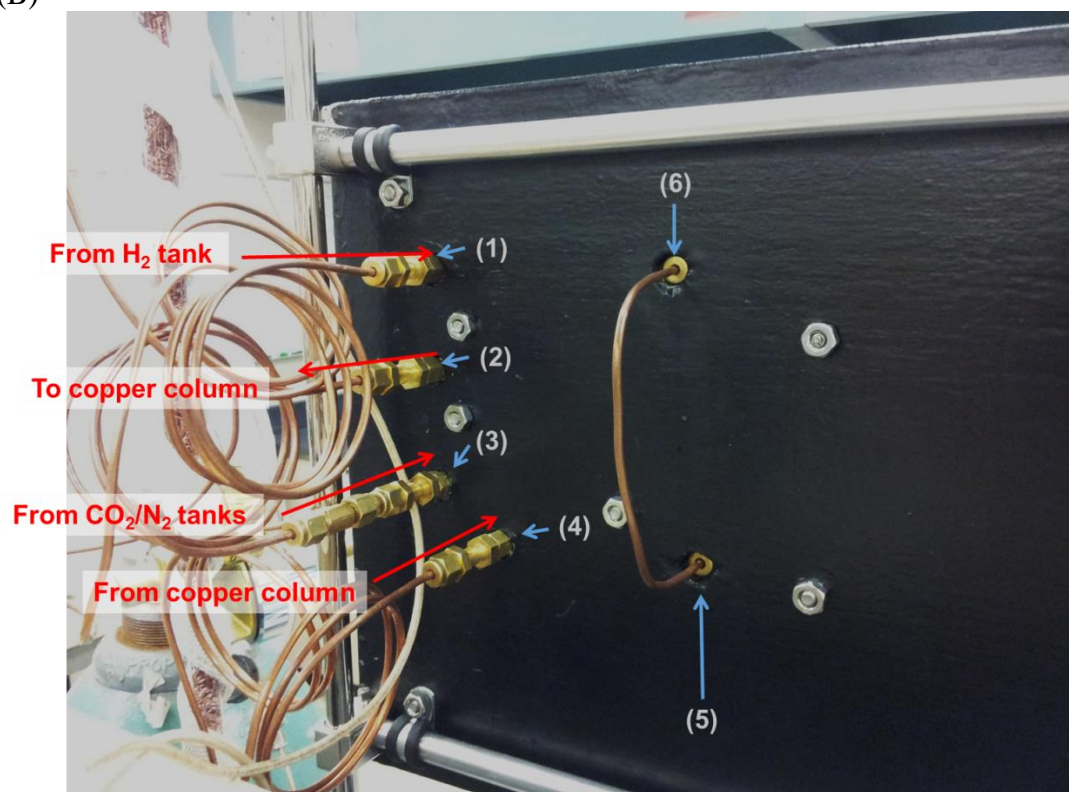


Fig 6. Frame components. For components visible from rear only, see Fig 4B.

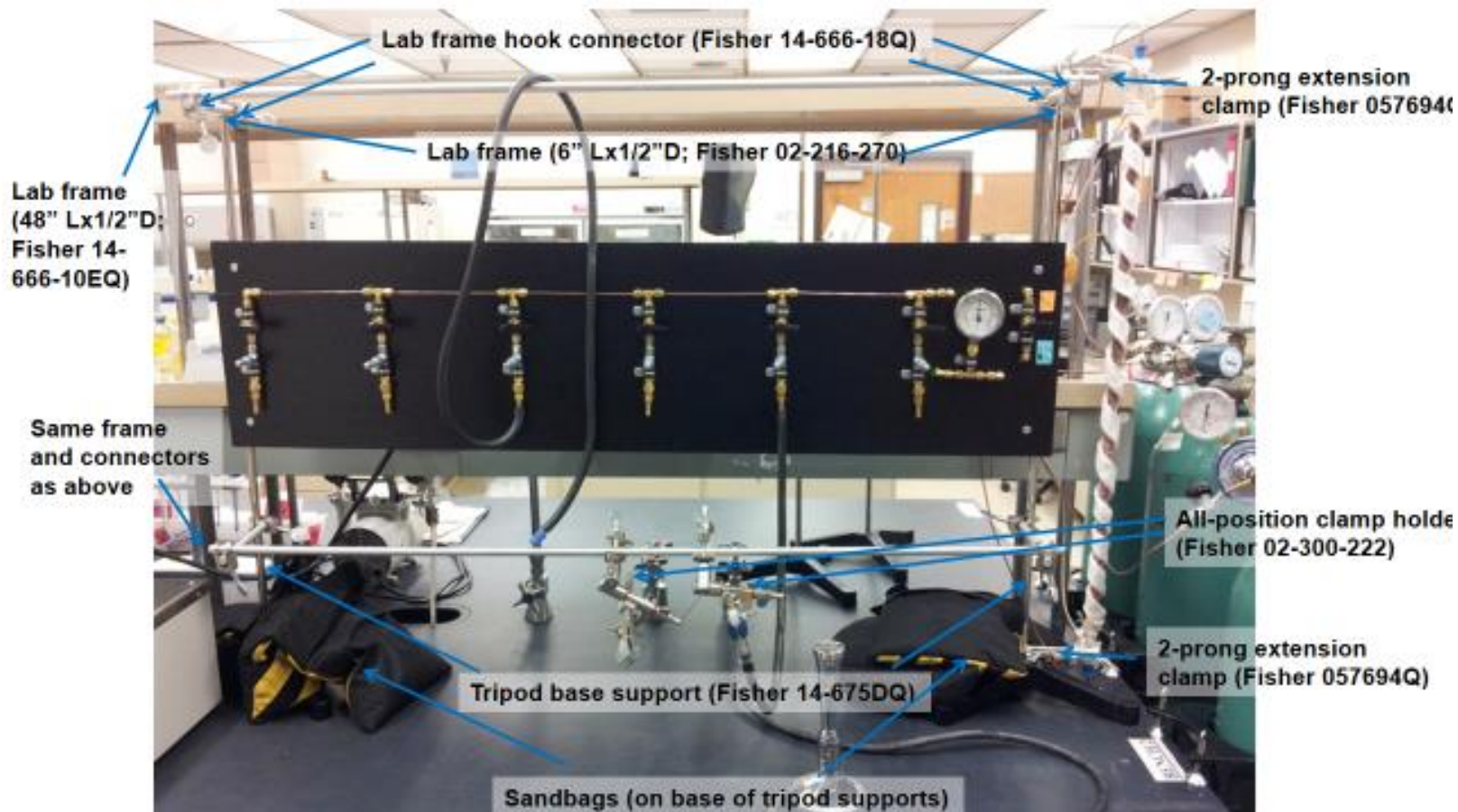


Fig. 7. Close-up of copper column.

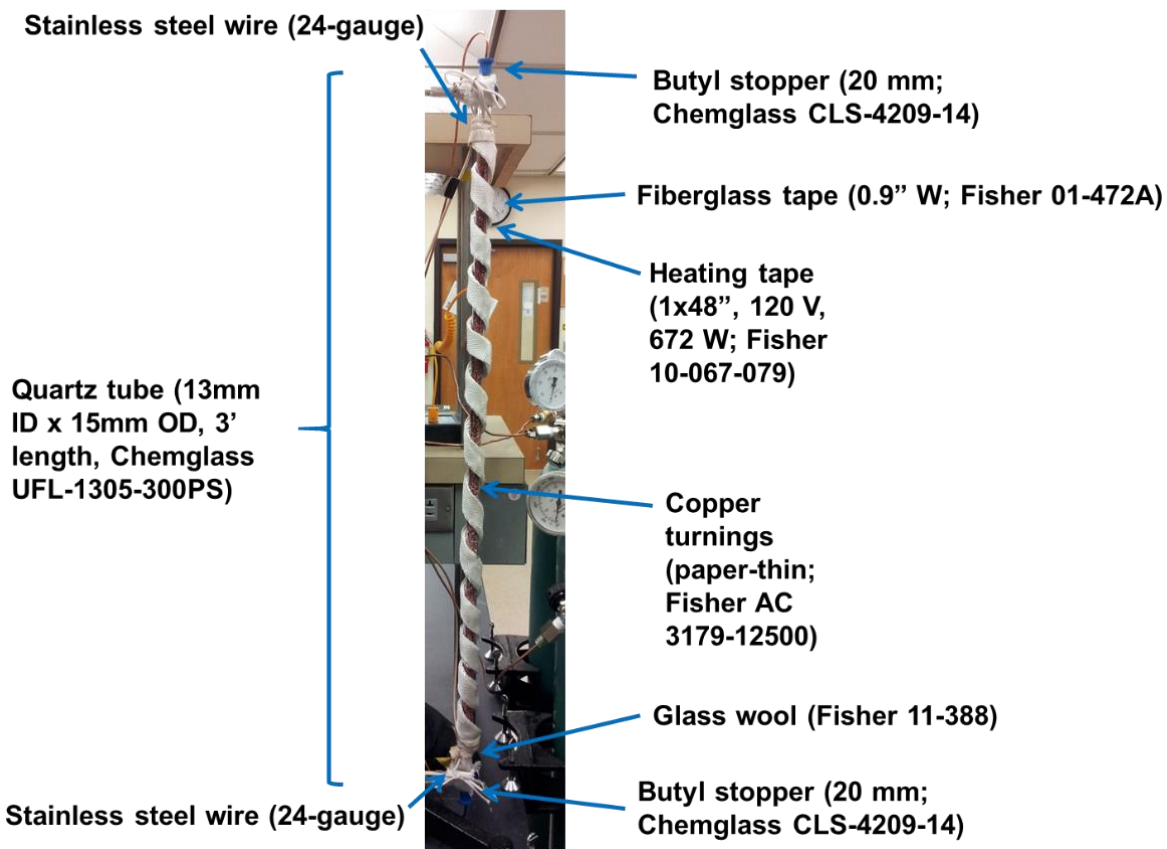


Fig 8. Packing column with copper turnings.



Fig 9. Flash arrestor and compression fitting on H₂ tank.



Fig 10. Compression fitting on N₂ tank (identical for CO₂ tank).



Fig 11. Gassing probe.

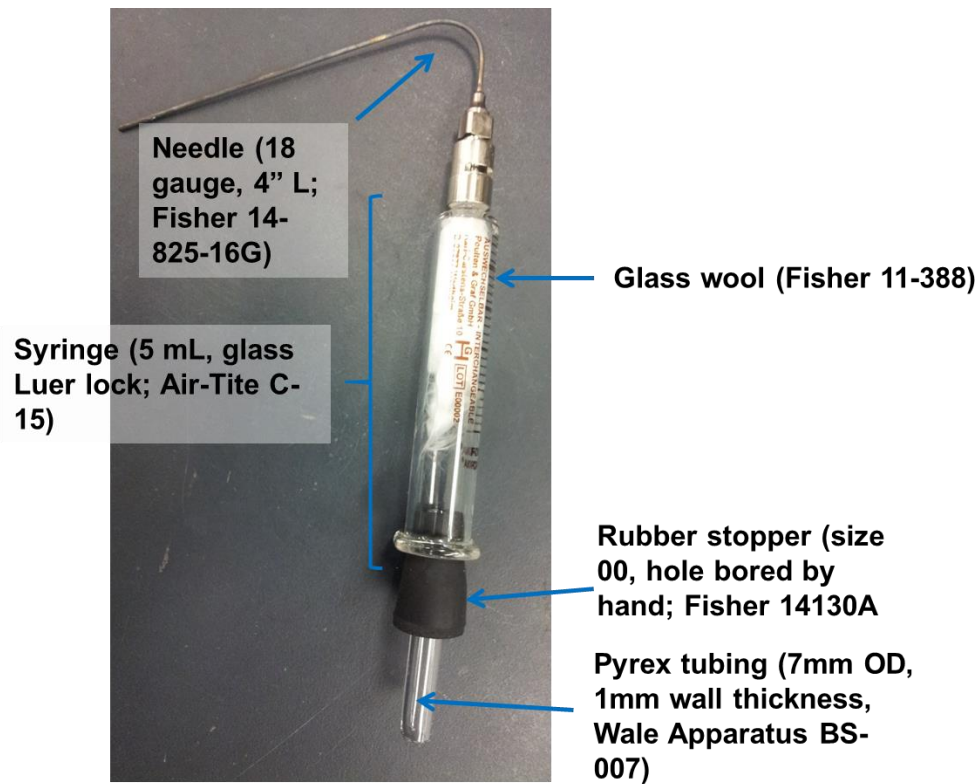


Fig 12. Bending needle of gassing probe. (A) Flaming needle above hub. (B) Bending needle around metal stand.

(A)



(B)

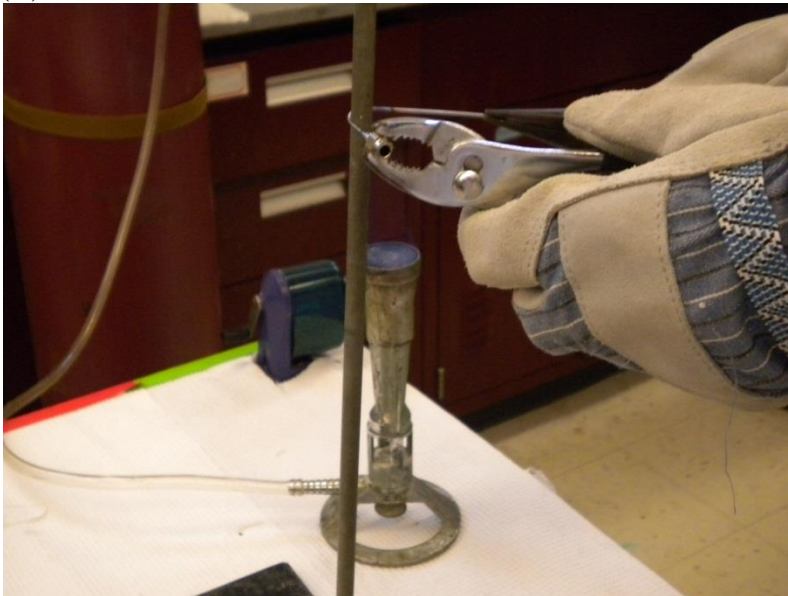


Fig. 13. Airstone bubbler. Airstone will later be inserted into rubber tubing.

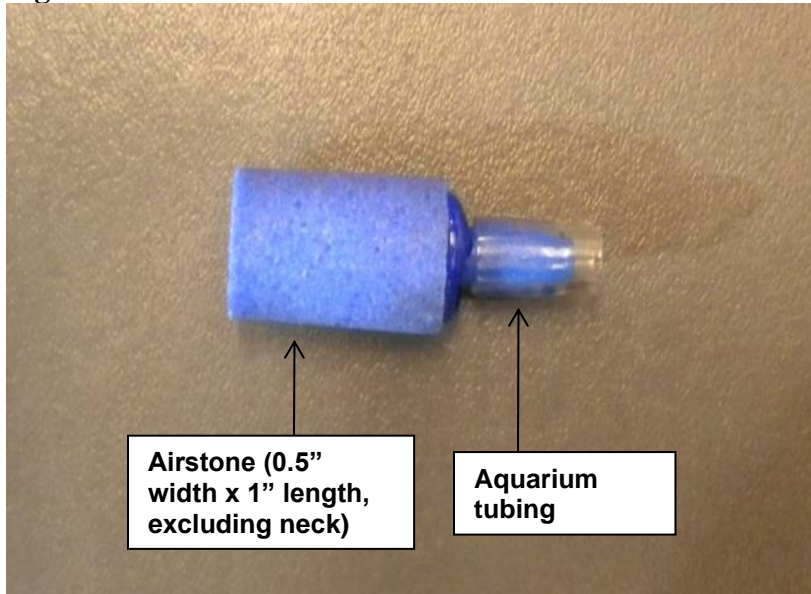


Fig. 14. Rheostat (140 V, 10A), with heat tape plugged in and adjusted to 72.5 V.



Culturing bacteria

From (1, 3-5), Burk Dehority (personal communication), Paul Weimer (personal communication)

Growth in broth

Use of broth is the easiest way to grow and transfer cells, though single colonies cannot be isolated to assess culture purity.

- 1.) Prepare 9-mL aliquots of broth media in Balch tubes. Follow *Making buffer and media* and *Media and buffer recipes*.
- 2.) Transfer 0.1 mL from a stock culture tube to 2 new tubes prepared in #1.
 - (a) Set up autoclaved gassing probe on clamp. Flame sterilize.
 - (b) Flame sterilize rubber bung of tubes using ethanol. Keep under a lit Bunsen burner.
 - (c) Flush 22-gauge needle of 1-mL syringe with CO₂ by inserting it into gassing probe (see *Making buffer and media*). Withdraw 0.3 mL CO₂ (same volume as culture removed in next step).
 - (d) Withdraw ~0.3 mL culture from stock tube after injecting 0.3 mL CO₂.
 - (e) Inject 0.1 mL culture into each of the 2 new tubes. Use the remaining culture to prepare a microscope slide to check purity.
- 3.) Incubate tubes at 39°C for 1 to 2 days (until good growth). Monitor growth according to *Measuring optical density for monitoring growth of bacterial cultures*.
- 4.) Remove tubes from incubator and keep at room temperature (viability decreases rapidly at 39°C once cells reach stationary phase).
- 5.) Repeat #2 to 4 at least once every 2 weeks. Repeating more frequently will shorten lag time of growth.

Growth on bottle plates

Bottle plates (**Fig. 1**) allow single colonies to be isolated, and their use is analogous to Petri dishes. Bottle plates are custom-ordered through Bellco (catalog #2535-S0027)

Preparation of bottle plates

- 1.) Prepare agar medium by adding 9-mL broth to 0.18 g agar in Balch tubes under CO₂. Agar may be individually weighed in tubes. Seal with rubber bung and aluminum lid then autoclave at 121°C/15 lb pressure for 20 min. Cool.
- 2.) Flush bottle plate with CO₂. Insert needle of bottle plate gassing probe (**Fig. 2**) into rubber bung of plate, turn on CO₂ to the probe, and insert a 22-gauge needle into rubber bung to exhaust gas. Continue flushing for several minutes, then remove needles and probe. Autoclave at 121°C/15 lb pressure for 20 min.

- 3.) Melt the agar medium prepared in #1 by placing Balch tube in 100°C water bath. Transfer tubes to ≥ 50 °C water bath, which will keep agar molten.
- 4.) With ethanol, flame sterilize upper rubber bung of bottle plate and the bung of agar medium tube. Keep under flame.
- 5.) With 10-mL syringe and needle flushed with CO₂, withdraw most agar medium from tube. Do not withdraw all agar in the tube (leave ~0.2 mL) to avoid introducing bubbles into the agar withdrawn in the syringe.
- 6.) Inject agar into the upper chamber of the bottle plate. Lay the plate flat, inject agar slowly, and then tilt the plate around to help spread the agar evenly. Do not eject all agar from the syringe (leave ~0.2 mL) or bubbles will be introduced. Allow several minutes to solidify.

Inoculation (standard method)

- 1.) With ethanol, flame sterilize lower rubber bung of bottle plate. Insert a sterile bottle-plate gassing probe that is connected to CO₂ from gassing station.
- 2.) With ethanol, flame sterilize upper rubber bung of bottle plate. Remove bung with curved-end hemostats (previously autoclaved and flame-sterilized with ethanol). Place bung and hemostats on the bench, keeping the small end of the bung face-up to minimize contamination.
- 3.) Following ***Growth in broth*** above, remove ~0.2 mL liquid from stock culture. Withdraw liquid using a 22-gauge 3-1/2" needle attached to a syringe. Use a flame-sterilized, straight-end hemostats to assist insertion of needle into culture.
- 4.) Orient needle into bottle plate and eject a large drop of culture into the top corner (**Fig. 1**). Be careful not to touch interior of bottle plate with needle.
- 5.) Tilt plate upwards to spread drop down to bottom of plate. Tilt the plate to the side, also, to keep drop at the wall of the plate.
- 6.) Streak the drop following the zig-zag pattern according to **Fig. 1**. Use the tip of a sterile colony picker (**Fig. 3**) for streaking. Between each streak, flame the tip of the colony picker. Be careful not to touch the neck, walls, or top face of the plate. To prepare the picker, see ***Preparing colony picker*** below. Before streaking, fuse the tip of the colony picker by holding it in the flame of the Bunsen burner.
- 7.) Re-insert upper bung into plate. Flame sterilize it and the hemostats after immersing in ethanol. Push bung in half-way, remove hemostats, flame sterile exposed end of bung with ethanol, and push bung in completely.
- 8.) Remove the gassing probe from lower bung.

9.) Incubate bottle plate at 39°C. Incubate upside down (with agar face on top) to permit water to drain from agar.

Picking colonies and transferring to broth

- 1.) Inoculate a bottle plate following ***Inoculation (standard method)***. Once colonies show good growth (≥ 0.5 mm in diameter), repeat #1 and 2 of ***Inoculation (standard method)***.
- 2.) With ethanol, flame sterilize bung of broth tube to receive colony.
- 3.) Attach a 22-gauge 3-1/2" needle to a 1-mL syringe. Insert into bottle plate without touching neck, walls, or top face of the plate (**Fig. 4**).
- 4.) Flush needle by drawing plunger in and out several times.
- 5.) Touch the colony of interest with the flat open, flat end of the needle. Scrape as much of the colony off the plate (without touching neighboring colonies) (**Fig. 4**).
- 6.) Remove needle from plate and quickly eject colony into broth tube. Use straight-end hemostats to aid insertion of needle into tube. Draw syringe plunger in and out to ensure colony was ejected and mixed.
- 7.) Incubate tube at 39°C overnight.

Picking colonies and transferring to bottle plate

Follow ***Picking colonies and transferring to broth***, but use colony picker, not a needle, to pick colony. Transfer to bottle plate and streak using zig-zag pattern in Fig. 1.

Inoculation (serial dilutions)

This method is similar to the standard method of inoculation, but it accommodates serial dilutions.

- 1.) Prepare a 10^{-1} dilution of culture. Load a 1-mL syringe with 1 mL culture, then inject remaining 1 mL fluid into a Balch tube containing 9-mL anaerobic dilution solution (ADS; see ***Buffer and media recipes***).
- 2.) Prepare a 10^{-2} dilution. Withdraw 1-mL of the 10^{-1} dilution tube prepared in #2, then inject liquid into a new Balch tube containing 9-mL ADS. Repeat to prepare 10^{-3} to 10^{-9} dilutions.
- 3.) Prepare bottle plates according to ***Preparation of bottle plates***. Before autoclaving, place 5 to 10 glass beads (3 mm) in the lower chamber. After injecting agar and solidification, roll beads up into upper chamber.

- 4.) Inoculate bottle plates with 10^{-1} to 10^{-9} dilutions. Inject 0.1 mL of dilution with an autoclaved 0.1 mL Hamilton syringe (#81020).
- 5.) Spread dilution with beads. Shake bottle plate in circular motions thoroughly to allow beads to spread dilution evenly. Roll beads into lower chamber.
- 6.) Incubate bottle plate at 39°C . Incubate upside down (with agar face on top) to permit water to drain from agar.

Growth on roll tubes

Similar to bottle plates, roll tubes allow single colonies to be isolated. Though serial dilutions are possible with bottle plates, they less laborious with roll tubes. Quantitative dilutions—the gold standard for assessing purity—is thus done with roll tubes.

Quantitative dilutions

- 1.) Prepare agar medium by adding 9-mL broth to 0.18 g agar in Balch tubes under CO_2 . Agar may be individually weighed in tubes. Seal with rubber bung and aluminum lid then autoclave at $121^{\circ}\text{C}/15$ lb pressure for 20 min. Cool.
- 2.) Before use, re-melt agar in roll tubes by placing in 100°C water bath. Transfer tubes to 45°C water bath, which will keep agar molten.
- 3.) Inoculate a roll tube. With syringe, withdraw 1-mL liquid from culture tube and inject into roll tube. Mix contents by rocking tube back-and-forth (careful not to introduce bubbles). Use this 10^{-1} dilution to inoculate another roll tube (10^{-2} dilution), and place 10^{-1} dilution back into 45°C water bath (to prevent solidification).
- 4.) Further prepare 10^{-3} dilution to 10^{-9} dilutions following #3.
- 5.) Roll the tube until agar solidifies. Keep tube nearly horizontal, and roll in container with dilute ice water. For best results (smoothest solidification of agar), roll tube quickly and with only the lower surface of the tube touching the water (do not completely submerge). Be careful not to tip agar towards stopper, where it can form a plug around the stopper. Incubate at 39°C for 1 to 3 days. Roll tube after incubation is shown in **Fig. 5**.
- 6.) Assess culture purity.
 - (a) Count colonies in 10^{-1} to 10^{-9} dilutions. Determine if colonies decrease in accordance with dilution. Rough counts, particularly at low dilutions, are often sufficient.
 - (b) Examine colonies under dissecting microscope for colony morphology (color, size, shape). Determine if only 1 colony morphology appears. Be aware that colonies growing in agar will be smaller and less diffuse than colonies growing on surface of agar, even in pure culture. **Fig. 6** compares morphology of colonies from mixed and pure cultures.

(c) Examine cells under conventional microscope. Determine if only 1 cell type appears. To obtain a sample, use a syringe to collect a small amount of water of syneresis from the bottom of a roll tube (invert tube to obtain sample).

(d) Perform sequencing and phenotypic test. Follow *Sequencing and taxonomic assignment of bacteria*. Phenotypic tests should also be done.

Qualitative dilutions

For rapid assessment of colony morphology, qualitative dilutions may be used in place of the more rigorous quantitative dilutions outlined above.

1.) Follow #1 to #3 of *Quantitative dilutions* above to prepare a 10^{-1} dilution. Do not remove needle from 10^{-1} dilution tube.

2.) Draw and expel liquid agar into syringe repeatedly. To avoid introducing bubbles, expel liquid from syringe only when tube is upright (liquid is at bottom).

3.) With the syringe fully depressed (agar only in the deadspace of the syringe and needle), insert the needle into a new roll tube. Mix and repeat for a total of 5-7 serially-diluted tubes. The same syringe and needle can be used throughout.

4.) Assess colonies and cells for purity according to *Colony picking and subculture*. Because quantitative (10-fold dilutions) are not used, do not assess colonies numbers.

Picking colonies

1.) Prepare roll tube for picking colony. Remove rubber bung aseptically by removing aluminum cap, flaming bung with ethanol, and twisting off bung with curved-end hemostats. Before use, autoclave hemostat and flame sterilize after dipping in ethanol. Immediately insert gassing probe (autoclaved and flame-sterilized) and clamp tube into place (**Fig. 7**). Be careful not to touch agar with probe.

2.) Pick colony. Attach pipette bulb to colony picker, and insert colony picker into roll tube (careful not to touch agar) (**Fig. 8**). For best control, hold colony picker with 2 hands and rest elbows on table. Squeeze bulb to force out air; stab pipette tip into agar region containing colony; cut agar with pipette tip by twisting; release pipette bulb to create suction; and remove picker. Small agar plug will be lodged in colony picker (as far as several centimeters from tip).

3.) Eject plug with colony into another roll tube. Before picking colony, remove bung from recipient roll tube, and insert sterile gas probe. Keep upright in 45°C water bath until plug is ejected. Re-insert bung into tube after flame sterilizing. Mix contents by rocking tube back-and-forth. Do not yet roll tube.

4.) Prepare quantitative or qualitative dilutions of the tube in #3. Roll the tubes.

Growth on slants

Slants allow bacteria to be grown on agar—not all bacteria grow well on broth—and slants are less laborious than bottle plates or roll tubes. Single colonies cannot be isolated.

1.) Prepare slants by adding 8 mL of media (see *Media and buffer recipes*) into 10 mL serum tubes containing 0.16 g agar. Seal with rubber bung and aluminum lid then autoclave at 121°C/15 lb pressure for 20 min. After autoclaving, before agar is melted, put serum bottle on rack to solidify at an appropriate angle (**Fig. 9**).

2.) Transfer culture from stock slant to new slant.

(a) Prepare a Balch tube with 10 mL ADS (*Media and buffer recipes*).

(b) Load a syringe with 0.5 mL ADS after flushing needle with CO₂ (see *Growth on broth*), then inject the 0.5 mL ADS into stock slant. Here and before every injection, flame sterilize the rubber bung of the tube using ethanol. Gently tilt tube back and forth to dislodge bacteria on agar surface.

(c) Withdraw 0.05 mL liquid from slant using a 22-gauge 3-1/2” needle attached to a syringe. Use a flame-sterilized hemostat to assist insertion of needle.

(d) Inject 0.05 mL liquid into the bottom agar of new slant.

3.) Incubate slants at 39°C. Transfer to new slant every week to keep viability.

Preparing colony picker

1.) Prepare colony picker from 9” disposable Pasteur pipette (**Fig. 9**). Bend end of pipette at an angle over flame of lighter. Position narrow end of pipette over flame, leaving 3-5 mm overhang. Pipette will bend under force of gravity. Do not use Bunsen burner as flame is too strong.

2.) Pack wide end with glass wool (to filter out particulates from gas entering pipette). Use needle from gassing probe to aid packing.

3.) Autoclave at 121°C/15 lb pressure for 30 min with 10 min dry time.

Fig. 1. Bottle plate and streaking pattern.

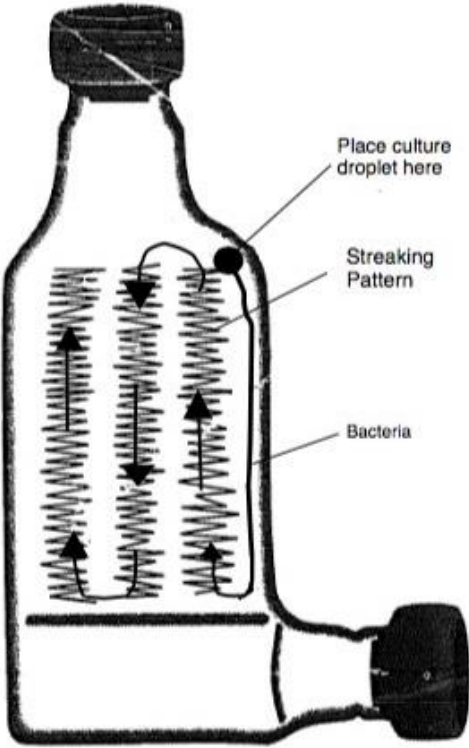


Fig. 2. Bottle plate gassing Probe

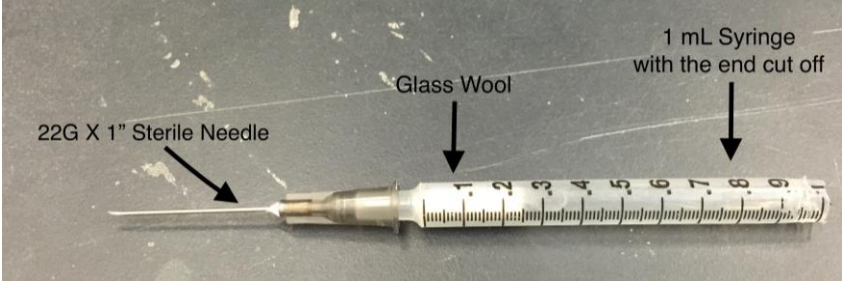


Fig. 3. Colony picker.



Fig. 4. Picking colony from bottle plate

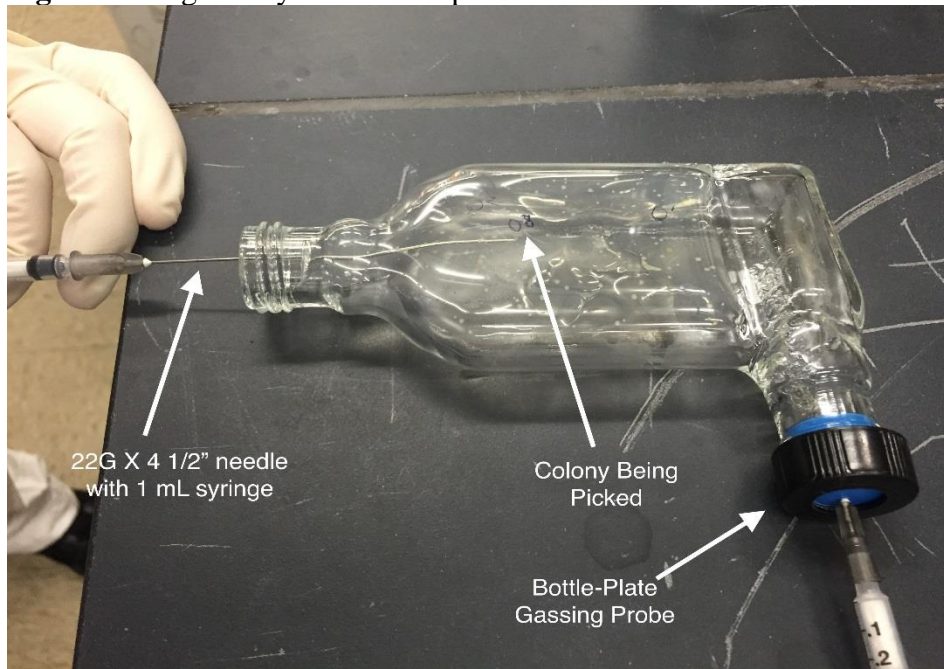


Fig. 5. Roll tubes after incubation for 2 d.

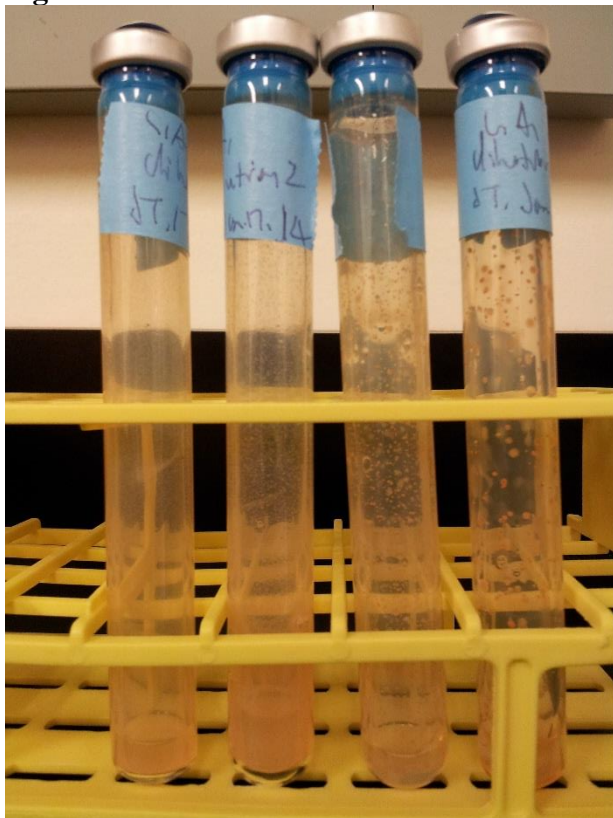


Fig. 6. Colonies of mixed and pure cultures.



Fig. 7. Preparation of roll tube for picking colony, showing placement of roll tube and gassing probe.

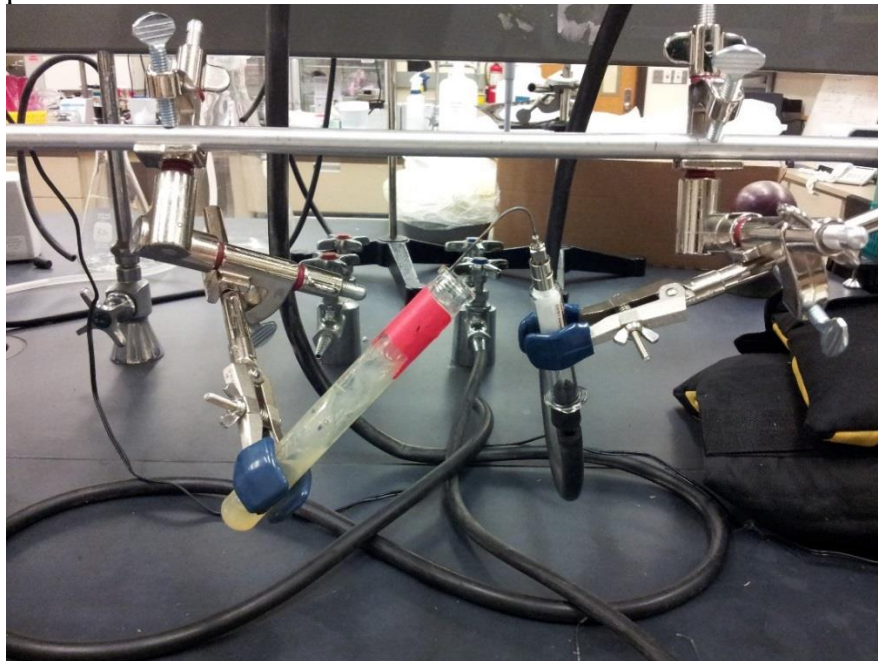


Fig. 8. Picking colony.



Fig. 9. Inoculated slant bottle, showing appropriate angle of solidified agar.



Measuring optical density for monitoring growth of bacterial cultures

- 1.) Set up Thermo Scientific Genesys 20 spectrophotometer. Make sure that measurement is set to 600 nm (default value) and COD vial holder (Fisher 14-385-449) is place.
- 2.) Inoculate a Balch tube containing media with a culture.
- 3.) Insert tube in spectrophotometer. Remove gray cap from spectrophotometer, slide tube into vial holder, and replace gray cap.
- 4.) Read optical density at 600 nm (OD_{600}).
- 5.) Place Balch tube in incubator to allow culture to grow.
- 6.) At intervals, remove Balch tube from incubator and repeat measurement of OD_{600} to monitor growth.

Notes

- (i) For highest precision, make sure tube is oriented in spectrophotometer the same way for each reading. The arrow on the aluminum cap provides a good reference for orienting the tube.

Isolation of pure cultures of bacteria from rumen fluid

From (3, 4)

Dilution and inoculation

- 1.) Collect fluid according to *Collecting rumen fluid*.
- 2.) Prepare 10^{-1} dilution of fluid. Load a 1-mL syringe with ~1.1 mL rumen fluid. Discard the first ~0.1 mL fluid (to flush the needle deadspace of any air), then inject remaining 1 mL fluid into a Balch tube containing pre-warmed 9-mL anaerobic dilution solution (ADS; see *Buffer and media recipes*).
- 3.) Prepare 10^{-2} dilution. Withdraw 1-mL of the 10^{-1} dilution tube prepared in #2, then inject liquid into a new Balch tube containing 9-mL ADS. Repeat to prepare 10^{-3} to 10^{-9} dilutions.
- 4.) Inoculate roll tubes with 10^{-7} dilution prepared above. With syringe, withdraw 1 mL liquid from 10^{-7} dilution tube, and then inject into roll tube containing 9 mL RGCSA media (kept at 45°C until just prior to inoculation; see *Buffer and media recipes*). See *Growth on roll tubes* in *Culturing bacteria* for use of roll tubes.
- 5.) Roll tube until agar solidifies. Be careful not to tip agar towards stopper, where it can form a plug around the stopper that hinders colony picking later.
- 6.) Repeat #4 and #5 for 10^{-8} and 10^{-9} dilutions.
- 7.) Incubate tubes at 39°C for 7 d.
- 8.) Pick a new colony from the highest dilution showing good growth (>5 colonies), subculture into a new RGCSA tube, and prepare a new set of 10^{-1} to 10^{-9} dilutions from that new subculture.
- 9.) Repeat subculture until culture is pure (see *Assessment of purity* in *Culturing bacteria*). Culture may be pure upon first subculture, but subculturing an additional few times will increase confidence of purity.
- 10.) When roll tube culture is pure, inoculate broth tube for routine maintenance. Inject 0.5 mL ADS into roll tube to wash colonies from walls, and inject 0.1 mL into broth tube.

Notes

- (i) When assessing colony purity, be aware that RGCSA contains dead bacteria from rumen fluid. These dead bacteria may appear to contaminate sample. Their concentration is low, as agar “traps” them.

Ordering and growing bacteria from culture collections

Adapted from DSMZ website

(https://www.dsmz.de/fileadmin/Bereiche/Microbiology/Dateien/Kultivierungshinweise/Kultivierungshinweise_neu_CD/Opening_14.pdf)

- 1.) Order the needed bacterial strain from DSMZ (<https://www.dsmz.de/>) or ATCC (<http://atcc.org/>). DSMZ is less costly and has wider selection of anaerobes.
- 2.) Make medium recommended by supplier following *Making buffer and media*.
- 3.) Order the strain, choosing the freeze-dried form if available.
- 4.) After the culture arrives, open the ampoule and rehydrate the freeze-dried pellet (**Fig. 1**).
 - (a) Set up autoclaved gassing probe on clamp. Flame sterilize.
 - (b) Holding the bottom of the outer vial with pliers, heat the top of the vial over the flame.
 - (c) Using spray bottle, squirt small amount of water on the heated glass to crack it.
 - (d) Strike off the top with a metal file (previously flame-sterilized with ethanol).
 - (e) Remove cotton plug with a straight-end hemostat (autoclaved and flame-sterilized).
 - (f) Immediately gas vial with gassing probe.
 - (g) With 1-mL syringe, withdraw 0.5 mL supplier-recommended medium from a culture tube, transfer to vial, and resuspend the pellet by drawing contents in and out of syringe. It is helpful for a second person to hold the vial while doing this step.
 - (h) Transfer 0.5 mL resuspended pellet back to the culture tube. This is the primary culture.
 - (i) Transfer the remaining pellet (~0.1 mL) to a second culture tube of supplier-recommended medium. This is the backup primary culture.
 - (j) Transfer 1 mL from the primary tube to a second type of medium. This second type of medium is any that is likely to give good growth, and PC - VFA or PC + VFA are common choices.
- 5.) Monitor growth by measuring optical density at 600 nm (see *Measuring optical density for monitoring growth*).
- 6.) Once good growth is achieved, transfer to new culture tube (secondary culture).
- 7.) Cryopreserve the first culture possible (preferably the primary culture) according to *Cryopreservation of bacteria*.
- 8.) Isolate colonies with first culture possible (preferably primary or secondary culture) according to *Growth on bottle plates* or *Growth on roll tubes*.
- 9.) Transfer isolated colony to broth. Use this broth culture for subsequent experiments and for cryopreservation.

Notes

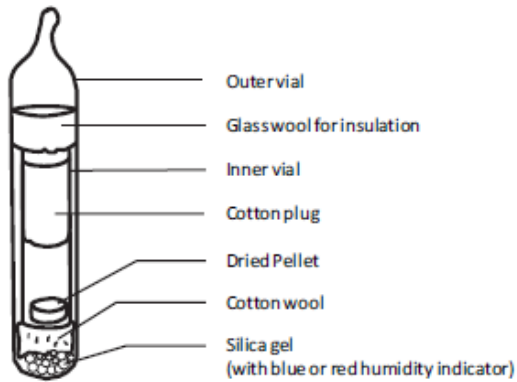
- (i) The supplier will not guarantee the viability of the culture if the recommended medium is not used.

(ii) If no growth occurs in primary or other cultures, contact supplier for replacement vial.

(iii) The first culture that is cryopreserved (usually the primary culture) should be kept but not used unless all subsequent cultures die or are compromised.

Fig. 1. Opening the ampoule containing the freeze-dried pellet. (A) Diagram of the vial. (B) Heating the vial under flame. (C) Striking off tip of vial. (D) Removing cotton plug. In (C), forceps are shown, but we have found a heavy metal file is ideal.

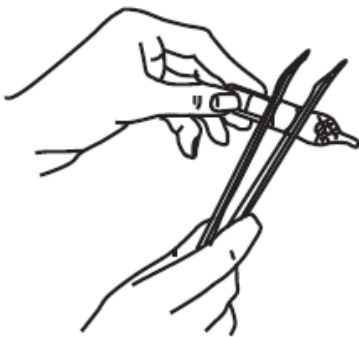
(A)



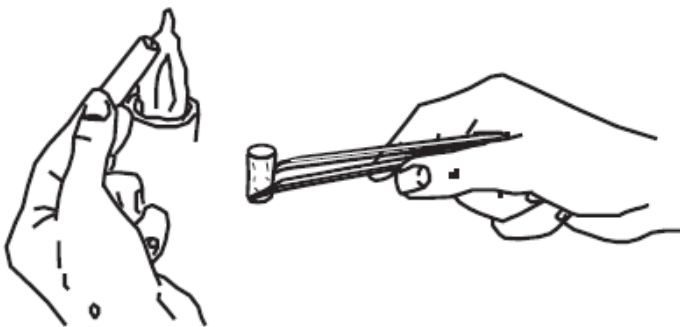
(B)



(C)



(D)



Cryopreservation of bacteria

Modified from (6), Michael Flythe (personal communication), and Paul Weimer (personal communication)

Freezing

- 1.) Inoculate 9 mL broth tube with a pure culture (see *Culturing bacteria*). Incubate broth culture for 12-24 h at 39°C.
- 2.) Add anaerobic dilution solution (ADS) with 50% glycerol (see *Media and buffer recipes*). Release gas pressure from tube with syringe. Add 6 mL ADS with 50% glycerol. Final concentration of glycerol will be 20%.
- 3.) Store at -20 or -80°C.

Revival

- 1.) Thaw tubes at 4°C.
- 2.) Transfer 0.4 mL to fresh 9 mL broth tube. Incubate at 39°C. Growth may be apparent after a several hours of lag.
- 3.) Place cryopreserved tube back into freezer (optional). Tube can be freeze-thawed multiple times without effect on viability (6).

Notes

- 1.) Frozen cultures may retain viability for years (6), but they are normally revived and re-frozen every year to ensure viability (Michael Flythe and Paul Weimer, personal communication).
- 2.) Viability may be longer at -80°C than -20°C (Michael Flythe, personal communication), though either temperature will suffice if cultures are revived and re-frozen every year.

Culturing methanogens

Procedure is similar to *Culturing bacteria*, except tubes and bottles must be pressurized with 80% H₂/20% CO₂ (Airgas Z02HY80320077M5), and growth is much slower.

Growth in broth

- 1.) Prepare 9-mL aliquots of broth media in Balch tubes. Make sure that atmosphere is 80% H₂, not 100% CO₂.
- 2.) Transfer 0.1 mL from a stock culture tube to 2 new tubes prepared in #1. Do transfer on lab bench and using CO₂ for flushing syringe.
 - 3.) Pressurize tubes with 80% H₂.
 - (a) Flame sterilize rubber bung of tubes using ethanol. Keep under a lit Bunsen burner.
 - (b) Move gassing probe to clamp in fume hood (requires long rubber tubing).
 - (c) Turn on 80% H₂ to 1 bar and allow to circulate for a few minutes. To ensure that H₂ is circulating, it can be combusted by holding a lighter up to the gassing probe (flame will appear).
 - (d) At lab bench and under flame, attach 22-gauge needle to 60-mL syringe. Keep cap on needle.
 - (e) Take syringe to fume hood, remove needle cap, and quickly insert into gassing probe needle. To prevent contamination, do this step quickly and avoid touching outside of gassing probe needle.
 - (f) Flush syringe by drawing plunger in and out several times, and then fill with H₂. Replace needle cap.
 - (g) Take syringe back to bench, and, under flame, change needle with new one (to prevent contamination). Depress plunger by a few milliliters (away from flame) to flush out air in new needle.
 - (h) Move a tube away from under the flame and inject 17 mL of 80% H₂.
 - 3.) Incubate tubes at 39°C until good growth is achieved. Monitor growth according to Measuring optical density for monitoring growth of bacterial cultures. Several days may be required to detect any growth, and final optical density will be approximately 0.2.
 - 4.) Remove tubes from incubator and keep at room temperature.
 - 5.) Repeat #2 to 4 at least once every month. Repeating more frequently will shorten lag time of growth.

Growth on bottle plates

Preparation of bottle plates

Steps are as described in *Culturing bacteria* with one exception. In step #1, agar medium is prepared under 80% H₂, not 100% CO₂. Bottle plate is still flushed with CO₂.

Inoculation (standard method)

Inoculation of bottle plates is as described in *Culturing bacteria* with two exceptions.

In step #4, the plate is inoculated by ejecting 6 or 7 drops of culture, not one drop. The larger inoculum is needed due to low density of methanogens.

After step #7, the bottle plate is flushed with 80% H₂. Flame sterilize the upper stopper and insert an empty needle, which will allow bottle to be flushed with gas. Move plate into hood and turn on 80% H₂ at 1 bar. Allow to flush for a few minutes, then remove needle from upper stopper to stop. Proceed with step #8.

Cryopreservation

Freezing

- 1.) Inoculate 9 mL broth tube with a pure culture (see *Growth in broth*). Incubate broth culture at 39°C until maximum optical density is achieved (may require several days).
- 2.) Add anaerobic dilution solution (ADS) with 50% glycerol (see *Media and buffer recipes*). Release gas pressure from tube with syringe. Add 1 mL ADS with 50% glycerol. Final concentration of glycerol will be 5%.
- 3.) Store at -20 or -80°C.

Revival

- 1.) Thaw tubes at 4°C.
- 2.) Transfer 0.4 mL to fresh 9 mL broth tube. Incubate at 39°C. Growth will be apparent after 10 days of lag.
- 3.) Place cryopreserved tube back into freezer (optional).

Feeding and transferring protozoa in culture tubes

From Dehority (personal communication) and (1, 7-10)

Schedule

- 1.) When feeding daily, feed 0.1 mL substrate prepared as described below. Transfer twice weekly.
- 2.) For feeding 5 d/wk, feed 0.12 mL substrate Monday through Thursday and 0.5 mL on Friday. Do not feed on Saturday or Sunday. Transfer on Monday and Friday.

Feeding

- 1.) From freezer, remove substrate tubes. Place in rack in 39°C water bath and thaw.
- 2.) Remove protozoal tubes from 39°C incubator. Place in rack in 39°C water bath.
- 3.) After following all steps from *Constructing and using O₂-scrubbing copper column*, light Bunsen burner. Flame metal needle of gassing probes until red-hot. Flame needle (**Fig. 1**) across its entire length of needle.
- 4.) Remove stopper from substrate tube and insert needle of gassing probe (**Fig. 2A**). Flame stopper and lip of culture tube. Hold culture tube with left hand, placing end of tube against body (waist) for support. Loosen stopper with thumb and forefinger of left hand while continuing to hold tube with remaining fingers of left hand. Hold gassing probe with right hand. While removing stopper, slide needle into tube, keeping it between stopper and inner wall of tube.
- 5.) While gassing, place culture tube containing substrate in clamp (**Fig. 3**). Place stopper upside down (to avoid contaminating tube upon re-insertion) near Bunsen burner. Place 1 mL, blunt-tip glass pipette in tube. Before each use, pipette should be sterilized with 80% ethanol or placed in 100°C oven then cooled. Using pipette bulb, flush pipette with CO₂ by placing the pipette in the upper part of the feeding tube and running CO₂ in and out of the pipette.
- 6.) Remove stopper from protozoal tube then insert needle of gassing probe, following #4. Mix then aspirate substrate (between 0.1 to 0.5 mL, depending on feeding schedule) with blunt-tip pipette. While continuing to hold tube open (**Fig. 2B**), dispense substrate into protozoal tube.
- 7.) Replace stopper in protozoal tube essentially by reversing steps of #4. Place stopper into tube while removing gassing probe (**Fig. 2C**). After one hand is free, flame the stopper and lip of the tube, then firmly seat stopper by twisting it firmly into place; inverting the tube to wet the stopper can help. Replace stopper on feeding tube using same procedure.

8.) Place protozoal tubes in 39°C incubator at a 10° angle (**Fig. 4**). Replace substrate tube in -20°C freezer.

Transferring

- 1.) Do #1-3 from **Feeding**.
- 2.) Remove stopper from protozoal tube, following #4 from **Feeding**. Place tube in clamp, as in #5 from **Feeding**. Insert wide-mouth, 10 mL glass pipette with pipette bulb or barrel attached. Before each use, glass pipette should be sterilized with 80% ethanol or placed in 100°C oven then cooled. Flush pipette with CO₂ as #6 from **Feeding**.
- 3.) Remove stopper from tube with fresh media (5 mL), again following #4 from **Feeding**. Using pipette, mix then aspirate 5 mL of culture from protozoal tube then dispense in tube with fresh media. Final volume of this new tube should be 10 mL (5 mL fresh media, 5 mL culture). Replace stopper on new tube.
- 4.) Dispose of the remaining 5 mL of culture from protozoal tubes if unwanted. If wanted, remove stopper from another tube with fresh media. Do reverse of #3; use pipette to aspirate 5 mL of media then dispense into protozoal tube. Doing #3 in reverse is not strictly necessary but is usually simpler.
- 5.) Feed new tubes, following **Feeding**.

Preparing blunt-tip pipette

Breaking flanged tip off a normal serological pipette. Score tip with file, wet scored part with finger with saliva, and break swiftly. Flame broken end over Bunsen burner to smooth out edges around break.

Other recommendations

- 1.) Maintain at least 2 tubes of each species to protect against accidents.
- 2.) To avoid cross-contamination when caring for both *Epidinium caduatum* and *Entodinium caudatum*, transfer and feed *Epi. caduatum* before *Ento. caudatum*, the latter being excellent at colonizing new tubes. Maintain separate substrate tubes for each species to further prevent cross-contamination. One can color-code tubes using different colors of lab tape.
- 3.) Media M should be used for *Epidinium caduatum*, and medium SP should be used for *Entodinium caudatum* (see Table 1 of **Buffer and Media Recipes**).

Making protozoal substrate

- 1.) Fill 500-mL Pyrex bottle with 100 mL dH₂O. Bubble dH₂O with CO₂ for 15 to 20 min to help remove O₂ from dH₂O.
- 2.) Make a 1.5% w/v ground wheat and 1% w/v ground orchardgrass suspension. Add 1.5 g ground wheat and 1 g ground orchardgrass to dH₂O. Continue to bubble with CO₂. Prepare ground wheat and ground orchardgrass by grinding through 425 µm screen (or one of next closest size).
- 3.) Add small stir bar to bottle and stir on medium speed to keep wheat and orchardgrass in suspension.
- 4.) Dispense 3 mL of substrate into a 23 mL culture tube. Hold tube with left hand tube and gas with metal gassing probe. Aspirate 3 mL substrate from round-bottom flask using wide-mouth, 10 mL glass pipette. Transfer to 23 mL culture tube. Insert stopper in culture tube, following #7 of *Feeding*.
- 5.) Freeze substrate tubes at -20°C. Do not autoclave.

Growing large volumes of protozoa

Volumes of up to 320 mL of protozoa have been grown in 1-L round-bottom flasks and 1-L Pyrex Roux culture bottles. Cultures can also be grown in culture bottles.

- 1.) Progressively double volume from 10 to 20, 40, 80, 160, and finally 320 mL by placing culture (10 mL) in flask or bottle then adding equal volume of fresh medium (10, 20, 40, 80, then 160 mL) during each “transfer” (no culture will in fact be removed and transferred). Feed 0.12 mL of substrate solution per 10 mL of culture.
- 2.) For transfers after 320 mL is reached, remove 160 mL using a vacuum aspirator while bubbling culture vigorously. Use sterile pipettes for aspirating and bubbling. Bubble from the bottom of the culture by position bubbling pipette at bottom.

Fig. 1. Flaming needle of gassing probe. Only tip is flamed in this image; be sure to move needle to flame entire length.

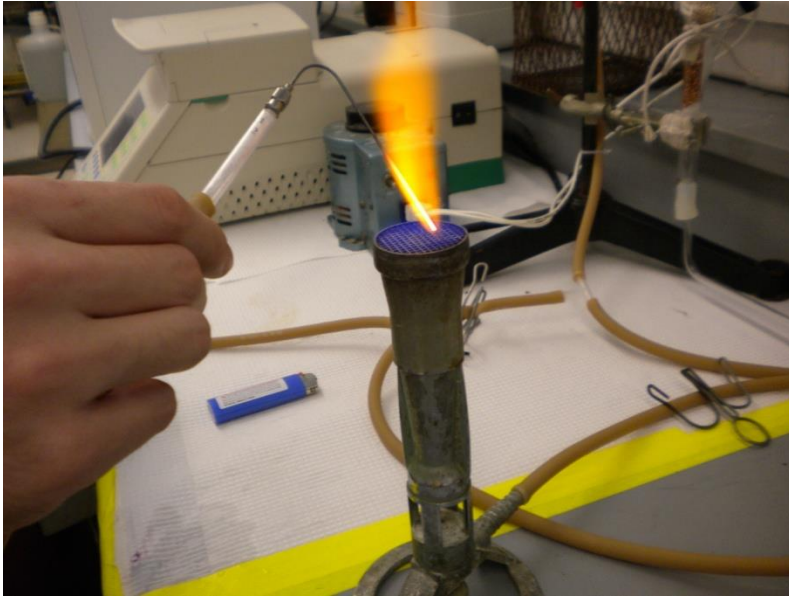


Fig. 2. Method of holding culture tubes, stopper, and gassing probe. (A) Position for opening tube. Needle is held near lip of culture tube for immediate insertion after stopper is removed. (B) Position for holding open tube for feeding and other manipulation. (C) Position for stoppering tube. Needle is kept between wall of tube and stopper, and needle is slid out as stopper is slid in.

(A)



(B)



(C)



Fig. 3. Placement of substrate tube in clamp. Feeding pipette and gassing probe also in place.

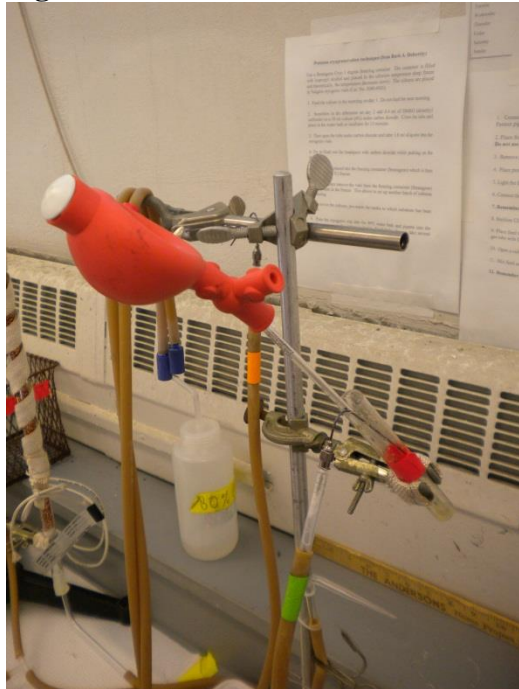
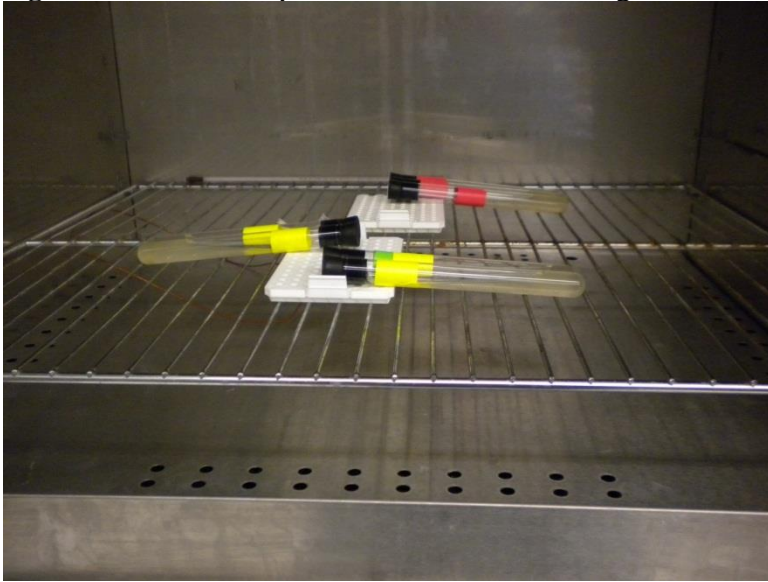


Fig. 4. Incubation of protozoal tubes at a 10° angle.



Cryopreservation of rumen protozoa

Adapted from Burk Dehority (personal communication) and (9)

Freezing

- 1.) Feed culture tubes in the morning of day 1. Do not feed on day 2.
- 2.) In afternoon of day 2, add 0.4 mL dimethylsulfoxide to 10 mL culture (~4% final concentration). Close tube under CO₂ and incubate in 39°C water bath for 15 min.
- 3.) Fill cryovials with culture. Open 2 mL screw-top cryovial and flush with CO₂ by placing it under a gassing probe clamped to a metal stand. Open culture tube and aliquot 1.8 mL culture into cryovial. Screw on top of cryovial while removing from gassing probe to keep the headspace full of CO₂. Repeat for more cryovials.
- 4.) Place vials in freezing container (Stratogene Cryo 1). Place container in -80°C freezer.
- 5.) After 24 h, remove vials from freezing container. Keep vials in -80°C freezer until ready for use. Thaw freezing container for next batch of protozoa.

Revival

- 1.) Prepare culture tubes with 8 mL media. Pre-warm and feed.
- 2.) Place cryovials in 39°C water bath. Wait until contents just becomes liquid (2-3 min), then dump into culture tube. Gas culture tube but do not attempt to gas cryovial.
- 3.) Feed daily. Up to 10 d may be needed to see viable cells (density is initially too low to see), and not all tubes will produce viable cells. Sometimes viable cells can be seen a few hours after revival, however.

Notes

- (i) Tubes produce viable cells for 3 to 6 months after freezing. Consequently, cryopreserved cells need to be periodically revived and cultured to maintain viability.
- (ii) The Stratogene Cryo 1 freezing container is filled with isopropyl alcohol and is designed to achieve a slow (-1°C/min) rate of cooling.

Collecting rumen fluid

- 1.) Verify that cannulated cows are available, particularly if it has been some time since last collecting fluid.
- 2.) Bring following to barn
 - cheesecloth (c. 17"x17" layers, 4)
 - nitrile/latex gloves (4)
 - veterinary sleeves with rubber bands (2)
 - centrifuge bottle (250 mL, 1) with hot water
 - beverage cooler (screw-top, 1.9 L) with water at 39°C
 - buckets (2)
 - funnel (1)
- 3.) At barn, find a cannulated cow and tie it in a stall using the halter, if needed. (Usually the cows are calm and it is not necessary to tie them.)
- 4.) Put on one pair of gloves, then sleeves, then another pair of gloves on top.
- 5.) Remove the cannula, and place handfuls of rumen contents into a bucket. Collect contents from across rumen. Approximately 8 handfuls of rumen content is enough to fill a 250 mL centrifuge bottle.
- 6.) Empty centrifuge bottle of hot water. Strain a homogeneously handful of contents through 4 layers of cheesecloth into centrifuge bottle with funnel in neck, avoiding excessive force. Repeat until bottle is filled completely (to exclude O₂) and seal the bottle.
- 7.) Immerse centrifuge bottle in cooler with water at 39°C and seal cooler.
- 8.) Put cannula back and untie cow, if necessary.
- 9.) Bring fluid to lab as quickly as possible.

Notes

- (i) A layer of cheesecloth refers to a single, unfolded ply. In the box, cheesecloth is usually folded to form multiple layers. Unfold the cheesecloth before use.
- (ii) During winter, lab should be used for straining rumen fluid. Straining fluid in the barn during cold weather sharply reduces viability of microbes in fluid.

Preparing and culturing mixed rumen microbes from rumen fluid

Adapted from (11, 12)

- 1.) Follow Fig. 1. Collect fluid according to *Collecting rumen fluid*.
- 2.) Place strained rumen contents in 500-mL separatory funnel attached to ring stand, pre-warmed in 39°C incubator, and pre-gassed with CO₂. Add Simplex buffer (to stabilize pH) and continue bubbling with CO₂ (using air-stone bubbler) for a few minutes. Place stopper on separatory funnel and place in 39°C incubator.
- 3.) Prepare particle free fluid
 - (a) Wait for feed particles to flocculate (requires about 45 min) (**Fig. 1**).
 - (b) Drain particle-free fluid through stopcock and into 250-mL centrifuge bottle. To avoid disturbing particle layer, do not move separatory funnel from incubator. Gas centrifuge bottle with CO₂ using air-stone bubbler (connected to gassing station through long tubing). Do not completely drain fluid; leave some to avoid draining particles.
 - (c) Place bottle in water bath (39°C) and continue bubbling with air-stone bubbler. Place metal ring around of bottle neck to stabilize the bottle and prevent it from tipping.
- 4.) Using a glass graduated pipette, transfer particle-free rumen fluid to 40 mL screw-cap centrifuge tubes. Pre-warm centrifuge tubes to 39°C and open and close them under a stream of O₂-free CO₂.
- 5.) Centrifuge according to **Fig. 2**. Pre-warm rotor (JA-17) in 39°C incubator to help maintain physiological temperature. Set temperature of centrifuge to 39°C (centrifuge will climb to that temperature ~5 min after start of spinning). Remove supernatant by aspiration with vacuum aspirator; simply decanting leads to loss of some pellet.
- 6.) Resuspend in 20 to 40 mL Simplex buffer to give concentration similar or less than rumen fluid. Transfer to 60 mL culture bottle and stopper with rubber bung.

Notes

- (i) Wait up to 90 min for particles to flocculate. If many particles still settle at bottom of funnel, collect fluid on another day. If no improvement is achieved after several attempts, modify the cow's diet (increase concentrate and select higher-quality forage). Some cows never achieve perfect flocculation, and results similar to **Fig. 2B** may represent the best possible.
- (ii) Up to 500 mL particle-free fluid can be prepared by using 250 mL of fluid and Simplex buffer, instead of the 75 mL recommended in **Fig. 1**.
- (iii) For mixed rumen microbes, mean (SD) recovery of prokaryotic cells from particle-free rumen fluid was nearly complete [mean [SD]; 96.8 (16.9) %, n = 8] as was protozoa [98.0 (12.4) %, n = 8].

(iv) Metabolic activity of microbes was normal. To verify that centrifugation indeed does not alter metabolic activity of microbes, we compared heat production of (i) particle-free rumen fluid that was not centrifuged and (ii) particle-free rumen fluid that was centrifuged. Fluid that was centrifuged was done so according to **Fig. 1**, except the pellet formed was re-suspended in its own supernatant rather than being discarded or re-suspended in buffer. Heat production of the centrifuged fluid was found as 104.9 (12.3) % that of the non-centrifuged fluid ($n = 6$). This finding supports that centrifugation did not alter the metabolism of microbes.

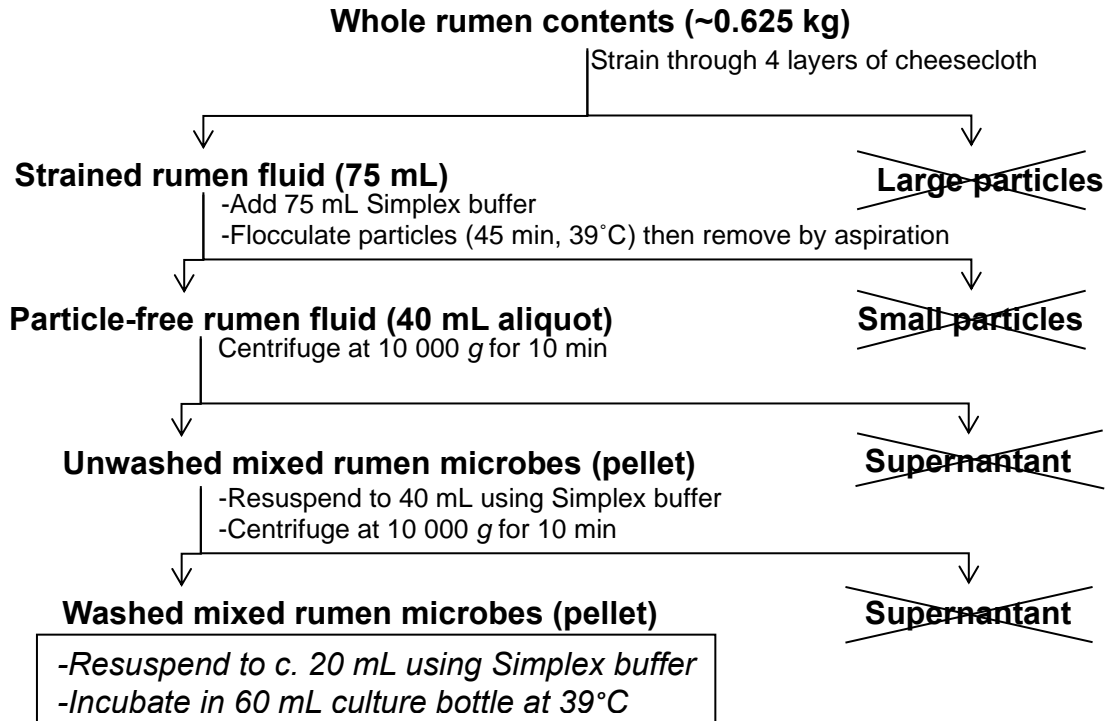
Fig. 1. Flocculation of rumen fluid in separatory funnel. (A) Complete flocculation. Particles form a foamy layer lying on top, and white layer at bottom (mostly protozoa) are free of particles. (B) Incomplete flocculation, showing some particles settling on top of bottom white layer.



(B)



Fig. 2. Flowchart for preparation of mixed rumen microbes.



Preparing and culturing mixed bacteria from rumen fluid

Adapted from (11, 12)

- 1.) Follow Fig. 1 along with #1 to 4 of *Preparing mixed rumen microbes from rumen fluid*.
- 2.) Centrifuge particle-free fluid for 2,500 x g for 5 min.
 - (a) Pre-warm centrifuge by setting temperature of centrifuge to 39°C and running until 5 min.
 - (b) Place tubes in centrifuge and start spin. Do not spin more than 3 tubes at a time, or step (c) takes too long to complete (pellets become loose).
 - (c) Remove supernatant by aspiration using the device shown in **Fig. 2** connected to a vacuum pump. Do this step quickly or the pellet become loose, releasing protozoa into the supernatant.
 - (d) Transfer the supernatant into a new 40-mL centrifuge tube under stream of O₂-free CO₂. Do this step quickly to minimize exposure of supernatant to air.
 - (e) Bring tubes to 40-mL volume using Simplex buffer.
- 3.) Centrifuge supernatant in #2 according to **Fig. 1**. After removing supernatant, pool the pellets of multiple tubes together into a single 40-mL tube. Do this by resuspending one pellet in 10 mL of Simplex buffer, transferring the suspension to the next tube, resuspending the next pellet in the suspension, and repeating sequentially for all tubes. Bring to 40-mL volume using Simplex buffer.
- 7.) Centrifuge and resuspend pellet in 23 mL using Simplex buffer according to Fig. 1. Transfer 22 mL aliquot to a culture bottle and incubate in 39°C incubator.

Notes

- (i) Nine tubes (360 mL) of particle-free fluid are needed to yield 1.5 to 2 g bacteria/L (concentration typically used for experiments). Centrifuge the nine tubes by working with three sets of three tubes. This is accomplished best by two people (one person loads a set of tubes in the centrifuge while the other person removes supernatant from a second set).
- (ii) Mean recovery of prokaryotic cells was 22% of particle-free rumen fluid ($n = 6$). Incomplete recovery owed mainly to those bacteria unavoidably pelleted with protozoa during the 2 500 g centrifugation. Contamination with ciliate cells was $\leq 0.6\%$.
- (iii) The speed of the first spin needs to be adjusted for each centrifuge. With a Rotina 38R centrifuge and 1720 rotor, a relatively fast speed (2,500 g) was needed because slower speeds (500 g and 1000 g) led to incomplete removal of protozoa in preliminary experiments. With a Beckman JA-17 rotor and J2-21 centrifuge, however, 1000 g was adequate.
- (iv) The mixed bacteria, as prepared, contain methanogens. As a result, they are technically mixed prokaryotes, not bacteria.

Fig. 1. Flowchart for preparation of rumen bacteria.

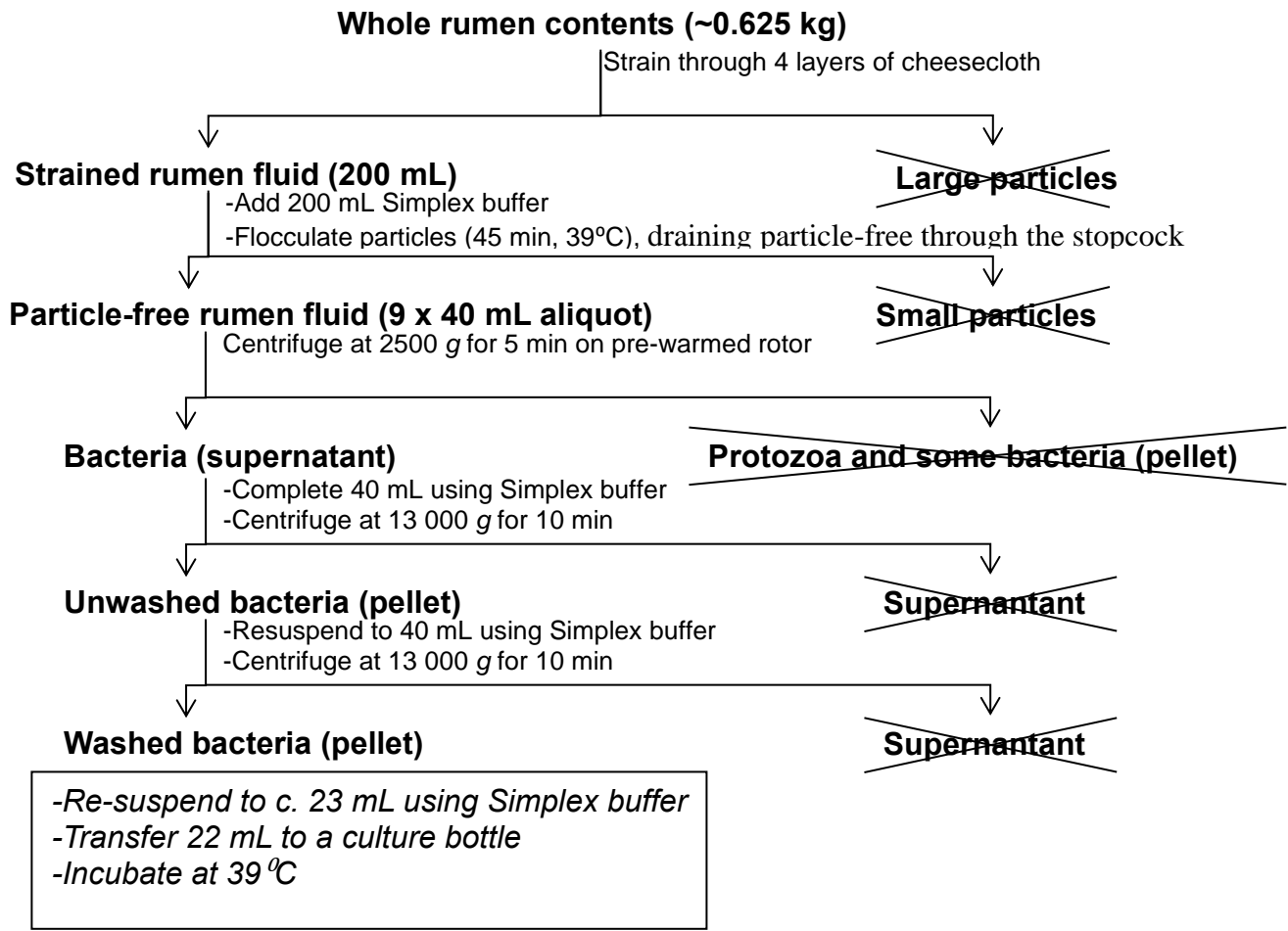
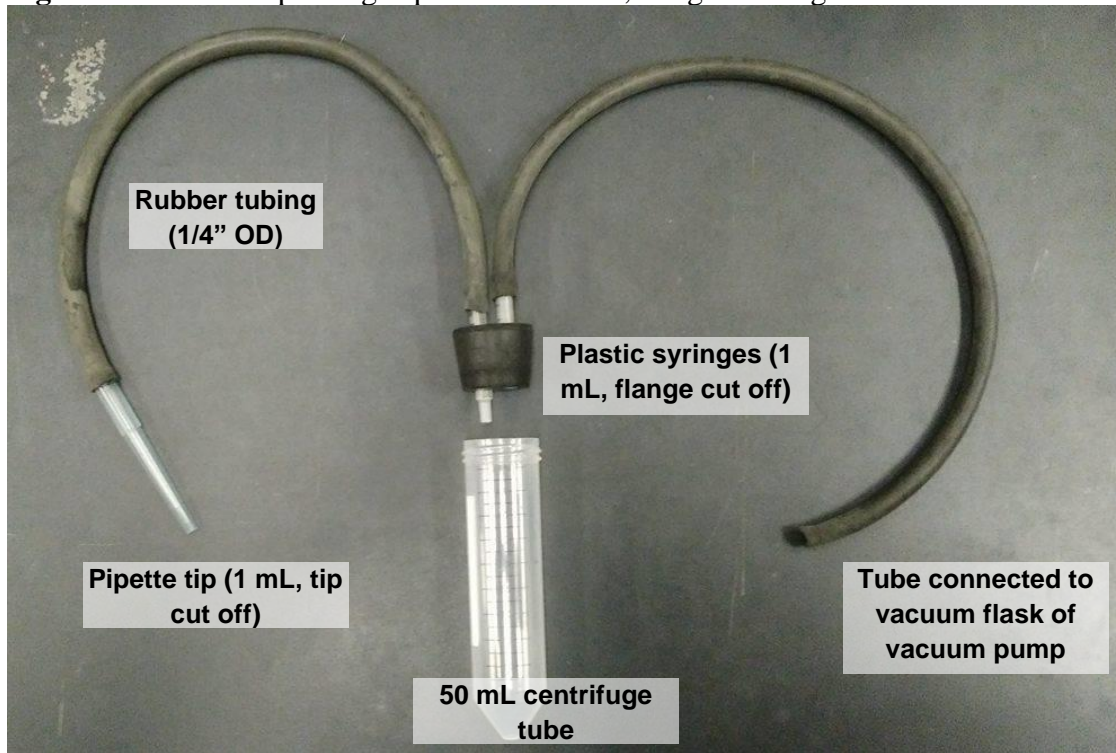


Fig. 2. Device for aspirating supernatant after 2,500 g centrifugation.



Preparing and culturing mixed protozoa from rumen fluid

Adapted from (11, 12)

- 1.) Follow **Fig. 1** along with steps #1-3 from *Preparing and culturing mixed rumen microbes from rumen fluid*.
- 2.) Place 20 μm nylon mesh filter (Small Parts 7050-1220-000-10) between upper and lower pieces of Buchner funnel (**Fig. 1**) and place funnel on 2 L plastic Erlenmeyer flask. Allow filter to sag about 1 to 2 cm in center (do not pull taut). Turn on CO_2 to maximum setting.
- 3.) Turn on CO_2 to rinse bottle assembly (**Fig. 2**) and add 250 mL Simplex buffer. Keep tubing with clamp open (to avoid dispensing Simplex buffer).
- 4.) Using wide-mouth pipette, apply 25 mL of particle-free, diluted rumen fluid (largest quantity that can be easily managed during washing) to filter. Fluid should pool in the center of the filter (due to sag in filter).
- 5.) Use Simplex buffer to wash the protozoa free of bacteria.
 - (a) Start the stream of Simplex buffer by attaching clamp to tubing on rinse bottle assembly (**Fig. 2**). Flow rate should be approximately 150 mL/min.
 - (b) Direct the stream of buffer around the outer edges of the rumen fluid pool.
 - (c) Once the rumen fluid begins to drain through filter, move the stream of Simplex buffer towards the center, following a spiral pattern. Use the stream to keep protozoa in suspension (otherwise protozoa will form mat that will retain bacteria) and push protozoa towards the center as fluid continues to drain.
 - (d) Once fluid turns clear (revealing the whitish-brown protozoa on the filter) and buffer drains quickly, move stream back to edges of pooled fluid. This will wash the protozoa gently, without forcing too many through the filter. Difficult samples may never turn clear.
- 6.) Recover protozoa from filter.
 - (a) Use 1-mL pipette to eject a small aliquot (e.g., 0.5 mL) of Simplex buffer on filter.
 - (b) Pipette up and down to lift protozoa off of filter.
 - (c) Dispense suspension into a culture tube under CO_2 . Use culture tube with a mark added at 23 mL.
 - (d) Repeat with additional aliquots until filter appears clean of protozoa. Some small, contaminating feed particles may remain.
- 7.) Repeat #4 to 6 if needed to obtain more protozoa. Keep culture tube with protozoal suspension at 39°C and under CO_2 in interim.
- 8.) Bring volume of suspension to 23 mL with Simplex buffer, transfer 22 mL to culture bottle, and incubate at 39°C .

Notes

(i) Three aliquots of particle-free fluid (60 mL total) are needed to yield 1.5 to 2 g protozoa/L (concentration typically used for experiments).

(ii) Mean (SD) recovery of protozoal cells was 70.77 (8.28) % of particle-free rumen fluid ($n = 4$). Contamination of protozoa with bacteria was 0.77 (0.38) g/100 g microbial protein ($n = 8$ [3 cows with $n = 2$ or 3 each] using $1.79 \cdot 10^{-13}$ g protein/prokaryotic cell measured on separate samples).

Incomplete recovery of protozoa owed to small *Entodinium* sp. (those $<20 \mu\text{m}$) passing through the 20- μm filter. Recovery of larger, non-*Entodinium* species was nearly complete (94.9 [15.6]%). In preliminary experiments, a smaller pore filter (10 μm) was attempted to minimize losses of small protozoa, but prokaryotic contamination was unacceptably high (>5 g/100 g microbial protein) and length of separation unacceptably long (>10 min).

(iii) Metabolic activity of protozoa is normal. To verify that filtration indeed does not alter metabolic activity of protozoa, we compared heat production of (i) particle-free rumen fluid that was not filtered and (ii) particle-free rumen fluid that was filtered. Fluid that was filtered was done so according to Fig. 1, except the filtrate was collected and recombined with the retentate. Fluid that was not filtered was diluted to 275 mL with Simplex buffer, to match the volume of filtered fluid. Both filtered and non-filtered fluid were then centrifuged at 10,000 g for 10 min to concentrate cells for measurement of heat production. Heat production of the filtered fluid was found as 100.2 (3.56) % that of the non-filtered fluid ($n = 3$). This finding supports that filtration did not alter the metabolic activity of fluid and the protozoa therein.

(iv) Do not apply negative pressure (vacuum) to filter. Protozoa will become lodged and difficult to remove.

Fig. 1. Flowchart for preparation of rumen protozoa.

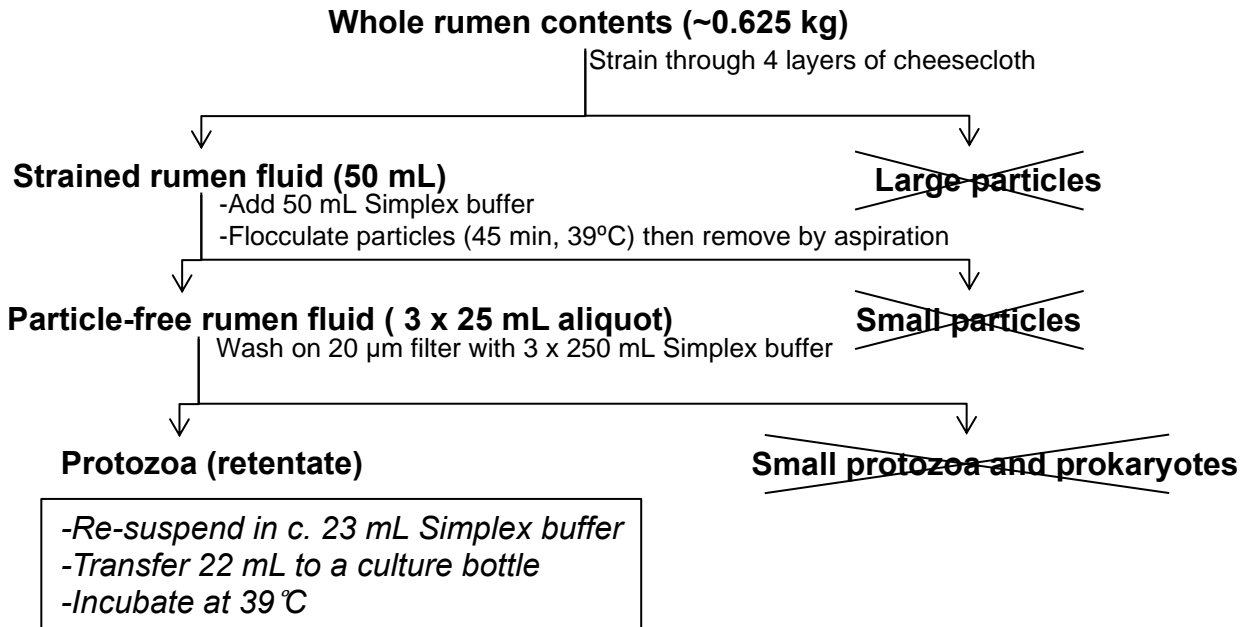


Fig. 2. Filtration apparatus for separation of rumen protozoa. Ten-micron filter is pictured but 20-µm is recommended.

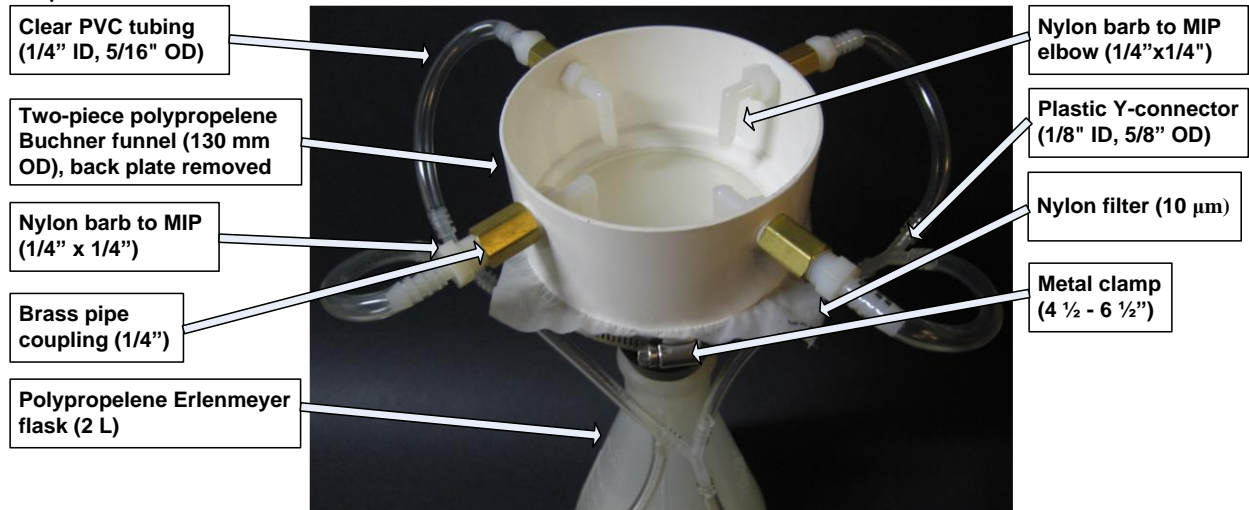
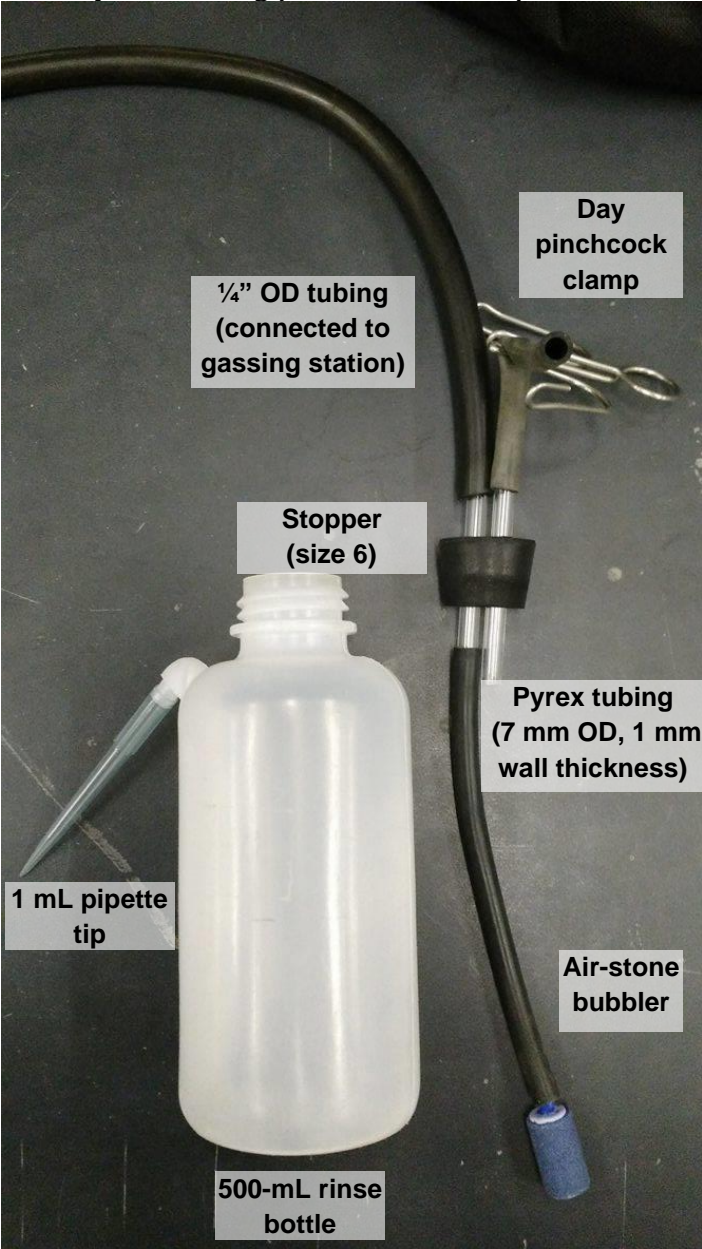


Fig. 3. Rinse bottle assembly for washing protozoa with Simplex buffer.



Preparing and culturing isolated protozoa from single-species cultures

Adapted from (13)

Follow **Fig. 1** or **Fig. 2** along with steps #1-3 from *Preparing and culturing protozoa from rumen fluid*.

Notes

(i) Flocculation cannot be used to remove feed particles with cultures because fermentation of cultures is not vigorous enough for particles to rise. Filtration is effective except feed particles between the small and large filters (10 and 35 μm for *Ent. caudatum*) are invariably retained.

(i) Mean (SD) recovery of *Entodinium caudatum* cells was 95.5 (31.3 SD) % ($n = 3$). Contamination of protozoa with bacteria was 1.89 (0.18 SD) g/100 g microbial protein ($n = 3$), using $1.34 \cdot 10^{-10}$ ($2.18 \cdot 10^{-11}$ SD) mg/prokaryotic cell ($n = 8$) measured on separate samples. Protein mass of *Ent. caudatum* was $3.21 \cdot 10^{-9}$ ($1.23 \cdot 10^{-9}$ SD) g/cell. Not enough data is available for *Epi. caudatum* to give reliable values.

Fig. 1. Flowchart for preparation of *Entodinium caudatum* cells

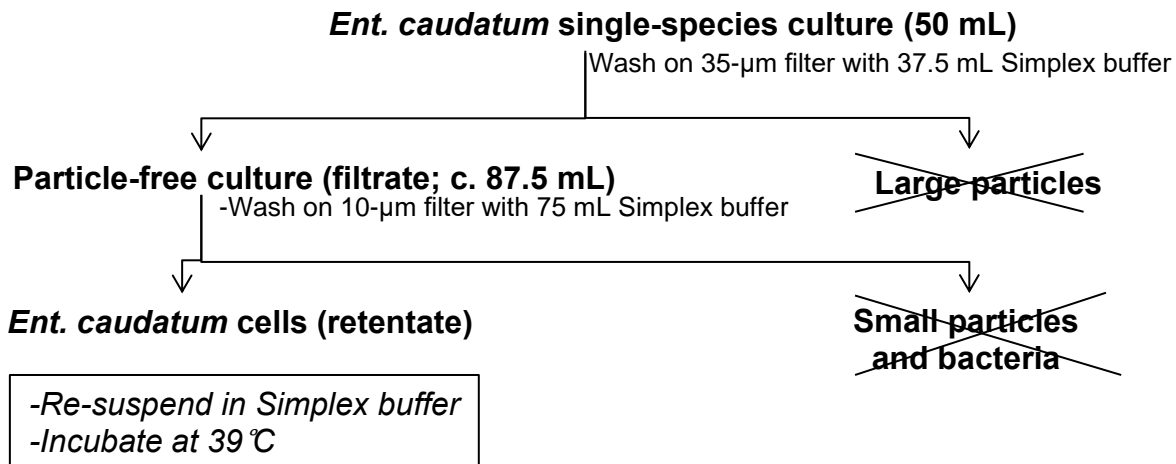
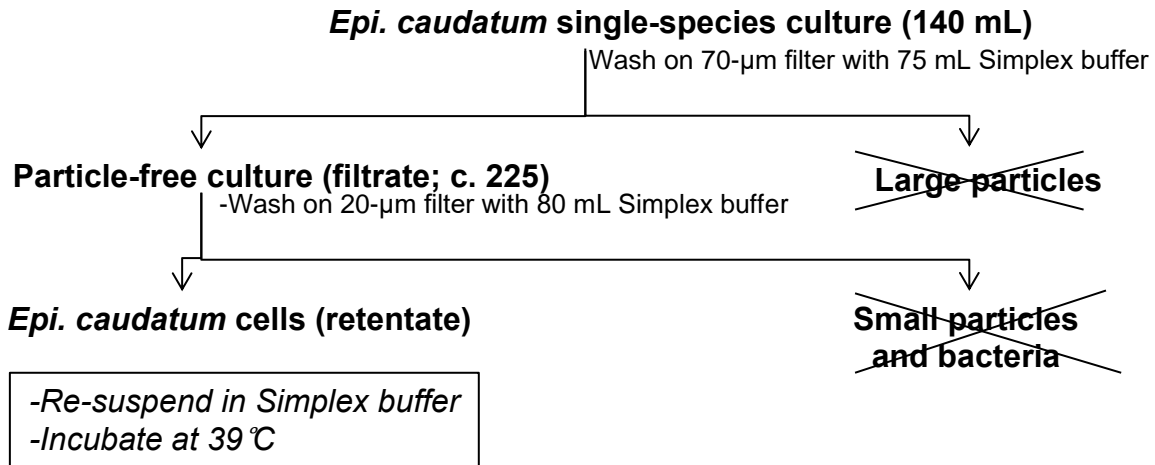


Fig. 2. Flowchart for preparation of *Epidinium caudatum* cells



Making buffer and media

Adapted from (1, 2, 14)

The procedure explains the general principles of preparing buffer/media. For details about specific types of buffer/media, see *Buffer and media recipes*.

Preparing small volumes of buffer/media

This procedure is used for buffer/media when the post-boiling volume is ≤ 600 mL.

1.) Add buffer/media components (dH₂O, salts, rumen fluid, resazurin,) to 1-L round bottom flask in quantities directed in *Buffer and media recipes*. Do not include NaCHO₃ or cysteine·HCl at this step.

2.) Boil buffer/media to remove most O₂ (**Fig. 1**). Attach round bottom flask on ring stand and rest of ceramic gauze (Fig. 1). Light Bunsen burner and bring to boil (requires 4 to 6 minutes). Continue to boil for 5-7 min.

Placing flask in water bath, adding ice to the bath to cool. After cooled, slowly add NaCHO₃ in quantity directed in individual procedures. Buffer/media must be cool before adding NaHCO₃ because NaHCO₃ will boil off as CO₂ if media is hot.

3.) Bubble until blue color imparted by resazurin changes to light purple or pink (indicating buffer is well-reduced); see *Buffer and media recipes* for approximate times.

Add cysteine solution in volumes directed in *Buffer and media recipes*. Use a syringe with 22-gauge needle attached (**Fig. 2**). Flame-sterilize a gassing probe attached to a stand. Flame-sterilize bung of culture bottle after applying small amount of 80% ethanol on bung. Keep bottle underneath flame. Insert the syringe needle into the gassing probe, flush syringe with CO₂ by drawing plunger in and out several times, and fill syringe with volume of CO₂ equal to amount of cysteine to be withdrawn. Puncture bung of culture bottle with syringe needle, inject CO₂, and withdraw cysteine. Dispense cysteine in round-bottom flask. Add cysteine only after media is well-reduced, or cysteine (a reducing agent) will be oxidized.

4.) Continue bubbling until colorless (indicating buffer is reduced); see *Buffer and media recipes* for approximate times.

Dispensing and autoclaving

1.) For <10-mL aliquots of buffer/media, dispense into 18x150 mm Balch tubes. While bubbling buffer with CO₂, hold culture tube with left hand and gas with metal gassing probe (see Fig. 2 in *Feeding and transferring protozoa in culture tubes*). Flush a wide-mouth, 10 mL glass pipette with CO₂ by placing pipette in the upper part of the round-bottom flask tube and drawing CO₂ in and out of the pipette. Aspirate buffer/media from round-bottom flask using pipette. Dispense buffer/media in tube, holding pipette tip at bottom of tube to flush out gas. Remove gassing probe

from culture tube while simultaneously inserting rubber bung. Seal with aluminum lid and autoclave.

Alternatively, dispense into 16x150 mm culture tubes. Insert stopper into tube, place tubes in metal rack (**Fig. 3**), and autoclave. Metal rack prevents stoppers from blowing out of tubes during autoclaving. Allow tubes to cool after autoclaving before removing from rack.

2.) For ≤ 95 mL aliquots of buffer/media, dispense into 160 mL culture bottles. Flush culture bottle with CO₂ by placing it under a gassing probe clamped to a metal stand. Flush volumetric pipette with CO₂ by drawing placing the pipette in the upper part of the round-bottom flask tube and drawing CO₂ in and out of the pipette. Withdraw buffer from round-bottom flask and dispense into culture bottles, seal with rubber bung and aluminum lid, and autoclave.

3.) For ≤ 600 mL aliquots of buffer, autoclave directly into 1-L flask. Stopper the flask.

Wire stopper in place (**Fig. 4**). Loop an 8" length of 18-gauge steel wire and tightly twist ends together to secure wire to flask. Make one end much longer than the other. Ends should be twisted tightly enough that wire cannot slip down neck of flask. Repeat 3 more times to secure 4 wires in total. Position 2 wires so that their long ends are at 180° angles (12 and 6 o' clock). Pull the long ends of above stopper. Secure stopper by twisting the ends of the wires together. The wires should be twisted tightly enough to make depressions in the stopper. Repeat with the 2 remaining wires for additional security; position these wires at 3 and 9 o' clock.

Alternatively, the stopper can be held in place using a press (**Fig. 5**).

Autoclave.

Preparing large volumes of buffer or media

This method is used for buffer/media volumes that have a post-boiling volume of ≤ 1.8 L.

1.) Assemble boiling and dispensing apparatus, if not already done, following instructions below

2.) Add buffer/media components (salts, resazurin, rumen fluid, dH₂O) to 3-L round bottom flask in amounts directed in individual procedures. Do not include NaCHO₃ or cysteine·HCl at this step.

3.) Secure 3-L flask on stand and insert tubing assembly, fitting snugly (**Fig. 6**). Clamp dispensing tubing but keep vent tubing open. Open CO₂ tubing and bubble solution with CO₂.

4.) Boil solution for 30 min. Light Bunsen burner and turn up as high as possible. Boiling will require 8-10 minutes to commence. Watch for steam from the vent tubing, and once observed, start timer for boiling for 30 min.

5.) Cool by placing flask in room-temperature water bath, gradually adding ice to water bath to speed cooling. Add NaCHO₃ in amount directed in individual procedures.

6.) Add cysteine hydrochloride in volume directed in individual procedures. Use a syringe with 22-gauge needle attached. Dispense in round-bottom flask. Continue bubbling until colorless (indicating buffer/media is reduced).

6.) Dispense into 1-L flask. Pre-mark graduations on 1-L flask for easy dispensing and follow **Fig. 7**.

(i) Insert tubing assembly into 1-L flask and flush 1-L flask with CO₂ for 4 min (**Fig. 7A**).

(ii) Flush dispensing tube (**Fig. 7B**). Raise 3-L tubing assembly so end is above liquid (but CO₂ is still flowing into 3-L flask), remove clamp from dispensing tube, and clamp vent tubing on 1-L tubing assembly. Flush tubing 60-90 sec.

(iii) Dispense buffer (**Fig. 7C**). Re-insert 3-L tubing assembly into 3-L flask and fit stopper snugly into neck of flask. Remove clamp from vent tubing on 1-L tubing assembly. Clamp vent tubing on 3-L assembly. Buffer will begin to flow in 1-L flask

(iv) When desired volume is reached, pinch dispensing tube to stop flow and remove clamp from vent tubing on 3-L tubing assembly. Up to 500-mL can be added.

(v) Repeat (iii) and (iv).

(iv) For 75 mL or smaller volumes, dispense into culture bottles directly with volumetric pipette. Dispensing apparatus is not efficient for use in this case.

7.) Autoclave at 121°C/15 lb pressure for 20 min.

Assembly of boiling and dispensing apparatus

1.) Prepare stoppers. Bore 3 holds in size 10 stopper, and insert 3 plastic syringes with flange cut off (**Fig. 8**). Repeat for a size 5 stopper (follow **Fig. 8**).

2.) Prepare tubing assemblies. Follow **Fig. 9** and **Fig. 10**. Cut tubing and attach to syringes. Insert glass tubing into dispensing tubing for 1-L flask (to weigh down and stabilize tubing during dispensing). Attach air stones to CO₂ tubing. Dispensing tubing will connect 3-L and 1-L flask assemblies.

3.) Prepare flask stand (**Fig. 6**). Clamp cast iron ring and flask clamp to rectangular stand. Place iron gauze on ring. Place Bunsen burner below ring. Ring should be 3 cm above flask. Clamp flask to stand and rest on ring.

4.) Connect CO₂ tubing to gassing station.

Preparing buffer or media by distillation

Procedures above remove O₂ mostly by boiling. To remove even more O₂, water can be distilled under O₂-free CO₂. It is useful for Simplex buffer, which is hard to reduce by boiling alone. It cannot be applied to buffer or media that contain non-water liquid components, which may not be easily distilled.

- 1.) Assemble distillation unit as shown (**Fig. 11**).
- 2.) Add buffer/media components (minus the dH₂O) to the 500-mL Erlenmeyer flask in quantities directed in *Buffer and media recipes*. NaCHO₃ can be included, but do not include cysteine at this step.
- 3.) Add 600 mL of dH₂O to 1-L flat bottom flask on ring stand. Light Bunsen burner and turn up heat as high as possible.
- 4.) Place tubing from the bottom of the Graham condenser into a 100-mL graduated cylinder (not shown in Fig. 11). Allow water in the flask to begin to boil and discard the first 100 mL of distillate (which contains O₂).
- 5.) After removing the graduated cylinder, attach the 500-mL Erlenmeyer flask to the tubing from the Graham condenser (as shown in Fig. 11).
- 6.) Remove the Erlenmeyer flask once volume of liquid reaches 300 mL.
- 7.) Follow #3 to 4 for *Preparing small volumes of buffer/media*.

Notes

(i) Never autoclave a flask more than 60% full with media (e.g., never fill 1-L flask with more than 500 mL media); 30% or less full is ideal.

(ii) After autoclaving, buffer/media is generally stable for a few months in culture tubes. It is stable much longer (months to years) in culture bottles and round-bottom flasks.

(iii) When preparing small volumes of buffer/media, a flask may crack even when taking all precautions indicated in the procedure.

(iv) Iron gauze evenly distributes heat from burner. No flasks have been observed to crack when using gauze.

Fig. 1. Technique for gassing and boiling buffer/media.

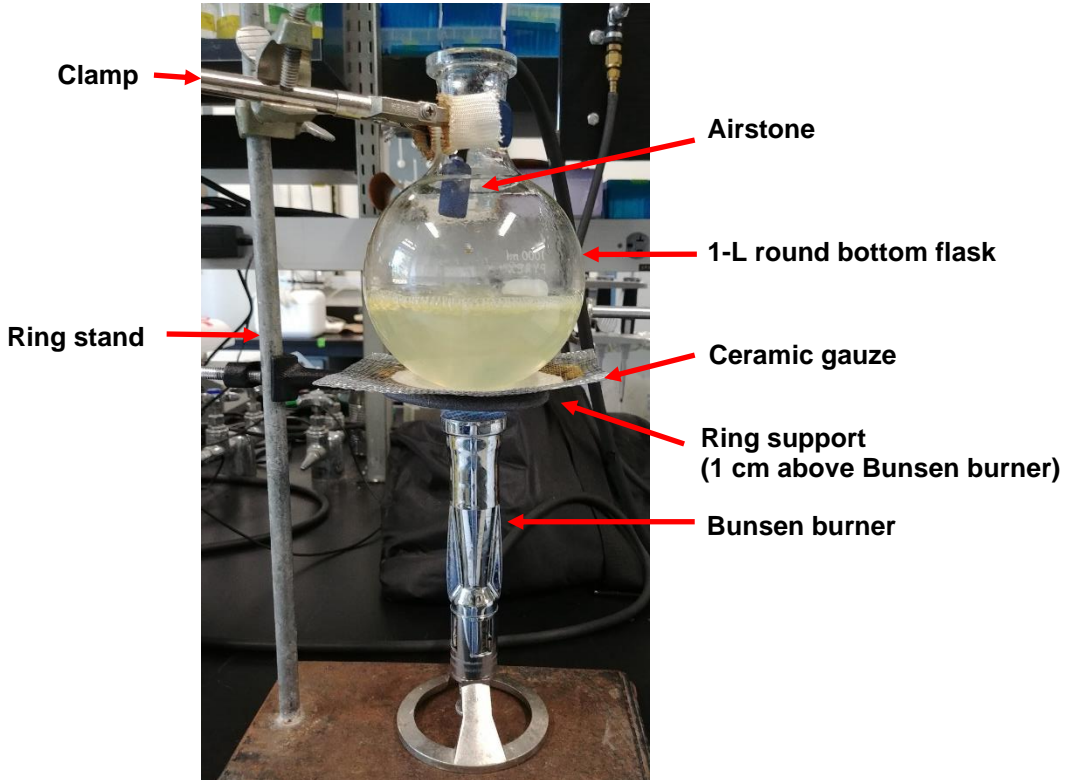
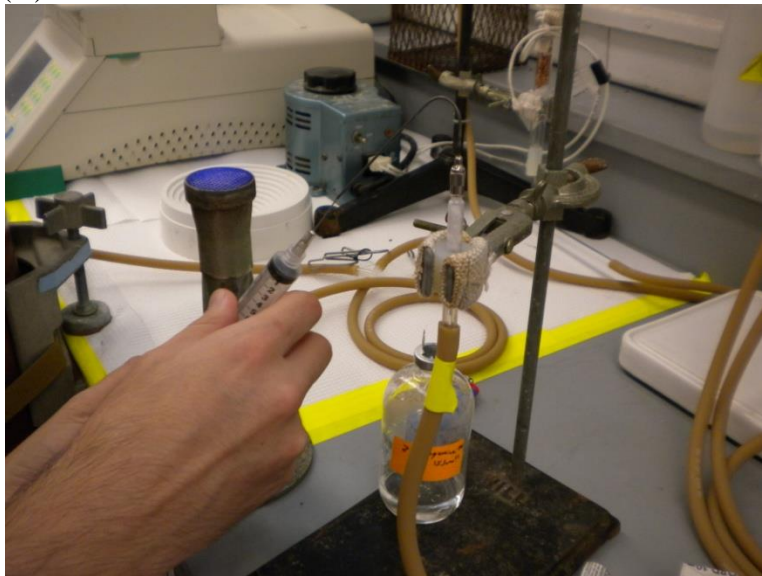


Fig. 2. Technique for withdrawing cysteine from culture bottle. (A) Flushing syringe with CO₂ via gassing probe clamped to stand. (B) Withdrawing cysteine.

(A)



(B)

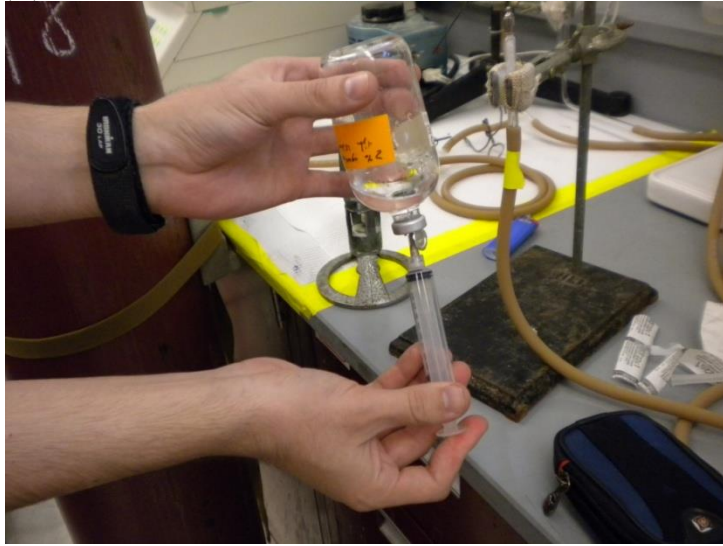


Fig. 3. Rack for autoclaving culture tubes.

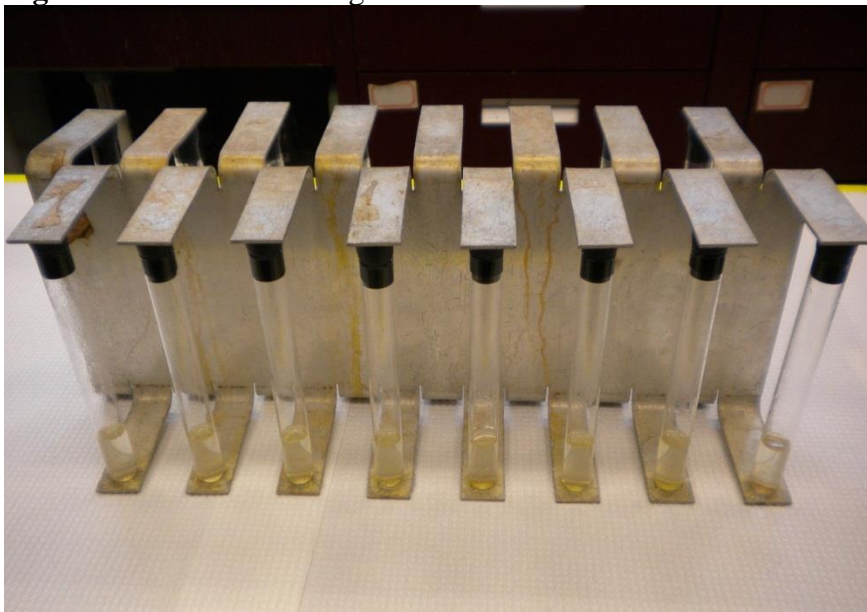
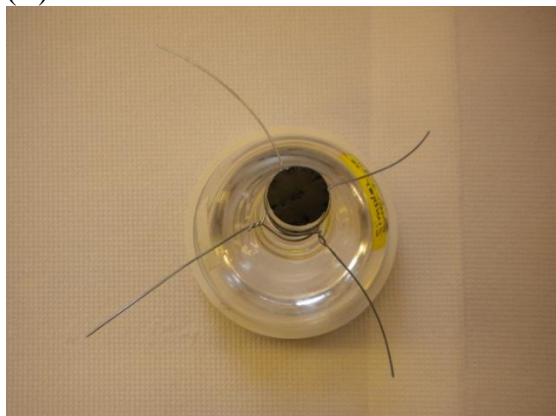


Fig. 4. Wiring stopper in place. (A). Securing 1 wire around neck of flask. (B) Repeating 3 more times to give 4 secured wires. Wires are positioned for securing around stopper; they are at 12, 3, 6, and 9 o'clock. (C) Securing 2 wires around stopper. (D) Securing remaining 2 wires around stopper.

(A)



(B)



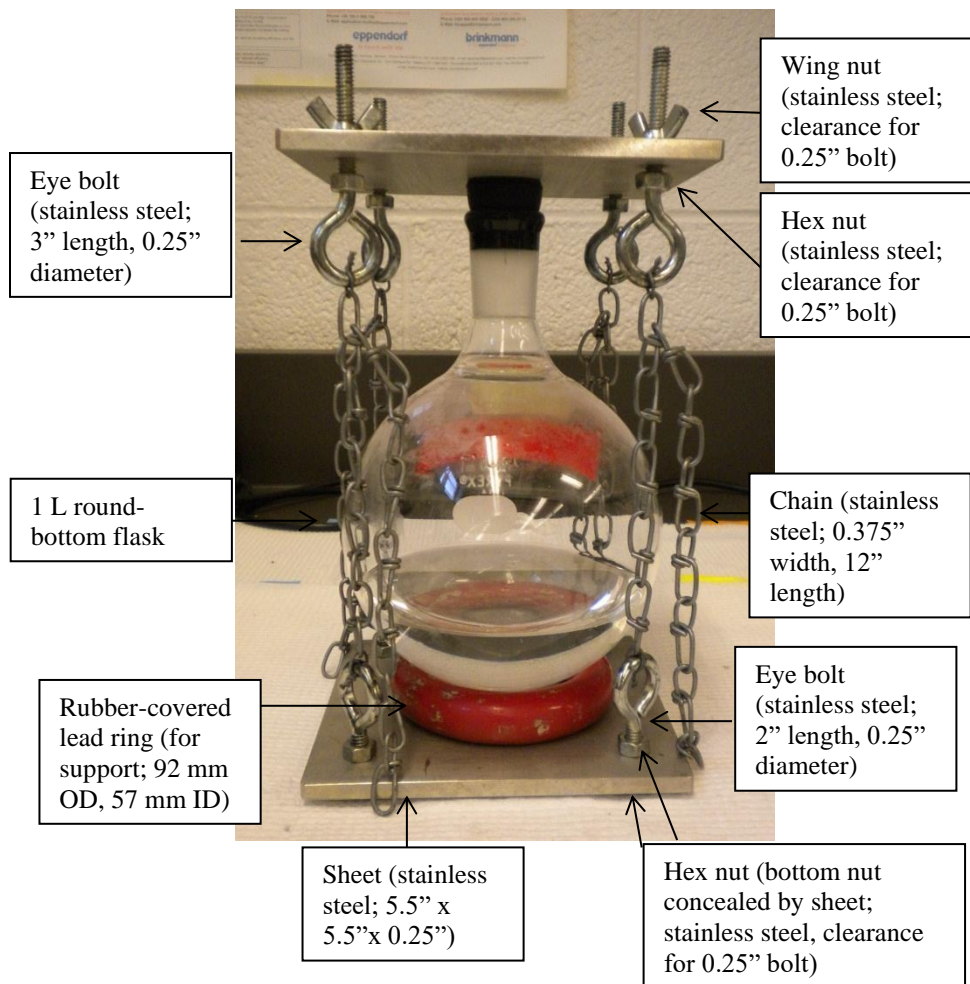
(C)



(D)



Fig. 5. Press for holding stopper in place during autoclaving. (A) Press with round-bottom flask in place. (B) Dimensions of steel sheet.



(B)

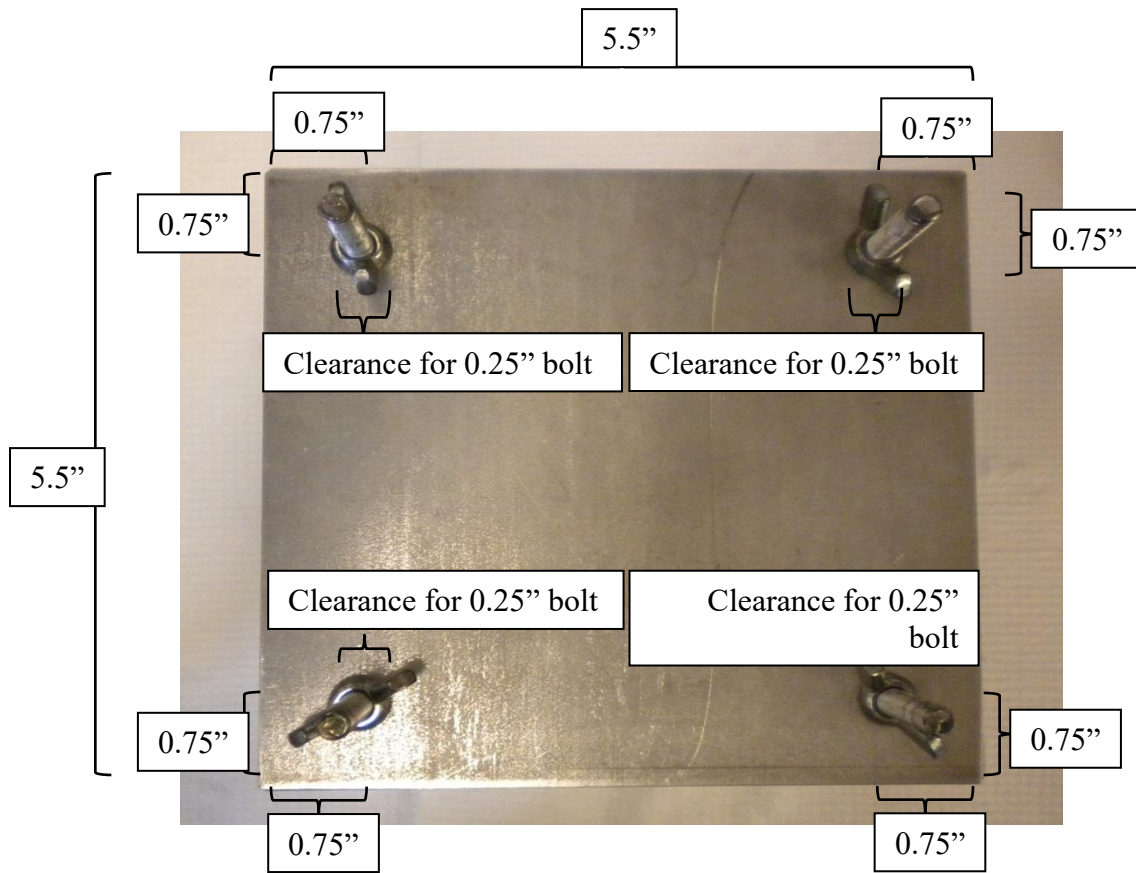


Fig. 6. Boiling buffer in 3-L flask.

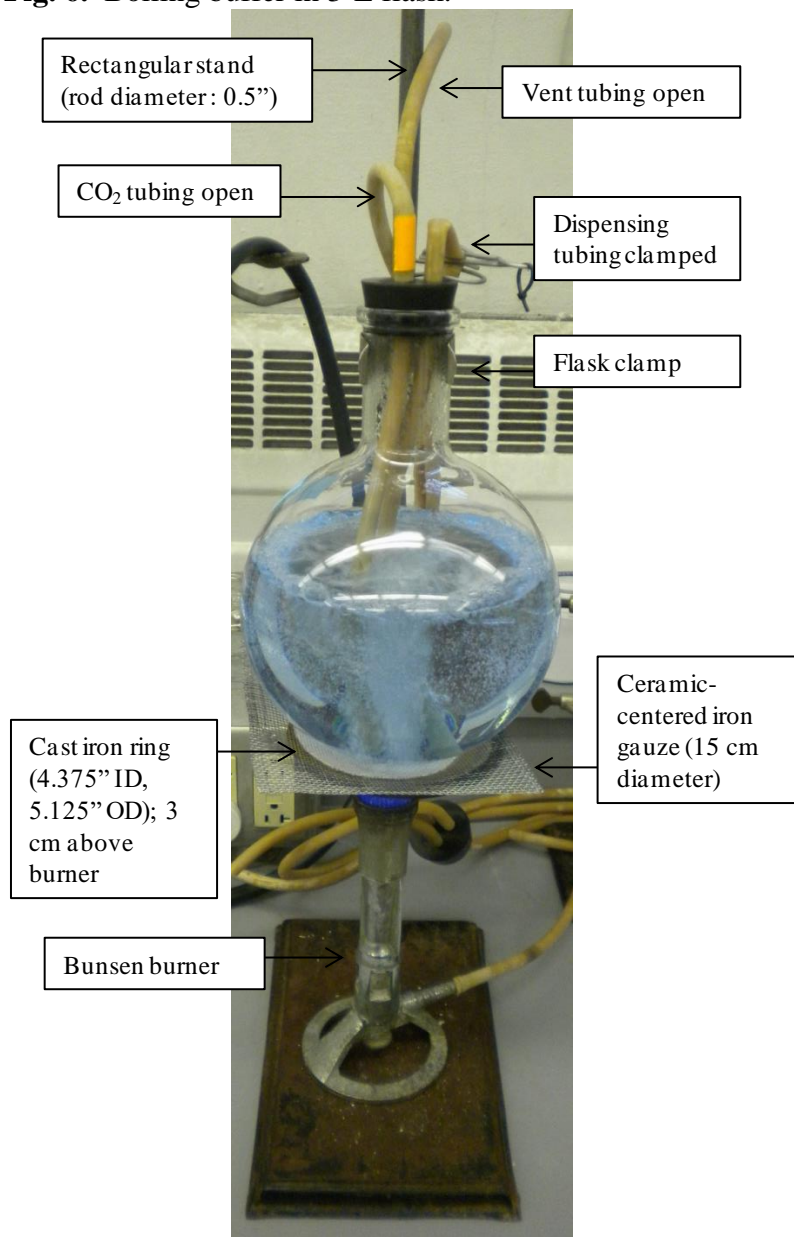
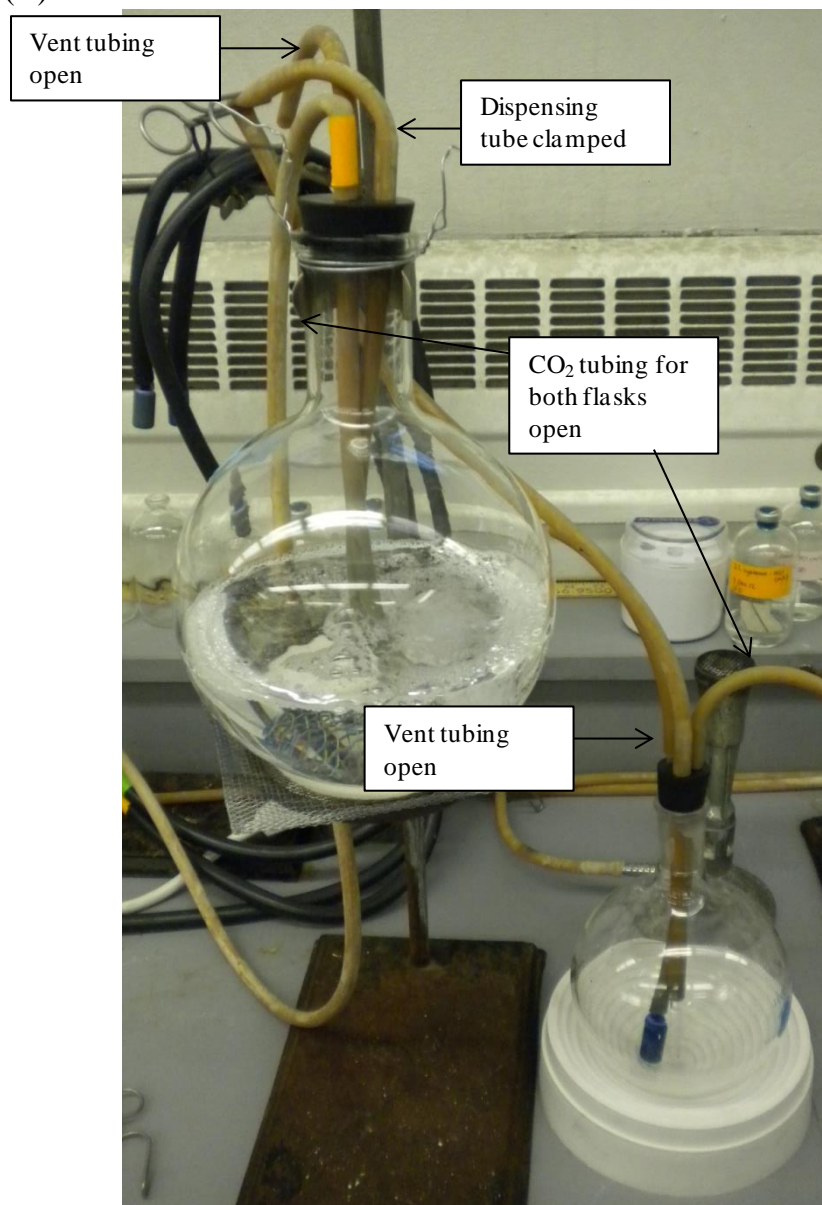
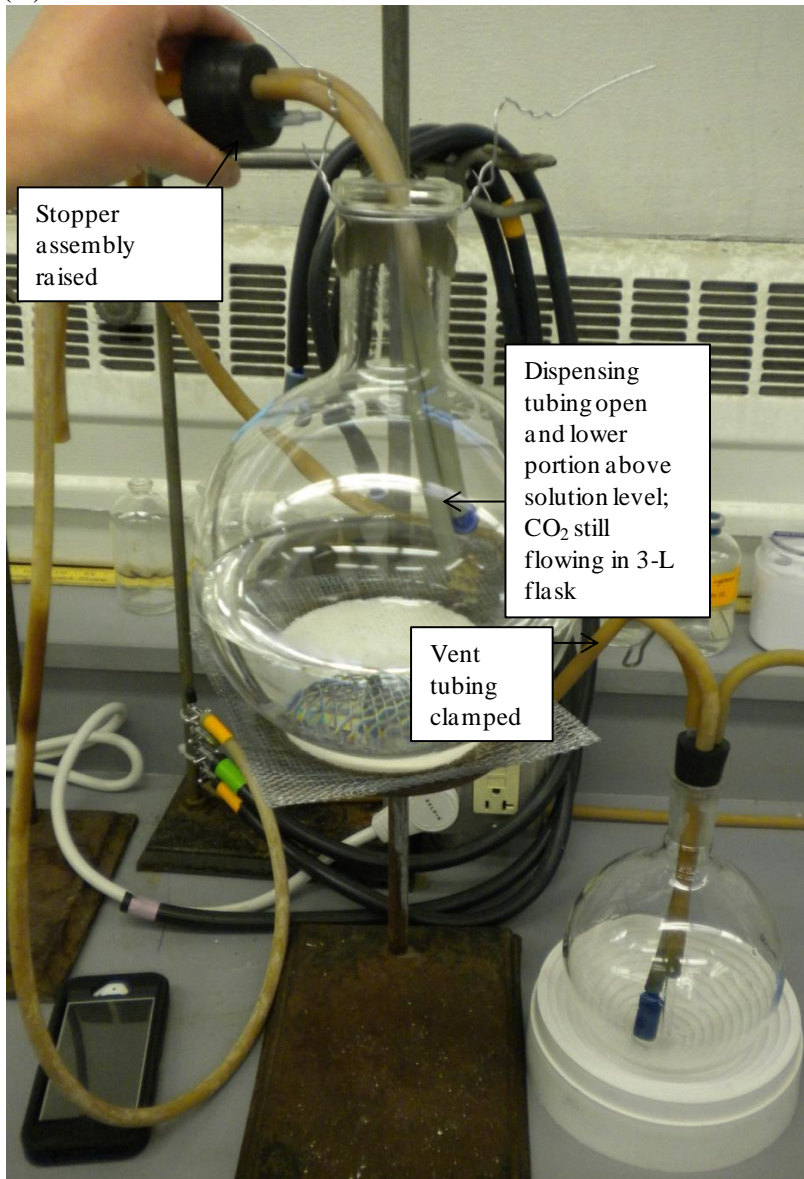


Fig. 7. Dispensing buffer into 1-L flask. (A) Flushing 1-L flask. (B) Flushing dispensing tube. (C) Dispensing buffer.

(A)



(B)



(C)

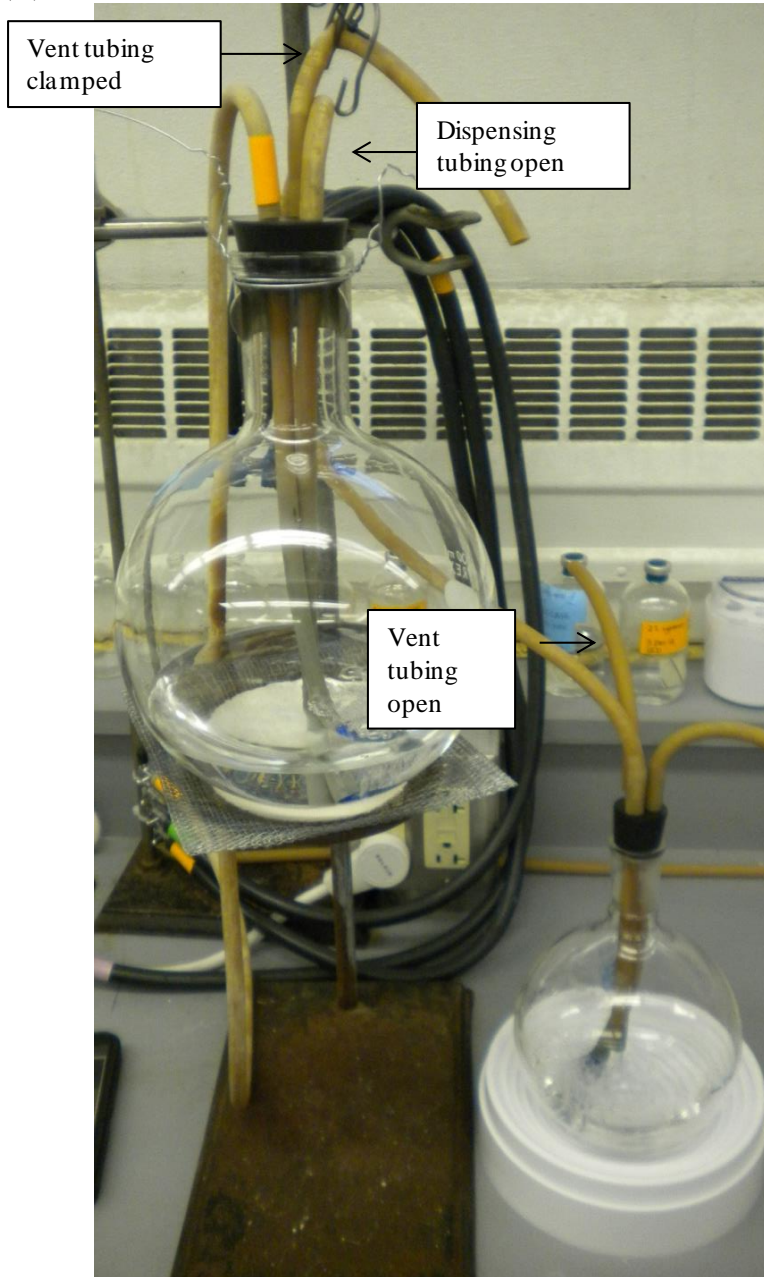


Fig. 8. Stopper with syringe barrels inserted.

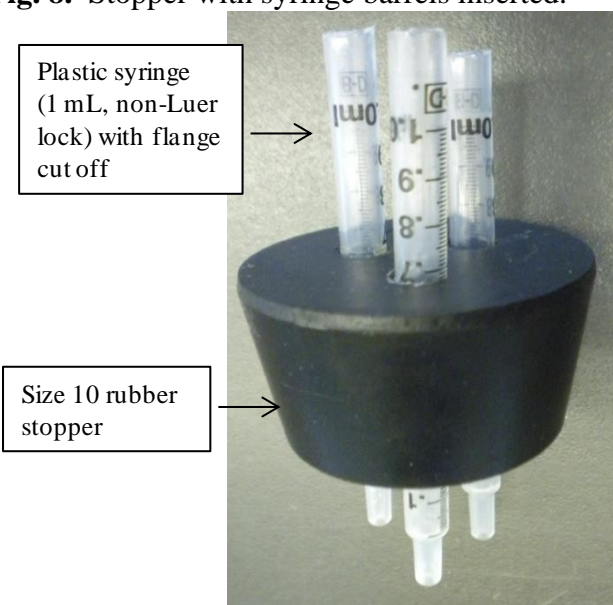


Fig. 9. Tubing assembly for 3-L flask.

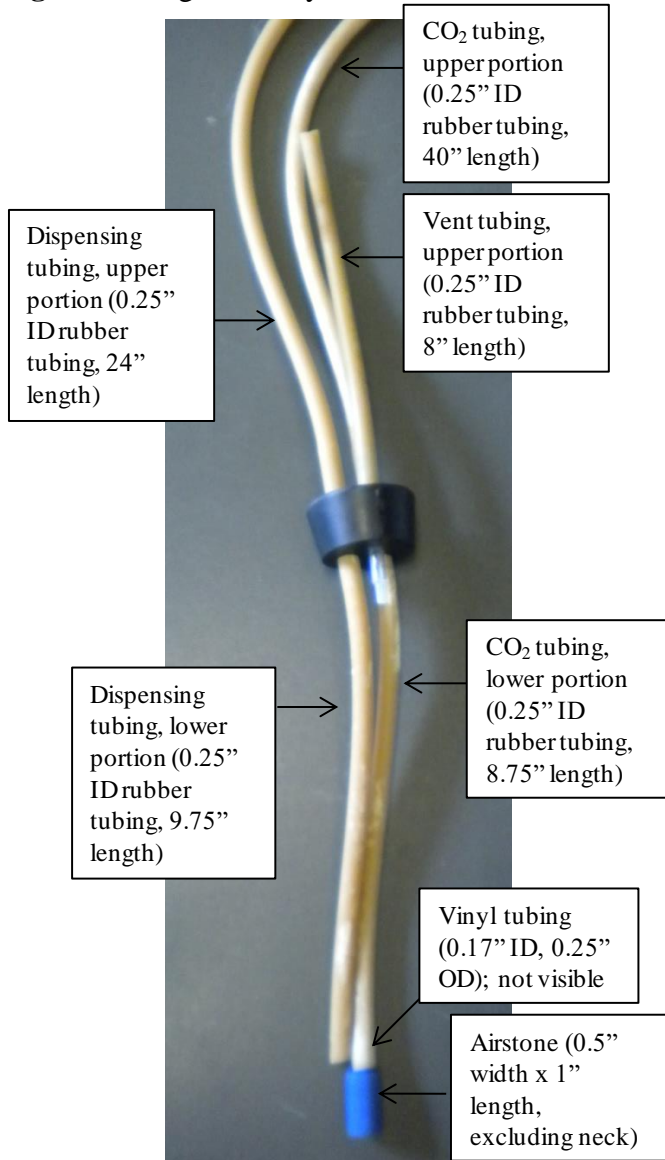


Fig. 10. Tubing assembly for 1-L flask.

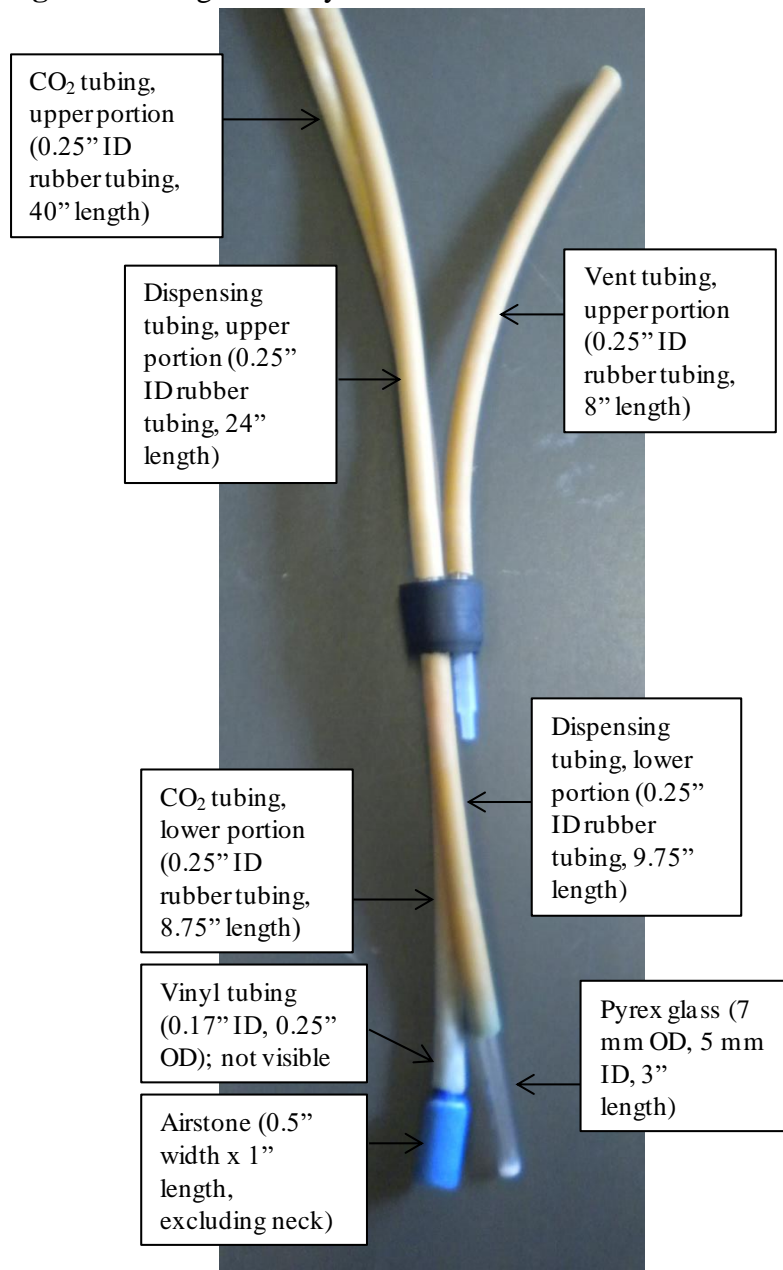
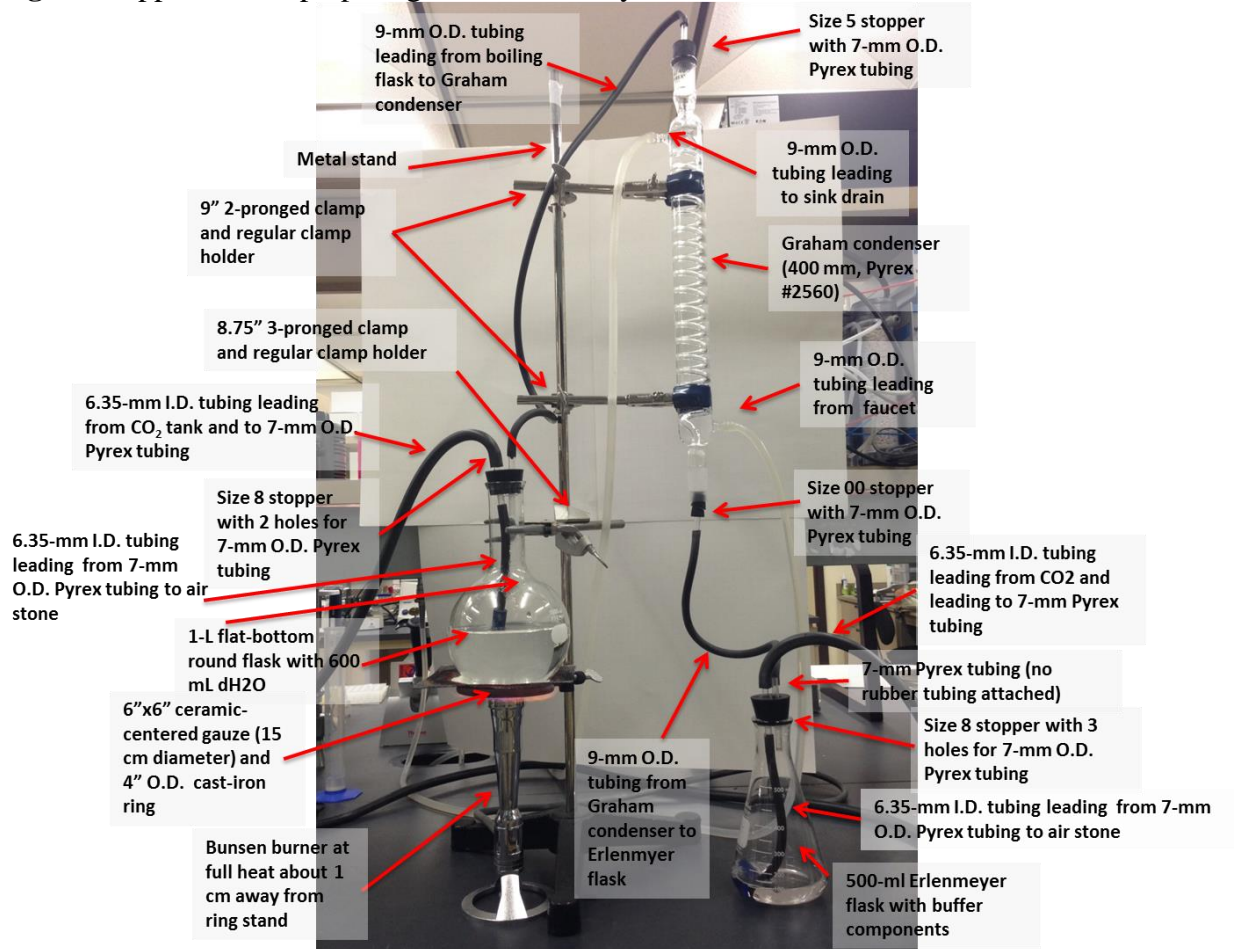


Fig. 11. Apparatus for preparing buffer/media by distillation.



Buffer and media recipes

See *Making buffer and media* for detailed procedure for preparing media anaerobically. See **Table 1** for compatibility of media with different bacterial and protozoal species.

6SG330 media

Adapted from (15)

Add the following to a 1-L round-bottom flask

- 350 mL dH₂O
- 2.7 g glucose
- 0.6 g xylan from corn ore (TCI X0078)
- 0.6 g pectin from apple (Sigma 93854-100g)
- 0.6 g trypticase peptone (BD 211921)
- 0.6 g yeast extract (BD 212750)
- 0.18 g K₂HPO₄
- 0.135 g KH₂PO₄
- 0.135 g (NH₄)₂SO₄
- 0.27 g NaCl
- 0.0563 g MgSO₄
- 0.036 g CaCl₂·2H₂O
- 0.6 mL hemin solution
- 3 mL VFA solution
- 3 drops of 0.1% resazurin

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na₂CO₃

Continue bubbling until redox no longer decreases (requires 2 to 3 h). Then add

- 2.0.1667 g L-cysteine HCl·H₂O

Continue bubbling until redox no longer decreases (requires 1 h). Color is tan. Dispense and autoclave.

Use media within a few weeks. Old media turns deep brown, forms a precipitate, and supports growth poorly.

Acid trace element solution

From (16)

Add the following ingredients to a 250 mL beaker

- 1.4911 g FeCl₂·4H₂O 7.5 mM

0.0618 g H₃BO₄ 1 mM
0.0682 g ZnCl₂ 0.5mM
0.0169 g CuCl₂·2H₂O 0.1mM
0.0989 g MnCl₂·4H₂O 0.5 mM
0.1189 g CoCl₂·6H₂O 0.5mM
0.0238 g NiCl₂·6H₂O 0.1mM
4.13 mL HCl 50mM

Dissolve in dH₂O and bring to 1 L using volumetric flask. Transfer to 1 L Pyrex bottle. Store at 4°C under dark (wrap bottle in aluminum foil).

Alkaline trace element solution

From (16)

Add the following ingredients to a 250 mL beaker

0.0173 g Na₂SeO₃ 0.1mM
0.0255 g Na₂WO₄ 0.1 mM
0.0206 g Na₂MoO₄ 0.1 mM
0.4 g NaOH 10mM

Dissolve in dH₂O and bring to 1 L using volumetric flask. Transfer to 1 L Pyrex bottle. Store at 4°C under dark (wrap bottle in aluminum foil).

Anaerobic dilution solution (ADS)

Modified from (3, 17)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
0.135 g K₂HPO₄
0.135 g KH₂PO₄
0.27 g (NH₄)₂SO₄
0.27 g NaCl
0.027 g MgSO₄
0.0358 g CaCl₂·2H₂O
3 drops of 0.1% resazurin

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

0.9 g Na₂CO₃

Bubble overnight (until color turns pink). Then add

0.1667 g L-cysteine HCl·H₂O

Continue bubbling until colorless (usually requires 1 to 4 h). Dispense and autoclave.

Anaerobic dilution solution (50% glycerol)

Prepare as anaerobic dilution solution (ADS), but add 150 mL glycerol and 200 mL dH₂O instead of 350 mL dH₂O. After adding cysteine, up to 6 hours may be needed for solution to turn clear/light pink. After autoclaving, the solution should be clear.

ATCC media 1015 for *Anaerovibrio lipolytica*

From ATCC (<https://www.atcc.org/~media/00CF8781BD5244E9B963A4999C0E2517.ashx>).

Add the following to a 1-L round-bottom flask

- 300 mL meat fluid
- 9.0 g trypticase peptone (BD 211921) or casamino acids (BD 223120)
- 1.5 g Yeast extract
- 1.5 g K₂HPO₄
- 0.3 g cellobiose
- 0.3 g maltose
- 0.3 g starch
- 3 drops of 0.1% resazurin

Boil under CO₂ for 5 minutes.

After boiling, the color change from dark brown to orange. Bubble until redox reaches approximately -160 mV (no color change will occur). Then add

- 4.2 mL of 3% (w/v) cysteine HCl

Continue bubbling until redox is below -250mV. Dispense 5.6 mL liquid aliquots into Balch tubes containing approximately 1.4 g of meat particles each. Autoclave.

Basal Media

Adapted from (18) and Michael Flythe (personal communication)

Add the following to a 1-L round-bottom flask

- 350 mL dH₂O
- 1.2 g glucose
- 0.0876 g K₂HPO₄
- 0.0876 g KH₂PO₄
- 0.1440 g (NH₄)₂SO₄
- 0.1440 g NaCl
- 0.0147 g MgSO₄
- 0.0192 g CaCl₂·2H₂O
- 3 drops of 0.1% resazurin
- 0.3 mL hemin solution
- 3.0 mL Russell vitamin solution

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na₂CO₃

Bubble for one hour (color turns yellow). Then add

0.2 g cysteine HCl·H₂O

Continue bubbling until colorless (usually requires 1 to 4 h). Dispense and autoclave.

Branch-chained VFA solution

Adapted from Paul Weimer (personal communication)

Add the following components to a previously-autoclaved 125-mL Pyrex bottle

20 mL n-valeric acid

20 mL isovaleric acid

20 mL isobutyric acid

20 mL DL- α -methyl butyric acid

Store at room temperature in flammables cabinet. Storing in styrofoam cooler will reduce odor.

Cellulolytic media

Adapted from Paul Weimer (personal communication)

Add the following to a 1-L round-bottom flask

350 mL dH₂O

0.3375 g KH₂PO₄

0.309 g NaCl

0.309 g (NH₄)₂SO₄

0.03 g MgCl₂·6H₂O

0.0225 g CaCl₂·2H₂O

0.00943 g MnCl₂ 4H₂O

0.00686 g FeSO₄ 7 H₂O

0.00324 g ZnCl₂

0.00072 g CoCl₂ 6H₂O

1.125 g Sigmacell 50 cellulose (0.034 g per tube)

3 drops of 0.1% resazurin

0.45 mL branched-chained VFA solution

1 mL Schaefer's vitamin solution + THF

0.94 mL 0.02% hydrocinnamic acid (*R. albus* 7 only)

0.375 g yeast extract (*R. albus* 7 and *R. flavefaciens* FD-1 only)

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g NaCHO₃

Color will turn pink. Bubble until redox reaches around -150 mV, then add

12.0.1667 g L-cysteine HCl·H₂O

Continue bubbling until color turns to light golden brown. Dispense and autoclave.

Chen media

Adapted from (19) and Michael Flythe (personal communication)

Add the following to a 1-L round-bottom flask

350 mL dH₂O

4.5 g trypticase peptone (BD 211921) or casamino acids (BD 223120)

0.0876 g K₂HPO₄

0.0876 g KH₂PO₄

0.144 g Na₂SO₄

0.144 g NaCl

0.0146 g MgSO₄

0.0192 g CaCl₂·2H₂O

1.5 mL Pfennigs heavy metal solution

3 mL Russell vitamin solution

3 drops of 0.1% resazurin

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

Bubble until color changes to orange. Then add

6 mL of 3% (w/v) L-cysteine HCl

Continue bubbling until color changes to golden. Dispense and autoclave.

Chen media with yeast extract

Adapted from (19) and Michael Flythe (personal communication)

Prepare as standard Chen media include yeast extract (0.15 g) in place of Pfennigs heavy metal solution and Russell vitamin solution. Do not carry strains on this medium for more than a few transfers as strains may lose ability to grow on standard Chen as a result.

Clarified rumen fluid

From (3)

Strain whole rumen contents through 2 layers of cheesecloth. Centrifuge at 1 000 g for 10 min. Save supernatant and dispense in 30 mL aliquots in plastic bottles. Freeze at -20°C.

3% (w/v) cysteine HCl

Add ~100 mL dH₂O to a 1-L round-bottom flask, bubble with CO₂, and boil for 5 to 7 min. Transfer 75 mL dH₂O to 160-mL culture bottle that is being flushed with gassing probe. Add

2.507 g L-cysteine HCl·H₂O

Place rubber bung in neck of culture bottle; place aluminum lid and seal with crimp sealer; then autoclave.

0.1 M Dithiothreitol (DTT)

Adapted from (20, 21)

Add ~100 mL dH₂O to a 1-L round-bottom flask, bubble with CO₂, and boil for 5 to 7 min. Transfer 75 mL dH₂O to 160-mL culture bottle that is being flushed with gassing probe. Add

1.569 g dithiothreitol

Filter through 0.2 μm membrane into 160-mL culture bottle (previously autoclaved and flushed with CO₂). Add after autoclaving medium.

Glucose (10% w/v) (anaerobic)

Add 1 g glucose to a Balch tube. Flush with N₂ for 10 min. While continuing to flush, add 9.25 mL water (anaerobic). Stopper tube and shake to dissolve glucose. Filter sterilize into a sterile Balch tube previously flushed with N₂ and autoclaved.

The volume of water (9.25 mL) was determined by adding 1 g of glucose to a 10-mL volumetric flask, then weighing the amount of water (9.3 g) needed to bring it to volume.

Hemin solution

Adapted from (22, 23)

Add following to 100 mL volumetric flask

0.28 g KOH

25 mL 95% ethanol

0.1 g hemin

Dissolve, then and bring to volume with dH₂O. Store at 4°C.

Hemin solution (0.05%)

Dissolve 50 mg hemin in 1 ml 1 N NaOH; make up to 100 ml with distilled water. Store refrigerated.

LH media

Adapted from Mackie and Heath (1)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
10 g sodium lactate (60% w/w)
6 g trypticase peptone (BD 211921)
0.6 g yeast extract (BD 212750)
0.135 g K₂HPO₄
0.135 g KH₂PO₄
0.27 g (NH₄)₂SO₄
0.27 g NaCl
0.027 g MgSO₄
0.0358 g CaCl₂·2H₂O
1.5 mL Pfennings heavy metal solution
6 mL hemin solution (0.05%)
3 mL VFA solution
3 drops of 0.1% resazurin

Adjust pH to 6.7 to 6.8 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.911 g NaHCO₃

Continue bubbling until redox no longer decreases (requires 2 h). Then add

0.1705 g L-cysteine HCl·H₂O

Continue bubbling until redox no longer decreases (requires 75 min). Dispense and autoclave.

Immediately before inoculation, add 0.205 mL of 2.5% Na₂S·9H₂O to each tube.

The composition of the media follows ref. (1), except 1.5 mL Pfennings heavy metal solution is added. Ref. (1) calls for a trace element solution, but does not report the composition. Instead, it cites another paper, which does not report a trace element solution (only the final concentration of trace elements for a different medium). Pfennings heavy metal solution contains the same trace elements as the cited paper (plus NiCl₂·6 H₂O), and its inclusion here is typical for other media.

Media M

Make mineral mix M by adding following to 1-L volumetric flask

6 g NaCl
0.2 g MgSO₄
0.26 g CaCl₂·2 H₂O
2.0 g KH₂PO₄ g/L

Bring to volume with dH₂O.

Add the following to a 1-L round-bottom flask

150 mL mineral mix M

30 mL clarified rumen fluid
15 mL 1.5% CH₃COONa
128 mL dH₂O
2 drops 0.1% resazurin

Approximately 50 mL dH₂O will be lost during boiling, giving 273 mL final volume. After boiling and cooling, add
25 mL of 6% NaHCO₃

Bubble for 15 or 20 (until color turns pink). Then add
2 mL of 3% (w/v) cysteine HCl

Continue bubbling until pale pink/brownish (usually requires 1 to 4 h). Dispense and autoclave. After autoclaving, media should be brownish, with no tinge of pink.

Media SP

Make mineral mix SP-2 by adding following to 1-L volumetric flask

16 g KH₂PO₄
4 g NaCl
0.212 g CaCl₂·2 H₂O
0.154 MgSO₄

Bring to volume with dH₂O.

Add the following to a 1-L round-bottom flask

75 mL 20 g/L K₂HPO₄
75 mL SP-2
30 mL clarified rumen fluid
128 mL dH₂O
2 drops 0.1% resazurin

Approximately 50 mL dH₂O will be lost during boiling, giving 273 mL final volume. After boiling and cooling, add
25 mL of 6% NaHCO₃

Bubble for 15 or 20 (until color turns pink). Then add
2 mL of 3% (w/v) cysteine HCl

Continue bubbling until pale pink/brownish (usually requires 1 to 4 h). Dispense and autoclave. After autoclaving, media should be brownish, with no tinge of pink.

Modified Methanobacterium medium (DSMZ 1523)

Adapted from DSMZ

(https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1523.pdf) and ref. (2)

Add the following to a 1-L round-bottom flask

0.15 g KH_2PO_4
0.0586 g MgSO_4
0.12 g NaCl
0.12 g NH_4Cl
0.015 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
1.8 g brain heart infusion (BD 237500; stored at 4°C)
1.8 g proteose peptone (BD 211684)
0.6 g yeast extract (Oxoid LP0021B)
0.3 g sodium acetate
0.6 g sodium formate
0.3 mL trace element solution SL-10
0.3 mL seven vitamins solution
3 drops 0.1% resazurin

Boil under CO_2 . Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na_2CO_3

Transfer to fume hood and gas with 80% H_2 :20% CO_2 . After redox no longer decreases, add
0.1667 g L-cysteine $\text{HCl} \cdot \text{H}_2\text{O}$

Continue bubbling until redox no longer decreases. Dispense and add to each tube of 9 mL medium

0.18 mL 2.5% (w/v) Na_2S

Do not add Na_2S until tubes are stoppered (to prevent release of toxic H_2S gas). Autoclave.

Modified Methanobacterium medium for Methnobrevibacter ruminantium (DSMZ 1523)

Adapted from DSMZ

(https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1523.pdf) and ref.

(24)

Prepare as Modified Methanobacterium medium (DSMZ 1523), but after autoclaving add to each tube of 9 mL medium

0.9 mL 0.5% (w/v) 2-mercaptoethanesulfonic acid

Alternatively, the same amount of 2-mercaptomethanesulfonic acid can be added before autoclaving; this option is more convenient if all medium tubes are being used for *Methnobrevibacter ruminantium* only.

Modified Peptone Yeast Extract Glucose (PYG; ATCC 1237)

Adapted from ATCC

(<https://www.atcc.org/~media/5A86796703884E068DBDB2B74E1346DB.ashx>)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
3 g trypticase peptone (BD 211921)
3 g yeast extract (BD 212750)
1.5. Glucose
0.15g (NH₄)₂SO₄
0.012g K₂HPO₄
0.012g KH₂PO₄
0.0024g MgSO₄
0.00318g CaCl₂ 2H₂O
0.024g NaCl
3 drops of 0.1% resazurin
0.93 ml VFA solution
3 mL of Vitamin K₃-Hemin solution

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g NaHCO₃

After boiling, color is golden. Bubble until the redox reaches approximately -150 mV. Then add 0.15 g L-cysteine HCl·H₂O

Continue bubbling until color turns amber. Dispense and autoclave.

Modified Peptone Yeast Extract Glucose (PYG; DSMZ 104)

Adapted from DSMZ

(http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium104.pdf)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
1.5 g trypticase peptone (BD 211921)
1.5 g peptone (BD 211677)
3 g yeast extract (BD 212750)
1.5 g glucose
1.5 g beef extract (LP0029)
0.612 g K₂HPO₄
0.012 g KH₂PO₄
0.00293 g MgSO₄
0.003g CaCl₂·2H₂O
0.024 g NaCl
0.3 mL Tween80
3 ml hemin solution (0.05%)
0.06 mL vitamin K1 solution

3 drops of 0.1% resazurin

Adjust pH to 6.7 with HCl.

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add
1.2 g NaHCO₃

After boiling, color is golden. Bubble until the redox reaches approximately -150 mV. Then add
0.15 g L-cysteine HCl·H₂O

Continue bubbling until color turns amber. Dispense and autoclave.

Mucin medium for *Akkermansia muciniphila*

From (25)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
0.12 g KH₂PO₄
0.159 g Na₂HPO₄
0.09 g NH₄Cl
0.09 g NaCl
0.03 g MgCl₂·6H₂O
0.044 g CaCl₂·2H₂O
0.3 mL alkaline trace element solution
0.3 mL acid trace element solution
0.3 mL *Akkermansia* vitamin solution
4 drops resazurin
0.75 g mucin purified by ethanol

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add
1.2 g NaCHO₃

After boiling, the color is a turbid pink. Bubble until the redox reaches approximately -150mV. Then add

0.1667 g L-cysteine HCl·H₂O

Continue bubbling until colorless (media will remain turbid). Dispense and autoclave.

Immediately before inoculation, add 0.9 mL of 2.5% Na₂S·9H₂O to each tube.

Meat fluid and particles

From ATCC (<https://www.atcc.org/~media/00CF8781BD5244E9B963A4999C0E2517.ashx>).

Add the following to a 1-L round-bottom flask

150 g ground beef (93% lean)
7.5 mL 1 N NaOH
350 mL dH₂O

Attach the flask to a metal stand, and boil under CO₂ for 8 to 10 minutes (until meat browns). Stir contents gently while boiling. Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume.

Remove fluid from the meat particles using aspiration, then transfer to a separatory funnel. Allow fat to rise to top, then aspirate off fat. Drain the remaining fluid and add sufficient dH₂O to restore the 300 mL volume. Preparation of fluid does not need to be done anaerobically (it will be boiled again).

Keeping the meat particles in the round-bottom flask and under CO₂, transfer approximately 1.4 g of meat particles to a Balch tubes also kept under CO₂. Transfer quickly with a spatula to minimize exposure to air. Stopper tube after adding meat. Repeat with more tubes.

To judge how much to meat to transfer to each tube, accurately weigh 1.4 g meat particles in a tube, and use the level of meat in this tube as a reference.

5% (w/v) 2-mercaptoethanesulfonic acid

Adapted from (24)

Add ~100 mL dH₂O to a 1-L round-bottom flask, bubble with CO₂, and boil for 5 to 7 min. Transfer 75 mL dH₂O to 160-mL culture bottle that is being flushed with gassing probe. Add 0.375 g 2-mercaptoethanesulfonic acid sodium salt. Place rubber bung in neck of culture bottle; place aluminum lid and seal with crimp sealer; then autoclave.

Minimal media for *Streptococcus bovis*

From (26-29) and Michael Flythe (personal communication)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
1.2 g glucose
0.0876 g K₂HPO₄
0.0876 g KH₂PO₄
0.1440 g (NH₄)₂SO₄
0.1440 g NaCl
0.0147 g MgSO₄
0.0192 g CaCl₂·2H₂O
3 drops of 0.1% resazurin

1.5 mL Pfennigs heavy metal solution
0.3 mL hemin solution
3.0 mL Russell vitamin solution

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

Bubble for one hour (color turns yellow). Then add

0.2 g cysteine HCl·H₂O

Continue bubbling until colorless (usually requires 1 to 4 h). Dispense and autoclave.

The composition of the medium follows ref. (26), but this reference did not provide a complete description. Composition of buffer salts is from ref. (26), with carbonate added as in ref. (28, 29). Composition and inclusion rate of vitamins is from ref. (28). Composition of minerals is from ref. (28), and their inclusion rate is the maximum recommended by the Russell “spell book” (M. Flythe, personal communication). Ref. (1, 2) would suggest carbonate should not be added, but it is added here as a cautionary measure and because it is mentioned in other references (4). Ref. (2) would suggest that the riboflavin should be higher (double), but that recommendation disagrees with ref. (3) and the spell book.

Minimal media plus VFA

Adapted from Minimal media for *Streptococcus bovis*

Add the following to a 1-L round-bottom flask

350 mL dH₂O

1.2 g glucose

0.0876 g K₂HPO₄

0.0876 g KH₂PO₄

0.1440 g (NH₄)₂SO₄

0.1440 g NaCl

0.0147 g MgSO₄

0.0192 g CaCl₂·2H₂O

1.5 mL Pfennigs heavy metal solution

0.3 mL hemin solution

3.0 mL Russell vitamin solution

0.93 mL VFA solution

3 drops of 0.1% resazurin

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

Continue bubbling until redox no longer decreases (requires 30 min). Then add

0.2 g cysteine HCl·H₂O

Continue bubbling until redox no longer decreases (requires 2 h). Color will be golden. Dispense and autoclave.

Mucin purified by ethanol

Adapted from (25, 30)

Add 0.75 g mucin (from porcine stomach; Type III; M1778-10G) and 60% w/v cold ethanol to 250 mL plastic centrifuge bottle. Keep on ice. Centrifuge (15,000 g, 15 min, 4°C), wash twice with 60% ethanol, and dry under N₂ before use.

10% (w/v) NaOH

Add ~100 mL dH₂O to a 1-L round-bottom flask, bubble with N₂, and boil for 5 to 7 min. Transfer 75 mL dH₂O to 160-mL culture bottle that is being flushed with gassing probe. Add

7.5 g NaOH

Place rubber bung in neck of culture bottle; place aluminum lid and seal with crimp sealer.

2.5% (w/v) Na₂S·9 H₂O

Adapted from (31, 32)

Obtain high purity Na₂S·9 H₂O (≥99.99%; Sigma 431648-50G). Lower purity will not support growth of methanogens, likely due to toxic oxidation products. After opening, store Na₂S·9 H₂O under N₂ in Pyrex bottle with stopper.

Flush 160-mL culture bottle with N₂, then add

1 drop 10% NaOH

75 mL water (anaerobic)

1.875 g Na₂S·9 H₂O

Add the NaOH first to make solution basic and loss of H₂S. Do not flush bottle with CO₂, which will acidify the solution and increase loss of Na₂S as H₂S (toxic).

Immediately seal with bung and aluminum seal (to minimized exposure to any H₂S), shake to dissolve crystals, and autoclave.

After autoclaving, solution will have some white precipitate. Add immediately before inoculation.

Orpin's Medium C with cellobiose

Add the following to a 1-L round-bottom flask

305 mL dH₂O

1.5 g cellobiose

3.0 g trypticase peptone (BD 211921)

0.75 g yeast extract (BD 212750)

0.135 g K₂HPO₄
0.135 g KH₂PO₄
0.27 g (NH₄)₂SO₄
0.27 g NaCl
0.027 g MgSO₄
0.027 g CaCl₂·2H₂O
3 drops of 0.1% resazurin
45 mL rumen fluid (supernatant from 16,000 g centrifugation)

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.8 g Na₂CO₃

After boiling, color is pink. Continue bubbling until reach reaches approximately ? mV. Then add

0.3 g cysteine HCl

Continue bubbling until colorless (usually requires ? h). Dispense and autoclave

Orpin's Medium C with reed canary grass

Prepare Orpin's Medium C with cellobiose, but replace cellobiose with reed canary grass. Grass is ground through 1 mm screen and included at 10 g/L (e.g., 0.09 g/9 mL). For uniformity, weigh out grass directly in the culture vessel (tubes or bottles), not the round bottom flask.

Orpin's Medium C with 15% glycerol

Prepare Orpin's Medium C with cellobiose, but replace 45 mL water with glycerol.

PC minus VFA media

Adapted from (18) and Michael Flythe (personal communication)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
1.2 g glucose
0.3 g trypticase peptone (BD 211921)
0.15 g yeast extract (BD 212750)
0.0876 g K₂HPO₄
0.0876 g KH₂PO₄
0.1440 g (NH₄)₂SO₄
0.1440 g NaCl
0.0147 g MgSO₄
0.0192 g CaCl₂·2H₂O
3 drops of 0.1% resazurin

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

After boiling, color is pink. Bubble until redox is around -150mV Then add

0.2 g cysteine HCl·H₂O

Continue bubbling until colorless (usually requires 1 to 4 h). Dispense and autoclave.

PC plus VFA media

Adapted from (18)

Add the following to a 1-L round-bottom flask

350 mL dH₂O

1.2 g glucose or 0.9 g cellobiose or 0.6 mL 0.2% glycerol

0.3 g trypticase peptone (BD 211921)

0.15 g yeast extract (BD 212750)

0.0876 g K₂HPO₄

0.0876 g KH₂PO₄

0.1440 g (NH₄)₂SO₄

0.1440 g NaCl

0.0147 g MgSO₄

0.0192 g CaCl₂·2H₂O

0.93 mL VFA solution

3 drops of 0.1% resazurin

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

After boiling, color is pink. Continue bubbling until reach reaches approximately -150mV. Then add

0.2 g cysteine HCl·H₂O

Continue bubbling until colorless (usually requires 1 to 4 h). Dispense and autoclave

Pfennigs heavy metal solution

Adapted from (33)

Add the following 1-L volumetric flask

0.5 g EDTA disodium salt

0.2 g FeSO₄·7 H₂O

0.2 g $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$
0.01 g $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$
0.03 g H_3BO_4
0.02 g $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$
0.001 g $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$
0.002 g $\text{NiCl}_2 \cdot 6 \text{H}_2\text{O}$
0.003 g $\text{NaMoO}_4 \cdot 2 \text{H}_2\text{O}$

Bring to 1 L with dH_2O . Store at 4°C.

Phosphate buffer for phosphotransferase assays

Adapted from (34-36)

Add the following to a 1-L round-bottom flask

3.054 KH_2PO_4
1.155 Na_2HPO_4
0.0305 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$
3 drops of 0.1% resazurin

Adjust pH to 7.2.

After boiling and cooling, bubble until color turns pink. Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. Dispense and autoclave. When required, add DTT after autoclaving (see *Phosphotransferase assay of rumen bacteria*).

Prins semi-defined medium

Adapted from (37)

Add the following to a 1-L round-bottom flask

350 mL dH_2O
3g trypticase peptone (BD211921)
3g Yeast extract (BD 212750)
0.15g K_2HPO_4
0.15g KH_2PO_4
0.3g NaCl
0.15g $(\text{NH}_4)_2\text{SO}_4$
0.015g MgSO_4
0.03g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
0.6ml glycerol (0.2%)
3 drops of 0.1% resazurin

Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1 g NaHCO_3

After boiling, color is orange. Continue bubbling until stops decreasing and stabilizes. Then add 0.1667 g L-cysteine $\text{HCl} \cdot \text{H}_2\text{O}$

Continue bubbling until color changes to golden. Dispense and autoclave.

Rumen bacteria medium (DSMZ 330)

From DSMZ (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium330.pdf)

Add the following to a 1-L round-bottom flask

350 mL dH₂O
0.6g trypticase peptone (BD211921)
0.15g yeast extract (BD 212750)
0.15 g glucose
0.15 g maltose
0.15 g cellobiose
0.15 g starch
0.09 g K₂HPO₄
0.0684 g KH₂PO₄
0.0684 g (NH₄)₂SO₄
0.1368 g NaCl
0.0139 g MgSO₄
0.0182 g CaCl₂·2H₂O
0.12 ml glycerol (0.2%)
3 drops of 0.1% resazurin
0.93 mL VFA solution
3 mL hemin solution

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na₂CO₃

After boiling, color is amber. Bubble until redox stops decreasing and stabilizes. Then add
2.0.1667 g L-cysteine HCl·H₂O

Continue bubbling until color changes to copper. Dispense and autoclave. Before inoculation, add

0.09 mL 2.5% Na₂S·9H₂O
to individual tubes.

Rumen fluid-glucose-cellobiose-starch-agar media (RGCSA)

From (3)

Add the following to a 1-L round-bottom flask

270 mL dH₂O
0.45 g glucose
0.45 g cellobiose

0.15 soluble starch
0.1349 g K_2HPO_4
0.1349 g KH_2PO_4
0.2697 NaCl
0.2697 g $(NH_4)_2SO_4$
0.02697 $MgSO_4$
0.0357 g $CaCl_2 \cdot 2H_2O$
3 drops of 0.1% resazurin.

120 mL rumen fluid (supernatant from 1000 g centrifugation)

Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

0.9 g $NaCHO_3$

Bubble for 20 min (until color turns pink-brown). Then add

0.1667 g L-cysteine HCl·H₂O

Continue bubbling until pink color disappears (usually requires 1 to 4 h). Pipette 9 mL aliquots into Balch tubes containing 0.18 g agar. Autoclave.

In advance of inoculation, melt agar by placing tubes in boiling water bath. Wait several minutes, until contents are fully liquid. Transfer to 45°C water bath, where agar will stay molten until ready for inoculation.

Agar can be withheld to prepare broth.

Russell vitamin solution

Adapted from (28) and Micheal Flythe (personal communication)

Add following to 1-L Pyrex bottle

0.1 g pyridoxamine dihydrochloride (stored at -20°C) (Fisher 436250010)
0.1 g pyridoxal hydrochloride (stored at 4°C) (Fisher 352710010)
0.1 g pyridoxine HCl (Sigma P6280-10G)
0.2 g riboflavin (Sigma R4500-25G)
0.2 g thiamine HCl (Sigma T1270-100G)
0.2 g nicotinamide (Sigm 72340-250G)
0.2 g calcium pantothenate (Sigma 21210-100G-F)
0.1 g lipoic acid (stored at 4°C) (Fisher 138720050)
0.01 g 4-aminobenzoic acid (Sigma 100536-250G)
0.005 g folic acid (stored at 4°C) (Fisher 216630100)
0.005 g biotin (stored at 4°C) (Fisher BP232-1)
0.005 g coenzyme B₁₂ (stored at 4°C) (Sigma V6629-1G)

Prepare 1 L 0.1 M KH_2PO_4 (pH = 6). Add 1.1994 g K_2HPO_4 and 11.8123 g KH_2PO_4 to a 1 L volumetric flask and bring to volume with dH_2O .

To the Pyrex bottle containing the vitamins, add the 1 L 0.1 M KH_2PO_4 (pH = 6) and a stir bar. Mix by stirring (solution will not completely dissolve). Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil). Stir before use. Solution is stable no longer than 1 yr.

Schaefer's vitamin solution + THF

Adapted from Paul Weimer (personal communication)

Add following to 1-L Pyrex bottle

- 0.075 g thiamine HCl
- 0.075 g Ca-D-pantothenate
- 0.075 g nicotinamide
- 0.075 g riboflavin
- 0.075 g pyridoxine HCl
- 0.00375 g para-amino benzoic acid
- 0.00188 g biotin (stored at 4°C)
- 0.00076 g cyanocobalamin (stored at 4°C)
- 0.00047 g folic acid (stored at 4°C)
- 0.00047 g tetrahydrofolic acid (stored at -20°C)

Store protected from light (wrap bottle in aluminum foil) at 4°C.

Selenomonas ruminantium medium (DSMZ 181)

Adapted from DSMZ

(http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium181.pdf).

Add the following to a 1-L round-bottom flask

- 350 mL dH_2O
- 1.5 g Trypticase
- 0.6 g Yeast extract
- 0.3 g glucose
- 0.3 g KH_2PO_4
- 1.2 g Na-acetate
- 0.03 mL n-Valeric acid
- 3 drops of 0.1% resazurin

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na_2CO_3

Bubble until color changes. Then add

- 0.1667 g L-cysteine HCl·H₂O

Continue bubbling until colorless. Dispense and autoclave

Seven vitamins solution

Adapted from DSMZ

(https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium503.pdf) and ref. (2)

Add following to 100-mL Pyrex bottle

- 0.01 g cyanocobalamin (stored at 4°C)
- 0.008 g 4-aminobenzoic acid
- 0.002 g biotin (stored at 4°C)
- 0.02 g nicotinic acid
- 0.01 g calcium pantothenate
- 0.03 g pyridoxine hydrochloride (stored at -20°C)
- 0.02 g thiamine-HCl
- 100 mL ddH₂O

Dissolve by gentle swirling. Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil). Stir before use.

Simplex-type buffer

Modified from (38)

Small volumes

Add the following to a 1-L round-bottom flask

- 350 mL dH₂O
- 1.905 g K₂HPO₄
- 1.5 g KH₂PO₄
- 0.195 NaCl
- 0.027 MgSO₄·7H₂O
- 0.0045 g CaCl₂·2H₂O
- 3 drops of 0.1% resazurin.

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 2.25 g NaCHO₃

Bubble overnight or until color changes to purple then pink. Then add
5 mL of 3% (w/v) cysteine

Continue bubbling until colorless (usually requires 1 to 4 h).

Williams and Coleman (38) call for 3.3 mL of 2% (w/v) cysteine, but we add more to make more resistant to oxidation and be consistent with concentration found in other types of media. The inclusion of the Coleman-type salts (K₂HPO₄, KH₂PO₄, NaCl, MgSO₄·7H₂O, CaCl₂·2H₂O) is also higher than that in Williams and Coleman (38).

Large volumes

Add the following to a 3-L round-bottom flask:

2.1 L dH₂O
11.430 g K₂HPO₄
9.0 g KH₂PO₄
1.170 g NaCl
0.162 g MgSO₄ 7H₂O
0.027 g CaCl₂·2H₂O
18 drops of 0.1% resazurin

Bubble solution and boil for 30 min. The post-boiling volume should be 1.8 L. Cool flask in an ice bath. After cooled, slowly add

13.5 g NaCHO₃

Bubble overnight or until color changes to purple then pink. Then add

31.2 mL 3% (w/v) cysteine

Continue bubbling until colorless (usually requires 1 to 4 h).

Titanium (III) nitrilotriacetate

Adapted from (39)

Add ~500 mL dH₂O to a 1-L round-bottom flask, bubble with N₂, and boil for 5 to 7 min.

Prepare saturated Na₂CO₃ by adding approximately

20 g Na₂CO₃

to 160-mL culture bottle flushed with N₂ gas from a gassing probe. Transfer 75 mL of boiled dH₂O. Seal with rubber bung and aluminum cap. Autoclave.

Transfer 45 mL dH₂O to a 125-mL Erlenmeyer flask flushed with N₂ gas from a gassing probe. Add

1.44 g nitrilotriacetate

Measure pH continuously and stir on stirplate. Adjust pH to 9 with 10% NaOH (requires approximately 7 mL). Add

1.44 mL 20% TiCl₃

Keep pH above 2 by adding TiCl₃ slowly and alternating with saturated Na₂CO₃ (otherwise precipitate will form). Adjust to pH = 7 with saturated Na₂CO₃. Approximately 3.8 mL Na₂CO₃ will be used in total. Solution begins as green, but it will turn violet-blue as TiCl₃ is progressively added.

Adjust to 75 mL volume with boiled dH₂O. Filter through 0.2 μm membrane into 160-mL culture bottle (previously autoclaved and flushed with N₂). When added to medium, reagent will form precipitate. This precipitate can interfere with experiments, and removal of precipitate eliminates reducing ability of the reagent.

Trace element solution SL-10

Adapted from DSMZ

(https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf)

Add following to 500 mL beaker

0.07 g ZnCl₂
0.1 g MnCl₂·4H₂O
0.006 g HBO₃
0.19 g CoCl₂·6H₂O
0.002 g CuCl₂·2H₂O
0.024 g NiCl₂·6H₂O
0.036 g Na₂MoO₄·2H₂O

Some components stick to weigh paper and must be washed into the beaker with a small volume of dH₂O.

Add the following ingredients to another 500 mL beaker

10 mL 7.7 M HCl
1.5 g FeCl₂·4H₂O

Dissolve FeCl₂ completely by gently swirling.

Combine contents of the two beakers, dilute in ~250 mL dH₂O to dissolve, and then bring to 1 L using volumetric flask. Transfer to 1 L Pyrex bottle. Store at 4°C protected from light (wrap bottle in aluminum foil).

Treponema bryantii medium (DSMZ 159)

Adapted from DSMZ

(http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium159.pdf)

Add the following to a 1-L round-bottom flask

260 mL dH₂O
90 mL rumen fluid, clarified
2 g glucose
0.135 g K₂HPO₄
0.135 g KH₂PO₄
0.27 g (NH₄)₂SO₄
0.27 g NaCl
0.026 g MgSO₄
0.036 g CaCl₂·2H₂O
3 drops of 0.1% resazurin

Adjust pH to 7.0 with NaOH

Approximately 50 mL dH₂O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

5 g NaHCO₃

After boiling, color is pinkish-orange. Bubble until redox reaches approximately -150 mV. Then add

10 mL of 3% (w/v) cysteine HCl

Continue bubbling until color turns orange. Dispense and autoclave

Vitamin K1 solution

Dissolve 0.1 ml of vitamin K1 in 20 ml 95% ethanol and filter sterilize. Store refrigerated in a brown bottle.

Vitamin K₃- Hemin solution

Adapted from ATCC

(<https://www.atcc.org/~media/5A86796703884E068DBDB2B74E1346DB.ashx>)

Part A

100 mg menadione (vitamin K₃)

30 mL 95% (w/v) ethanol

Filter sterilize. Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil).

Part B

50 mg Hemin

1 mL N NaOH

Dissolve hemin in NaOH. Bring to 100 mL with ddH₂O. Autoclave. Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil).

Aseptically add 1 ml Part A to 100 mL Part B. Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil).

Akkermansia vitamin solution

From (16)

Add the following to a 250 mL beaker

0.02 g biotin (stored at 4°C)

0.2 g niacin

0.5 g pyridoxine

0.1 g riboflavin

0.2 g thiamine HCl

0.1 g coenzyme B₁₂ (stored at 4°C)

0.1 g 4-aminobenzoic acid

0.1 g pantothenic acid

Dissolve in dH₂O and bring to 1 L using volumetric flask. Store at 4°C and protected from light (wrap Pyrex storage bottle in aluminum foil).

VFA solution

Adapted from (18, 40)

Add following to previously-autoclaved 125 mL Pyrex bottle

- 6 mL propionic acid
- 4 mL n-butyric acid
- 1 mL n-valeric acid
- 1 mL isovaleric acid
- 1 mL isobutyric acid
- 1 mL DL- α -Methyl butyric acid
- 17 mL acetic acid

Store at room temperature in flammables cabinet. Storing in styrofoam cooler will reduce odor.

Table 1. Media compatibility

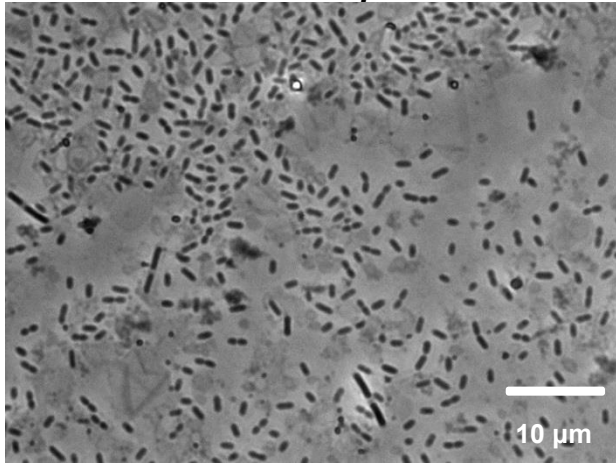
Strain	Medium ¹
<i>Akkermansia muciniphila</i> BAA-835	Mucin medium > PYG (Anaerobe Systems)
<i>Anaerovibrio lipolytica</i> 5S	Prins semi-defined medium > PC + VFA + glycerol
<i>Butyrivibrio fibrisolvens</i> D1	PC+VFA = PC – VFA = minimal media + VFA > rumen bacteria medium
<i>Butyrivibrio proteoclasticus</i> B316	6SG330 > minimal media + VFA = PC + VFA
<i>Clostridium aminophilum</i> F	Chen w/ yeast extract ² > Chen
<i>Clostridium sticklandii</i> SR	Chen w/ yeast extract ² > Chen
<i>Fibrobacter succinogenes</i> S85	PC + VFA + cellobiose ² > PC+VFA ² > cellulolytic medium
<i>Lachnobacterium bovis</i> YZ87	PC+VFA = PC – VFA > rumen bacteria medium
<i>Lachnospira multipara</i> D32	PC+VFA > PC – VFA
<i>Lactobacillus ruminis</i> RF1	MRS > PYG (DSMZ) > PC+VFA.
<i>Megasphaera elsdenii</i> B159	PC – VFA
<i>Megasphaera elsdenii</i> T81	PC – VFA
<i>Mitsuokella jalaludinii</i> M9	PYG (DSMZ) > PC+VFA = PC – VFA > rumen bacteria medium
<i>Prevotella bryantii</i> B14	Treponema bryantii medium > PC+VFA = minimal media + VFA > PC – VFA
<i>Prevotella albensis</i> M384	Treponema bryantii medium > PC+VFA = PC – VFA
<i>Pseudobutyrvibrio</i> sp. B1A1	RGCSA
<i>Ruminococcus albus</i> 7	cellulolytic medium = PC+VFA+cellobiose ²
<i>Ruminococcus albus</i> 8	cellulolytic medium > PC + VFA + cellobiose ²
<i>Ruminococcus flavefaciens</i> FD-1	cellulolytic medium = PC + VFA + cellobiose ²
<i>Selenomonas bovis</i> WG	PYG (DSMZ) > PC-VFA > PC + VFA = rumen bacteria medium
<i>Selenomonas ruminantium</i> GA-192	PC + VFA > <i>Selenomonas ruminantium</i> medium > PC – VFA
<i>Selenomonas ruminantium</i> HD4	PC-VFA
<i>Streptococcus bovis</i> JB1	PC - VFA = PC + VFA = RGCSA
<i>Streptococcus</i> sp. C1A1	PC - VFA = PC + VFA = RGCSA
<i>Succinimonas amylolytica</i> 13846	PC + VFA = rumen bacteria medium
<i>Succinivibrio dextrinosolvens</i> 24	PC + VFA > PC – VFA.
<i>Succinivibrio dextrinosolvens</i> 226	PC+VFA
<i>Entodinium caudatum</i>	medium SP
<i>Epidinium caudatum</i>	medium M

¹Media are ranked according to ability to support growth; “=” indicates equal performance

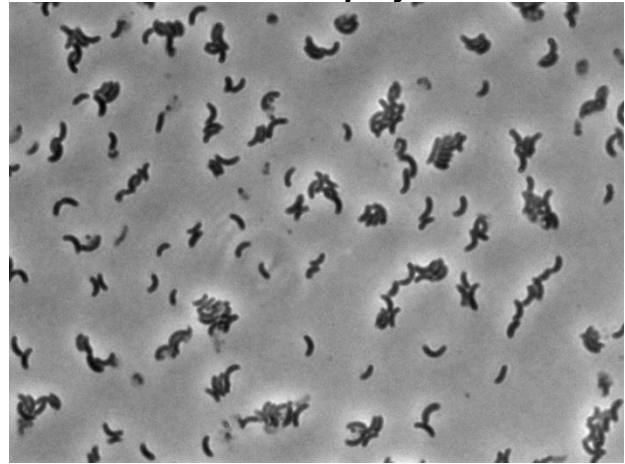
²These media should be avoided for long-term maintenance because they may cause biosynthetic mutants (loss of the wild-type function)

Images of pure cultures of bacteria and methanogens

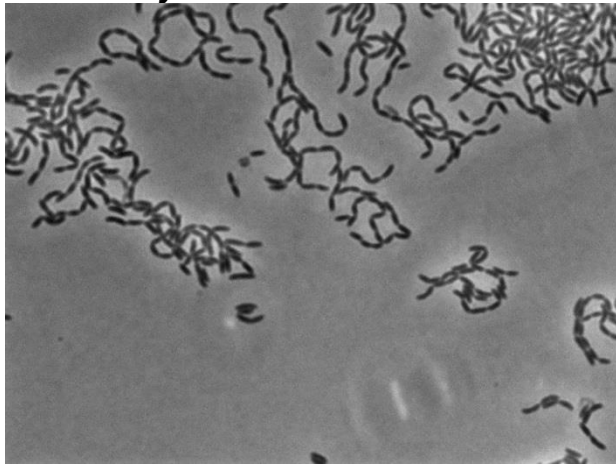
Akkermansia muciniphila BAA-835



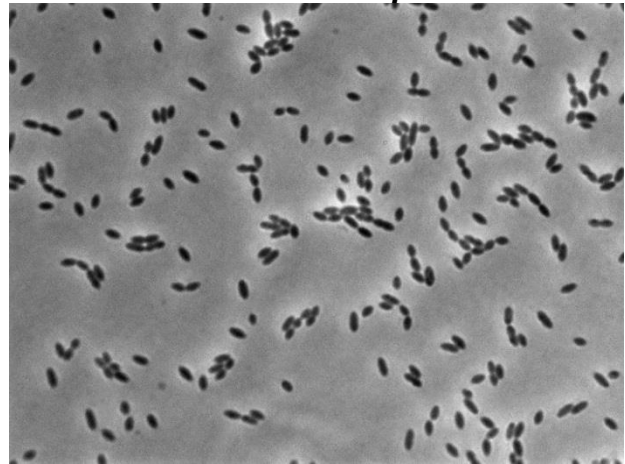
Anaerovibrio lipolytica 5S



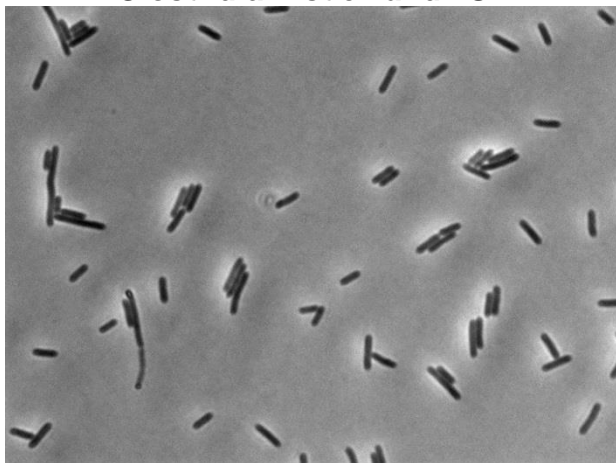
Butyrivibrio fibrisolvens D1



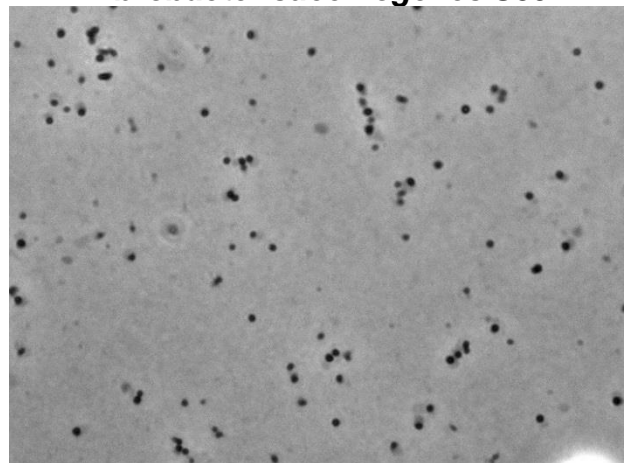
Clostridium aminophilum F



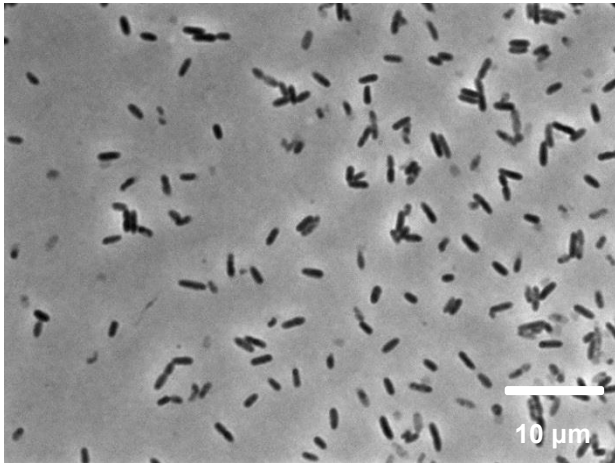
Clostridium sticklandii SR



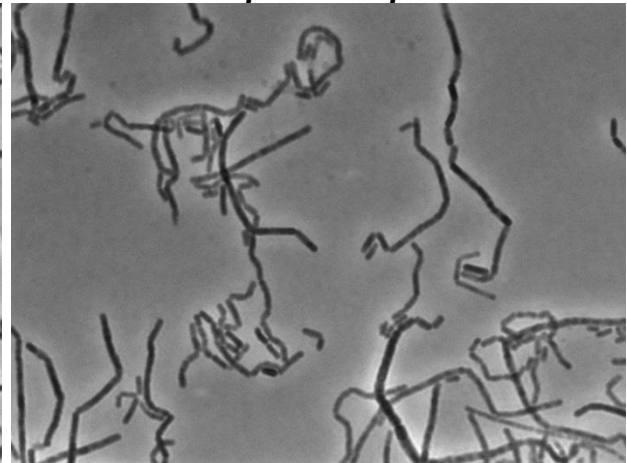
Fibrobacter succinogenes S85



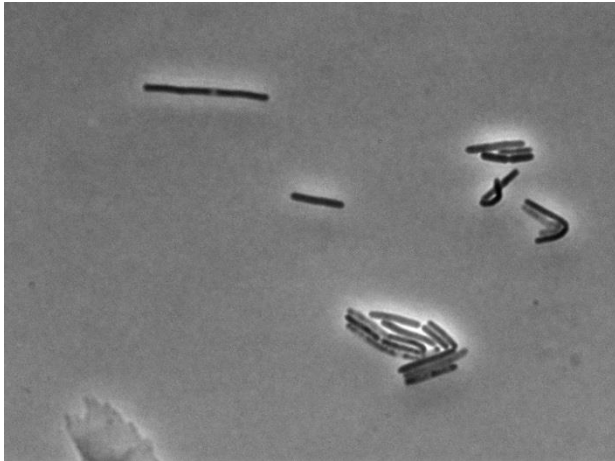
Lachnobacterium bovis YZ87



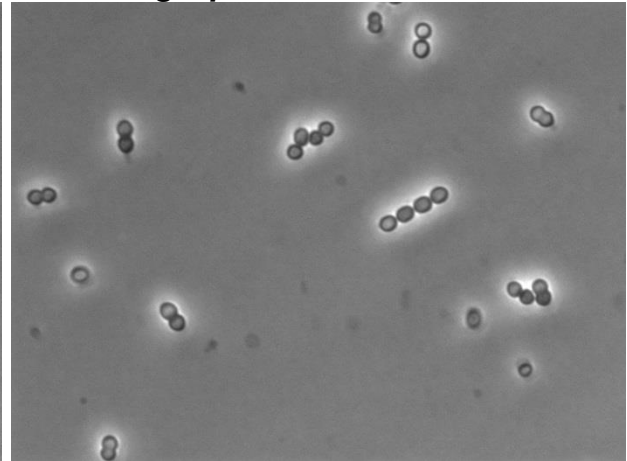
Lachnospira multipara D32



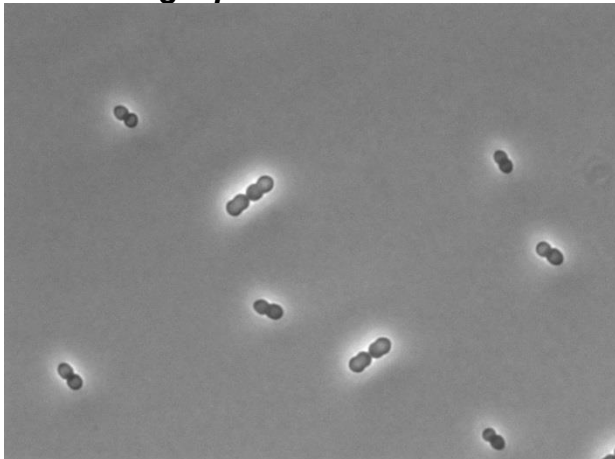
Lactobacillus ruminis RF1



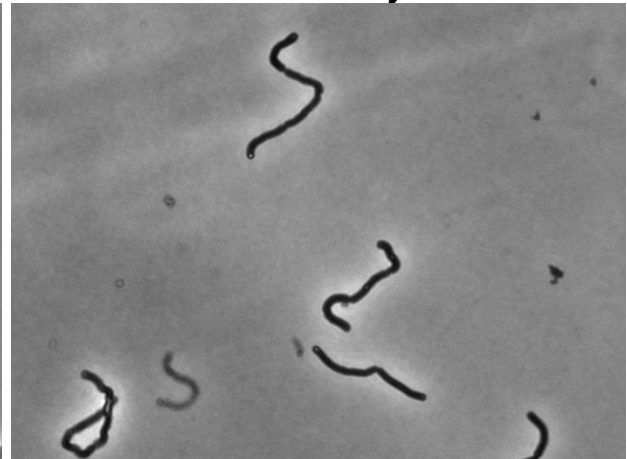
Megasphaera elsdenii B159



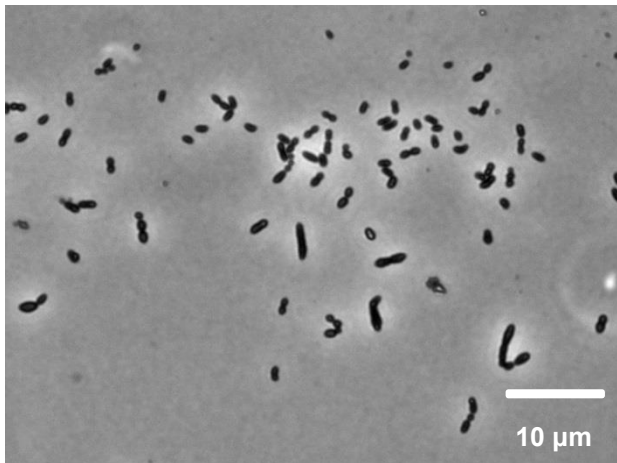
Megasphaera elsdenii T81



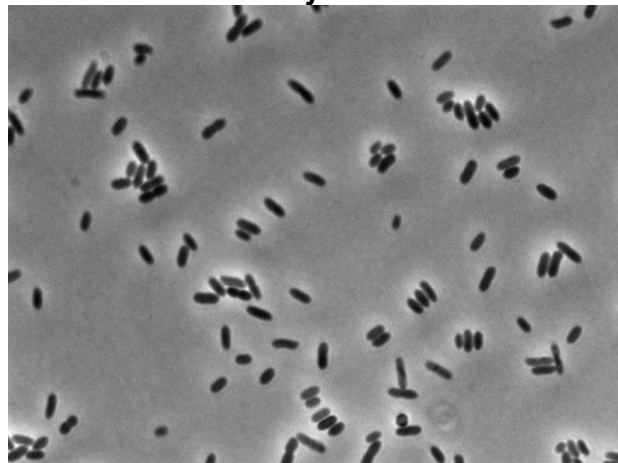
Methanobacterium bryantii M.o.H.



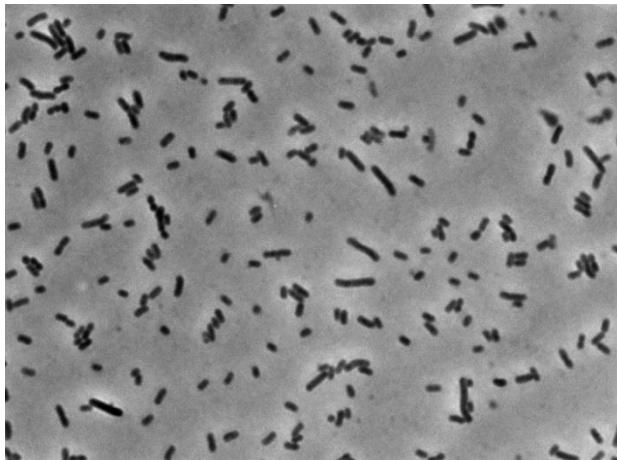
***Methanobrevibacter ruminantium* M1**



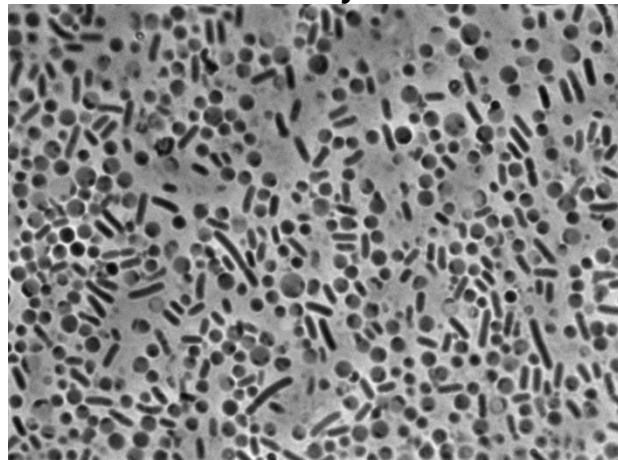
***Mitsuokella jalaludinii* M9**



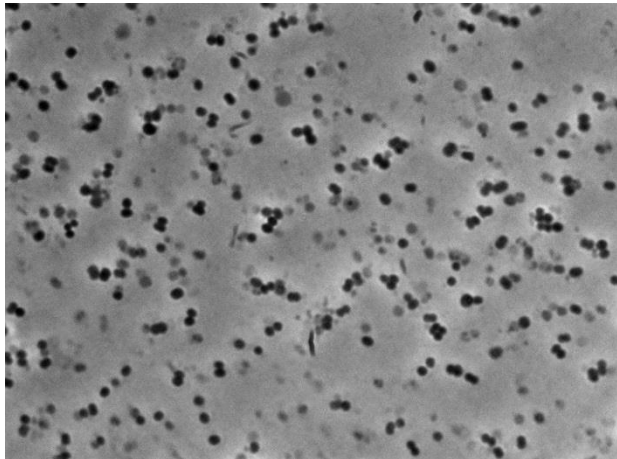
***Prevotella albensis* M384**



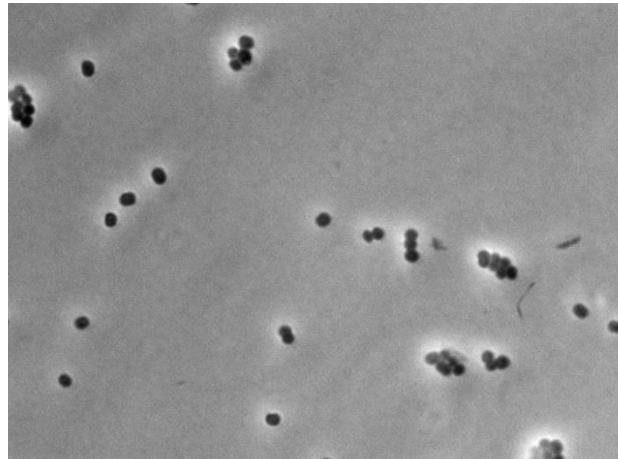
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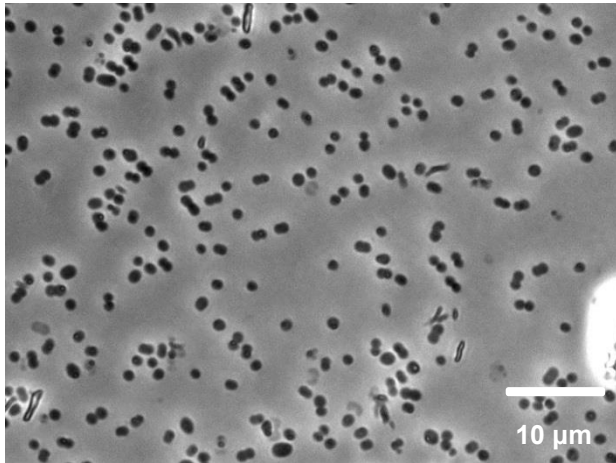
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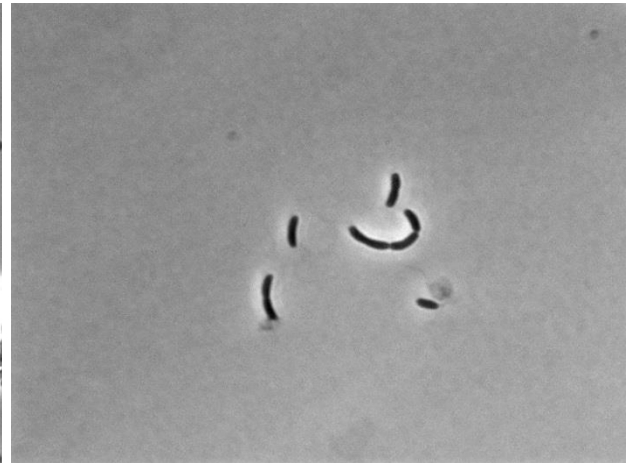
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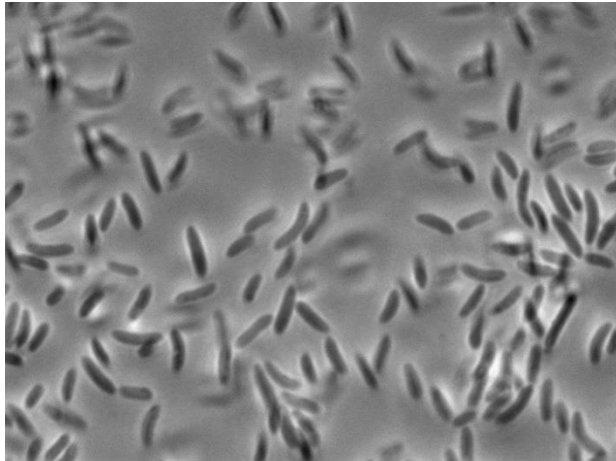
Ruminococcus flavefaciens FD1



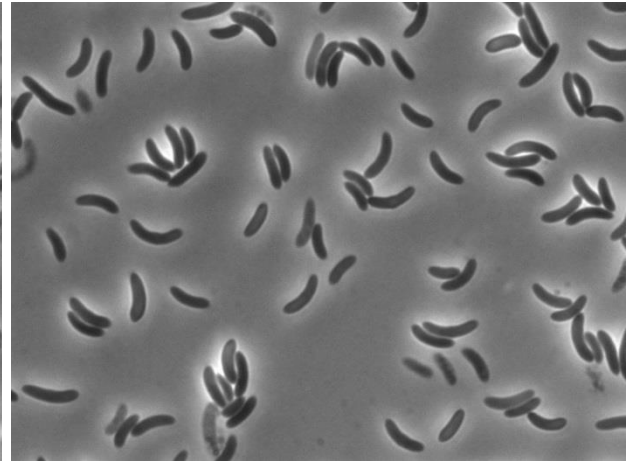
Selenomonas bovis WG



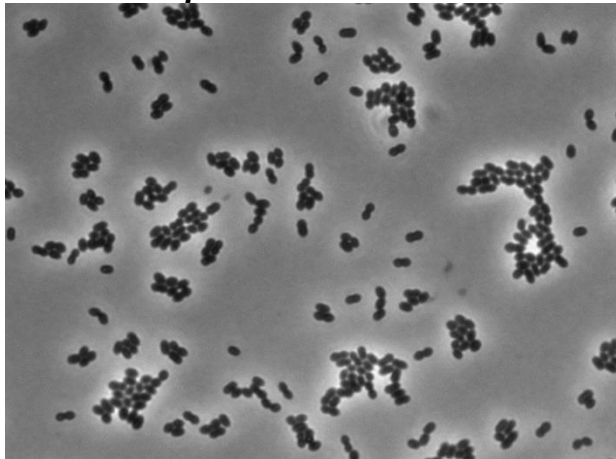
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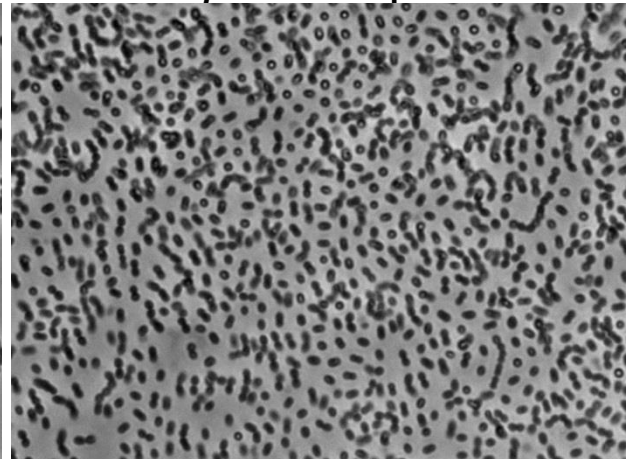
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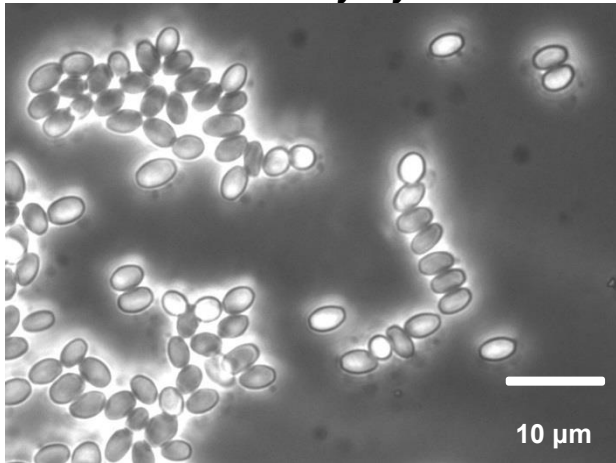
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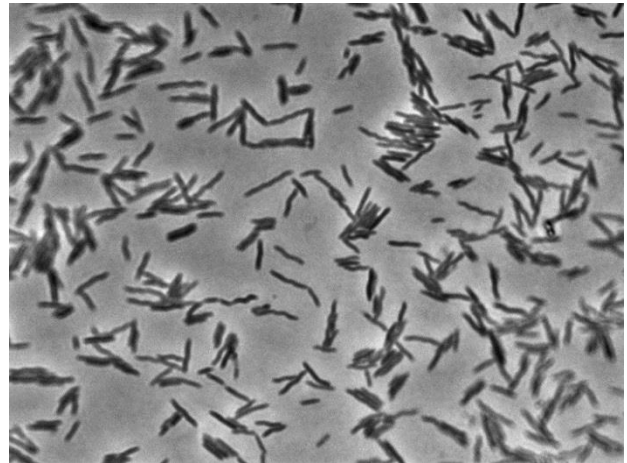
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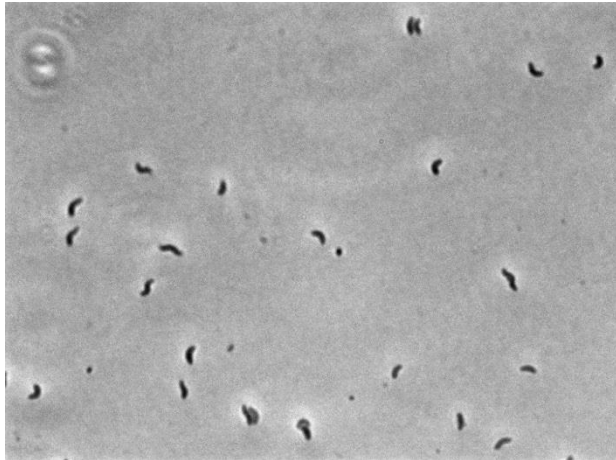
***Succinimonas amylolytica* 13846**



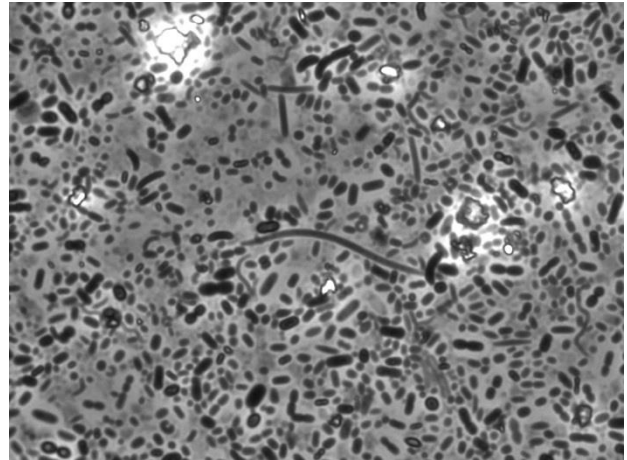
***Succinivibrio dextrinosolvens* 24**



***Succinivibrio dextrinosolvens* 226**



Mixed rumen bacteria

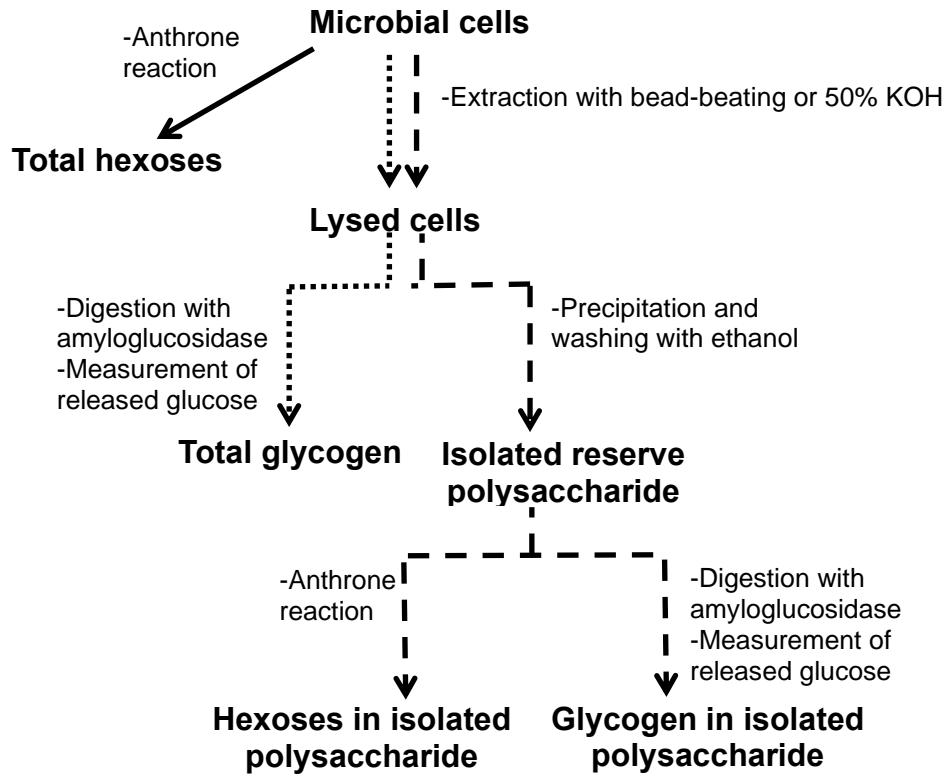


SECTION II: ANALYSIS OF MICROBIAL SAMPLES

Both chemical and microscopic analyses are described here. Unlike culturing, no special techniques need to be employed to analyze anaerobic microbes, and methods are adapted from general microbiology texts.

One area particularly emphasized here is analysis of reserve polysaccharide, which is often the largest chemical component of rumen microbes. Several different methods have been used for analysis, but these methods have usually not been compared, and no method has been evaluated extensively. This manual compares and evaluates these methods (Fig. 1, Table 1). For routine and quantitative measurement of reserve polysaccharide—the most common application—total hexoses measured by the anthrone reaction is the best.

Fig. 1. Flowchart showing methods for measuring reserve polysaccharide.



Procedure in manual

- *Total hexoses of microbial cells by the anthrone reaction*
-→ *Total glycogen of microbial cells by amyloglucosidase*
- -→ *Isolation of reserve polysaccharide and measurement of hexoses and glycogen*

Table 1. Comparison of methods to measure glycogen.

Trait	Type of measurement			
	Total hexoses	Total glycogen	Hexoses in isolated polysaccharide	Glycogen in isolated polysaccharide
Quantitative measurement	Yes	No	No	No
Specific for reserve polysaccharide	No	Yes (if minimal free glucose)	Yes (if purity of isolated material high)	Yes

Preparing cells and culture supernatant for chemical analysis

Collecting cells and supernatant

1.) Prepare washed cell pellets and supernatant according to **Fig. 1**. Work in 2-mL graduated centrifuge tubes throughout. Remove supernatant by aspiration with pipette; simply decanting leads to loss of some pellet. As indicated, keep the supernatant from the first centrifugation and in a separate 2-mL tube, but discard the supernatant from the second centrifugation. Keep sample on ice when not centrifuging.

2.) Store at -20°C .

Resuspending cell pellet with dH_2O

1.) Immediately prior to chemical analysis, resuspend cell pellets to 1 mL final volume using dH_2O . With micropipette, add dH_2O to the 1 mL graduation on centrifuge tube. Less than 1 mL dH_2O should be added because the pellet occupies some volume.

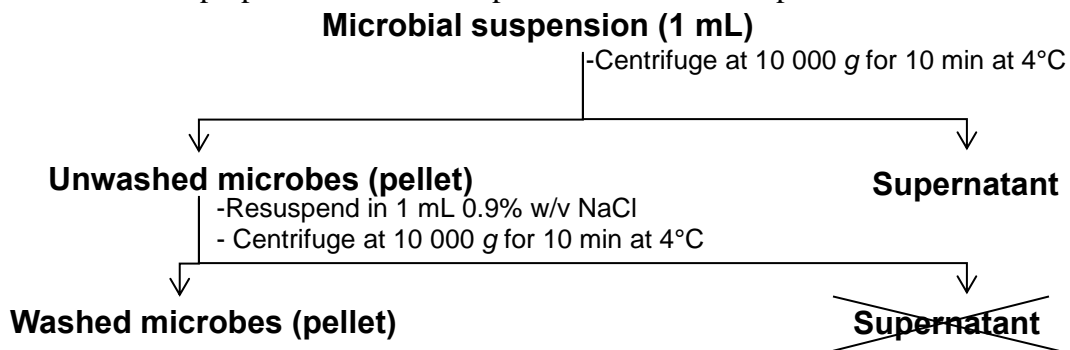
2.) After resuspension, immediately boil for 15 min to inactivate degradative enzymes.

Notes

(i) For some analyses, resuspended pellets can be frozen and later thawed for further analysis. For other analyses, freezing/thawing a resuspended pellet cannot be done because doing so lowers the concentration of the analyte.

(ii) For accurate work, weigh the tube to ensure that the correct amount of dH_2O is added during resuspension of cell pellet. This requires the weighing the empty tube (i.e., before preparing cell pellet). The increase in mass should be 1 g (assuming density of resuspended cell pellet is 1 g/L).

Fig. 1. Flowchart for preparation of washed protozoa and bacteria pellets.



Wet, dry, and organic matter content of microbial cells

- 1.) Dry clean, small aluminum pans (~1 g mass) in 105°C oven to constant mass. Quickly transfer to analytical balance and, immediately after balance reading stabilizes, record mass of pans (hot pan mass). Cool pans to room temperature and record mass again (cold pan mass).
- 2.) Transfer bacterial or protozoal pellets to pan. Around 10 to 30 mg dry matter (DM) are needed (41). Routinely, three to five pellets can be prepared according to Fig. 1 of *Preparing cells and culture supernatant for chemical analysis*, then transferred to pan using 1 mL dH₂O and a pipette (0.5 mL for initial transfer, and 2 × 0.25 mL additional water to wash remaining residue from tubes and pipette tip). Record mass (cold pan + sample mass), correcting for mass of water used in transferring (c. 1 g).
- 3.) Dry to constant mass in 105°C oven (usually overnight) and record mass (hot pan + sample mass).
- 4.) Incinerate dry matter at 550°C in muffle furnace overnight. Cool sample enough to transfer to 105 °C oven. Once equilibrated in oven, record mass (ash pan + sample mass).
- 5.) Calculate wet matter (WM) as (cold pan + sample mass) - (cold pan mass), DM as (hot pan + sample mass) – (hot pan mass), and organic matter (OM) as (hot pan + sample mass) – (ash pan + sample mass). Often these values will be expressed as mg/mL culture.

Protein content of microbial cells

Adapted from (3, 7) and Pierce BCA protein assay kit

Following procedure is done with a Pierce BCA protein assay kit (product # 23227).

1.) Prepare resuspended cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.

2.) Lyse cells by boiling them in 0.2 N NaOH. In 2-mL boil-proof microcentrifuge tube, add the following:

Expected protein mass/pellet (μg)	Resuspended cell pellet (μL)	2 N NaOH (μL)	dH ₂ O (μL)
<2700	167	50	283
2700 to 8100	56	50	394
8100 to 22500	20	50	430

Follow the row corresponding to the expected protein mass in the resuspended pellet. Heat tubes in 100°C water bath for 15 min then cool on ice.

3.) Prepare standards in 2 mL tubes using 2 000 $\mu\text{g}/\text{mL}$ BSA stock from Pierce BCA assay kit according to the following:

Tube	Volume and source of BSA (μL)	Volume of H ₂ O (μL)
A	300 stock	0
B	375 stock	125
C	325 stock	325
D	175 B tube	175
E	325 C tube	325
F	325 E tube	325
G	325 F tube	325
H	100 G tube	400
I	0	400

To the indicated tube, add 2 N NaOH in the following amounts: A, 33 μL ; B, 36 μL ; C, 36 μL ; D, 39 μL ; E, 36 μL ; F, 36 μL ; G, 61 μL ; H, 56 μL ; and I, 44 μL .

Heat standards in 100°C water bath for 15 min then cool

4.) Dilute samples and standards 1:5 using dH₂O as the diluent. For example, 100 μL of sample can be diluted to 500 μL .

5.) Prepare working reagent by mixing 50 parts of BCA reagent A with 1 part BCA reagent B (e.g., 20 mL A with 0.4 mL B) from Pierce BCA assay kit. Volume needed is 0.2 mL for each

replicate of standard or sample. That is, 5.4 mL is needed for all standards (9 in total) and 0.6 mL for each sample if run in triplicate. Make reagent daily.

6.) Pre-read microplate at wavelength 562 nm following *Measuring sample absorbance for microplate assays*.

7.) Load microplate. Pipette 25 μ L sample or standard in triplicate. Then add 200 μ L working reagent to each well; an 8-chambered pipette is helpful.

8.) Cover plate with aluminum foil, then incubate at 37°C in air incubator for 30 min.

9.) Read absorbance with spectrophotometer and calculate actual protein content of pellet (prepared in #1).

10.) Calculate protein concentration. For diluted standards, protein concentrations are (μ g/mL) 360 for A, 270 for B, 180 for C, 135 for D, 90 for E, 45 for F, 22.5 for G, 4.5 for H, and 0 for I. For samples, make sure to multiply concentration by 5 (dilution factor for dH₂O in step #4 of the main procedure), then multiply by either 3 (dilution factor for expected protein mass <2700 μ g/tube in step #3 of the main procedure), 8.93 (2700 to 8100 μ g/tube), or 25 (8100 to 22500 μ g/tube).

Notes

(i) Pellets can be frozen and re-thawed for this analysis because doing so does not affect concentration of protein.

(ii) Protein mass for microbial cells is (mean [SD]) 1.29 (0.28) 10^{-8} g/protozoal cell ($n = 12$ total across 5 cows) and 1.79 (0.39) $\cdot 10^{-13}$ g/prokaryotic cell ($n = 16$ total across 8 cows).

(iii) The step for lysing cell pellets uses volumes of cell pellet, NaOH, and dH₂O that gives (i) final concentration of NaOH of 0.2 N; (ii) cell protein between 300 to 900 μ g/tube (which will give readings that fall within standard curve if actual protein mass is between 1/3 to 2x of expected mass); and (iii) a final volume of 0.5 mL (similar to standards).

(iv) NaOH (2 N) is added to standards to give final concentration of 0.2 N NaOH. Standards must be heated in 0.2 N NaOH because doing so lowers absorbance (samples will have artificially low protein if they are heated but standards are not).

(v) Cell pellets and standards are diluted 1:5 with dH₂O after boiling in 0.2 N NaOH to lower NaOH to its maximum compatible concentration of 40 mM (100 mM $\cdot 8/20$, calculated from concentration reported for test-tube procedure and ratios of working reagent to sample in microplate and test-tube procedures).

RNA and DNA content of microbial cells

Adapted from (41-45)

Extraction of nucleotides

Prepare resuspended cell pellets according to *Preparing cells and culture supernatant for chemical analysis*, but resuspend to 1 mL using 0.5 N perchloric acid instead of dH₂O. Heat at 70°C for 20 min to extract nucleic acids. Because RNA degrades while pellet thaws, do re-suspension and transfer to 70°C immediately. Chill on ice. Extract nucleotides immediately before analysis.

Measurement of DNA using diphenylamine

1.) Prepare aqueous acetaldehyde. Transfer 1 mL of acetaldehyde to 50 mL dH₂O. Chill acetaldehyde to 4°C before opening because boiling point is 21°C. Likewise, chill dH₂O and transfer pipette. Use only in fume hood. This solution is stable in a stoppered bottle for several months at 4°C.

Prepare diphenylamine solution without aqueous acetaldehyde. Dissolve 1.5 g diphenylamine in 100 mL glacial acetic acid, then add 1.5 mL concentrated H₂SO₄. This solution is stable for 3 months at 4°C. It will solidify at 4°C because of the acetic acid.

Prepare diphenylamine solution. Add 0.25 mL aqueous acetaldehyde to 50 mL diphenylamine reagent without aqueous acetaldehyde (thaw in warm water before use). This solution should be prepared immediately before use.

Prepare 5 mM NaOH. Dilute 0.625 mL 2 N NaOH to 250 mL using dH₂O.

Prepare DNA stock solution. Bring 0.04 g DNA (from herring sperm) to 100 mL with chilled 5 mM NaOH. Stock is stable for 6 months at 4°C. Make sure to measure DM of DNA and report concentration of stock accordingly.

Prepare DNA standards:

Standard (g DNA/L)	Stock (μL)	dH ₂ O (μL)	1 N perchloric acid (μL)
0	0	1000	1000
0.025	125	875	1000
0.05	250	750	1000
0.075	375	625	1000
0.1	500	500	1000

Immediately after adding perchloric acid, heat at 70°C for 15 min. Standards are stable for 2 wk at 4°C.

- 2.) Using 0.5 M perchloric acid, dilute nucleotide extract to fall within range of DNA standards; a 5 to 10-fold dilution usually will do. Transfer 0.25 mL of this diluted extract and standards to fresh 2-mL screw-top centrifuge tubes in triplicate.
- 3.) Add 0.5 mL diphenylamine solution. Incubate at approximately 30°C in large water bath for 16 to 20 h (or overnight).
- 4.) Centrifuge at 10000 g for 15 min (room temperature) to remove unhydrolyzed protein (which can cause cloudiness).
- 6.) After pre-reading microplate at 600 nm (see *Measuring sample absorbance for microplate assays*), transfer 200 µL to each well, read absorbance at 600 nm, and calculate total DNA content. Correct for dilution with 0.5 M perchloric acid in #2.

Measurement of RNA using orcinol

- 1.) Prepare FeCl₃ solution. Dissolve 0.540 g FeCl₃ in 1 L concentrated HCl. Solution is stable indefinitely.

Prepare aqueous orcinol. Dissolve 0.5 g orcinol in 50 mL dH₂O. This solution is stable for a few weeks at 4°C.

Prepare orcinol solution. Add 1 volume of aqueous orcinol (e.g., 25 mL) to 4 volumes of FeCl₃ solution (e.g., 100 mL). Prepare immediately before use. Chill on ice.

Prepare RNA stock. Bring 0.02 g RNA (from torula yeast) to 100 mL with chilled 5 mM NaOH. Dissolve by vigorous shaking (~15 min) and trituration with glass pipette. Stock is stable for at least 4 d at 4°C (longer times are untested). Make sure to measure DM of RNA and report concentration of stock accordingly.

Prepare RNA standards:

Standard (g RNA/L)	Stock (µL)	dH ₂ O (µL)	1 N perchloric acid (µL)
0	0	1000	1000
0.025	250	750	1000
0.05	500	500	1000
0.075	750	250	1000
0.1	1000	0	1000

Immediately after adding perchloric acid, heat at 70°C for 15 min. Keep on ice. Prepare immediately before use; long-term stability is unknown.

- 2.) Using 0.5 M perchloric acid, dilute nucleotide extract to fall within range of RNA standards. A 15 to 25-fold dilution usually will do. Chill on ice.

- 3.) Transfer 0.3 mL of this diluted extract and standards to boiling tubes (100 mm x 13 mm, 10 mL Kimax screw-cap tubes) in triplicate. Cap tubes. Place tubes in rack, and place the rack in ice bath until tubes are completely chilled (requires approximately 20 min).
- 3.) Add 0.9 mL orcinol solution using bottle-top dispenser. Re-cap tubes.
- 4.) Transfer tubes to 100°C water in large water bath for 10 min. Set water bath to shake at approximately 30 rpm.
- 5.) Transfer tubes back in ice bath. Cool completely (requires approximately 30 min).
- 6.) After pre-reading microplate at 672 nm (see *Measuring sample absorbance for microplate assays*), transfer 200 µL to each well, read absorbance at 672 nm, and calculate total DNA content. Correct for dilution with 0.5 M perchloric acid in #2.

Notes

- (i) The diphenylamine reaction is specific for DNA, but orcinol reaction is not nearly as specific for RNA. Both DNA and hexoses can interfere in the orcinol reaction. This procedure already attempts to minimize interference with hexoses by using a short (10 min) incubation time [after the recommendation of Herbert et al. (41)]. For accurate work, one must correct values given by the orcinol reaction by measuring DNA and hexoses and using correction factors for DNA (which gives 0.137 the absorbance as RNA) and hexoses (glucose gives 0.033 the absorbance as RNA) [see Table XII of Herbert et al. (41)].
- (ii) Concentrations of DNA and RNA stock solutions can be verified by using the NanoDrop instrument. This can be useful for checking the long-term stability of the stocks. The NanoDrop-measured concentration of DNA tends to be about 20% higher than expected, which probably owes to the greater extinction coefficient of oligonucleotides (which comprise the herring sperm source). Measured concentration of RNA tends to be about 5% higher than expected.
- (iii) Screw-cap centrifuge tubes are recommended with diphenylamine reaction because assay tubes usually must be centrifuged to remove cloudiness from unhydrolyzed protein. Boiling tubes are recommended with orcinol reaction because these are reusable. Presumably screw-cap centrifuge tubes could be used, too.
- (iv) For diphenylamine reaction, volumes of reagents and sample have been decreased about 4-fold compared to those recommended by Herbert et al. (41). For the orcinol reaction, volumes have been decreased 3.33-fold. These smaller volumes make the assays more manageable.

Lipid content of microbial cells

Adapted from (46) and procedure from lab of Dr. Konrad Dabrowski

1.) Prepare solvent mixture by combining 2 L chloroform, 1 L methanol, and 0.75 g butylated hydroxytoluene (0.25 g/L).

Prepare $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ solution by dissolving 6 g $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ in 1 L dH_2O .

2.) Transfer 0.5 g wet pellet to homogenization tubes (150 mm x 25 mm, Pyrex screw-cap tubes). Add 10 mL solvent mixture. Routinely, 5 to 10 pellets can be prepared according to Fig. 1 of *Preparing cells and culture supernatant for chemical analysis*, then transferred to tube using solvent. Close tube with Teflon-lined cap and keep on ice.

3.) Homogenize sample with homogenizer at mid-speed for 1 min. Run homogenizer tip throughout entire volume of sample. In Dr. Dabrowski's lab, an Omni homogenizer is used at speed 4. Between samples, wash teeth of homogenizer generator with dH_2O by running homogenizer for 10 s in beaker filled with dH_2O . Repeat similarly with solvent. Blot homogenizer tip dry with Kimwipe.

4.) Filter sample. Place Watman #1 filter (42.5 mm diameter) on small porcelain Buchner funnel (bottom inner diameter 41 mm) attached to Erlenmeyer vacuum flask (125 mL). Wet filter with small amount of solvent (1/2 volume of glass Pastuer pipette) to seat it. While continuing to wet filter (solvent dries quickly), pour sample on filter. Wash tube with small volume of solvent (c. 0.5 mL) twice, using vortex mixer to swirl solvent on walls of tube; add to filter.

5.) Transfer filtrate into large tubes (200 mm x 25 mm, Pyrex screw-cap tubes). Wash vacuum flask twice with small volume of solvent. Add to filtrate. Keep on ice.

6.) Add 2 mL $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ solution. Fill tube with N_2 gas and cap quickly. Vortex for 1 min. Store overnight at room temperature to allow phases to separate.

7.) Pipette lower phase into a fresh homogenization tube. Lower glass Pastuer pipette into lower phase and aspirate it. Be sure to expel all air from pipette before lowering into lower phase so that air does not escape and disturb upper phase above.

8.) Evaporate solvent by applying heat and stream of N_2 . Place boiling tube in warm (c. 40° C) water bath. Place tubing from N_2 tank into upper part of boiling tube (1-2 cm away from liquid surface), and turn on N_2 gas so that surface is slightly disturbed. A commercial nitrogen evaporator, which has N_2 tubing above a water bath, is used in Dr. Dabrowski's lab. Evaporate to near dryness or until sample turns cloudy.

Usually sample will turn cloudy due to water contamination. To remove water, add some chloroform (5 to 10 mL) and re-evaporate. Repeat as needed. Water (which forms upper phase) can also be removed with pipette if contamination is extensive.

9.) Transfer sample to a smaller glass tube (100 x 13 mm, Pyrex screw-cap tubes). Use small volume of chloroform to re-dissolve lipid and transfer with Pastuer pipette. Wash tube twice with chloroform and add to pre-weighed glass tube. Evaporate solvent by applying heat and stream of N₂ as in step #7.

10.) Transfer sample to autosampler vial (2 mL, screw-top). Vial should be cleaned, put in dessicator, and weighed before use. Evaporate solvent under stream of N₂. To keep tube cool, do not use water bath. A separate commercial nitrogen evaporator, which has no water bath, is used in Dr. Dabrowski's lab. Weigh vial (after drying in dessicator) and calculate lipid mass by difference.

11.) To keep lipid for further analysis, re-dissolve in ~ 1 mL chloroform, and cap tube under N₂ gas.

Notes

(i) Work under hood for all procedures. Chloroform and methanol are toxic, and chloroform is an anesthetic.

(ii) Avoid use of plastics because these can leech contaminants and absorb solvent.

(iii) An autosampler vial is used as a vessel to weigh lipid because it is smaller (lighter) than most other glass vessels and can lead to more accurate determinations of lipid mass. It also has a screw-top (useful for storage). A series of larger glass tubes is used during evaporation to progressively scale down the volume of solvent to the point that the volume can fit in an autosampler vial.

Total hexoses of microbial cells using the anthrone reaction

Adapted from (41, 45)

- 1.) Prepare resuspended cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Prepare anthrone reagent. To prepare 100 mL of reagent, add 0.2 g anthrone to 5 mL absolute ethanol in 100 mL volumetric flask. Bring to volume with 75% v/v H₂SO₄ and dissolve anthrone completely. Transfer to screw-top Pyrex bottle. Make fresh daily. Place in ice bath or in refrigerator until completely chilled (requires approximately 2 to 3 hours).
- 3.) Prepare glucose stock. Add 0.5 g glucose, 1 g benzoic acid (to inhibit microbial activity), and c. 400 mL dH₂O to a 1 L Berzelius beaker. Stir and heat gently to dissolve. Transfer quantitatively to 500 mL volumetric flask and bring to volume. Store stock at 4°C. Stock is stable for months (at least 6 months in our experience).
- 4.) Prepare standards in 2-mL microcentrifuge tubes.

Standard (mg glucose/L)	Stock (μL)	dH ₂ O (μL)
0	0	2000
50	100	1900
100	200	1800
150	300	1700
200	400	1600
350	700	1300
500	1000	1000
750	1500	500
1000	2000	0

Store at -20°C. Standards are stable for months (at least 3 months).

- 5.) Dilute cell pellets in a separate, boil-proof, 2-mL centrifuge tube with dH₂O so that values fall within the standard curve; a 2- to 10-fold dilution will usually do. Store on ice until ready for use.
- 6.) Add 0.12 mL standard or heated sample to boiling tubes (100 mm x 13 mm, 10 mL Kimax screw-cap tubes) or 2-mL screw-top centrifuge tubes. Cap tubes. Place tubes in rack, and place the rack in ice bath until tubes are completely chilled (requires approximately 20 min).
- 7.) Prepare tubes for addition of anthrone reagent.
 - (i) Place ice bath containing tube rack on cart next to hood (within arm's reach).
 - (ii) Place vortexer in hood.

(iii) If not already, place anthrone reagent in ice in cooler. Screw bottle-top dispenser (5 mL capacity) onto anthrone reagent bottle. Pack ice around bottle as completely as possible without touching dispenser. Place cooler in hood. Immediately before use, flush bottle-top dispenser completely with fresh, cold anthrone reagent.

8.) With bottle-top dispenser, add 0.6 mL anthrone reagent to a tube. Re-cap tube, vortex, and replace in ice bath immediately. After adding reagent, keep tubes on ice for 5 additional minutes. Vortex once more within the 5 minutes.

9.) Transfer tubes to 100°C water in large water bath for exactly 10 min. Set water bath to shake at approximately 30 rpm.

10.) Transfer tubes back in ice bath. Cool completely (requires approximately 30 min).

11.) After pre-reading a 96-well microplate at 625 nm (see *Measuring sample absorbance for microplate assays*), transfer 200 µL of standard or sample to microplate. Measure absorbance with spectrophotometer at 625 nm and calculate total carbohydrate concentration. Correct for dilution with dH₂O in step #3.

12.) If measuring mass of glycogen, express hexoses as glucose·0.9.

Notes

(i) An advantage of the anthrone method is that it measures reserve polysaccharide quantitatively (completely). When using the anthrone method, the resulting sum of most major cell components (polysaccharide, DNA, RNA, protein) approaches 100% (47), suggesting all polysaccharide was detected. Compared to the next best method, the anthrone method detected larger increases in reserve polysaccharide during carbohydrate excess and larger decreases during carbohydrate exhaustion (47). This suggests the method measures more polysaccharide that indeed acts as a reserve material (stored during glucose excess and used during glucose exhaustion).

(ii) A disadvantage of the method is that it is not specific. It measures non-glycogen hexoses (trehalose, cell wall glucans, galactose, mannose, rhamnose) that may not serve as reserve polysaccharide.

Anthrone also reacts weakly with DNA and RNA. For accurate work, one must correct values given by the anthrone reaction by measuring DNA and RNA and using correction factors for DNA (which gives 0.062 the absorbance as glucose) and RNA (which gives 0.047 the absorbance as RNA). See Table VIII and text of Herbert et al. (41) for more details.

(iii) After being thawed and resuspended in dH₂O, pellets should not be re-frozen/thawed because doing so lowers the concentration of hexoses. Incubating at 100°C partially protects against losses from freeze-thawing.

(iv) White precipitate forms if outside water (e.g., from water bath or from condensation) enters tube. This precipitate interferes with the assay by increasing absorbance. To prevent water from

entering, screw tube caps tightly and cool tubes completely before opening. Discard tubes that form excess white precipitate, especially if there is evidence that tube has accumulated water during boiling step.

Old H_2SO_4 can also cause formation of white precipitate because H_2SO_4 tends to accumulate water over time (it is hygroscopic). Use only relatively fresh H_2SO_4 , and change to a new bottle of H_2SO_4 if most tubes form white precipitate. Avoid using H_2SO_4 bottle used for Kjeldahl analysis or other bottles frequently open to air.

(v) Keep tubes and reagents chilled when indicated. Keeping them chilled prevents the further formation of color and seems to help prevent formation of white precipitate.

(vi) Anthrone should be stored at 4°C . Before storage, the headspace in its container should be flushed with N_2 .

(vii) Either boiling tubes or screw-top centrifuge tubes can be used and give similar results. An advantage of boiling tubes is that they are re-usable. Advantages of centrifuge tubes are that they (i) seal better than boiling tubes and lower incidence of white precipitate and (ii) can be centrifuged (10000 g, 10 min, 4°C) to remove precipitate if it does form.

(viii) Volumes shown here are 1/5 that recommended by Daniels et al. (45) because the small boiling tube used here is 1/5 the volume of the large tube recommended by Daniels et al. (45). Using small tubes makes the assay more manageable.

(ix) Beef liver glycogen (Sigma) can be used as a control. Be sure to measure dry matter content and consult Sigma's Certificate of Analysis for purity. Also recall that each glucose residue in glycogen is about 162 g/mol. Using boiling tubes, one sample of glycogen was found to contain (mean [SD]) 96.5 (6.5) % CHO on a DM basis ($n = 9$), which compares well with the 96% glucose assay given by Sigma.

Total glycogen content of microbial cells using amyloglucosidase

Adapted from (48-50)

Extraction

Bead-beating

- 1.) Prepare resuspended cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Prepare 0.2 N sodium acetate buffer (pH = 5.2) by adding 1.6406 g to 250 Berzelius beaker, bringing nearly to volume (~75 mL), and adding concentrated HCl (~12.1 N) drop-by-drop to adjust to pH = 5.2. Approximately 0.35 mL of HCl will need to be added. Transfer solution to 100 mL volumetric flask and add water to volume.

Prepare diluted amyloglucosidase solution by diluting amyloglucosidase (3260 U/mL from *Aspergillus niger*; MegaEnzyme, Ltd.) 100-fold in 0.2 N sodium acetate buffer (pH = 5.2).

Prepare bead-beating tubes. Add 0.3 g of 0.1 mm and 0.1 g of 0.5 mm zirconia beads to 2 mL screw-top centrifuge tubes.

- 3.) Heat resuspended cell pellets for 15 min at 100°C to inactivate glycosidic enzymes. Transfer 0.25 mL heated sample to bead-beating tubes. Add 0.75 mL sodium acetate buffer (pH = 5.2). If the sample contains more than 2 g/L glycogen, use less sample and more buffer (so that total glycogen in bead-beating tube is <500 mg).
- 5.) Disrupt cells by bead-beating for 45 min at maximum speed on a Mini-Beadbeater (in ZT's lab). Beat in 15x3 min intervals, and between each interval, incubate tubes on ice for 3 min to cool.

50% KOH

Instead of using bead-beating, glycogen can be extracted by digestion with 50% KOH.

- 1.) Prepare cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Prepare 50% KOH and 6 N HCl. Titrate 50% KOH with 6 N HCl to determine volume of HCl needed to neutralize KOH.
- 3.) Transfer cell pellets to 2-mL screw-top centrifuge tube using 300 μ L dH₂O. Add 120 μ L, 50% KOH to tube. Heat at 100°C for 3 h.
- 4.) Neutralize 50% KOH with 6 N HCl, referring to titration in #2 for the volume of 6 N HCl needed.

5.) Transfer 0.25 mL neutralized extract to fresh 2-mL screw top tube. Add 0.75 mL sodium acetate buffer (pH = 5.2). If the sample contains more than 2 g/L glycogen, use less sample and more buffer (so that total glycogen in bead-beating tube is <500 mg).

Amyloglucosidase digestion and measurement of glucose

1.) Add 56 μ L of diluted amyloglucosidase solution to each tube. Incubate at 57°C overnight using Lauda M3 circulating water bath.

2.) Centrifuge at 1000 g, 10 min, 25°C. Determine glucose released according to step #6 of *Free glucose content of culture supernatant*. Mass of glycogen is glucose \cdot 0.9.

Notes

(i) An advantage of this method is that it is specific to glycogen because amyloglucosidase digests glycogen only. A disadvantage is that it measures less polysaccharide than the anthrone method and probably does not measure all polysaccharide that can indeed serve as reserve material (see *Notes of Total hexoses of microbial cells using the anthrone reaction*).

Unlike the anthrone reaction, the procedure above probably does not detect all glycogen in microbes. Ref. (51) shows that measuring glycogen using the above procedure resulted in a sum of cellular components (glycogen, DNA, RNA, protein, lipid) that was below 100% for mixed rumen microbes.

(ii) Measurement of glycogen is higher for extraction by bead-beating vs. extraction with 50% KOH (47). Bead-beating requires special equipment (bead-beater) while 50% KOH does not.

(iii) After being thawed and resuspended in dH₂O, pellets should not be re-frozen/thawed because doing so lowers the concentration of glycogen. Incubating at 100°C partially protects against losses from freeze-thawing.

(iv) Beef liver glycogen (Sigma) can be used as a control. Be sure to measure the dry matter content and purity; purity can be determined by Sigma's Certificate of Analysis (available on website) or using the anthrone method. After correcting for water and impurities, recovery of glycogen should be quantitative; ratio of glycogen to anthrone-detectable CHO approached unity (mean = 0.979, SD = 0.0839, $n = 9$).

(v) This procedure uses 45 min bead-beating to disrupt cells. This time led to maximal detection of glycogen by this method.

Isolation of reserve polysaccharide and measurement of glycogen and total hexoses

Adapted from (45, 52, 53).

Extraction

Bead-beating

- 1.) Prepare resuspended cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Prepare 25% w/v trichloroacetic acid (TCA). Add 25 g TCA pellets (in refrigerator) to 100 mL volumetric flask then bring to volume. Store at 4°C, and chill on ice before use.
- 2.) Follow **Fig. 1**. Also refer to *Total glycogen content of microbial cells using amyloglucosidase* because that procedure is similar to the present, except in the present procedure (i) bead-beating is done on the pellet directly, not pellet suspended in NaAc buffer, (ii) protein is precipitated using TCA and (iii) glycogen is precipitate and washed with ethanol.

Only 0.9 mL resuspended pellet is added to tube containing beads so that transfer is quantitative; some pellet remains adhered to walls of the tube, preventing transfer of the full 1 mL.

50% KOH

Instead of using bead-beating, glycogen can be extracted by digestion with 50% KOH.

- 1.) Prepare cell pellets according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Follow **Fig. 2**. Also refer to *Total glycogen content of microbial cells using amyloglucosidase* because that procedure is similar to the present, except in the present procedure (i) no neutralization of KOH is needed and (ii) glycogen is precipitate and washed with ethanol.

Measurement of hexoses and glycogen

- 1.) Follow *Total hexoses of microbial cells using the anthrone reaction* to measure hexose content of the isolated polysaccharide.
- 2.) Alternatively, follow *Total glycogen content of microbial cells using amyloglucosidase* to measure glycogen content. Transfer 0.25 mL neutralized extract to fresh 2-mL screw top tube. Add 0.75 mL sodium acetate buffer (pH = 5.2). If the sample contains more than 2 g/L glycogen, use less sample and more buffer (so that total glycogen in bead-beating tube is <500 mg). Then digest with amyloglucosidase and measure glucose released. Mass of glycogen is glucose·0.9.

Notes

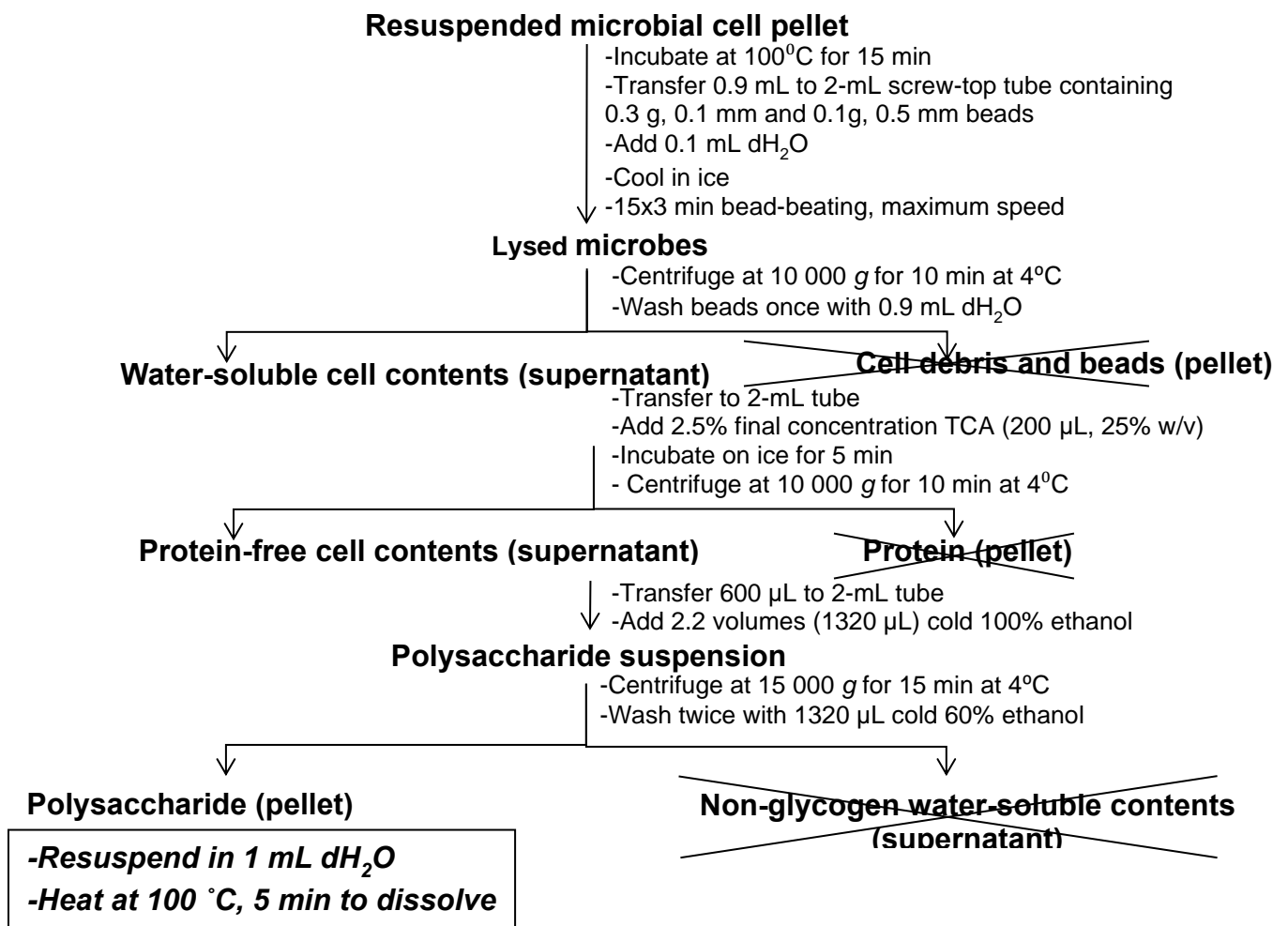
(i) An advantage of this method is that it is specific; non-reserve polysaccharide components are removed before measurement. A disadvantage is that it is not quantitative (47), probably because some glycogen is probably not solubilized by 50% w/v KOH boiling (50) and thus is removed during the first centrifugation.

(ii) Measurement of reserve polysaccharide is higher for extraction by bead-beating vs. extraction with 50% KOH (Appendix II). Bead-beating requires special equipment (bead-beater) while 50% KOH does not.

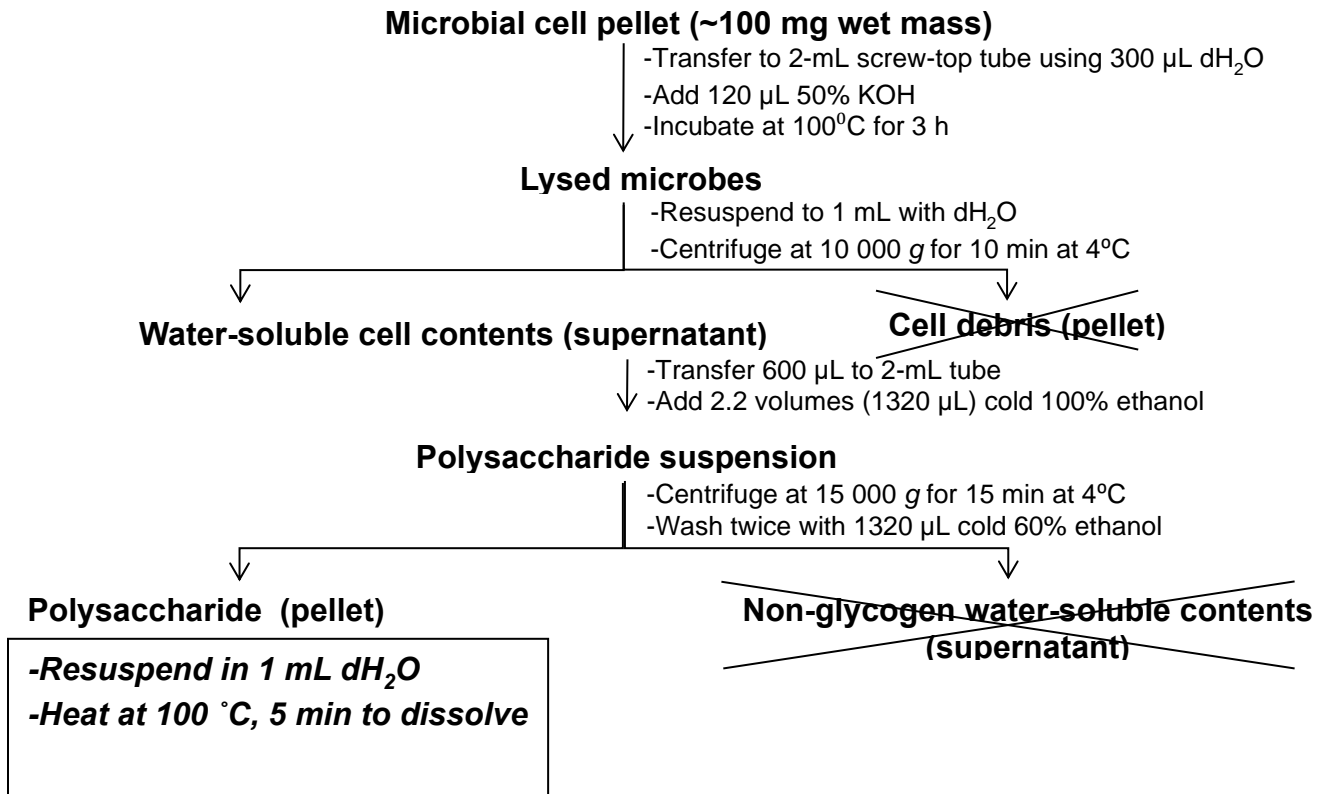
(iii) Polysaccharide is glycogen, as indicated by a ratio between and glycogen and total hexoses of effectively unity (0.994 [0.045 SD] for bead-beating extraction, 1.048 [0.038 SD] for KOH extraction, $n = 5$).

Fig. 1. Flowchart for isolating reserve polysaccharide. (A) Extraction by bead-beating. (B) Extraction with 50% KOH.

(A)



(B)



Thin-layer chromatography (TLC) of cytoplasmic carbohydrate and reserve polysaccharide

Based on (54-57)

Sample and standard preparation

- 1.) Prepare TLC-ready cytoplasmic carbohydrate. Prepare protein-free cell contents according to *Isolation of reserve polysaccharide and measurement of glycogen and total hexoses*. To prepare TLC-ready, digested cytoplasmic carbohydrate, follow **Fig. 1**. To prepare TLC-ready, undigested cytoplasmic carbohydrate, follow Fig. 1 but omit addition of 100-fold dilution amyloglucosidase. For solvent evaporation, use vacuum speed concentrator in Dr. Wick's lab.
- 2.) Prepare TLC-ready isolated reserve polysaccharide. Prepare protein-free cell contents according to *Isolation of reserve polysaccharide and measurement of glycogen and total hexoses*. To prepare TLC-ready, digested polysaccharide, follow Fig. 1 but omit neutralization with 0.2 N NaOH and extraction with diethyl ether. To prepare TLC-ready, undigested polysaccharide, follow Fig. 1 but omit neutralization with 0.2 N NaOH, extraction with diethyl ether omit, and addition of 100-fold dilution amyloglucosidase.
- 3.) Measure concentration of glucose in samples. For digested samples, dilute sample to <500 mg/L glucose and follow step #6 of *Free glucose content of culture supernatant*. For undigested samples, first digest with amyloglucosidase, then measure glucose concentration. Yield of glucose during sample preparation is typically 30 to 50%.
- 4.) Prepare standards (glucose, maltose, maltotriose, maltoheptaose, beef liver glycogen; 3 g glucose equivalents/L). For glucose standard, add 0.3 g glucose to 100 mL volumetric flask and bring to volume with dH₂O. For maltose, repeat but with 0.3 g maltose monohydrate. For maltotriose hydrate, add 0.02915 g maltotriose hydrate to 10 mL dH₂O (assuming 98% DM and 98% maltotriose; adjust for actual DM and purity on certificate of analysis). For maltoheptaose, add 0.02915 g to 10 mL dH₂O (assuming 94% maltoheptaose; adjust for actual purity). For beef liver glycogen, add 0.0314 g to 10 mL dH₂O (assuming 90.04% DM and 94% glucose; adjust for actual DM and purity).

Plate preparation

- 1.) Prepare solvent (1-propanol:acetone:dH₂O; 2:2:1 by volume).
- 2.) Cut plates (20 x 20 cm, Merck Si 60 F₂₅₄, 0.2 mm layer thickness) into two, 20 x 10 cm plates. Cut in the direction in which silica was applied (look for scratches on aluminum backing). Cut plate with scissors angled slightly right to the vertical.
- 3.) Mark plate for development. Follow **Fig. 2**. Using a pencil and ruler, mark sample application zones with a small point or hash mark. As shown, leave at least 1.5 cm between zones and horizontal edges of plates. Draw a horizontal line to mark the proposed distance of solvent front. Throughout, use pencil lightly (just enough to see) to avoid damaging plate.

4.) Wash plates. Fill development chamber (glass; 10" H, 11.5" W, 3.75" D) with solvent (see **Fig. 3**). Add 100 mL solvent (enough to give make solvent 0.5 to 1 cm deep). Help saturate chamber with solvent by adding filter paper (Whatman #1, 185 mm; 2 circles); fold filter paper in thirds, wet with solvent, and place one circle of filter paper per on each side of chamber. Place lid over tank and wait 1 h or more for chamber to fully saturate. Allow solvent to reach upper edge of plate (requires c. 3 h). Dry plate of solvent using hair dryer on low setting.

Development, charring, and visualization

- 1.) Prepare 2 L detection reagent (H₂SO₄:ethanol; 9:1 by volume).
- 2.) Apply standards and samples on starting line. Use a micropipette to apply 1 µL. Apply approximately 3 µg glucose equivalents. Dry samples (using hair dryer as needed).
- 3.) Develop plate (**Fig. 3**). Fill development chamber with fresh solvent and filter, and allow chamber to saturate. Place plate upright in chamber with tweezers. Position bottom edge of plate c. 2 cm away from back of chamber, and rest the top edge of plate against back of chamber. Do not allow solvent to splash past starting line or sides of plate to touch filter paper.
- 4.) Remove plate after solvent reaches 10 cm mark made earlier (requires c. 1 h). Dry plate using hair dryer.
- 5.) Detect spots on plate. Fill 2 L Berzulus beaker with 1.8 L detection reagent. Dip plate in reagent; grasp plate with tweezers, and lower and raise plate in reagent slowly and in one continuous motion (no pauses). Blot dry the back of plate with paper towel. Place plate in metal pan (9"x9") lined with aluminum foil, and cover top of plate loosely with aluminum foil. Allow front and back of plate to completely dry. Char in ash oven at 110°C for 10 min (prop open door of oven with hand to regulate temperature). Work in hood throughout.
- 6.) Immediately after charring, take image of plate using camera and software in ZT's lab. Place plate in camera cabinet. Open FluorChem 8000 program on computer next to camera. Click "Acquire" button in the main taskbar at top. In the "Camera Setup & Preview" window that appears, click the "Expose preview" button (green, in upper right corner). Check the "Auto Expose" box. Under "Sensitivity/Resolution" section, make sure "Normal/High" is selected. Under "Cabinet Controls" section, click the "White" button for "Reflective". Make sure that filter 2 ("EtBr, Colorimetric") is selected. Using black box next to camera, zoom and focus image as needed. Click "Acquire image" button (red, in upper right corner). Adjust contrast and rotate image using windows that appear, then save as *TIF.

Notes

(i) Handle the TLC plate carefully to avoid contamination or damage to front of plate. Wear gloves at all times. Handle plate by grasping it with tweezers at the top when possible; when hands must be used, grasp plate only by the edges. Place plate on clean surface (e.g., aluminum foil) and cover plate when not in use.

- (ii) Washing plates removes contaminating substances and lowers the background coloration of plate when charred.
- (iii) If the chamber were not saturated with solvent, solvent would quickly evaporate from the solvent front during development. Development would require more time, make the solvent front uneven, and increase R_F values of spots.
- (iv) Wash glassware and other materials contacting plate with both dH_2O and acetone.
- (v) During sample preparation, diethyl ether extraction removes TCA and TMD-8 (mixed bed resin) removes salt. If not removed, TCA and salt can cause tailing and streaking of spots, making their identification difficult.
- (vi) Circles of filter paper are used to saturate the chamber in this procedure, but sheets of filter paper are recommended in most other procedures. When available, sheets should probably be used.

Fig. 1. Flowchart for preparing digested cytoplasmic carbohydrate for TLC.

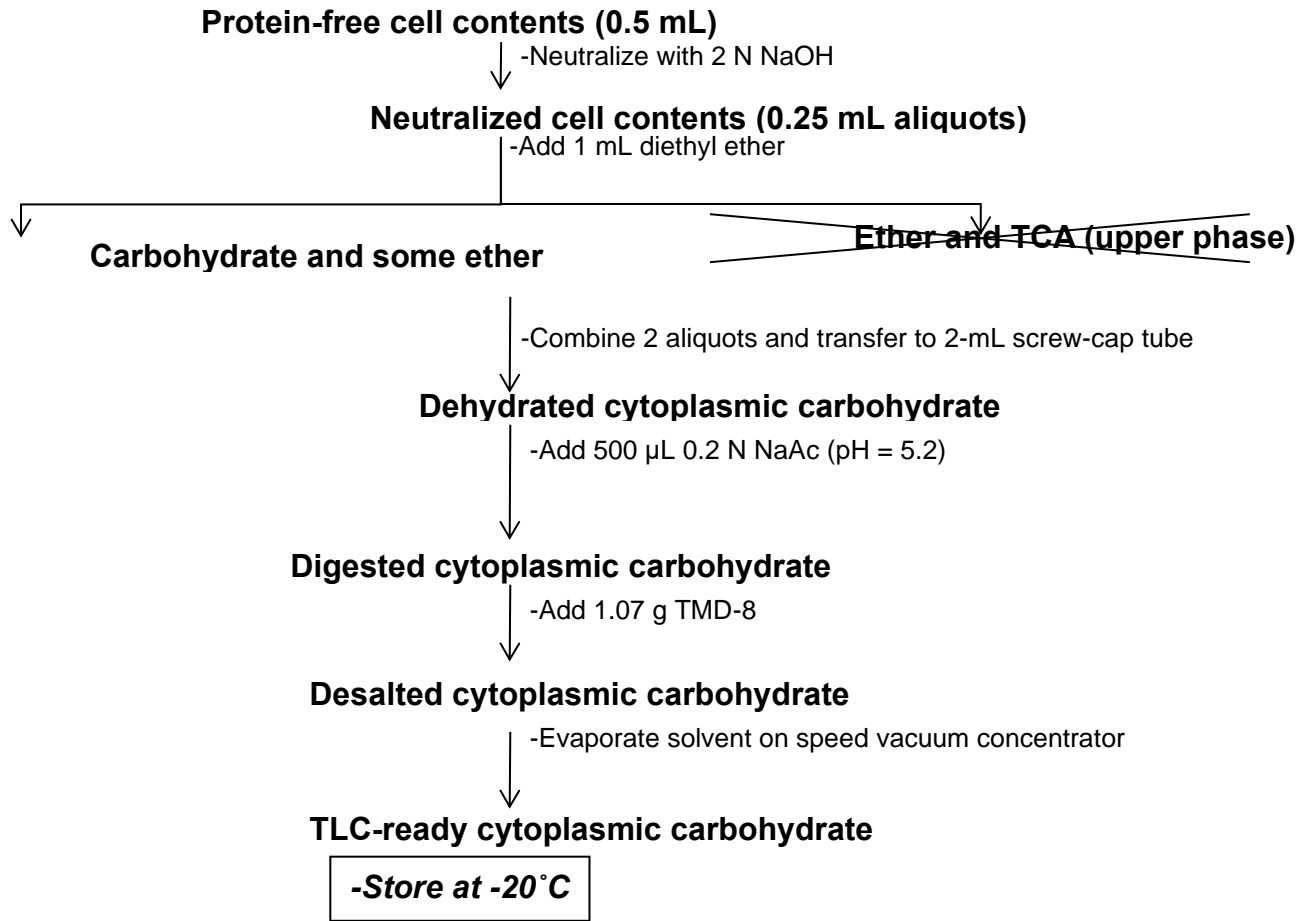


Fig. 2. Diagram of TLC plate marked for development.

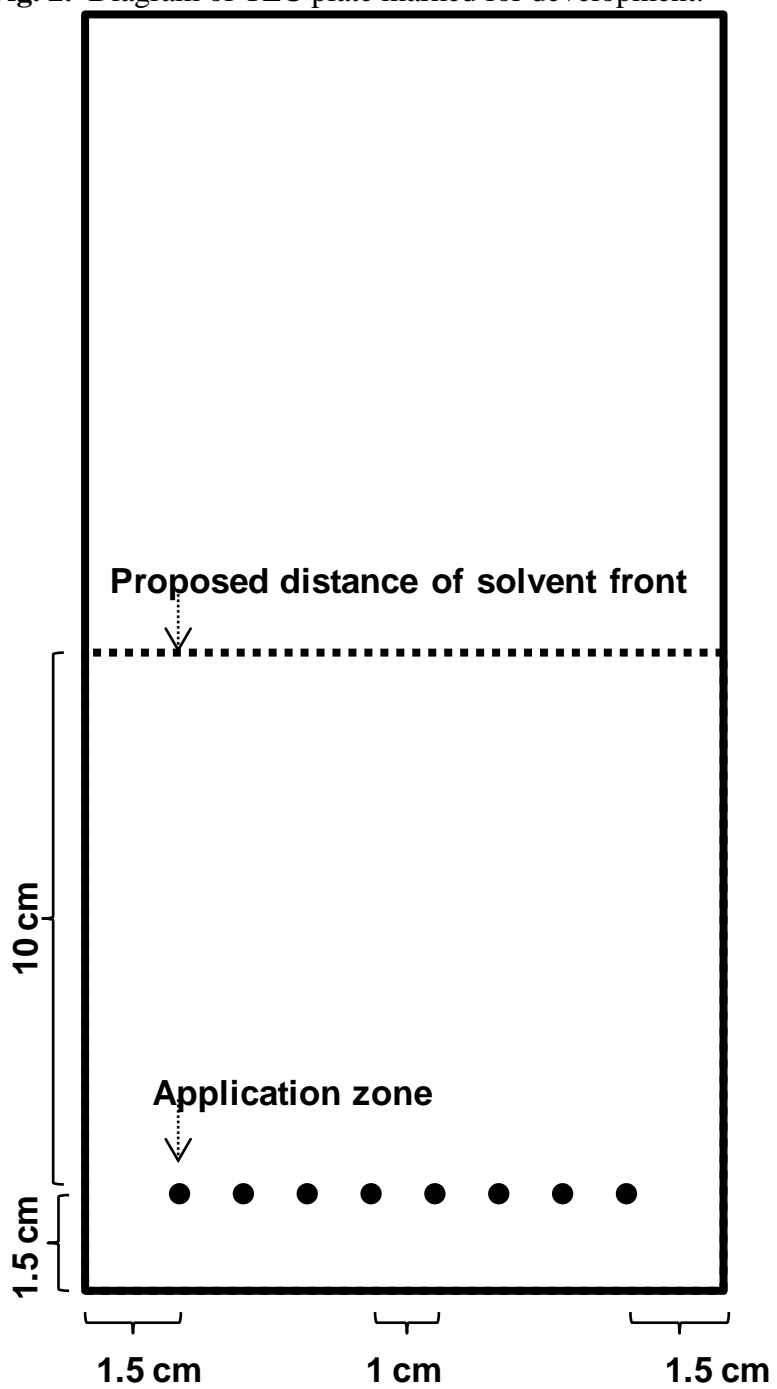
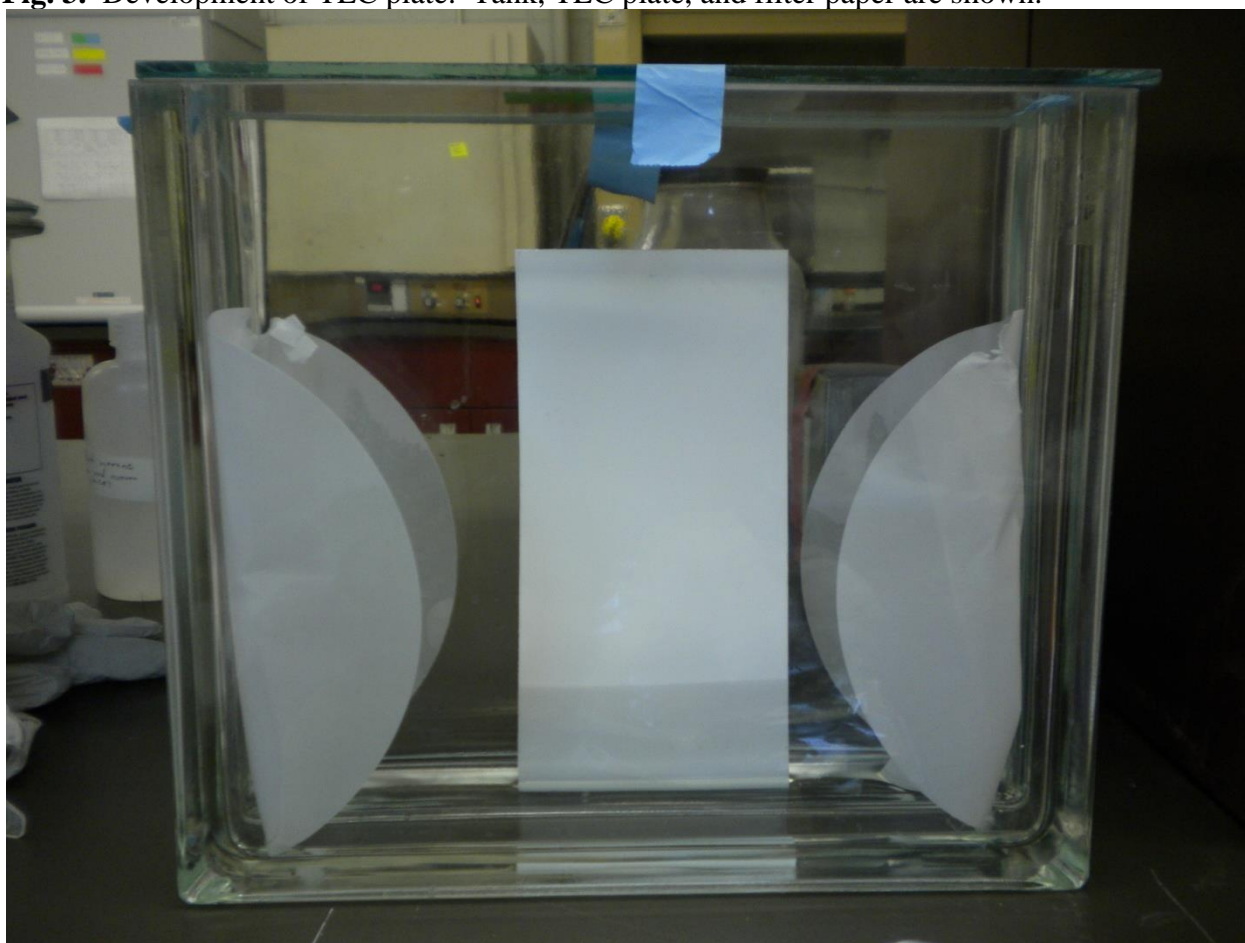


Fig. 3. Development of TLC plate. Tank, TLC plate, and filter paper are shown.



Iodine absorbance spectrum of reserve polysaccharide

Adapted from (58)

1.) Prepare isolated reserve polysaccharide according to *Isolation of reserve polysaccharide and measurement of glycogen and total hexoses*.

2.) Prepare iodine solution. Add 0.13 g I₂ and 1.3 g KI to 5 mL dH₂O. Store at RT. Prepare saturated CaCl₂. Add 30 g CaCl₂·2H₂O to 25 mL dH₂O and stir to dissolve. Heating is not necessary because reaction is exothermic. Cool on ice to fully crystallize CaCl₂. Warm to RT and stir to dissolve most of the crystals. Filter out remaining crystals on Buchner funnel. Store filtrate at RT.

Prepare 2x iodine reagent. Add 115.4 μL iodine solution to 15 mL saturated CaCl₂. Thaw saturated CaCl₂ before use (ensure all crystals dissolve). Store in store in amber jar or clear jar covered with aluminum foil at 4°C. Solution is stable for 1 week.

3.) Add 30 μL reserve polysaccharide and 97.5 μL saturated CaCl₂ to each well of a microplate. Include blank wells with 30 μL dH₂O and 97.5 μL saturated CaCl₂.

Read absorbances from 380 to 700 nm in 10 nm increments. Follow *Measuring sample absorbance for microplate assays*.

The set of absorbances is called A₁(reserve polysaccharide) for wells to which reserve polysaccharide was added, and A₁(dH₂O) for wells to which dH₂O was added.

4.) Add 97.5 μL 2x iodine reagent. Read absorbances from 380 to 700 nm in 10 nm increments. This set of absorbances is called A₂(reserve polysaccharide) for wells to which reserve polysaccharide was added, and A₂(dH₂O) for wells to which dH₂O was added.

5.) Determine the absorbance spectrum of the reserve polysaccharide. Determine [A₂(reserve polysaccharide) – A₁(reserve polysaccharide)] – [A₂(dH₂O) – A₁(dH₂O)].

Notes

(i) The absorbance spectrum formed has a peak from 490 to 510 nm. This peak is characteristic of glycogen-iodine complex and confirms that reserve polysaccharide is glycogen.

(ii) Cell suspensions can also be analyzed and produce a similar absorbance spectrum, particularly after bead-beating.

(ii) Subtracting A₁(reserve polysaccharide) in #5 corrects for absorbance of reserve polysaccharide and saturated CaCl₂. Further subtracting [A₂(dH₂O) – A₁(dH₂O)] corrects for absorbance of 2x iodine reagent.

(iii) Saturated CaCl₂ is added in #3 to bring solution to a volume readable by the spectrophotometer. Saturated CaCl₂, rather than dH₂O, must be used because CaCl₂ increases the absorbance of the iodine-glycogen complex.

(iv) Saturated CaCl_2 is made adding CaCl_2 in slight excess of its solubility at RT, heating to dissolve, and cooling to fully crystallize. Warming the solution to RT will produce an exactly saturated solution with large crystals that can be removed with filtration.

Free glucose content of culture supernatant

Adapted from (59, 60)

1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.

2.) Prepare glucose oxidase-peroxidase (GOP) reagent (9.1 g Na₂HPO₄, 5 g KH₂PO₄, 1 g phenol, 0.15 g 4-aminoantipyrine, 0.404 g glucose oxidase from *Aspergillus niger* [Type II, Sigma G6125], 0.0972 g peroxidase from horseradish [Type I, Sigma P8125] per liter). The masses of glucose oxidase and peroxidase listed assume 17300 U/g for glucose oxidase and 72 U/mg for peroxidase. See pack label for exact U/g and adjust masses of glucose oxidase and peroxidase accordingly (to give 7000 U/L final concentration).

Remove glucose oxidase and peroxidase from -20°C freezer and 4°C refrigerator to thaw. Completely dissolve Na₂HPO₄ and KH₂PO₄ in dH₂O (1/3 of the final volume of the solution) in volumetric flask. Add phenol and 4-aminoantipyrine and completely dissolve. Add glucose oxidase and peroxidase and dissolve by gentle swirling to avoid foaming. Bring to volume, filter through Whatman GF/A filter, and store in amber jar or clear jar covered with aluminum foil at 4°C. Solution is stable for 1 month.

Prepare 8 mM (1.001 g/L) *N*-ethylmaleimide. Add *N*-ethylmaleimide to Berzelius beaker and cap with watch glass. Add nearly all dH₂O. Stir vigorously and gently heat to dissolve (usually requires 15 min owing to poor solubility). Transfer to volumetric flask and bring to volume.

3.) Prepare glucose stock. Add 0.5 g glucose and 1 g benzoic acid (to inhibit microbial activity) to ~300 mL dH₂O in beaker. Swirl vigorously and heat gently to dissolve all of most benzoic acid crystals. Transfer quantitatively to 500 mL volumetric flask and bring to volume. Store stock at 4°C. Crystals will form. Stock is stable for at least 1 yr.

4.) Prepare standards in 2-mL microcentrifuge tubes.

Standard (mg glucose/L)	Stock (μL)	dH ₂ O (μL)
0	0	2000
50	100	1900
100	200	1800
150	300	1700
200	400	1600
350	700	1300
500	1000	1000

Be careful not to pipette any benzoic acid crystals into standards. Store at -20°C. Standards are stable for at least 4 months.

5.) Add 238 μL of 8 mM *N*-ethylmaleimide to 100 μL supernatant. This presumes supernatant is principally Simplex buffer (see note below).

- 6.) Dilute samples from #5 with dH₂O so that they fall within standard curve.
- 7.) After pre-reading microplate at 505 nm (see *Measuring sample absorbance for microplate assays*), add 40 μL standard or sample to a microplate (flat-bottom, 96-well). Add 200 μL GOP reagent. Incubate at 35°C in air incubator for 45 min. If 35°C incubator is unavailable, incubate at 37°C.
- 8.) Measure absorbance with spectrophotometer at 505 nm and calculate glucose concentration. Correct for dilution with *N*-ethylmaleimide and dH₂O.

Notes

- (i) Culture supernatant can be frozen and re-thawed for this analysis because doing so does not affect concentration of glucose.
- (ii) *N*-ethylmaleimide and phenol are toxic and should be handled under the hood. 4-aminoantipyrene is light-sensitive. It should be stored in an amber jar or clear covered with aluminum foil. Jar should be sealed and contain desiccant. Glucose oxidase and peroxidase should be stored at -20°C in a sealed jar containing desiccant.
- (iii) *N*-ethylmaleimide should be added to give >2-fold molar excess over cysteine·HCl in samples. The volume indicated above is for Simplex buffer and needs to be adjusted for other buffer types. *N*-ethylmaleimide is included because cysteine·HCl interferes with GOP assay by lowering absorbance. *N*-ethylmaleimide reacts with cysteine·HCl (specifically, sulfhydryl groups therein) and prevents interference with the assay.
- (iv) After incubating samples, Karkalas (59) recommends cooling samples for 10 min. Cooling was found unnecessary, as absorbance changed nominally during cooling.
- (iv) Reagent and sample volumes have been decreased 25-fold from Karkalas to accommodate a 96-well plate format.

Lactic acid content of culture supernatant by enzymatic analysis

Adapted from instructions for R-Biopharm 11 112 821 035 and (61)

Following procedure is done with a D-lactic acid/L-lactic acid kit from R-Biopharm (product code 11 112 821 035).

- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve a concentration of D-lactic acid of ≤ 0.03 g/L (0.33 mM) and L-lactic acid of ≤ 0.03 g/L (0.33 mM). If supernatant has 0.06 g/L D-lactic acid and 0.06 g L-lactic acid, for example, dilute by combining 200 μ L supernatant and 200 μ L dH₂O.
- 4.) Make lactic acid standards (Table 1). Make fresh (long-term stability is unknown).
- 5.) Make solution 2. Add 6 mL dH₂O to bottle 2 and dissolve contents. Make aliquots (0.25 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C.
- 6.) Prepare solutions 3 and 4. Aliquot a small volume of solution from bottle 3 into 0.5-mL microcentrifuge tube. Keep on ice, and return bottle to 4°C. Repeat for bottle 4 to prepare solution 4.
- 7.) Make working reagent. For each reaction (analysis of sample or standard), combine 100 μ L solution 1, 20 μ L solution 2, and 2 μ L solution 3. Include one extra reaction to avoid running out. Make fresh daily.
- 8.) Pipette 122 μ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction. Pop any bubbles that form by using tip of disposable Pasteur pipetted heated over Bunsen burner.
- 9.) Pipette 100 μ L sample or standard into wells. Use one well per reaction. Cover plate with aluminum foil, and incubate at room temperature or 39°C.
- 10.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 5 min (until absorbances stabilize), then read one final time. This set of absorbances is called A₁.
- 11.) Add 2 μ L solution 4 to each well. Incubate at room temperature.
- 12.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 180 min (until absorbances stabilize), then read one final time. This set of absorbances is called A₂.

13.) Add 2 μ L solution 5 to each well. Incubate at room temperature.

14.) Read absorbance at 340 nm. Read at intervals for approximately 180 min (until absorbances stabilize), then read one final time. This set of absorbances is called A_3 .

14.) Calculate total, D-, and L-lactic acid concentrations. Create a standard curve for total lactic acid by determining the $A_3 - A_1$ for each standard and plotting these differences against total (D- and L-lactic acid) concentrations. Correct for dilution with dH_2O in #2.

Repeat for D-lactic acid, except use the difference $A_2 - A_1$ throughout.

Calculate L-lactic acid concentration by difference between total and D-lactic acid concentration for each sample.

Notes

(i) Bottle 1 contains glycylglycine buffer (pH = 10) and L-glutamic acid (440 mg) in 30 mL total solution. Bottle 2 containing NAD lyophilizate (210 mg). Bottle 3 contains 0.7 mL glutamate-pyruvate transaminase (1100 U). Bottle 4 contains 0.7 D-lactate dehydrogenase (3800 U). Bottle 5 contains 0.7 mL L-lactate dehydrogenase (3800 U). Bottle 6 contains D-lactate assay control solution (c. 0.2 g/L). Bottle 7 contains L-lactate assay control solution (c. 0.2 g/L).

(ii) As shipped, bottle contents are stable at 4°C. After adding 6 mL dH_2O , contents of bottle 2 are stable for only 2 months at -20°C.

(iii) Because bottle 2 is not stable indefinitely, it may need to be replaced before the rest of the kit. It can be replaced with 1 g NAD Grade II (catalog #10127981001) from Roche Applied Science. Prepare solution 2 as 0.035 g NAD/mL dH_2O .

(iv) Incubation times are 180 min, corresponding to time observed for the reaction to reach completion. The kit's suggestion of only 30 min was found to be too short.

(v) Absorbance is linear up to 0.02 g total lactic acid /L (e.g., 0.01 g D-lactic acid + 0.01 g L-lactic acid). It is reasonably linear to 0.06 g/L. D-lactic acid produces the same amount of absorbance as L-lactic acid.

(vii) The kit instructs calculating L-lactic acid concentrations from $A_3 - A_2$. However, L-lactic acid concentration is directly proportional to $A_3 - A_2$ only under the condition that the standard curve is linear or the ratio of D- and L-lactic acids is 1:1 (same as standards). Calculation concentration by difference, as instructed above, does not require these conditions.

(viii) Simplex buffer does not cause interference with the assay.

Table 1. Lactic acid standards*

Standard	D-lactic acid solution (Bottle 6)	L-lactic acid solution (Bottle 7)	dH ₂ O
A	0	0	300
B	7.5	7.5	285
C	15	15	270
D	30	30	240
E	45	45	210

*Final concentrations of D-lactic acid (g/L) are 0 for A, 0.005 for B, 0.01 for C, 0.02 for D, and 0.03 for E (if bottle 4 contains 0.2 g/L D-lactic acid; see label for exact concentration). Same comment for L-lactic acid.

Formic acid content of culture supernatant by enzymatic analysis

Adapted from instructions for Megazyme K-FORM

Following procedure is done with a formic acid kit from Megazyme (product code K-FORM)

- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve formic acid concentration of ≤ 0.2 g/L (4.3 mM). If supernatant has 0.4 g/L, for example, dilute by combining 50 μ L supernatant and 50 μ L dH₂O.
- 4.) Make formic acid stock and standards. To make stock (0.2 g/L formic acid), add 0.03022 g bottle 4 (sodium formate) to a 100-mL volumetric flask and bring to volume with dH₂O. Make standards by diluting stock (Table 1). Store at -20°C or use fresh.
- 5.) Make solution 1. Add 4 mL dH₂O to bottle 1 and dissolve contents. Two bottles are supplied, and use only one at a time. Make aliquots (0.25 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C. Solution is stable for at least two years at -20°C.
- 6.) Make solution 2. Add 5.2 mL dH₂O to bottle 2 and dissolve contents. Make aliquots (0.25 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C. Solution is stable for at least two years at -20°C.
- 7.) Prepare solution 3. Aliquot a small volume of solution from bottle 3 into 0.5-mL microcentrifuge tube. Keep on ice, and return bottle to 4°C. Solution is stable for at least two years at 4°C.
- 8.) Make working reagent. For each reaction (analysis of sample or standard), combine 200 μ L dH₂O, 20 μ L solution 1, and 20 μ L solution 2. Include one extra reaction to avoid running out. Prepare immediately before use.
- 9.) Pipette 240 μ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 10.) Pipette 10 μ L of sample or standard into wells. Cover with aluminum foil, and incubate at room temperature.
- 11.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 3 min (until absorbances stabilize), then read one final time. This set of absorbances is called A₁.

This set of absorbances is called A₁.

12.) Add 5 μL solution 3 to each well. Mix by stirring with pipette tip until white precipitate is in suspension. Cover with aluminum foil, and incubate at room temperature.

13.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 30 min (until absorbances stabilize), then read one final time. This set of absorbances is called A_2 .

14.) Calculate formic acid concentrations. Create a standard curve for formic acid by determining the $A_2 - A_1$ for each standard and plotting these differences against formic acid concentrations. Calculate concentration for samples from this standard curve and $A_2 - A_1$ for each sample. Correct for dilution with dH_2O in #2.

Table 1. Formic acid standards

Standard	Formic acid stock μL	dH_2O μL
A	0	100
B	12.5	87.5
C	25	75
D	50	50
E	100	0

*Final concentration (g/L) is 0 for A, 0.025 for B, 0.05 for C, 0.1 for D, and 0.2 for E.

Succinic acid content of culture supernatant by enzymatic analysis

Adapted from instructions for Megazyme K-SUCC

Following procedure is done with a succinic acid kit from Megazyme (product code K-SUCC).

- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve succinic acid concentration of ≤ 0.4 g/L (3.4 mM). If supernatant has 0.8 g/L, for example, dilute by combining 50 μ L supernatant and 50 μ L dH₂O.
- 4.) Make succinic acid stock and standards. To make stock (0.4 g/L succinic acid), add 0.04 g bottle 6 (succinic acid) to a 100-mL volumetric flask and bring to volume with dH₂O. Make standards by diluting stock (Table 1). Store at -20°C or use fresh. Solution is stable at -20°C for at least 2 years.
- 5.) Make solution 2. Add 2.4 mL dH₂O to bottle 2 and dissolve contents. Make aliquots (0.25 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C. Two bottles are supplied, and use only one at a time. Solution is stable for at least 2 years at -20°C and at least 4 weeks at 4°C.
- 6.) Make solution 3. Add 2.4 mL dH₂O to bottle 3 and dissolve contents. Two bottles are supplied, and use only one at a time. Make aliquots (0.25 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C. Solution is stable for at least 4 weeks at -20°C.
- 7.) Prepare solutions 4 and 5. Aliquot a small volume of solution from bottle 4 into 0.5-mL microcentrifuge tube. Keep on ice, and return bottle to 4°C. Repeat for bottle 5 to prepare solution 5. Solutions are stable for at least 4 weeks at 4°C.
- 8.) Make working reagent. For each reaction (analysis of sample or standard), combine 200 μ L dH₂O, 20 μ L solution 1, 20 μ L solution 2, 20 μ L solution 3, and 2 μ L solution 4. Include one extra reaction to avoid running out. Prepare immediately before use.
- 9.) Pipette 262 μ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 10.) Pipette 10 μ L of sample or standard into wells. Cover plate with aluminum foil, and incubate at room temperature.
- 11.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 5 min (until absorbances stabilize), then read one final time. This set of absorbances is called A₁.

12.) Add 2 μL solution 5 to each well. Mix by stirring with pipette tip until precipitate clears. Incubate at room temperature.

13.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 15 min (until measurements stabilize), then read one final time. This set of absorbances is called A_2 .

14.) Calculate succinic acid concentrations. Create a standard curve for succinic acid by determining the $A_2 - A_1$ for each standard and plotting these differences against succinic acid concentrations. Calculate concentration for samples from this standard curve and $A_2 - A_1$ for each sample. Correct for dilution with dH_2O in #2.

Table 1. Succinic acid standards.

Standard	Succinic acid stock μL	dH_2O μL
A	0	100
B	12.5	87.5
C	25	75
D	50	50
E	100	0

*Final concentration (g/L) is 0 for A, 0.05 for B, 0.1 for C, 0.2 for D, and 0.4 for E.

Ethanol content of culture supernatant by enzymatic analysis

Adapted from instructions for Megazyme K-ETOH

Following procedure is done with an ethanol kit from Megazyme (product code K-ETOH).

- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve ethanol acid concentration of ≤ 0.12 g/L (2.6 mM). If supernatant has 0.24 g/L, for example, dilute by combining 50 μ L supernatant and 50 μ L dH₂O.
- 4.) Make ethanol standards (Table 1). Store at 4°C. Solution is stable at 4°C for up to 2 days.
- 5.) Make solution 2. Add 12.4 mL dH₂O to bottle 2 and dissolve contents. Make aliquots (0.5 mL volumes in 0.5 mL centrifuge tubes) and store at -20°C.
- 6.) Prepare solutions 3 and 4. Aliquot a small volume of solution from bottle 3 into 2-mL microcentrifuge tube. Keep on ice, and return bottle to -20°C. Repeat for bottle 4, but store bottle at 4°C.
- 7.) Make working reagent. For each reaction (analysis of sample or standard), combine 200 μ L dH₂O, 20 μ L solution 1, 20 μ L suspension 2, and 5 μ L solution 3. Include one extra reaction to avoid running out. Make fresh daily.
- 8.) Pipette 245 μ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 9.) Pipette 10 μ L of sample or standard into wells. Cover plate with aluminum foil, and incubate at room temperature.
- 10.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 10 min (until measurements stabilize), then read one final time. This set of absorbances is called A₁.
- 11.) Add 2 μ L solution 4 to each well. Mix by stirring with pipette tip until white precipitate clears. Incubate at room temperature.
- 12.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 50 min (until measurements stabilize), then read one final time. This set of absorbances is called A₂.

13.) Calculate ethanol concentrations. Create a standard curve for ethanol by determining the $A_2 - A_1$ for each standard and plotting these differences against ethanol concentrations. Calculate concentration for samples from this standard curve and $A_2 - A_1$ for each sample. Correct for dilution with dH_2O in #2.

Table 1. Ethanol standards.

Standard	Ethanol stock μL	dH_2O μL
A	0	1000
B	3	997
C	6	994
D	12	988
E	24	976

*Final concentration (g/L) is 0 for A, 0.015 for B, 0.03 for C, 0.06 for D, and 0.12 for E.

Concentration and taxonomic distribution of protozoa

Adapted from (62, 63)

- 1.) Prepare 50% formalin by diluting one volume of 37% formaldehyde with an equal volume of dH₂O. Prepare Brilliant green dye by diluting 2 g and 2 mL of glacial acetic acid to 100 mL with dH₂O. Prepare 30% v/v glycerol by diluting 300 mL glycerol to 1000 mL with dH₂O.
- 2.) Fix sample in formalin. Pipette 10 mL of sample (e.g., rumen fluid or culture) into a 23-mL culture tube. To store at room temperature, add 10 mL, 50% formalin to give 25% final concentration of formalin.
- 3.) Stain sample with Brilliant green dye. Transfer a 990 µL aliquot of fixed sample to a separate 23 mL culture tube. Add 10 µL Brilliant green dye to this aliquot. Mix gently and allow to stand for dye to color cells. Dehority (62) recommends allowing to stand for at least 4 h, but we have found only a few minutes are needed if intense color is not necessary.
- 4.) Dilute stained aliquot with 30% v/v glycerol. Prepare a 1→40 dilution when expecting 10⁵ cells/mL.
- 5.) Load diluted, stained sample into a Sedgewick-Rafter counting chamber. Pipette 1.01 mL into the chamber and spread the sample evenly across chamber using pipette tip. Mount the coverslip by sliding it flat across the chamber opening. Because chamber volume is 1 mL, some sample (0.01 mL) will be expelled from the opening while sliding the coverslip into place; overloading the chamber ensures it will stay full while sliding the coverslip into place.
- 6.) Count protozoa at 100x magnification and using camera. Count only those cells for which >50% of the cell falls within the field of view; exclude those for which the majority (>50%) of the cell falls outside the field of view. Make 2 counts by (i) counting 50 fields then (ii) rotating the chamber 180° then counting 50 more fields.
- 7.) If needed, differentiate protozoa into taxonomic groups *Isotricha*, *Dasytricha*, *Entodinium*, *Epidinium*, *Ophryoscolex*, and Diplodiinae. Use Fig. 1 and (62, 63) as a guide.
- 8.) Calculate #cells/mL for the formalized sample as

$$N \times A_t / (G \times A_g) / V \times d$$

where N is the number of cells counted, A_t is the area of the chamber (1000 mm²), G is the number of fields (100), A_g is the area of each field (0.3128 mm²), V is the volume of the sample (1 mL), and d is dilution (e.g., 40). Note count is per milliliter of formalized sample and stained sample, not original sample.

Notes

- (i) Because glycerol is viscous and mixes with difficulty, dilute glycerol in a 1000-mL graduated cylinder, and mix the glycerol by transferring it back and forth between the cylinder and the glass bottle in which it is to be stored. Store at 4°C.

(ii) To store at samples at 4°C for short periods (a few weeks), the sample need be fixed in only 1% final concentration of formalin.

(iii) The dilution needs to be adjusted to give approximately 200 to 300 cells per 100 fields. The 200 to 300 cell range ensures enough cells are present to give reasonable accuracy but not so many are present that counting becomes tedious.

(iv) To obtain a representative sample, culture tubes of sample need to be mixed well (a) by flipping the tube end-over-end, (b) drawing sample in and out of the pipette repeatedly, and (c) sampling quickly. (iii) To avoid shearing large protozoa, the opening of pipette tips should be enlarged by cutting off 1-2 mm of their pointed end.

(v) Glycerol is used to dilute samples because its viscosity prevents settling during mixing. The slow settling also means that one must wait 1 to 2 minutes to count protozoa after loading the counting chamber, to allow protozoa to settle at the bottom.

(vi) To help evenly count 50 fields, mark with pencil on the microscope stage 10 evenly-spaced lines in the horizontal direction and 5 lines in the vertical direction. These lines will form an imaginary grid with 50 points. Count 50 fields by aligning a corner of the slide at each of 50 points of the imaginary grid.

Fig. 1. Decision chart for classifying rumen protozoa

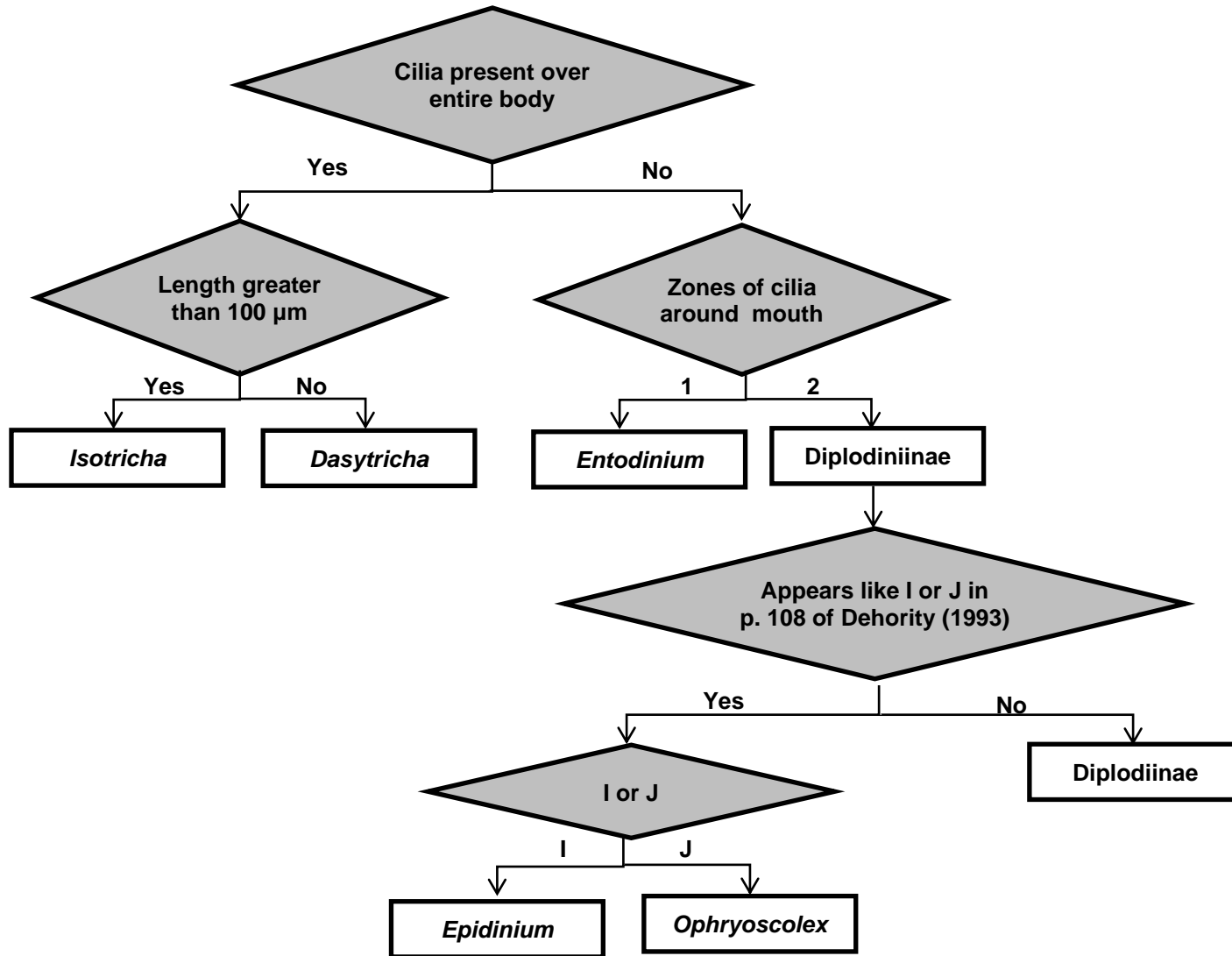


Fig. 1. Rules for counting protozoa inside counting grid. To avoid a bias that will lead to overcounting, protozoa touching the 2 sides marked with red X's should be excluded from counting. Protozoa touching the 2 sides marked with green O's should be included, however. Actually, the arrangement of the 2 X's and 2 O's in this the figure is arbitrary, and any 2 sides can be chosen for exclusion and the 2 sides chosen for inclusion.

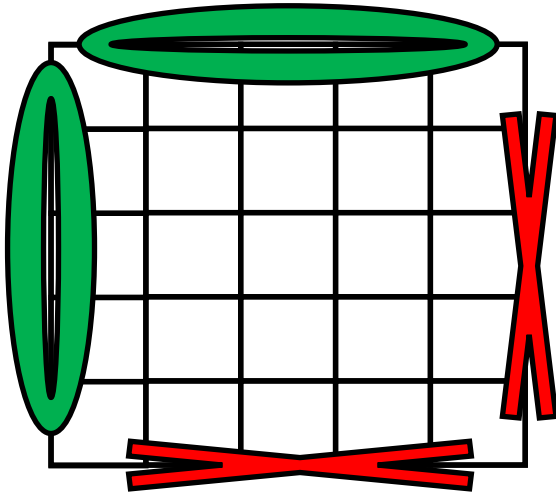
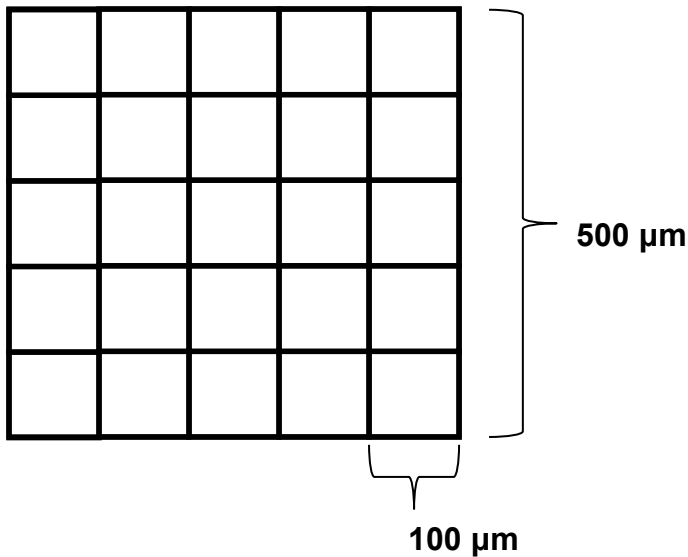


Fig. 2. Dimensions of counting grid at 150x (10x objective, 15x ocular) magnification. For 300x (not shown in figure), the corresponding dimensions are 250 μm and 50 μm ; for 600x, they are 125 μm and 25 μm ; and for 1500x, they are 50 μm and 10 μm .



Concentration of bacteria

Modified from (64, 65) and Life Technologies DAPI counterstaining protocol

Preparing DAPI stock and other reagents

- 1.) Prepare DAPI stock. Add 2 mL dH₂O to 10 mg DAPI dilactate (Life Technologies D3571) and dissolve. Aliquot 40 µL DAPI stock into 2-mL microcentrifuge tubes and store at -20°C protected from light.
- 2.) Prepare DAPI stain. Add 10 µL glutaraldehyde (50% w/v) and 1950 µL dH₂O to 40 µL DAPI stock in tube. Store at 4°C and protected from light. Solution is stable for at least 6 months.
- 3.) Prepare diluent and rinse water. Add 1 mL glutaraldehyde (50% w/v) to 20 mL dH₂O (sterilized through 0.2-µm filter and autoclaved). Make fresh daily.

Filtering and counting cells

- 1.) If cells are clumped (e.g., in centrifuged samples), incubate sample in Triton-X (0.1% final concentration) overnight to help unclump cells. If cells are not clumped (e.g., in uncentrifuged samples), proceed to #2.
- 2.) Dilute sample with diluent water (see composition below). Prepare 10⁴ dilution when expecting 10⁹ cells/mL.
- 3.) Connect filter holder (25-mm diameter; Kimble 953705-0000) to vacuum flask. Connect flask to vacuum pump. Place filter (black Nuclepore, 0.2 µm pore, 25 mm diameter; Whatman 110656) shiny side up on filter holder and pre-wet with 1 drop of diluent water.
- 4.) Perform staining in the filter holder. Bring all solutions and sample to room temperature. Add diluent water (1.48 mL), diluted sample (0.5 mL), and DAPI stain (20 µL) directly to the filter and in that exact order. Incubate for 7 min, occasionally mixing contents with pipette.
- 5.) Vacuum with slight pressure (<30 mmHg) to filter sample. Just before all sample passes the filter, apply rinse water (two, 1-mL aliquots). If filtration takes more than 1 to 2 min, dilute the sample.
- 6.) Place 1 drop of immersion oil to acetone-cleaned glass slide. Apply filter sample-side up. Place another drop of immersion oil on top of filter, and apply coverglass.
- 7.) Examine at 1000x with Zeiss AxioScope A.1 epifluorescent microscope and filter for DAPI. Count at least 400 cells within a minimum of 20 fields of view.
- 8.) Calculate #cells/mL for the formalized sample as

$$N \times A_t / (G \times A_g) / V \times d$$

where N is the number of cells counted, A_t is the effective area of the filter (25 mm^2), G is the number of fields, A_g is the area of each field (0.00346 mm^2), V is the volume of the sample (0.5 mL), and d is dilution (e.g., 10^5). Note count is per milliliter of formalized sample, not original sample.

Notes

(i) Do all procedures involving DAPI in a darkened room to mitigate photobleaching. Turn off main lights and leave on only emergency lights. Wrap filtration apparatus and tubes with aluminum foil.

(ii) Effective area of the filter (A_t) is that which is exposed to sample during filtration. The total area of the filter is 491 mm^2 , but not all filter is exposed because filter holder shields clamps down on edges of filter and shields this area from sample. The effective area reported here is that given by the manufacturer.

(iii) Area of the field of view is calculated using a stage micrometer. The diameter of the field of view at $1000\times$ magnification is $210 \mu\text{m}$.

Preparing wet mounts of bacteria for imaging

Adapted from (66)

- 1.) Prepare 2% w/v noble agar by adding 1 g noble agar (BD 214220) to 50 mL ddH₂O in a 250 mL Erlenmeyer flask. Cover with aluminum foil and autoclave at 121°C for 20 min.
- 2.) Cool agar to 45°C. While agar is still molten, transfer flask to circulating water bath set to 45°C. Place a rubber-coated lead ring on the flask neck to prevent the flask from tipping over.
- 3.) Prepare a dust-free environment for coating slides with agar. Under flame, clean bench with 70% ethanol and lay down clean aluminum foil. Do all subsequent steps on foil and under flame.
- 4.) Coat slides with agar. Transfer c. 1 mL of agar to slide with disposable Pasteur pipette. Spread agar across entire slide evenly. Allow agar to solidify into a pad and then store slides in slide box.
- 5.) Prepare the wet mount. Add 1 to 2 drops of culture to agar, apply coverslip, and image. Agar will absorb liquid and immobilize cells, giving a sharp image with high contrast.

Notes

- (i) Noble agar gives low and uniform background when imaging, whereas normal or washed agar gives a high background that resembles crumpled paper.
- (ii) Less agar (0.25 mL) can be applied to the slide if used immediately; drying overnight will cause agar pad to shrink and occupy only small region of slide.

Measuring sample absorbance for microplate assays

- 1.) Turn on plate spectrophotometer (Molecular Devices M3) and allow for instrument to connect (requires about 2 minutes).
- 2.) Open SOFTmax Pro on computer desktop.
- 3.) Set wavelength by clicking “Setup” button (in “Plate” tab appearing mid-screen) then typing the wavelength in the appropriate box. Refer to individual procedures for correct wavelength.
- 4.) Pre-read plate. Insert empty microplate (flat-bottom, 96-well) in drawer of spectrophotometer. Click “Read” button (on toolbar beneath menu bar), then click “Pre-read”.
- 5.) Load microplate with samples and standards. Refer to individual procedures for details.
- 6.) Read plate. With settings established in #3, click “Read” on toolbar, then click “Read”.
- 7.) Set up a standard curve and calculate concentration of samples.
 - (i) Label standards and samples in SoftMax by clicking “Template” button (in “Plate” tab). Highlight one or more squares (representing wells in the microplate) by clicking on it. Identify it as a standard or sample using the “Group” pull-down menu. Label samples and standards using “Sample” box. Insert the concentration of standards using the “Concentration” box.
 - (ii) Expand the “Graph” tab by clicking on it. Under “Fit” pull-down menu, select quadratic fit to fit standards. Remove outliers with “Template” button, clicking the square containing the outlier and clicking the “Clear” button.
 - (iii) Check concentration of samples by expanding the “Samples” tab. Remove outliers.
 - (iv) Export file as *.txt document using “File” pull-down menu. Record calculated sample concentrations from this *.txt document. Multiply these concentrations by any dilutions made during sample preparation.

Notes

- (i) Wells of an empty plate have similar but non-identical absorbance readings. Pre-reading the plate corrects for this non-uniformity.

SECTION III: GAS CHROMATOGRAPHY

Gas chromatography is used for measurement of many analytes, and it is especially useful for analysis of fermentation products from microbes. This procedure explains how to use gas chromatography for measurement of volatile fatty acids (VFA) and gases (H_2 , CH_4).

Operation of gas chromatograph

Risks of operation

Do not operate the gas chromatograph (GC) until understanding the risks. The GC will be damaged if

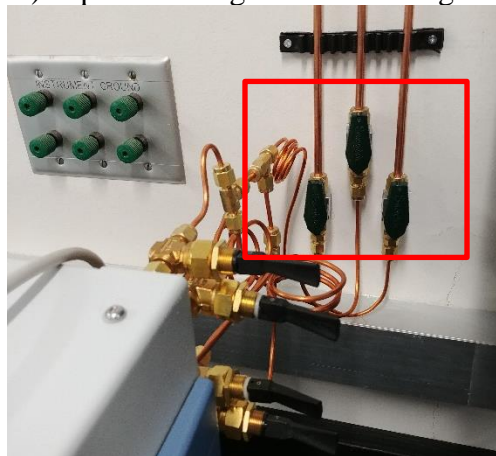
- Any component is heated above 240°C. This is the maximum temperature of the WAX column.
- The carrier gas is off, and the oven or detector is heated above 100°C. The columns are destroyed by a combination of oxygen and heat. It is safe to heat only after the carrier gas is on and has been flushed of oxygen.
- The TCD filament is turned on (or otherwise heated above 100°C) while the reference gas is turned off.

Turning on and placing into standby mode

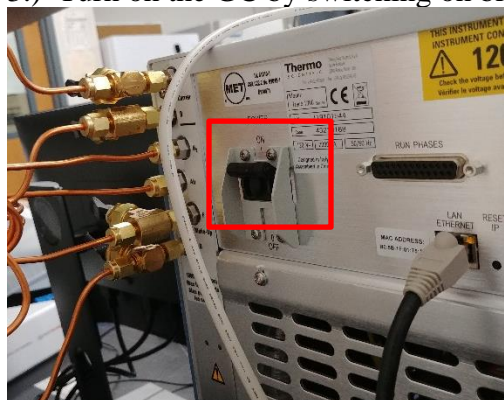
1.) Turn on compressed gases (nitrogen, air, hydrogen). Turn on cylinder valve, then turn on diaphragm at regulator to 100 psi.



2.) Open all Swagelok valves on gas lines leading to GC.

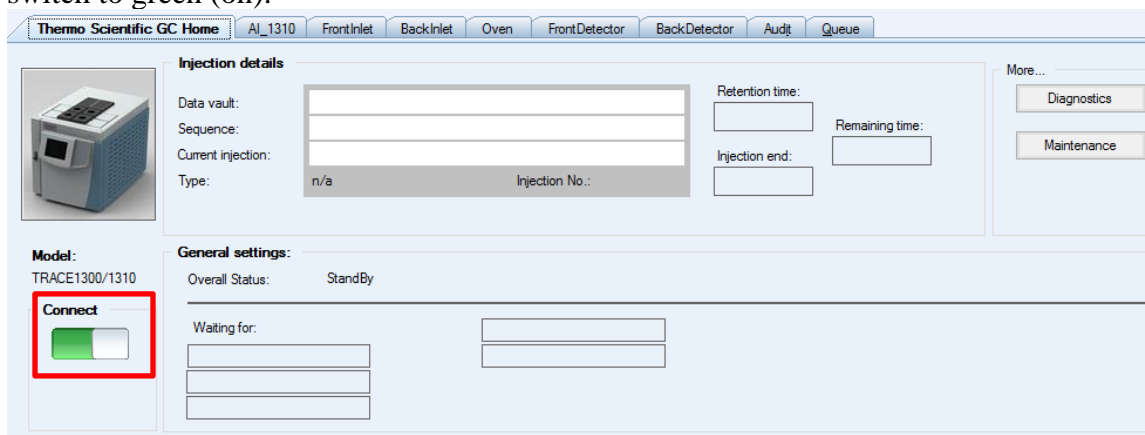


3.) Turn on the GC by switching on breaker on back of instrument.

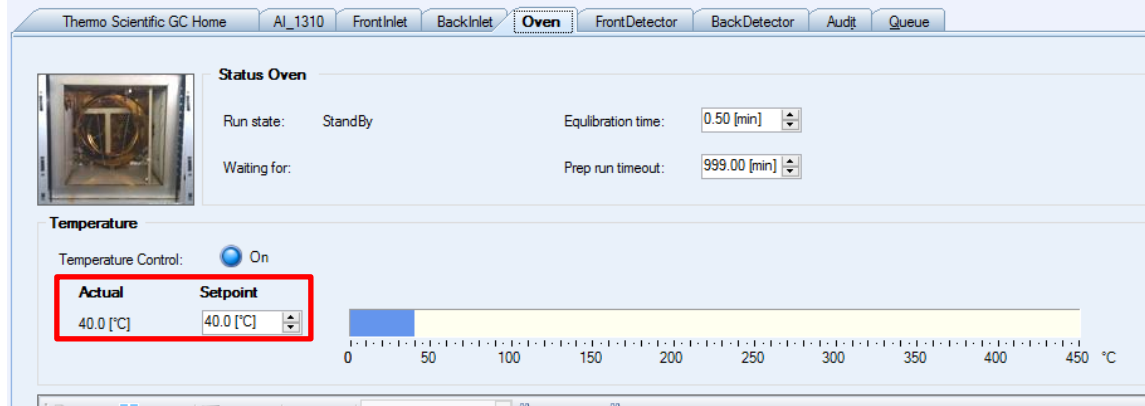


4.) Start Chromeleon 7 software on GC computer.

5.) Connect to Chromeleon to instrument. In Thermo Scientific GC Home tab, toggle Connect switch to green (on).



6.) Verify that oven is at safe temperature. In Oven tab, check that Actual and Setpoint temperatures are well below 100°C (e.g., 40°C). If above, immediately adjust the set point.



7.) Turn on carrier gas and temperature for front inlet. Go to FrontInlet tab, and turn Column flow to On. Repeat for Purge flow, Split flow, and Temperature. Choose setpoints shown (minimum needed to purge system and prevent condensation of water).

Thermo Scientific GC Home | AI_1310 | **FrontInlet** | BackInlet | Oven | FrontDetector | BackDetector | Audit | Queue

Status SSL

Inlet type: SSL

Gas saver: Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	52.18 [kPa]	
Column flow: <input checked="" type="radio"/> On	1.499 [ml/min]	1.500 [ml/min]
Purge flow: <input checked="" type="radio"/> On	2.000 [ml/min]	2.000 [ml/min]
Split flow: <input checked="" type="radio"/> On	5.0 [ml/min]	5.0 [ml/min]
Temperature: <input checked="" type="radio"/> On	110 [°C]	110 [°C]

Column Functions

Column Properties

Leak Check

Column Evaluation

8.) Turn on carrier gas and temperature for back inlet. Go to BackInlet tab and follow step 7 above.

Thermo Scientific GC Home | AI_1310 | FrontInlet | **BackInlet** | Oven | FrontDetector | BackDetector | Audit | Queue

Status SSL

Inlet type: SSL

Gas saver: Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	11.03 [kPa]	
Column flow: <input checked="" type="radio"/> On	1.498 [ml/min]	1.500 [ml/min]
Purge flow: <input checked="" type="radio"/> On	2.000 [ml/min]	2.000 [ml/min]
Split flow: <input checked="" type="radio"/> On	5.0 [ml/min]	5.0 [ml/min]
Temperature: <input checked="" type="radio"/> On	110 [°C]	110 [°C]

Column Functions

Column Properties

Leak Check

Column Evaluation

9.) Turn on reference gas for back detector (TCD).

Thermo Scientific GC Home | AI_1310 | FrontInlet | BackInlet | Oven | FrontDetector | **BackDetector** | Audit | Queue

Status TCD

Detector type: TCD

Temperature: Off 71 [°C]

Polarity: **Negative**

Filament power: **Off**

AutoZero

	Actual	Setpoint
Reference gas: <input checked="" type="radio"/> On	1.5 [ml/min]	1.5 [ml/min]

Signal

-9.259200 [mV]

10.) Continue to monitor the compressed gases, and adjust back to 100 psi as needed.

11.) Allow carrier and reference gas to circulate for 30 min to 1 hour. This will allow columns to be flushed of oxygen. If the oven and detector are heated above 100°C before completing this step, the columns will be destroyed in minutes.

Turning on detectors

There are two detectors (front and back). Only the detector that is needed should be turned on. The front detector is needed for VFA analysis, and the back detector is needed for gas analysis.

To turn on front detector, go to FrontDetector tab. Turn on Temperature, Flame, Hydrogen, Air, and Makeup gas. The flame will turn when the temperature reaches 150°C and hydrogen and air reach set flow rates. The flame will produce an audible pop.

	Actual	Setpoint
Temperature:	39 [°C]	240 [°C]
Flame:	On	
Hydrogen:	0.0 [ml/min]	35.0 [ml/min]
Air:	0.0 [ml/min]	350.0 [ml/min]
Makeup gas:	0.0 [ml/min]	40.0 [ml/min]

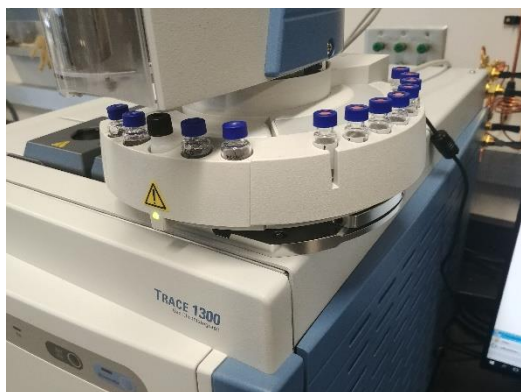
Allow signal from detector to stabilize. Signal will decrease quickly (from 1200 to 130 pA) in 3 h, when decrease more slowly (to 50 pA) overnight.

To turn on the detector, go to BackDetector tab. Verify that Reference gas is On, then turn Temperature and Filament power to On. The filament will be damaged if it is on while the reference gas is off. Avoid turning on detector unless needed; switching on filament on unnecessarily shortens life.

	Actual	Setpoint
Temperature:	101 [°C]	150 [°C]
Polarity:	Negative	
Filament power:	On	
Reference gas:	1.5 [ml/min]	1.5 [ml/min]

Setting up autosampler

- 1.) Fill wash vials. See individual procedures for specific wash solvent to be used.
- 2.) Place wash and sample vials in autosampler.



Running samples

Run samples according to individual experiments (e.g., *Measuring VFA content of culture supernatant*).

Placing back into standby mode

After samples are run, place the instrument in standby mode.

1.) Turn off the front detector (FID). In FrontDetector tab, turn off Temperature, Flame, Hydrogen, Air, and Makeup gas.

	Actual	Setpoint
Temperature:	Off	182 [°C]
Flame:	Off	
Hydrogen:	Off	0.0 [ml/min]
Air:	Off	0.0 [ml/min]
Makeup gas:	Off	0.0 [ml/min]

3.) Turn off back detector. In BackDetector tab, turn Temperature and Filament to Off. Do not turn off reference gas yet; filament will be damaged.

	Actual	Setpoint
Temperature:	Off	71 [°C]

Polarity: **Negative**
 Filament power: **Off**

Reference gas: **On** 1.5 [ml/min] 1.5 [ml/min]

4.) Adjust carrier gas and carrier gas and temperature for front inlet. Go to FrontInlet tab, and change Column flow to value below (1.5 mL/min). Repeat for Purge flow (2 mL/min), Split flow (5 mL/min), and Temperature (110°C). Turn off Gas saver. Turning down carrier gas flow to these values will conserve gas.

Thermo Scientific GC Home | AI_1310 | **FrontInlet** | BackInlet | Oven | FrontDetector | BackDetector | Audit | Queue

Status SSL

Inlet type: SSL

Gas saver: Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	52.18 [kPa]	
Column flow: <input checked="" type="radio"/> On	1.499 [ml/min]	1.500 [ml/min]
Purge flow: <input checked="" type="radio"/> On	2.000 [ml/min]	2.000 [ml/min]
Split flow: <input checked="" type="radio"/> On	5.0 [ml/min]	5.0 [ml/min]
Temperature: <input checked="" type="radio"/> On	110 [°C]	110 [°C]

Column Functions

Column Properties

Leak Check

Column Evaluation

5.) Adjust carrier gas and carrier gas and temperature for back inlet. Go to BackInlet tab and follow step #4 above.

Thermo Scientific GC Home | AI_1310 | FrontInlet | **BackInlet** | Oven | FrontDetector | BackDetector | Audit | Queue

Status SSL

Inlet type: SSL

Gas saver: Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	11.04 [kPa]	
Column flow: <input checked="" type="radio"/> On	1.499 [ml/min]	1.500 [ml/min]
Purge flow: <input checked="" type="radio"/> On	2.000 [ml/min]	2.000 [ml/min]
Split flow: <input checked="" type="radio"/> On	5.0 [ml/min]	5.0 [ml/min]
Temperature: <input checked="" type="radio"/> On	110 [°C]	110 [°C]

6.) Place oven at safe temperature. In Oven tab, put setpoint well below 100°C (e.g., 40°C).

Thermo Scientific GC Home | AI_1310 | FrontInlet | BackInlet | **Oven** | FrontDetector | BackDetector | Audit | Queue

Status Oven

Run state: Ready

Equilibration time: 0.50 [min]

Waiting for:

Prep run timeout: 999.00 [min]

Temperature

Temperature Control: On

Actual	Setpoint
40.0 [°C]	40.0 [°C]

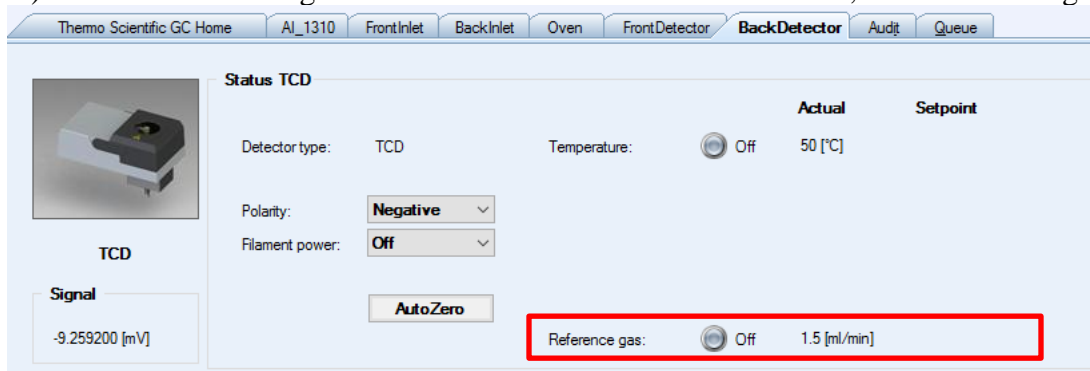
0 50 100 150 200 250 300 350 400 450 °C

7.) Allow temperature of oven and detectors to cool down to well below 100°C (e.g., 50°C).

8.) Keep in standby mode (if using within next day or so) or proceed with turning off.

Turning off

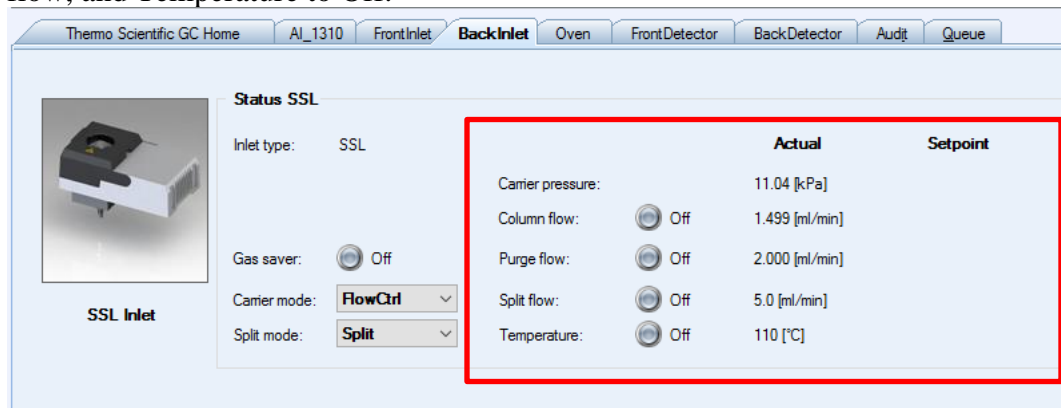
- 1.) Follow all steps for *Placing back into standby mode*. Verify detectors and oven are well below 100°C before proceeding.
- 2.) Turn off reference gas for back detector. In BackDetector tab, turn Reference gas to Off.



The screenshot shows the 'BackDetector' tab in the software interface. The 'Reference gas' parameter is highlighted with a red box and is set to 'Off' with a flow rate of 1.5 [ml/min].

Parameter	Actual	Setpoint
Reference gas	Off	1.5 [ml/min]

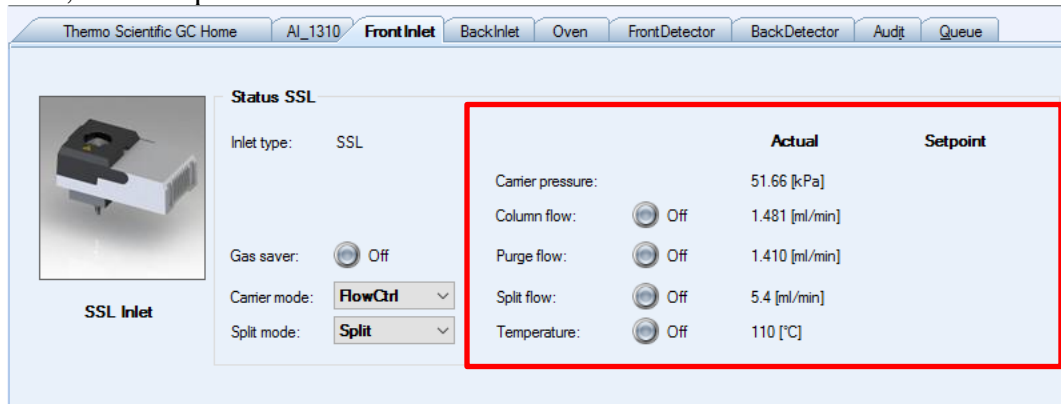
- 3.) Turn off carrier gas for back inlet. In BackInlet tab, turn Column flow, Purge flow, Split flow, and Temperature to Off.



The screenshot shows the 'BackInlet' tab in the software interface. The 'Column flow', 'Purge flow', 'Split flow', and 'Temperature' parameters are highlighted with a red box and are set to 'Off'.

Parameter	Actual	Setpoint
Carrier pressure	11.04 [kPa]	
Column flow	Off	1.499 [ml/min]
Purge flow	Off	2.000 [ml/min]
Split flow	Off	5.0 [ml/min]
Temperature	Off	110 [°C]

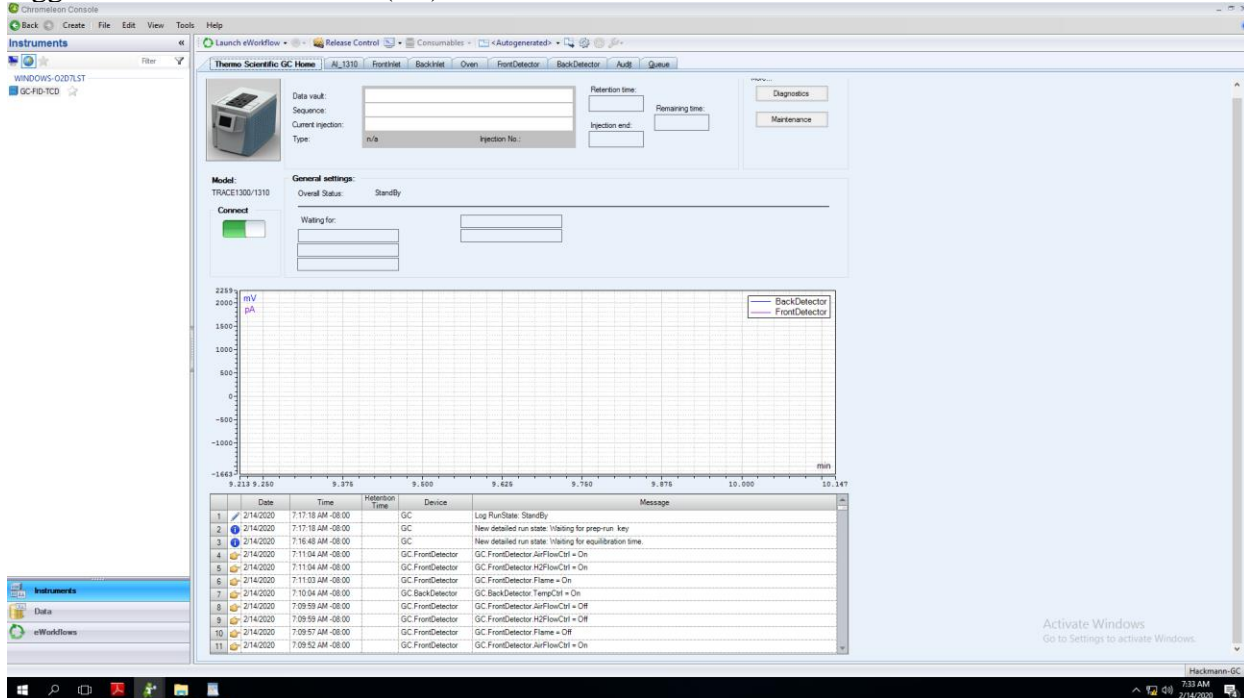
- 4.) Turn off the carrier gas for front inlet. In FrontInlet tab, turn Column flow, Purge flow, Split flow, and Temperature to Off.



The screenshot shows the 'FrontInlet' tab in the software interface. The 'Column flow', 'Purge flow', 'Split flow', and 'Temperature' parameters are highlighted with a red box and are set to 'Off'.

Parameter	Actual	Setpoint
Carrier pressure	51.66 [kPa]	
Column flow	Off	1.481 [ml/min]
Purge flow	Off	1.410 [ml/min]
Split flow	Off	5.4 [ml/min]
Temperature	Off	110 [°C]

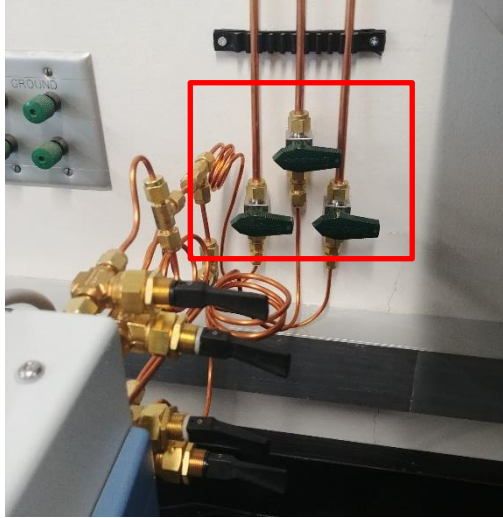
5.) Optional: disconnect Chromleon from instrument. In Thermo Scientific GC Home tab, toggle Connect switch to red (off).



6.) Turn off the GC by switching on breaker on back of instrument.



7.) Close Swagelok valves on gas lines leading to GC.



8.) Turn off compressed gases (nitrogen, air, hydrogen). Turn off diagram, then turn off cylinder valve.

Maintenance of gas chromatograph

Replacing injector septum

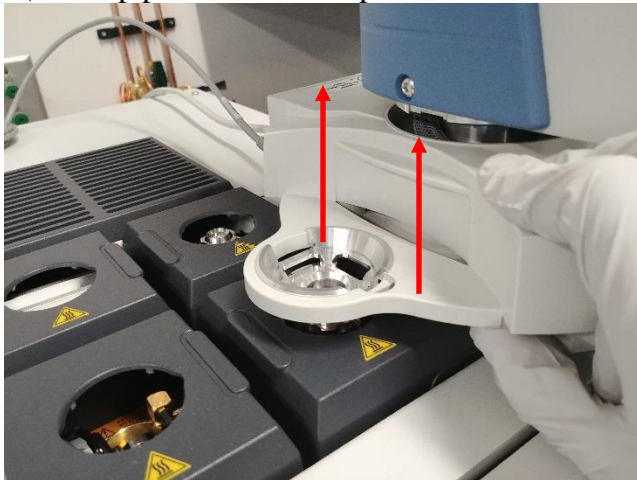
Injector septum will get dirty and should be replaced after a certain number of injections..

1.) Follow steps 1 to 4 for **Turning off**. Make sure that all components cool down to $\leq 60^{\circ}\text{C}$ before proceeding.

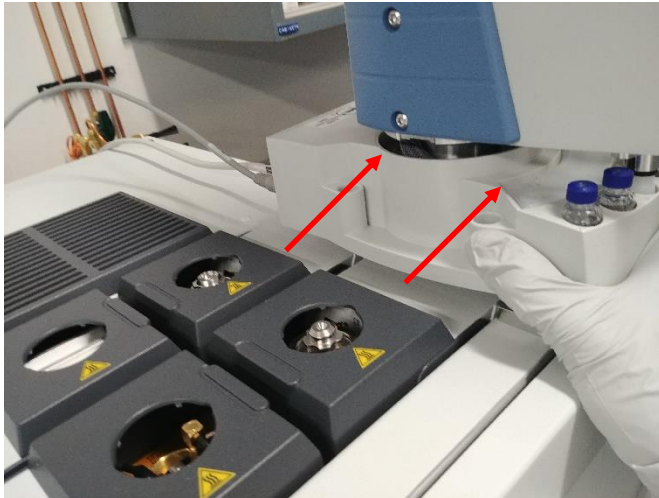
2.) Unplug autosampler.



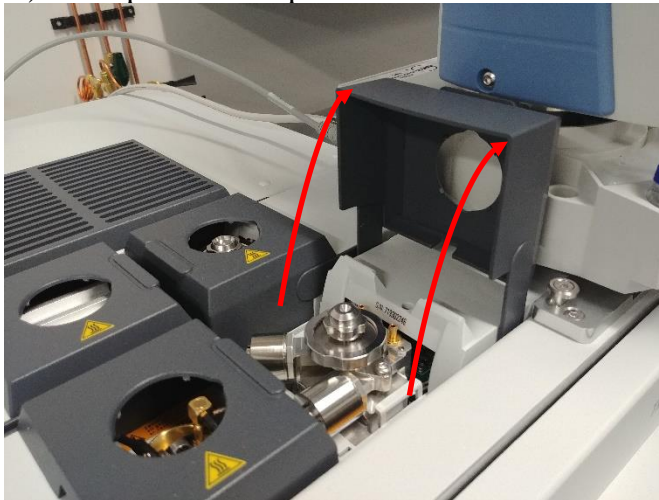
3.) Lift up plate on autosampler.



4.) Push autosampler away from injector.



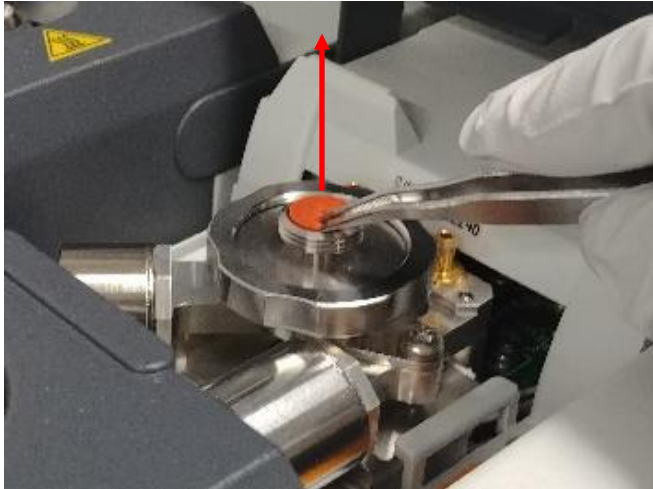
5.) Lift up module flap cover.



6.) Unscrew septum cap and lift up.



8.) Remove old septum.



9.) Put in new septum.

10.) Do steps above in reverse to restore system to original state. Make sure to tighten the septum cap firmly. Otherwise, gas will leak from it (detectable by an audible hiss).

Replacing injector liner

Injector liner will get dirty and should be replaced after a certain number of injections.

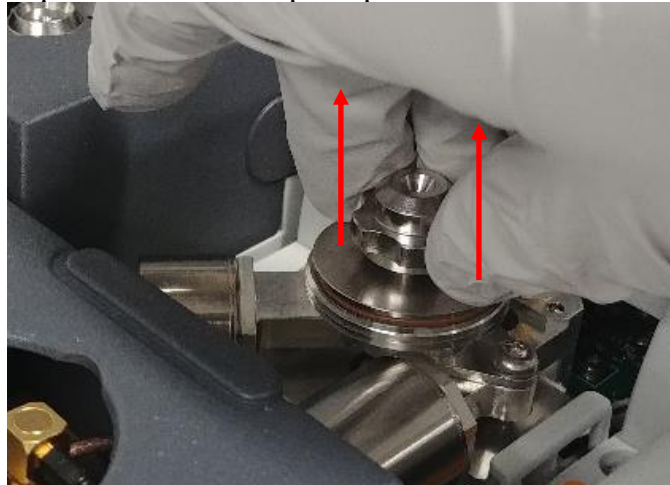
1.) Follow steps 1 to 3 for **Turning off**. Make sure that all components cool down to $\leq 60^{\circ}\text{C}$ before proceeding.

2.) Follow steps 2 through 5 **Replacing injector septum**.

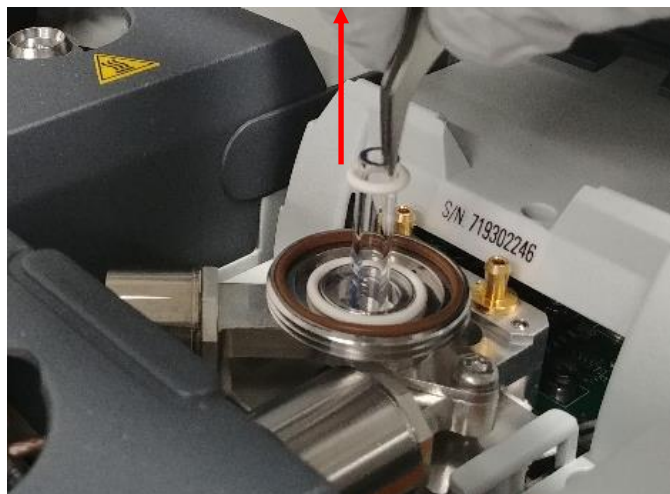
3.) Unscrew and lift up ring nut.



4.) Pull up the septum holder, using septum cap as a grip. If the septum holder is stuck, it may require extra effort to pull up.



5.) Pull up injector liner with forceps. The liner has a white o-ring near the top. Make sure that it is removed along with the liner. If it is not removed (remains stuck in the injector), pull it up separately.



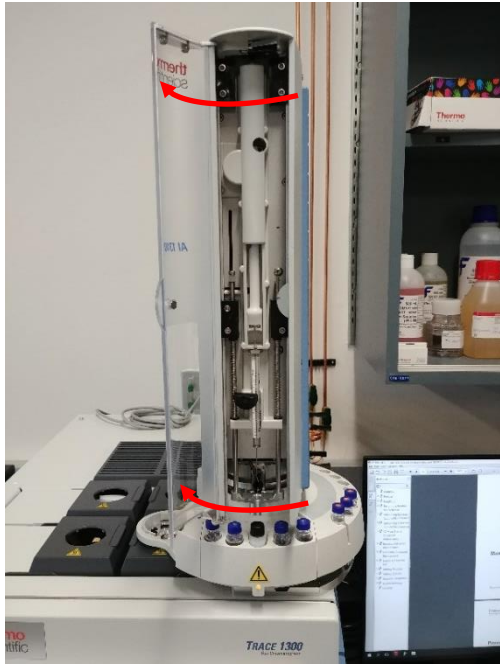
6.) Replace injector liner. Make sure to transfer white o-ring from old liner to new one.

7.) Do steps above in reverse to restore system to original state. Make sure to tighten the ring nut firmly. Otherwise, gas will leak from it (detectable by an audible hiss).

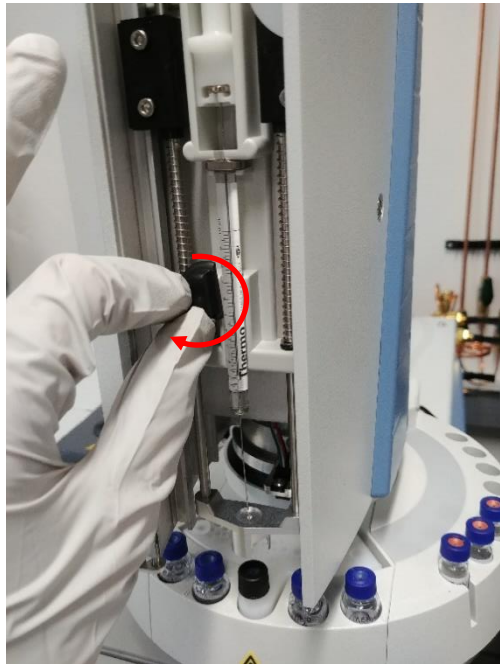
Replacing autosampler syringe

Autosampler syringe does not need to be replaced regularly, but it may need to be removed to be inspected or if broken.

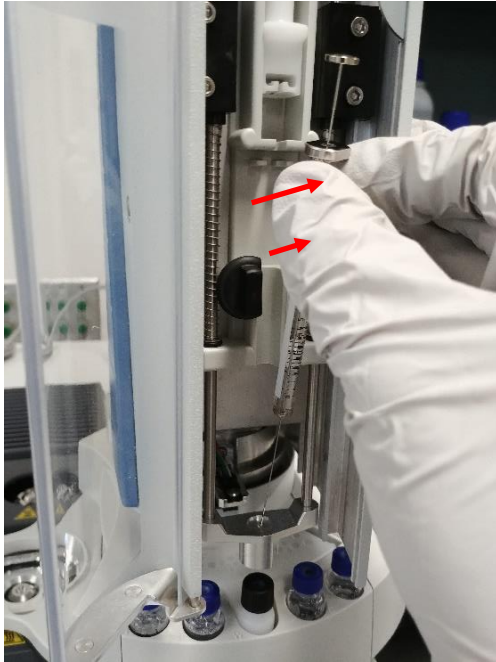
1.) Open door of autosampler.



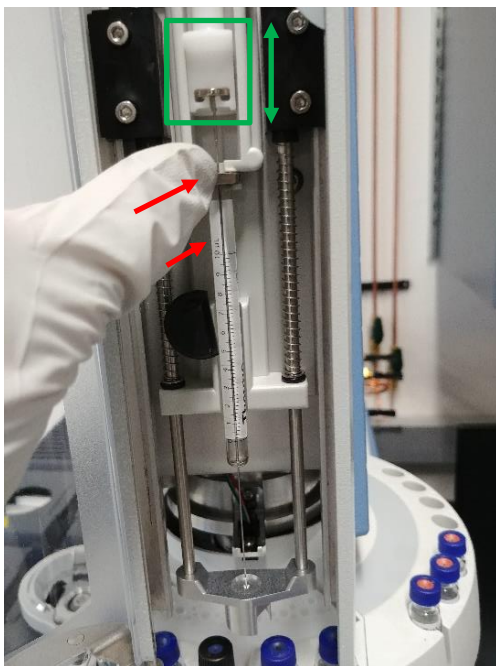
2.) Unlock syringe by turning black half circle clockwise.



3.) Remove syringe. Grip syringe by top of barrel, and pull out and up gently. Make sure the plunger comes out with barrel, and it does not get caught in the plunger head guide. Likewise, make sure that needle comes out, and it does not get caught by the vial capture device.

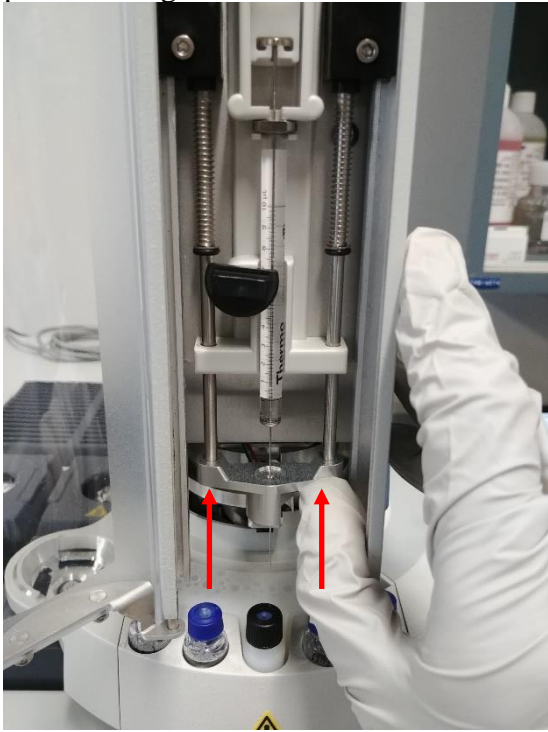


4.) Replace syringe. Slide needle through hole of vial capture device. Line up plunger in plunger head guide, sliding guide up or down by hand as needed. Push back into original position by pushing top of barrel.



5.) Lock syringe by turning black half circle counterclockwise.

6.) Verify that needle is positioned correctly. Pull up vial capture device and make sure needle passes through hole.



Selection of compressed gas and regulators for gas chromatography

Compressed gases must be of high purity. Gases routinely used are Airgas NI UPC300 (Ultra Pure Carrier 5.3 Nitrogen), Airgas HY UPC300 (Ultra Pure Carrier 5.5 Hydrogen), Airgas AI UZ300 (Ultra Zero Air). These gases meet requirements for the Trace 1300

If choosing different gases from above, verify that N₂ and H₂ have purity 99.999% or higher. They should have maximum of 1 ppm each for H₂O, O₂, and THC

Air should have maximum of 1 ppm for THC. Maximum of 1 ppm for H₂O is preferable, but up to 2 ppm is permissible.

Regulators must be dual stage and with nickel or stainless steel. Regulators routinely used are Airgas Y12N245E580-AG, Y12N245E350-AG, and Y12N245E590-AG.

Measuring VFA content of culture supernatant

Preparing standards and blanks

1.) Prepare internal standard stock (2-ethyl butyric acid, 10 mM). Weigh approximately 0.1162 g 2-ethyl butyric acid (a liquid) into a 100 mL volumetric flask. Record actual weight and calculate actual concentration. Add 10 mL 95% ethanol (to retard microbial growth) and bring to volume with ddH₂O. Store in autoclaved culture bottle with bung (to prevent volatilization). Solution is stable indefinitely at 4°C.

2.) Prepare external standard stock (60 mM acetate, 20 mM propionate, 2 mM isobutyrate, 15 mM butyrate, 3 mM isovalerate, 3 mM valerate). To a 100 mL volumetric flask, add the following

VFA	Volume to add (μL)	Approximate mass (g)
Acetic acid	343	0.36
Propionic acid	149	0.148
Isobutyric acid	18.8	0.018
Butyric acid	138	0.132
Isovaleric acid	33.3	0.031
Valeric acid	33.3	0.031

Record the actual masses added. Bring to volume with ddH₂O.

To prevent volatilization, add VFA to flask quickly, and stopper flask when not in use. From actual masses recorded, calculate actual concentrations. Store in autoclaved culture bottle with bung (to prevent volatilization). Solution is stable indefinitely at 4°C.

3.) Prepare external standard in 2-mL autosampler vials. Add following to 2-mL vials (Fisher 13-622-186):

Standard	External standard	ddH ₂ O	Internal standard	Formic acid	Methanol
1	0	350	50	100	500
2	10	340	50	100	500
3	50	300	50	100	500
4	100	250	50	100	500
5	200	150	50	100	500
6	300	50	50	100	500
7	350	0	50	100	500

4.) Prepare wash samples in autosampler vials. To a 2-mL vial, add 100 μL formic acid, 400 μL ddH₂O, and 500 μL of methanol.

5.) Prepare blank samples autosampler vials. To a 2-mL vial, add 500 μL ddH₂O and 500 μL of methanol.

Preparing samples

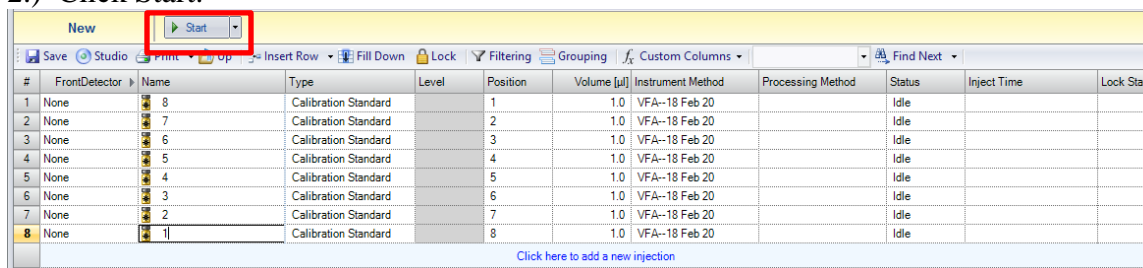
- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Prepare supernatant samples in autosampler vials. To each vial, add 350 μL supernatant, 50 μL internal standard, 100 μL formic acid, and 500 μL of methanol.

Preparing GC for analysis of samples and standards

- 1.) Start GC and set up for gas analysis according to *Turning on GC and placing into standby mode in Operation of gas chromatograph* procedure.
- 2.) Turn on FID detector according to *Turning on detectors in Operation of gas chromatograph* procedure.
- 3.) Set up autosampler according to *Setting up autosampler in Operation of gas chromatograph* procedure. Fill wash vial A with 2 mL of ddH₂O and 2 mL methanol (1:1 ratio). Fill vial C with 4 mL ddH₂O and vial D with 4 mL methanol.
- 4.) In Chromeleon software, create a sequence (list of samples to be analyzed). Follow *Setting up a sequence* below.

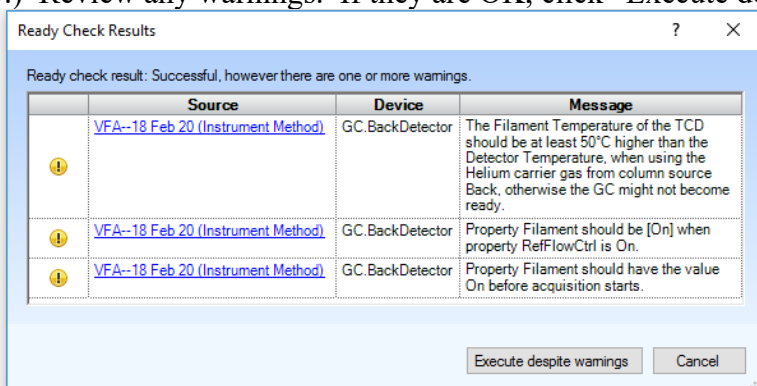
Analyzing samples and standards

- 1.) Open a sequence.
- 2.) Click Start.



#	FrontDetector	Name	Type	Level	Position	Volume [μL]	Instrument Method	Processing Method	Status	Inject Time	Lock Statu
1	None	8	Calibration Standard		1	1.0	VFA--18 Feb 20		Idle		
2	None	7	Calibration Standard		2	1.0	VFA--18 Feb 20		Idle		
3	None	6	Calibration Standard		3	1.0	VFA--18 Feb 20		Idle		
4	None	5	Calibration Standard		4	1.0	VFA--18 Feb 20		Idle		
5	None	4	Calibration Standard		5	1.0	VFA--18 Feb 20		Idle		
6	None	3	Calibration Standard		6	1.0	VFA--18 Feb 20		Idle		
7	None	2	Calibration Standard		7	1.0	VFA--18 Feb 20		Idle		
8	None	1	Calibration Standard		8	1.0	VFA--18 Feb 20		Idle		

- 3.) Review any warnings. If they are OK, click “Execute despite warnings”.



4.) Allow sequence to complete.

5.) Calculate VFA concentrations according to *Constructing a standard curve and quantifying VFA concentrations* below.

Shutting down GC after analysis of samples and standards

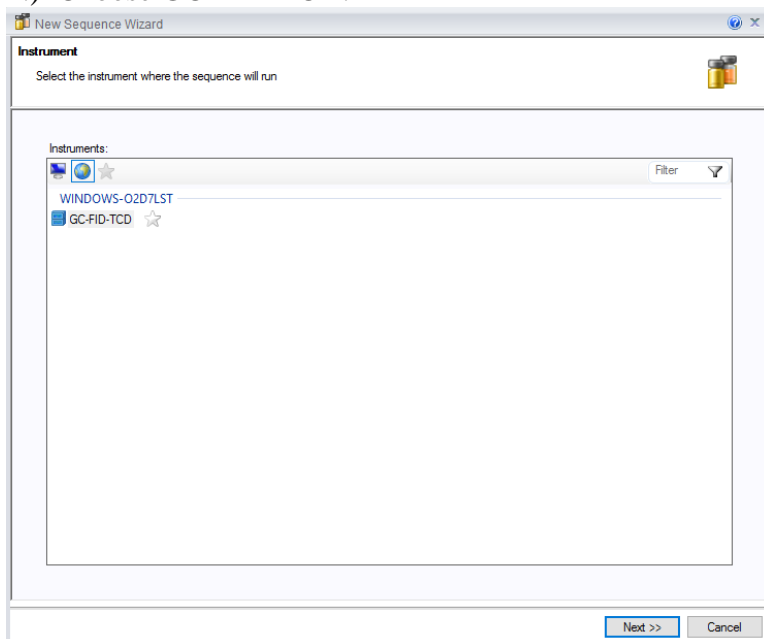
1.) Place the instrument back into standby mode according to *Placing back into standby mode*.

2.) If no samples will be run in the next day or two, proceed to turn off the instrument according to *Turning off*.

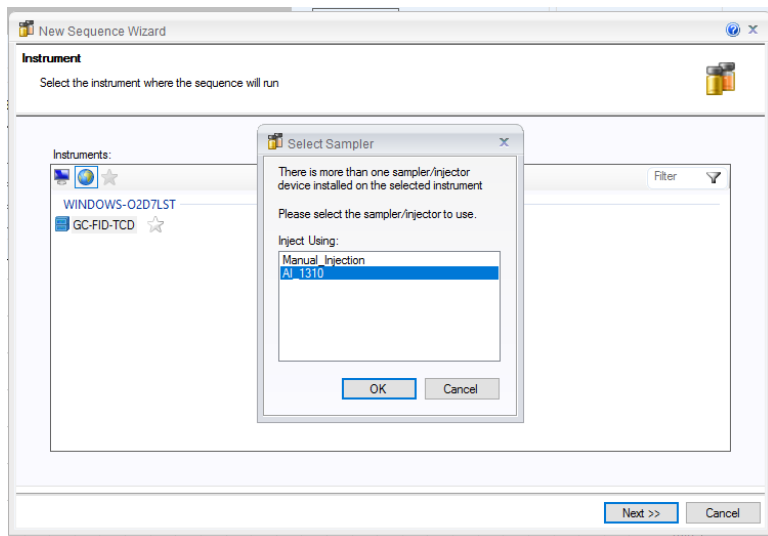
Setting up a sequence

1.) In Chromeleon 7 software, choose Create ->Sequence.

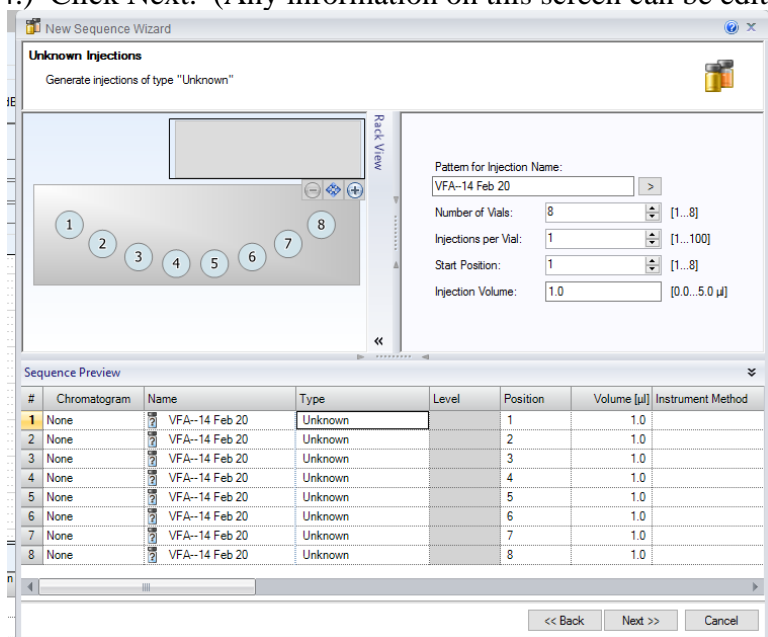
2.) Choose GC-FID-TCD.



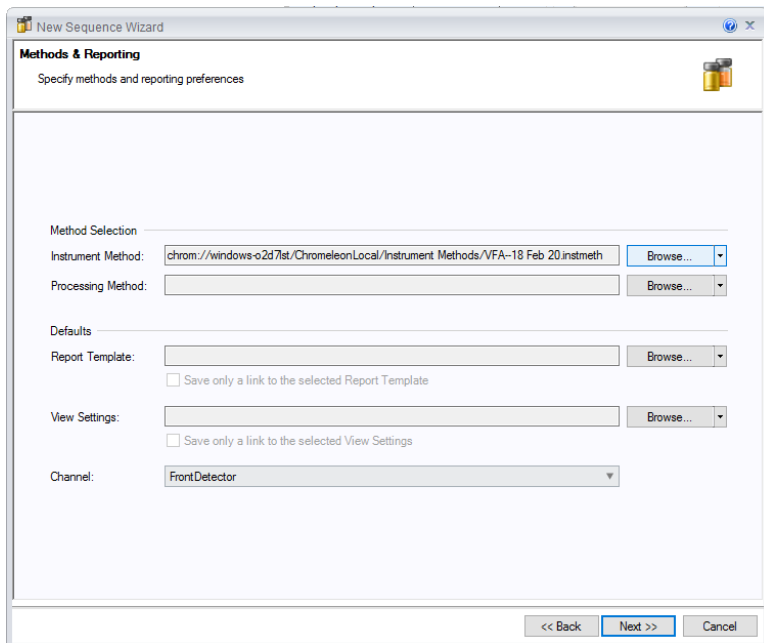
3.) Choose AI_1310 (the autosampler).



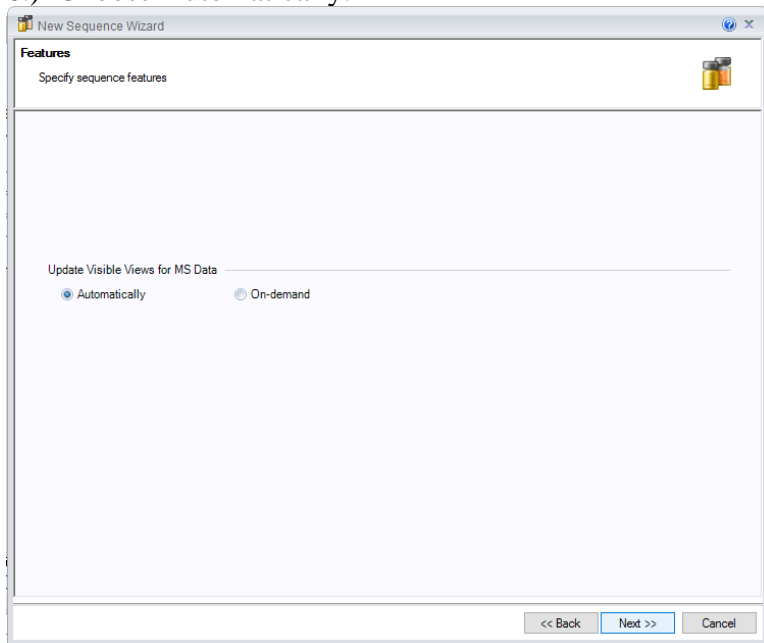
4.) Click Next. (Any information on this screen can be edited later.)



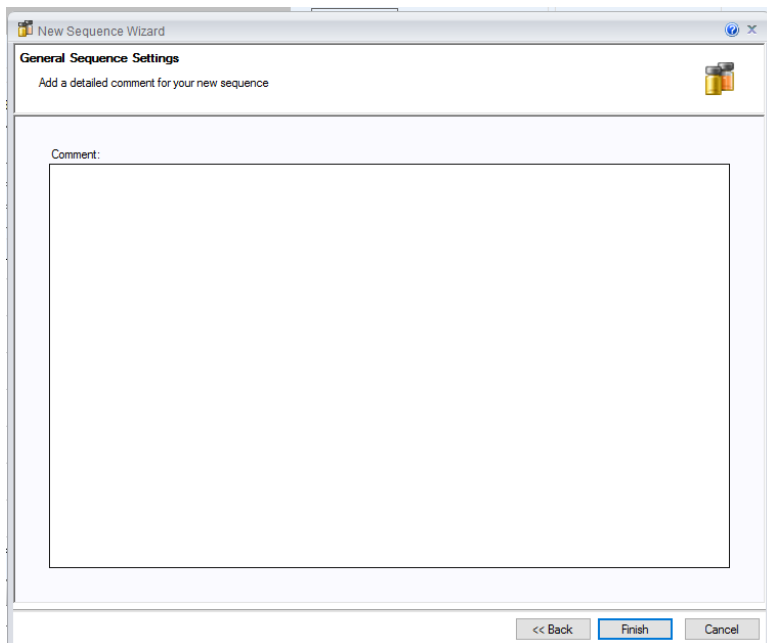
5.) Under Instrument Method, choose “VFA—18 Feb 20.instmethod”. See details for this method under *Setting up a method*.



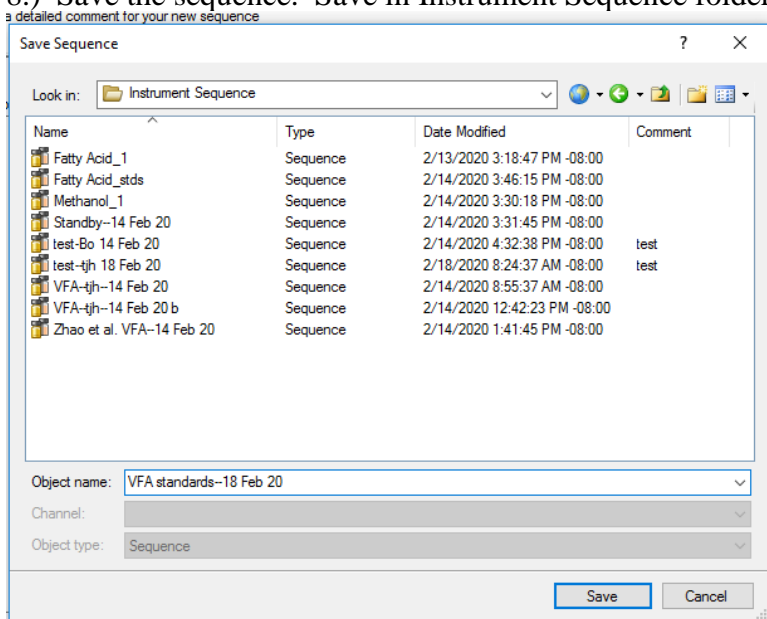
6.) Choose Automatically.



7.) Enter comments (optional).



8.) Save the sequence. Save in Instrument Sequence folder so that it is easy to find.



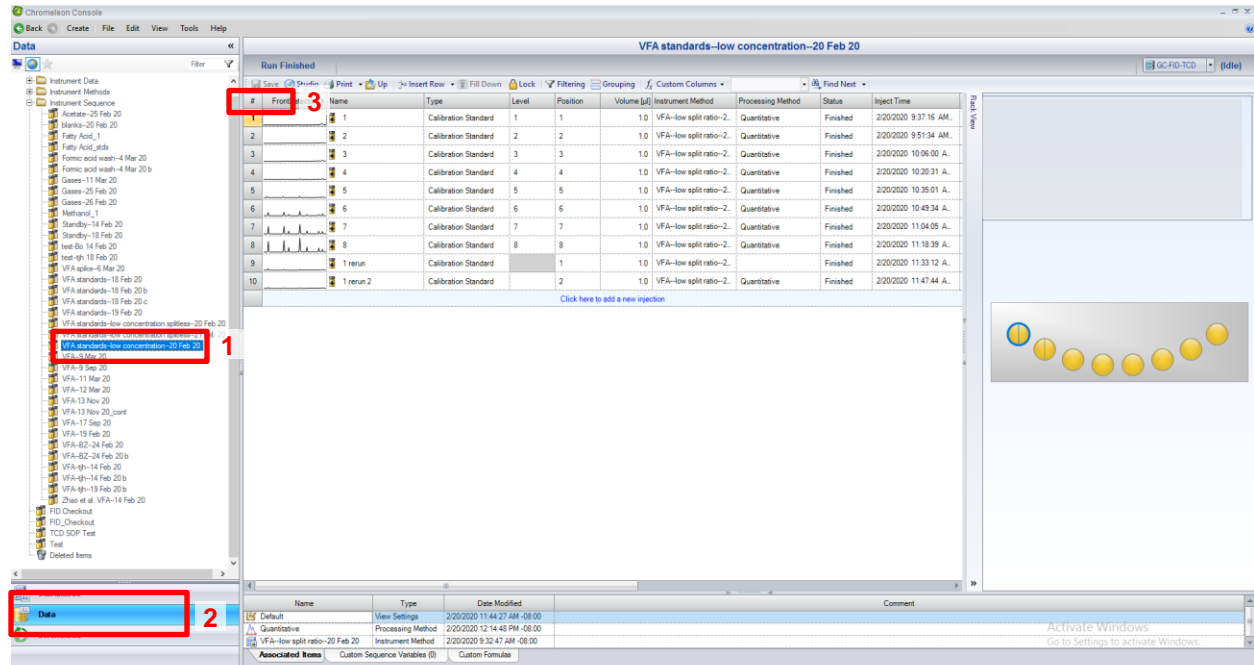
9.) Enter information for samples. See typical information below.

#	FrontDetector	Name	Type	Level	Position	Volume [µl]	Instrument Method	Processing Method	Status	Inject Time	Lock Statu
1	None	8	Calibration Standard		1	1.0	VFA--18 Feb 20		Idle		
2	None	7	Calibration Standard		2	1.0	VFA--18 Feb 20		Idle		
3	None	6	Calibration Standard		3	1.0	VFA--18 Feb 20		Idle		
4	None	5	Calibration Standard		4	1.0	VFA--18 Feb 20		Idle		
5	None	4	Calibration Standard		5	1.0	VFA--18 Feb 20		Idle		
6	None	3	Calibration Standard		6	1.0	VFA--18 Feb 20		Idle		
7	None	2	Calibration Standard		7	1.0	VFA--18 Feb 20		Idle		
8	None	1	Calibration Standard		8	1.0	VFA--18 Feb 20		Idle		

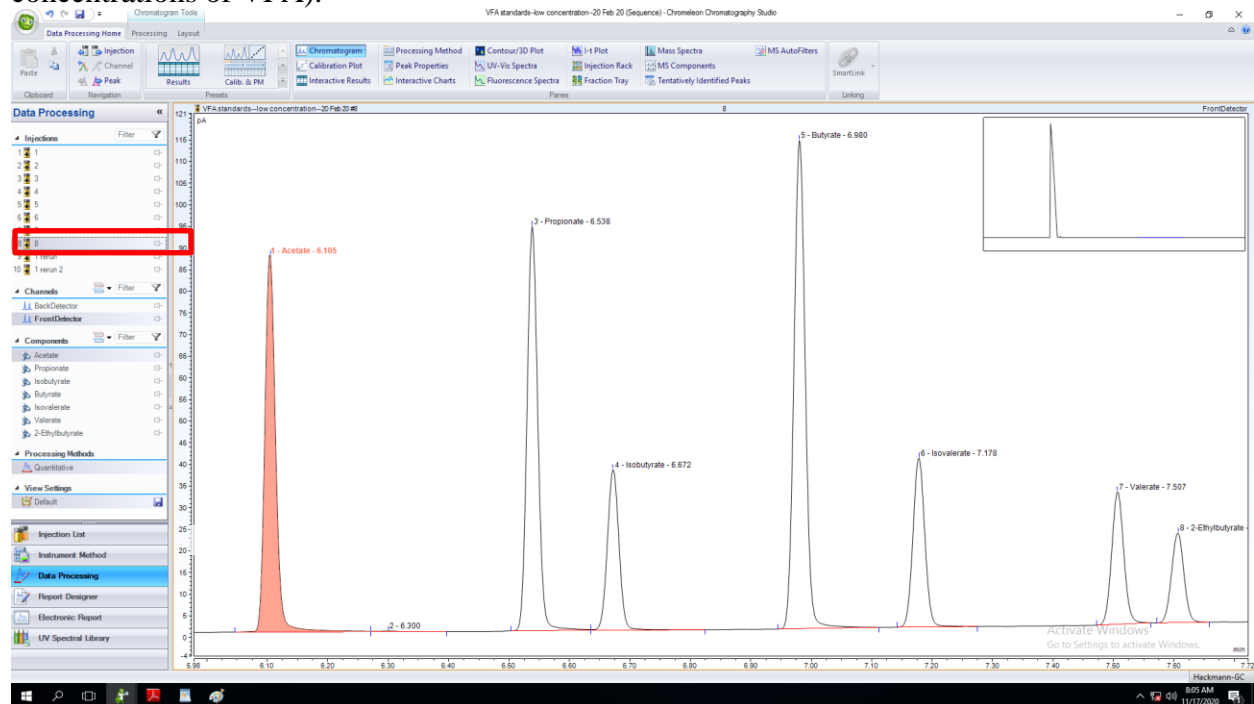
[Click here to add a new injection](#)

Constructing a standard curve and quantifying VFA concentrations

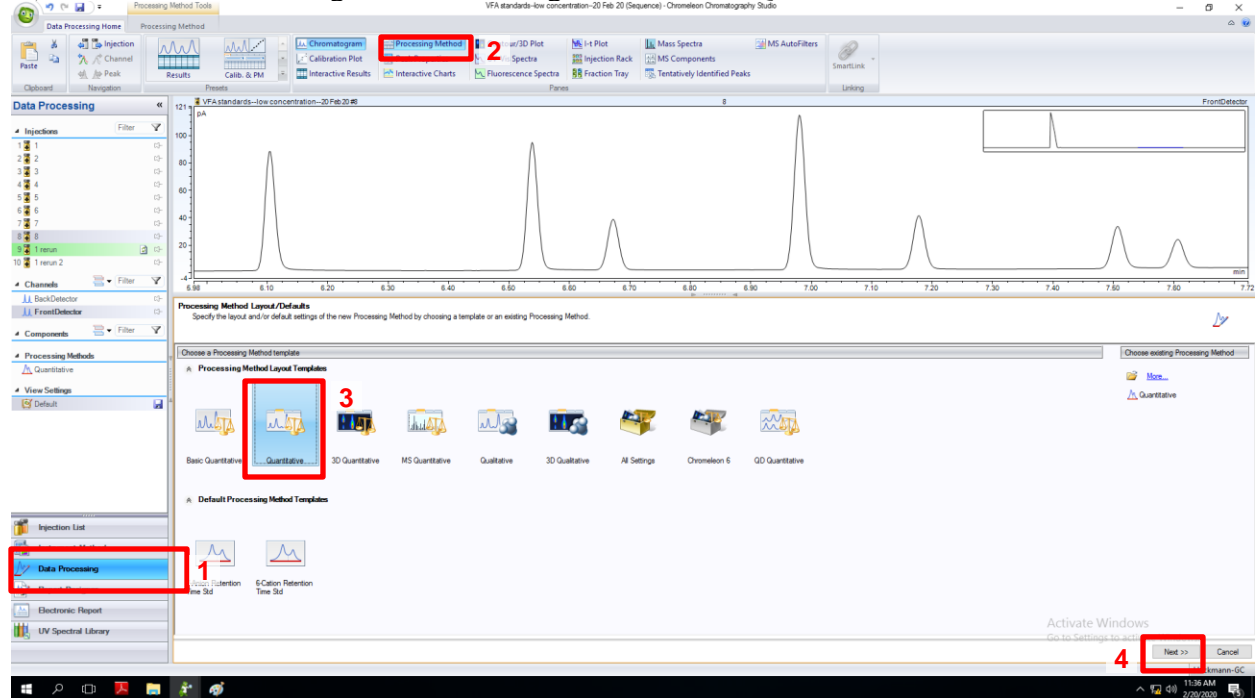
1.) In Chromeleon 7 software, click on the sequence that should be analyzed. Click on Data, then click on Studio.



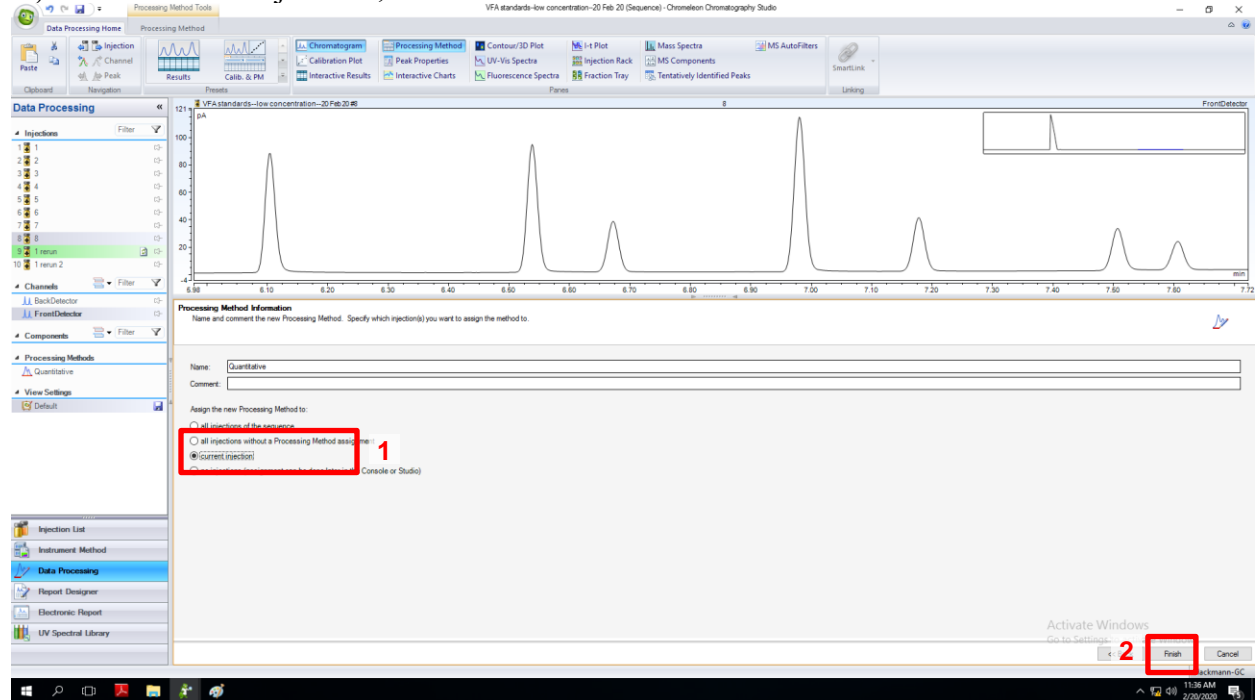
2.) In the window that appears, click on an injection of the last standard (the one with the highest concentrations of VFA).



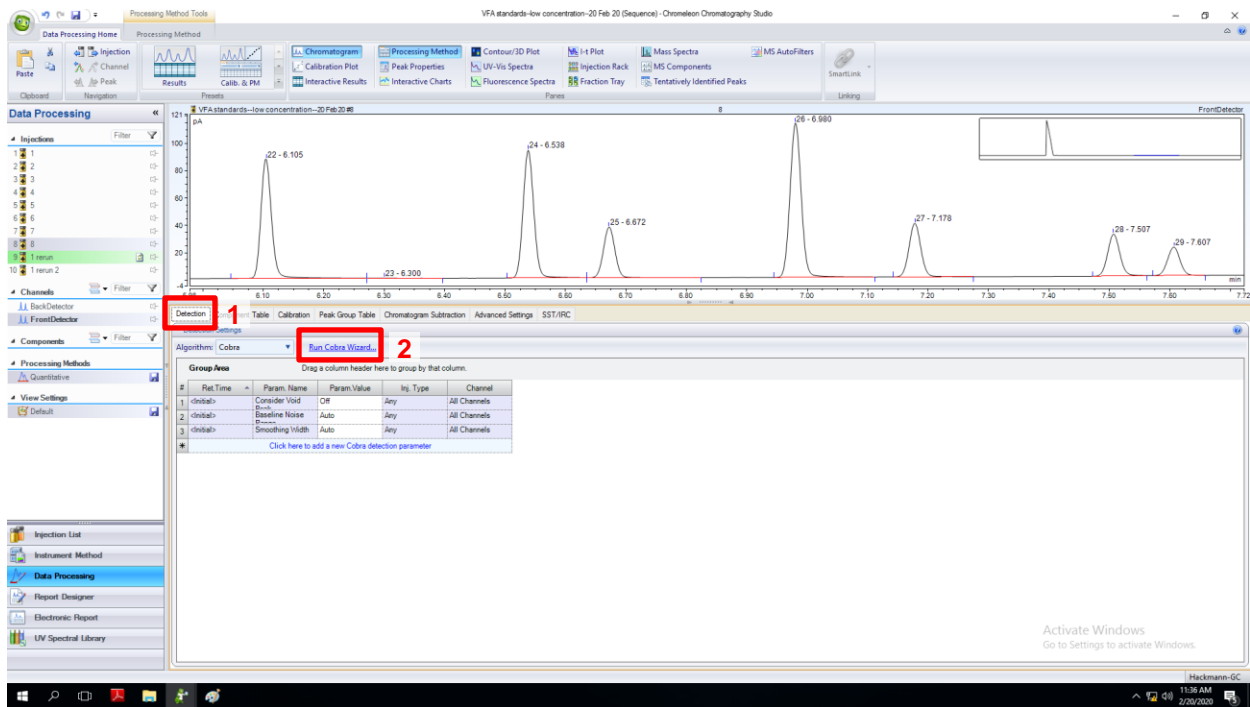
2.) Click Data Processing, then Processing Method, then Quantitative, then Next.



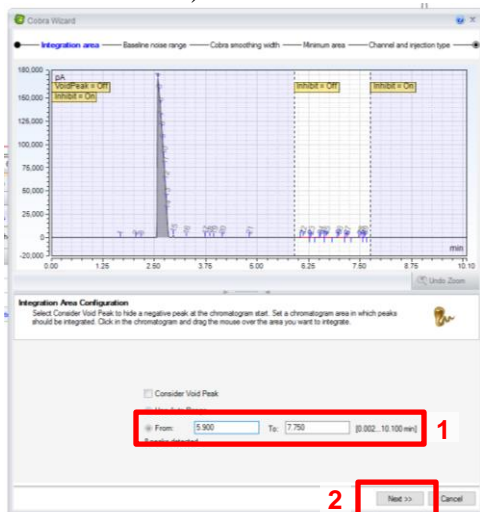
3.) Click "current injection", then click Finish.



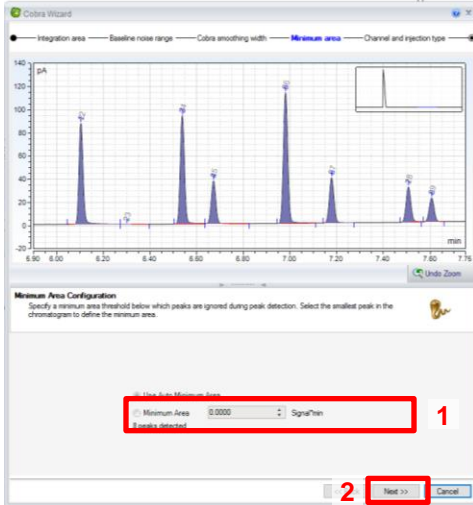
4.) Click Detection, then Click Run Cobra Wizard.



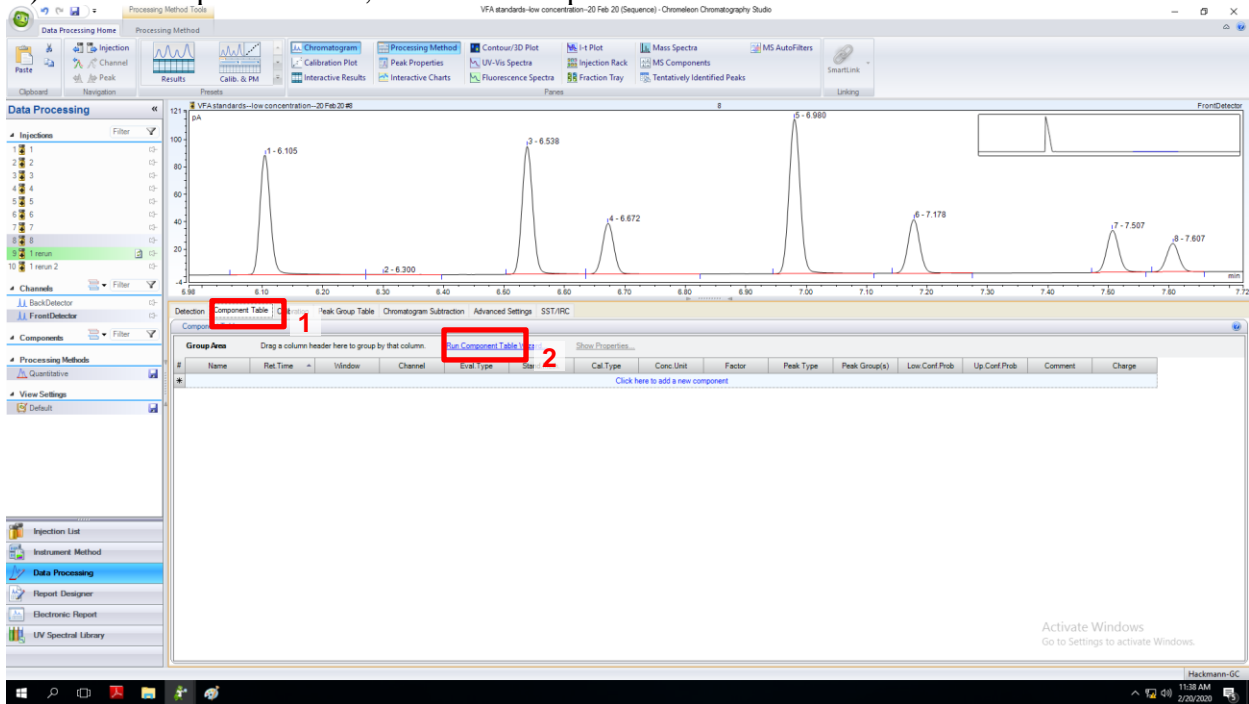
5.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next.



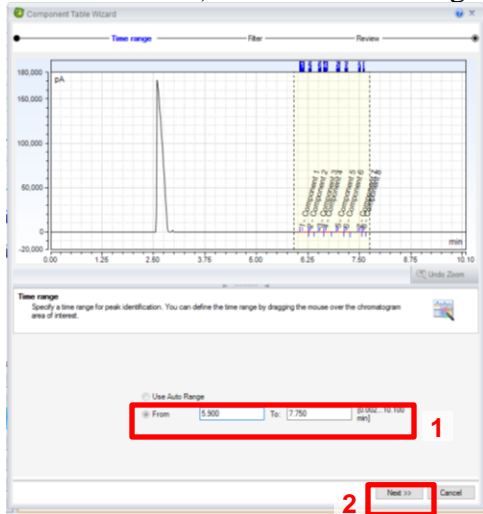
6.) Continue to click Next until reaching window below. If unexpected, small peaks are detected, enter a value of Minimum Area to suppress these small peaks. Otherwise, click Next, then click Finish.



7.) Click Component Table, then Run Component Table Wizard.



11.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next through all subsequent windows, then click Finish.



12.) Type in the component names as shown below.

Group Area	Ret Time	Inj/Inj	Channel	Eval Type	Stand Meth	Cell Type	Conc Unit	Factor	Peak Type	Peak Group(s)	Low Conf Prob	Up Conf Prob	Comment	Charge
1	6105	0.056 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
2	6538	0.033 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
3	672	0.042 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
4	6672	0.042 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
5	6980	0.033 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
6	6178	0.023 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
7	6507	0.023 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	
8	6507	0.022 AN	All Channels	Area	External	Lin, V&H Offset		1.000000	Autodetect		99.5 %	99.5 %	auto generated	

13.) Right click Processing Method, then click Assign to Injection. (If Processing Method is not visible, scroll down to the bottom of the Data Processing tab.)

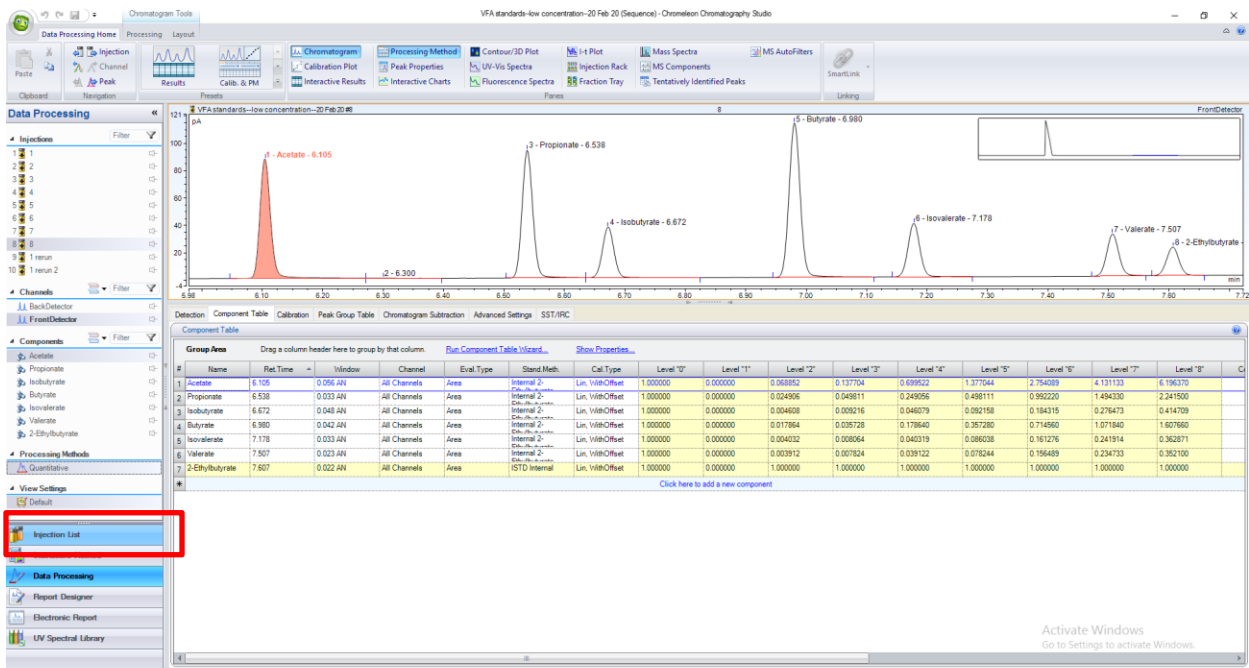
The screenshot shows the 'Data Processing' window in Chromleon. On the left, the 'Processing Methods' list includes 'Quantitative', which is highlighted. A context menu is open over 'Quantitative', with 'Assign to Injection...' selected. The main window displays a chromatogram with peaks labeled 1-8. Below the chromatogram is a 'Component Table' with the following data:

Group Area	Name	Ret Time	Injection	Channel	Eval. Type	Stand. Mark.	Cal. Type	Level '0'	Level '1'	Level '2'	Level '3'	Level '4'	Level '5'	Level '6'	Level '7'	Level '8'
1	Acetate	6.105	0.056 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.000000	0.137704	0.039532	1.377304	2.754309	4.131133	6.195370
2	Propionate	6.538	0.033 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.024606	0.049811	0.249056	0.498111	0.992220	1.494330	2.241500
3	Isobutyrate	6.672	0.048 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.004608	0.009216	0.046079	0.092158	0.184315	0.276473	0.414709
4	Butyrate	6.980	0.042 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.017864	0.035728	0.178640	0.357280	0.714560	1.071840	1.607660
5	Isovalerate	7.178	0.033 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.004032	0.008064	0.040319	0.080638	0.161276	0.241914	0.362871
6	Valerate	7.507	0.023 AN	All Channels	Area	Internal 2	Lin. V&H Offset	1.000000	0.000000	0.003912	0.007824	0.039122	0.078244	0.156488	0.234733	0.352100
7	2-Ethylbutyrate	7.607	0.022 AN	All Channels	Area	ISTD Internal	Lin. V&H Offset	1.000000	0.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000

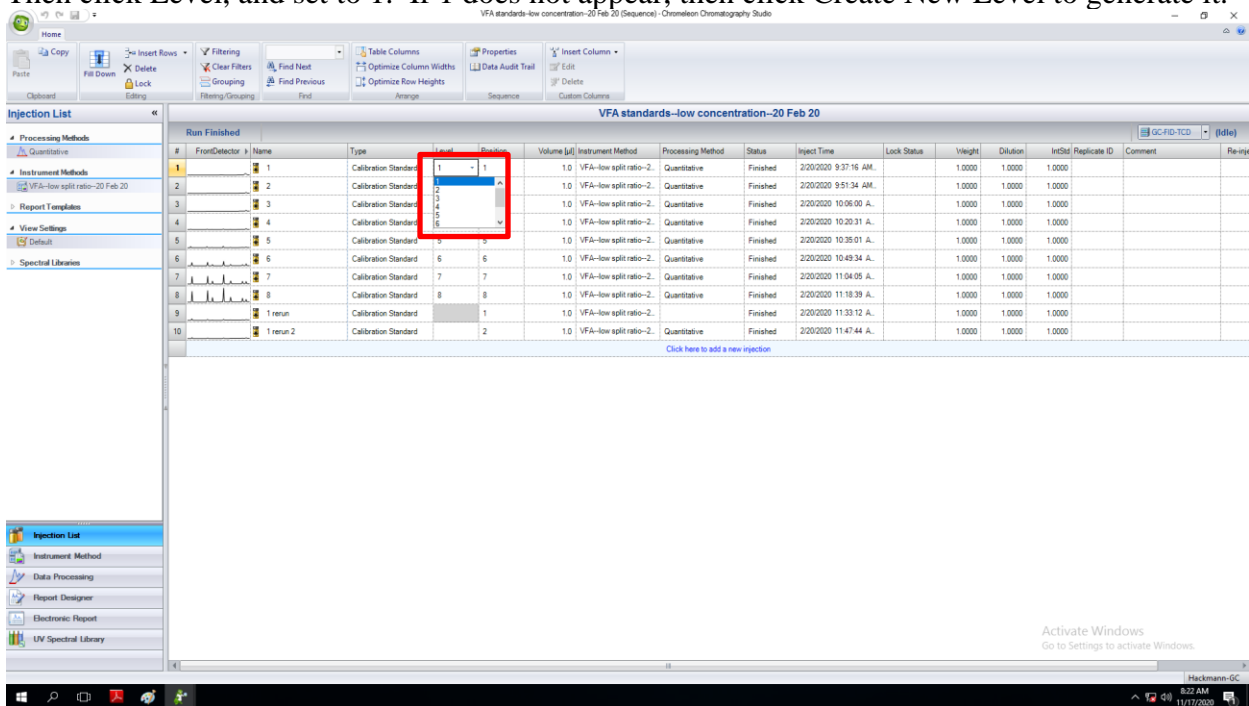
14.) In the window that appears, click “all injections of the sequence”, then click OK.

The screenshot shows the 'Assign Processing Method to Injection' dialog box. The 'all injections of the sequence' radio button is selected. The background shows the chromatogram and component table.

15.) Click Injection List.



16.) Click on the injection for the first standard (the one with the lowest concentrations of VFA). Then click Level, and set to 1. If 1 does not appear, then click Create New Level to generate it.



17.) Repeat for the second standard, but set Level to 2. Repeat similarly for all other standards. Go back by clicking Data Processing.

VFA standards-low concentration-20 Feb 20 (Sequence) - Chromelion Chromatography Studio

Run Finished

#	FrontDetector	Name	Type	Level	Position	Volume [μL]	Instrument Method	Processing Method	Status	Inject Time	Lock Status	Weight	Dilution	InStd	Replicate ID	Comment	Reinj
1		1	Calibration Standard	1	1	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 9:37:16 AM		1.0000	1.0000	1.0000			
2		2	Calibration Standard	2	2	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 9:51:34 AM		1.0000	1.0000	1.0000			
3		3	Calibration Standard	3	3	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:06:00 A.		1.0000	1.0000	1.0000			
4		4	Calibration Standard	4	4	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:20:31 A.		1.0000	1.0000	1.0000			
5		5	Calibration Standard	5	5	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:35:01 A.		1.0000	1.0000	1.0000			
6		6	Calibration Standard	6	6	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:49:34 A.		1.0000	1.0000	1.0000			
7		7	Calibration Standard	7	7	10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:04:05 A.		1.0000	1.0000	1.0000			
8		8	Calibration Standard			10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:18:39 A.		1.0000	1.0000	1.0000			
9		1 run	Calibration Standard			10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:33:12 A.		1.0000	1.0000	1.0000			
10		1 run 2	Calibration Standard			10	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:47:44 A.		1.0000	1.0000	1.0000			

Click here to add a new injection

Injection List

Data Processing

18.) Under Level, type in concentrations of standards (in mmol/L). Standard curve should appear; click Calibration Plot if it does not.

VFA standards-low concentration-20 Feb 20 (Sequence) - Chromelion Chromatography Studio

Data Processing

Chromatogram

Peak Properties

Calibration Plot

Interactive Results

Interactive Charts

Fluorescence Spectra

Fraction Tray

Tentatively Identified Peaks

Acetate 6.195

Propionate 6.538

Isobutyrate 6.672

Isovalerate 7.178

2-Ethylbutyrate 7.607

Acetate

External

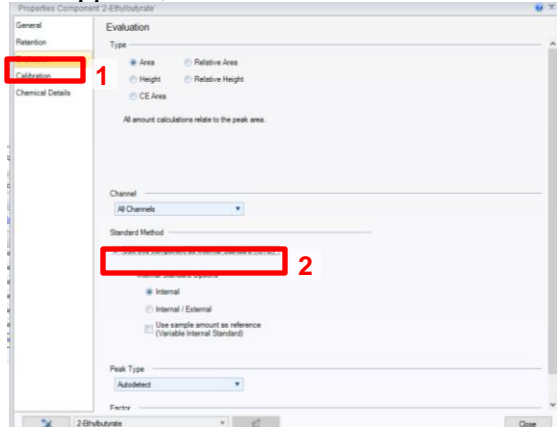
FrontDetector

Component Table

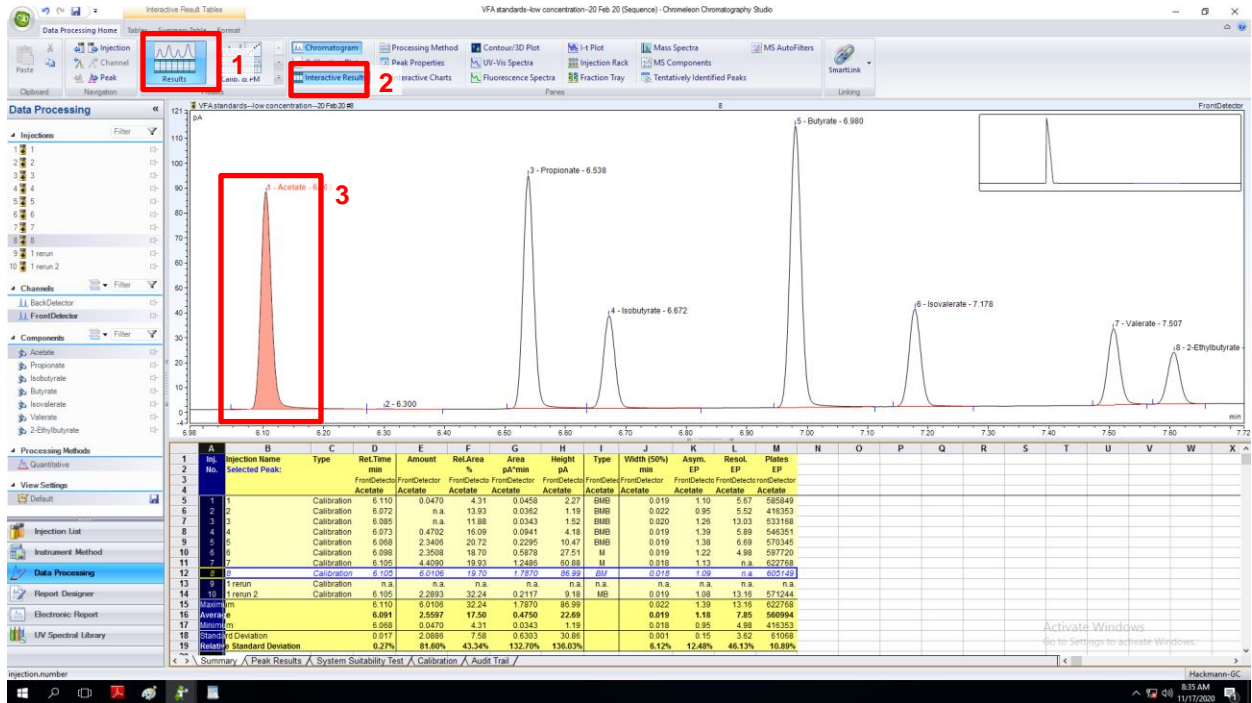
#	Name	Ret Time	InjVol	Channel	Eval. Type	Stand Meth.	Cal. Type	Level "1"	Level "2"	Level "3"	Level "4"	Level "5"	Level "6"	Level "7"	Level "8"	Conc. Unit
1	Acetate	6.195	0.092 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.048852	0.117104	0.199522	1.377044	2.746208	4.131133	6.196376	1.0000
2	Propionate	6.538	0.033 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.020808	0.049811	0.249596	0.498111	0.992220	1.484330	2.241800	1.0000
3	Isobutyrate	6.672	0.048 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.046608	0.093216	0.046079	0.092158	0.184315	0.276473	0.414709	1.0000
4	Butyrate	6.980	0.042 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.017864	0.035728	0.178640	0.357280	0.714560	1.071840	1.607960	1.0000
5	Isovalerate	7.178	0.033 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.004032	0.008064	0.040319	0.080638	0.161276	0.241914	0.362871	1.0000
6	Valerate	7.507	0.023 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	0.003912	0.007824	0.039122	0.078244	0.156489	0.234733	0.352100	1.0000
7	2-Ethylbutyrate	7.607	0.022 AN	All Channels	Area	External	Lin, VFA-Offset	0.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000	1.0000

Click here to add a new component

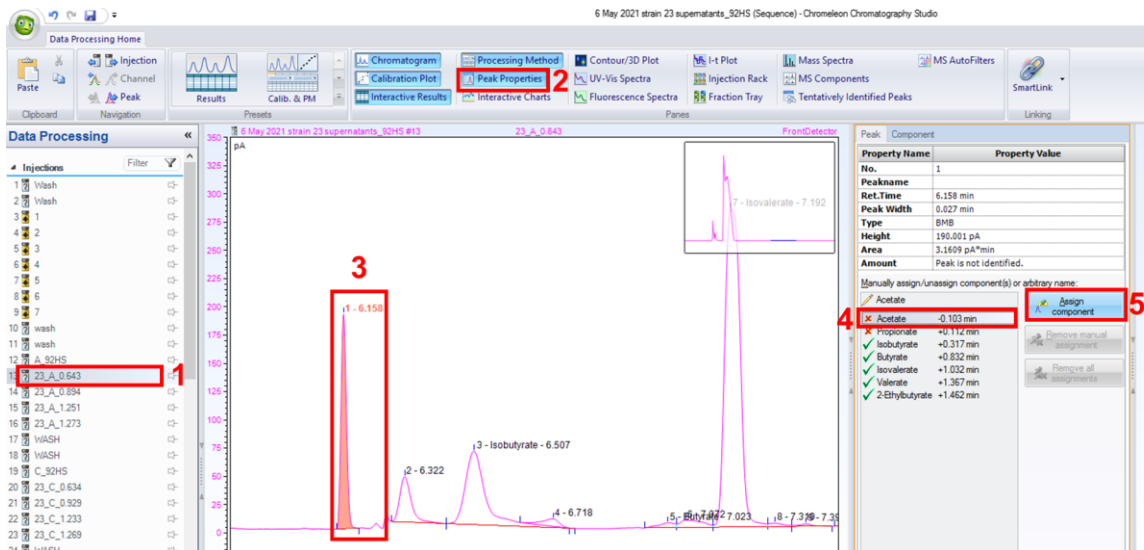
20.) Optional: set 2-ethylbutyrate as an internal standard by double clicking on it. In window that appears, click Evaluation and Use this component as Internal Standard (ISTD).



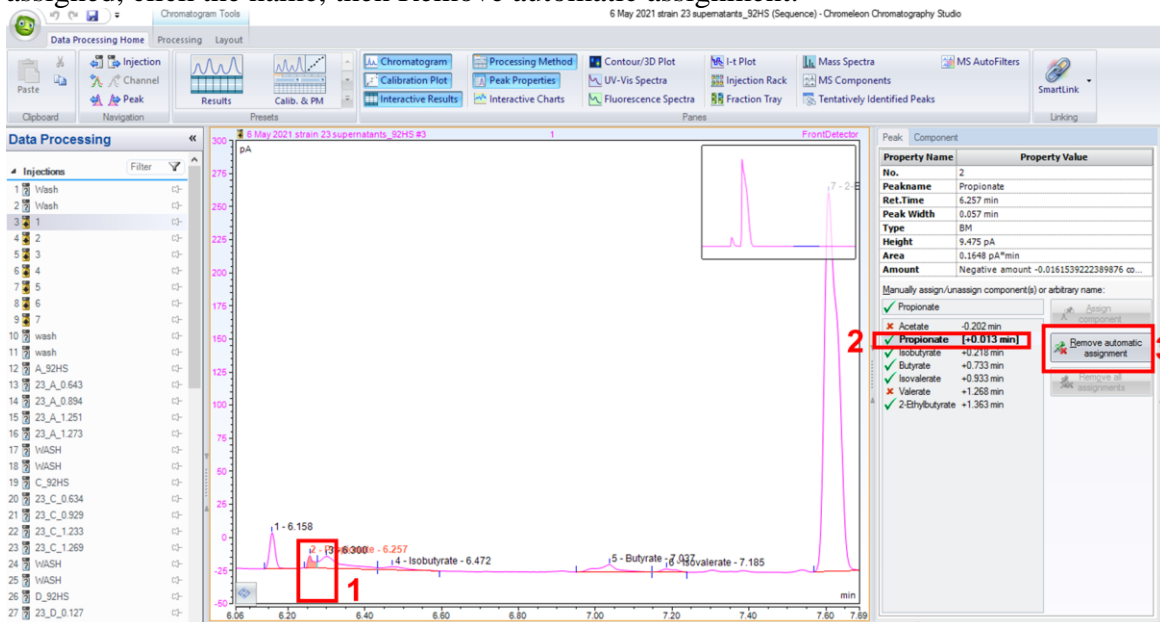
21.) Click Results, then Interactive Results, and then the acetate peak in the chromatogram. The concentration of VFA is reported under Amount. Click the remaining peaks to see their concentrations.



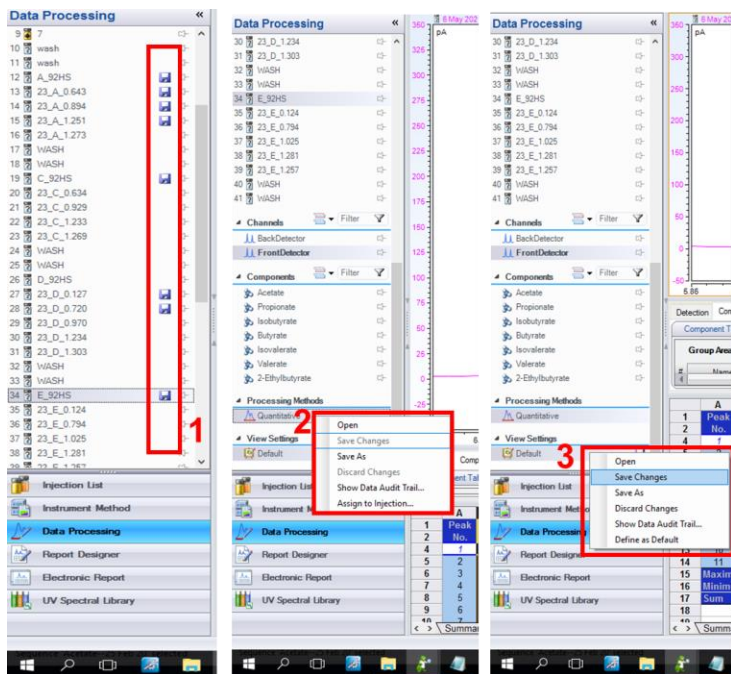
22.) Optional: Rename any peaks that are misidentified by the software. Click the sample, Peak Properties, the misidentified peak, the correct name, then Assignment component.



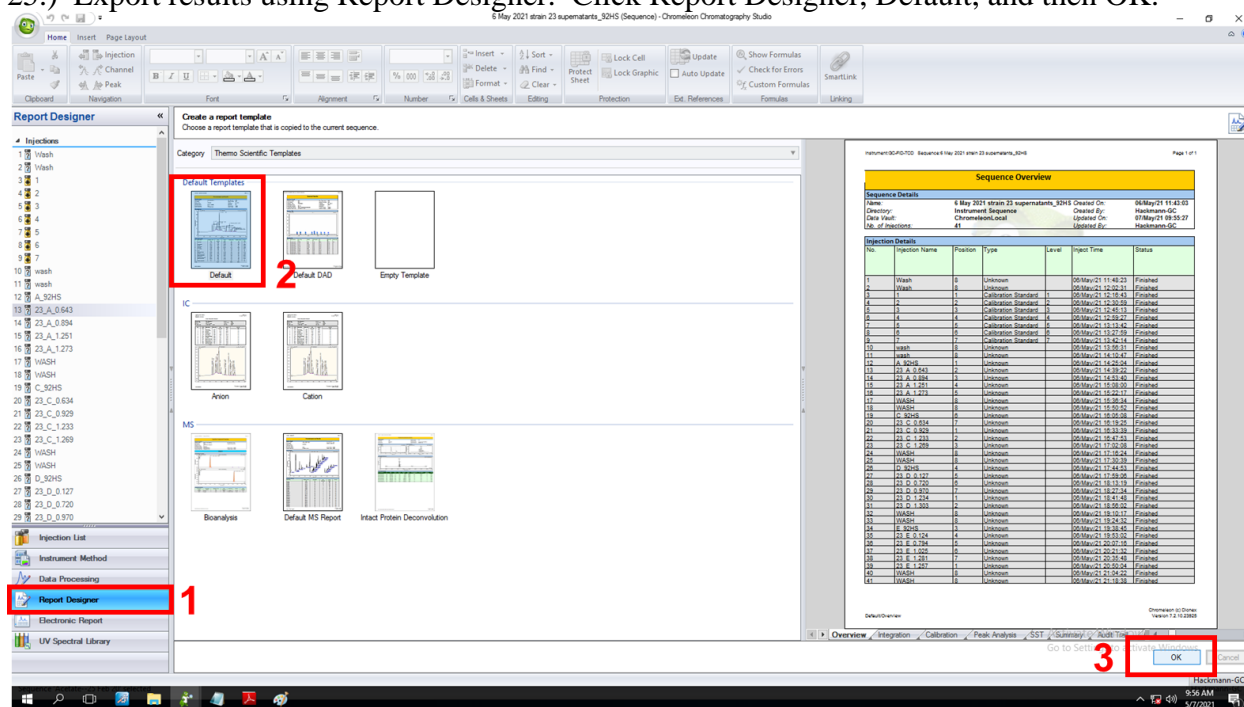
22.) Optional: Remove any peak assignments that are spurious. Click the peak that is wrongly assigned, click the name, then Remove automatic assignment.



24.) Save all changes.



25.) Export results using Report Designer. Click Report Designer, Default, and then OK.



26.) In the window that appears, click any injection. Scroll down, Click FrontDetector in Channels; click Acetate in Components; then click Summary.

6 May 2021 strain 23 supernatants_52HS (Sequence) - Chromleon Chrom

Report Designer

Summary Table

Summary

Sequence Details

Name: 6 May 2021 strain 23 supernatants_52HS Created On: 06/May/21 11:43:03
 Instrument Sequence: Chromleon_Local Created By: Hackmann-GC
 Data Vault: Chromleon_Local Updated On: 07/May/21 09:55:27
 No. of Injections: 41 Updated By: Hackmann-GC

By Component: Acetate

No.	Injection Name	Ret. Time min	Area pA*min	Height pA	Amount	Rel. Area %	Peak Type
1	Wash	6.073	0.954	44.956	0.052	33.60	BM
2	Wash	6.078	0.280	16.394	n.a.	23.58	BMB
3	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	Wash	6.077	2.606	152.005	1.291	13.33	BMB
5	Wash	6.075	12.264	687.438	8.471	24.97	BMB
6	Wash	6.147	25.646	1439.320	18.421	26.15	BMB*
7	Wash	6.062	49.406	2324.202	36.085	29.40	BMB
8	Wash	6.055	72.208	3091.417	53.038	30.11	BMB
9	Wash	6.055	83.593	3473.729	61.503	30.20	BMB
10	Wash	6.085	0.271	15.342	n.a.	19.80	BMB
11	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
13	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
16	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
18	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
19	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
20	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
21	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
23	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
26	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
27	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
28	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
29	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
30	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
31	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
32	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
33	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
34	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
35	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
36	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
37	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
38	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
39	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
40	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
41	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
42	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
43	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
44	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
45	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
46	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
47	Wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

27.) Click Chromeleon 7 icon, Export, choose Current Injection, select file address to save the result. Select Excel file format or other formats, then OK.

6 May 2021 strain 23 supernatants_52HS (Sequence) - Chromleon Chromatography Studio

Export

Apply to: Current Injection Current Sequence

Destination Path:

Parent Folder:

Sub Folder:

Export Formats and Filenames

Text format (Results) (*.txt)

Text format (Raw Data) (*.txt)

Excel file format (*.xls)

PDF file format (*.pdf)

GAML file format (*.gam)

AnDI/NetCDF file format (*.cdf)

Raw file format (*.raw)

Chromleon data file format (*.cmdb)

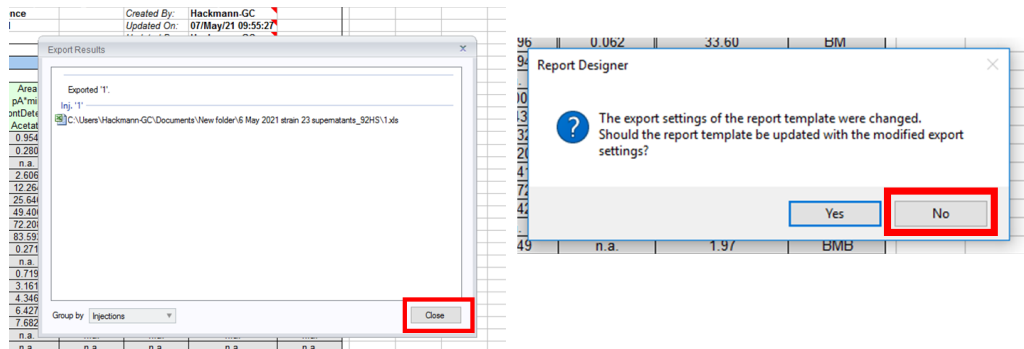
Allotrope data file format (*.adf)

External Program: Call external program

Notifications: Enable Notifications

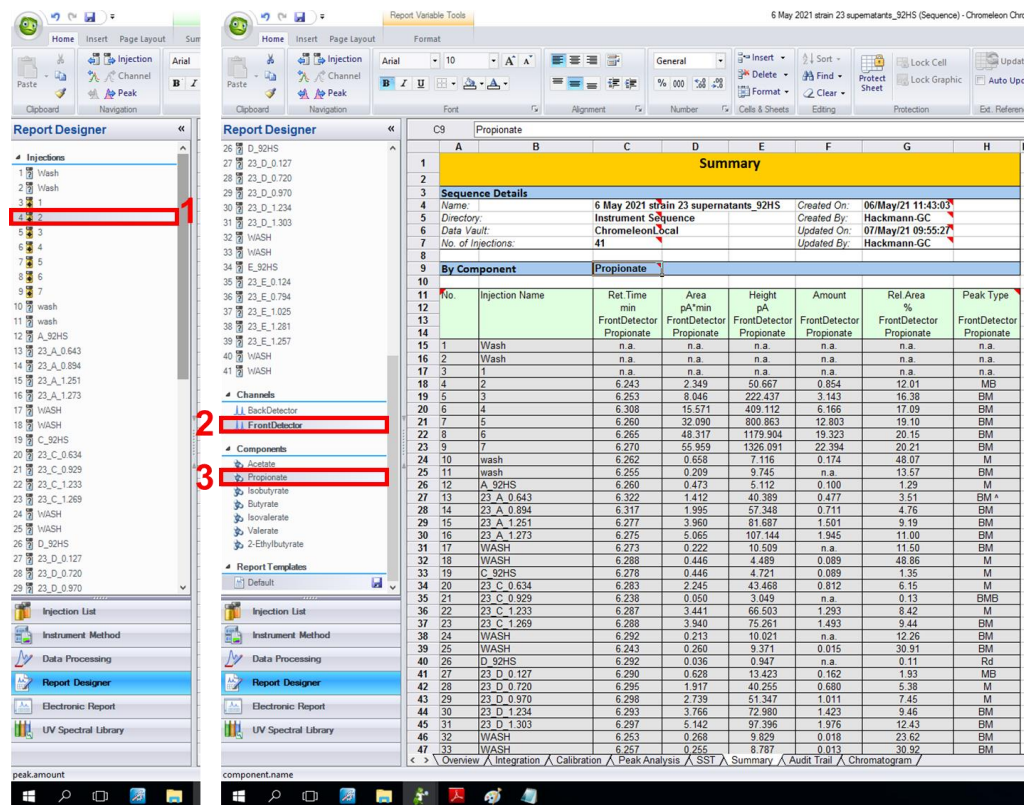
Make New For:

28.) In the window that appears, select Close. Then select No update.



29.) Alternatively, instead of following step 7 and 8, select the value shown in the sheet summary of step 6, copy the value to Wordpad or Excel for analysis.

30.) Repeat the step 6 to 8 for other VFA, but choose a different injection for each VFA if following step 7 and 8. No need to choose a different injection if process step 9.



31.) Save the changes.

Report Variable Tools

6 May 2021 stan 23 superstanis_32HS (Sequence) / Chromleon Chromatography Suite

Home Insert Page Layout Format

Clipboard Navigation Font Alignment Number Editing

General Insert Sort Lock Cell Update Show Formulas Check for Errors Custom Formulas SmartLink

Delete Find Format Clear Edit Protection

Protect Sheet Lock Graphic Auto Update Edit References

SmartLink

Report Designer

26 D_32HS
27 23_D_0_127
28 23_D_0_720
29 23_D_0_970
30 23_D_1_234
31 23_D_1_303
32 WASH
33 WASH
34 E_32HS
35 23_E_0_124
36 23_E_0_784
37 23_E_1_025
38 23_E_1_281
39 23_E_1_287
40 WASH
41 WASH

Channels

BackDetector
FrontDetector

Components

Acetate
Propionate
Isobutyrate
Butyrate
Isovalerate
Valerate
2-Ethylbutyrate

Report Templates

Default

Injection List

Instrument Method

Data Processing

Report Designer

Electronic Report

UV Spectral Library

component.name

Overview / Integration / Calibration / Peak Analysis / SST / Summary / Audit Trail / Chromatogram

Hackmann-GC

10:29 AM 5/7/2021

Activate Windows
Go to Settings to activate Windows.

Save Modifications

You have modified one Sequence and one Report Template. Do you want to save your changes?

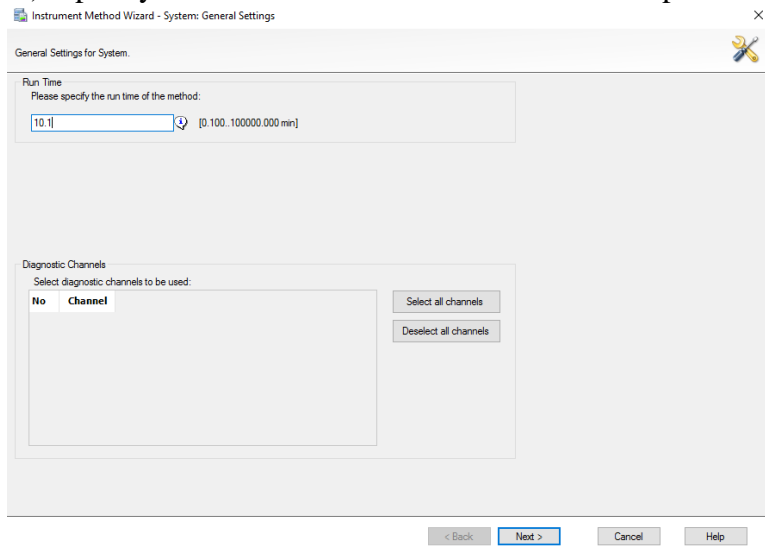
Modified Objects	Type	Status
4 May 2021 st...	Sequence	Created
Default	Report Template	Created

Save Cancel

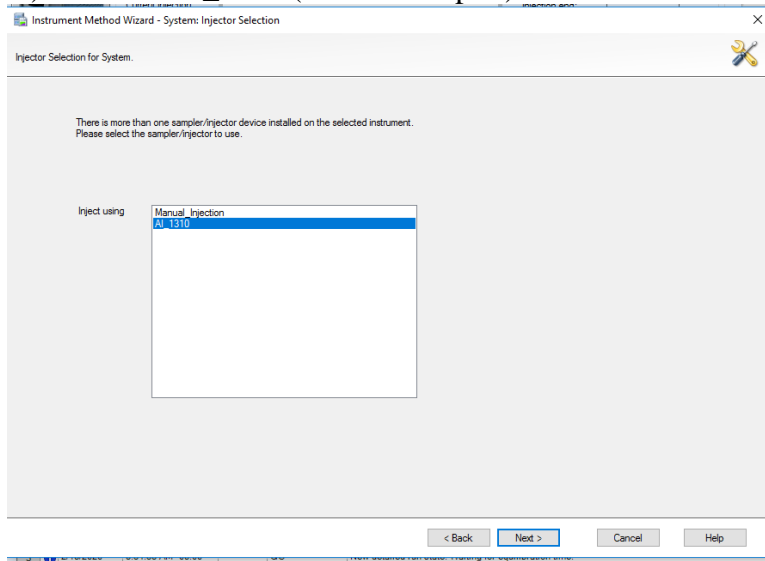
No.	Injection Name	Ret Time min	Area FrontDetector	Height FrontDetector	Amount FrontDetector	Ret Area %	Peak Type
1	Wash	6.073	Acetate	Acetate	Acetate	Acetate	Acetate
2	Wash	6.078					
3		n.a.					
4		6.077					
5		6.075					
6		6.147					
7		6.062					
8		6.055					
9		6.055					
10	wash	6.085					
11	wash	n.a.					
12	A_32HS	6.082					
13	23_A_0_843	6.158					
14	23_A_0_884	6.155					
15	23_A_1_251	6.083					
16	23_A_1_273	6.082					
17	WASH	n.a.					
18	WASH	n.a.					
19	C_32HS	6.088					
20	23_C_0_834	6.087					
21	23_C_0_929	6.030					
22	23_C_1_233	6.083					
23	23_C_1_269	6.082					
24	WASH	n.a.					
25	WASH	n.a.					
26	D_32HS	6.087					
27	23_D_0_127	6.087					
28	23_D_0_720	6.088					
29	23_D_0_970	6.088					
30	23_D_1_234	6.085					
31	23_D_1_303	6.087					
32	WASH	n.a.					
33	WASH	n.a.					
34	E_32HS	6.115					
35	23_E_0_124	6.055					
36	23_E_0_784	6.088					
37	23_E_1_025	6.090					
38	23_E_1_281	6.087					
39	23_E_1_287	6.087					

Setting up a method

- 1.) Go to Create -> Instrument Method.
- 2.) Specify run time. Value shown allows for complete elution of VFAs.



- 3.) Choose AI_1310 (the autosampler).



4.) Specify sampling and injection parameters for autosampler. Values shown are typical.

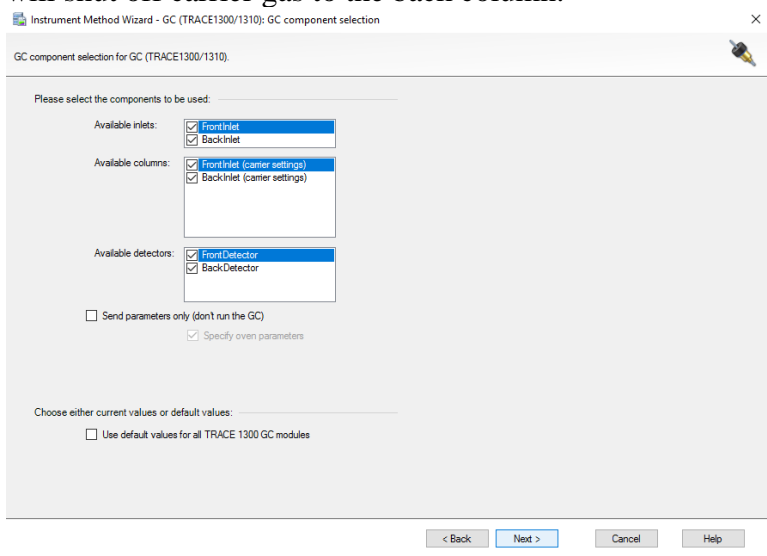
The screenshot shows the 'Instrument Method Wizard - AI_1310: Inject Options' dialog box. It is titled 'Inject Options for AI_1310.' and contains two main sections: 'Sampling Parameters' and 'Injection Parameters'.
Under 'Sampling Parameters':
- Draw speed: Slow (dropdown)
- Fill strokes: 3 (spin box, range [0..15])
- Air volume: 1.00 (spin box, range [0.00..3.00 µl])
- Sample depth: Bottom (dropdown)
Under 'Injection Parameters':
- Cold needle injection:
- GC type: TRACE_1300_1310 (dropdown)
- Pre injection delay time: 0.0 (spin box, range [0.0..63.0 s])
- Post injection delay time: 4.0 (spin box, range [0.0..63.0 s])
At the bottom are buttons for '< Back', 'Next >', 'Cancel', and 'Help'.

5.) Specify syringe wash program for autosampler. Values shown are typical.

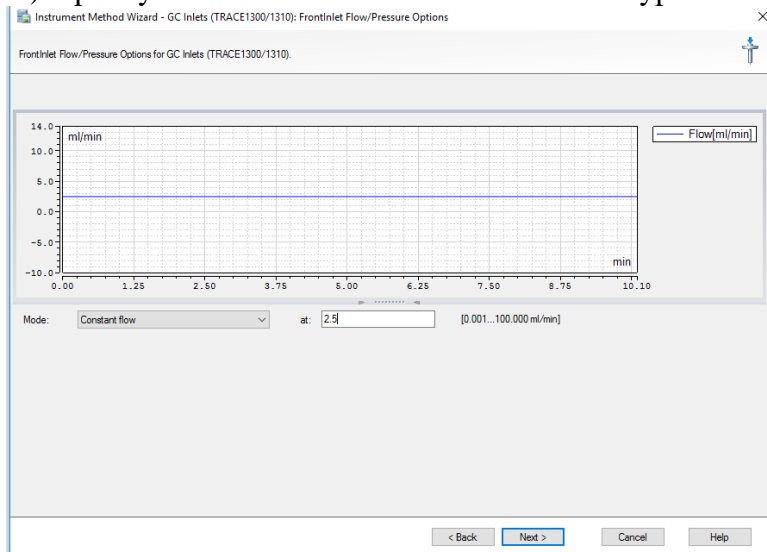
The screenshot shows the 'Instrument Method Wizard - AI_1310: Sampler Wash Program' dialog box. It is titled 'Sampler Wash Program for AI_1310.' and contains three sections: 'Pre Injection Washing Parameters', 'Sample Washing Parameters', and 'Post Injection Washing Parameters'.
Under 'Pre Injection Washing Parameters':
- Pre injection washing cycles: 8 (spin box, range [0..15])
- Pre injection washing vial(s): A (dropdown)
Under 'Sample Washing Parameters':
- Sample wash cycles: 0 (spin box, range [0..15])
Under 'Post Injection Washing Parameters':
- Post injection washing cycles: 3 (spin box, range [0..15])
- Post injection washing vial(s): C_D (dropdown)
At the bottom are buttons for '< Back', 'Next >', 'Cancel', and 'Help'.

6.) Select all GC components. Even though not all components are used for VFA analysis, they still need to be selected to avoid damaging the instrument. Unselecting BackInlet, for example,

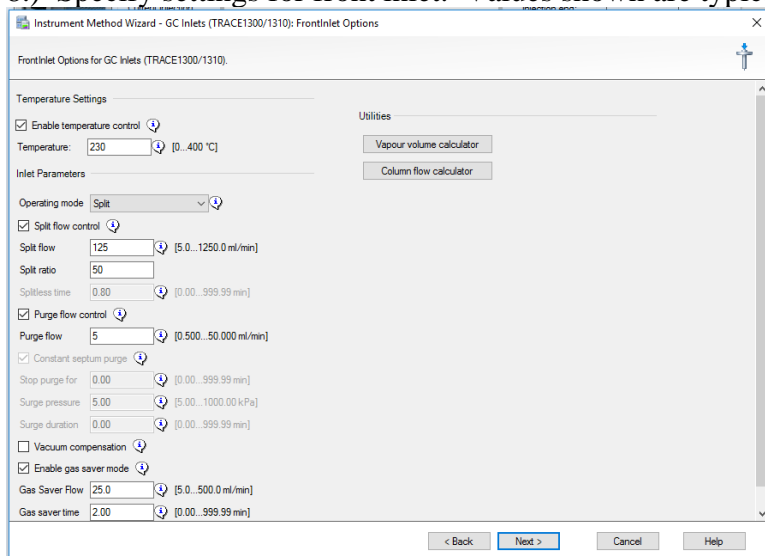
will shut off carrier gas to the back column.



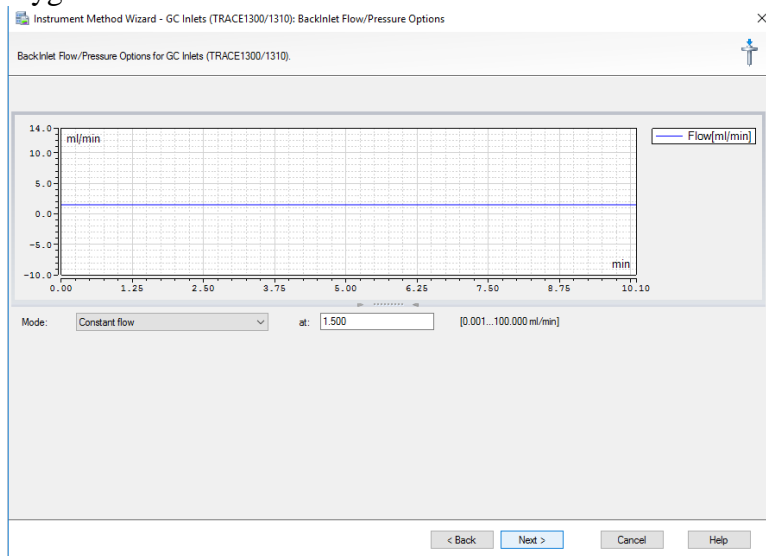
7.) Specify flow for front inlet. Value shown is typical.



8.) Specify settings for front inlet. Values shown are typical.



9.) Specify flow for back inlet. Value shown is minimum needed to purge the back column of oxygen.



10.) Specify settings for back inlet. Values shown are minimum needed to purge the system of oxygen and prevent condensation of water.

Instrument Method Wizard - GC Inlets (TRACE1300/1310): BackInlet Options

BackInlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

Enable temperature control

Temperature: 110 [0..400 °C]

Utilities

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

Split flow control

Split flow: 5.0 [5.0..1250.0 ml/min]

Split ratio: 3

Splitless time: 1.00 [0.00..999.99 min]

Purge flow control

Purge flow: 2.000 [0.500..50.000 ml/min]

Constant septum purge

Stop purge for: 0.00 [0.00..999.99 min]

Surge pressure: 5.00 [5.00..1000.00 kPa]

Surge duration: 0.00 [0.00..999.99 min]

Vacuum compensation

Enable gas saver mode

Gas Saver Flow: 15.0 [5.0..500.0 ml/min]

Gas saver time: 3.00 [0.00..999.99 min]

< Back Next > Cancel Help

11.) Specify oven temperature. Values shown led to good separation of VFAs.

Instrument Method Wizard - GC (TRACE1300/1310): GC Oven Settings

GC Oven Settings for GC (TRACE1300/1310).

Prep Run Timeout: 999.00 [0.00..999.99 min]

Oven equilibration time: 0.50 [0.00..999.99 min]

Ready delay: 0.00 [0.00..99.99 min]

Mode: Ramped temperature

Temperature [°C]

No	Retention time [min]	Rate [°C/min]	Target value [°C]	Hold time [min]
1	0.000	Run		
2	0.500	0.00	45.0	0.50
3	10.100	25.00	235.0	2.00
4		New Row		
5	10.100	StopRun		

Insert new row

Delete row(s)

< Back Next > Cancel Help

12.) Specify column settings. These are default values that should not be changed.

Instrument Method Wizard - GC (TRACE1300/1310): Column Configuration Settings

Column Configuration Settings for GC (TRACE1300/1310).

FrontColumn

Validate column configuration

Description

Length [0.10..200.00 m]

Diameter [0.050..0.999 mm]

Film thickness [0.00..20.00 µm]

< Back Next > Cancel Help

13.) Specify front detector settings. These are typical values.

Instrument Method Wizard - GC Detectors (TRACE1300/1310): FrontDetector Options

FrontDetector Options for GC Detectors (TRACE1300/1310).

Detector type: Flame Ionization Detector (FID)

Signal Settings

Data collection rate [1..600 Hz]

Detector Settings

Detector temperature control

Detector temperature [0..450 °C]

Flame

Enable flameout retry

Ignition threshold [0.0..10.0 pA]

Peak width

Gas Settings

Air flow control

Air flow [5.0..500.0 ml/min]

Makeup gas flow control

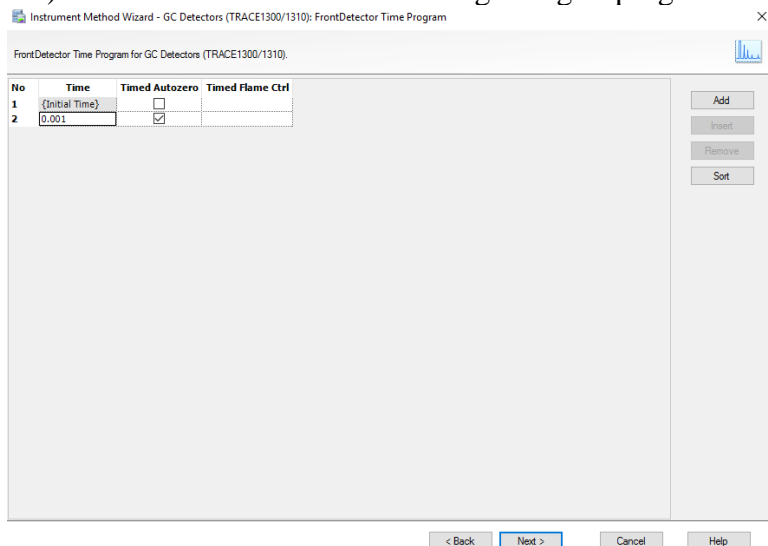
Makeup gas flow [1.0..50.0 ml/min]

Hydrogen flow control

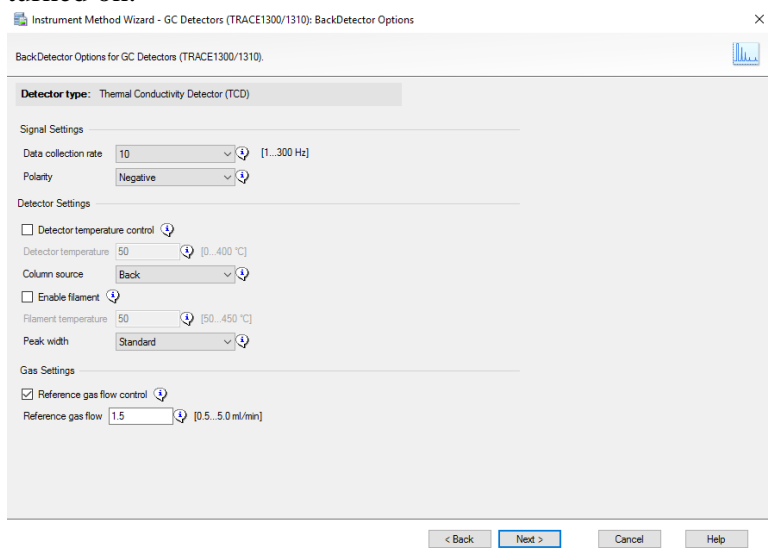
Hydrogen flow [1.0..100.0 ml/min]

< Back Next > Cancel Help

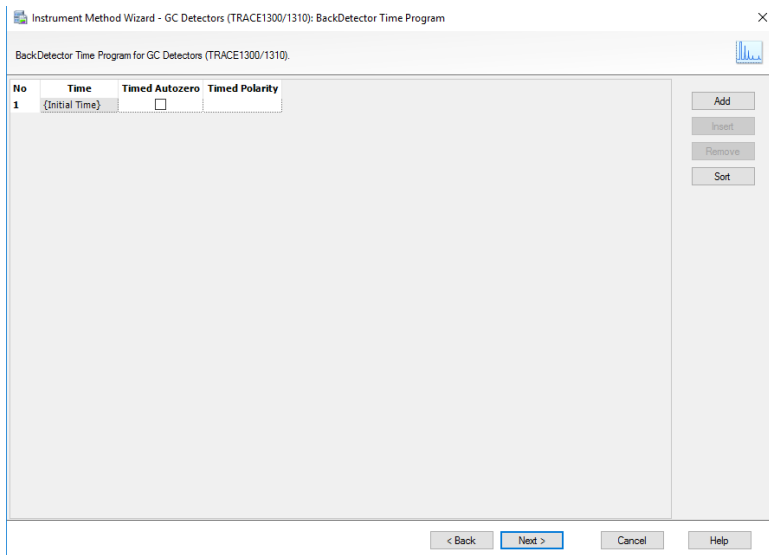
14.) Autozero front detector near beginning of program. This will set the signal to zero.



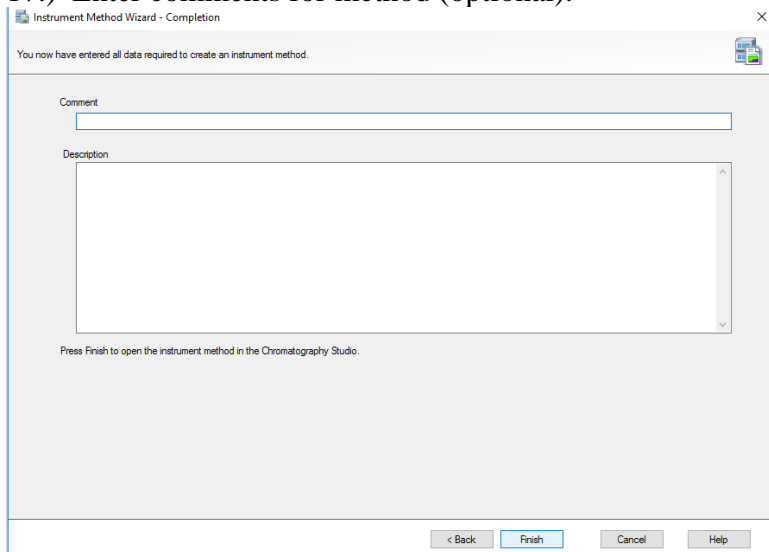
15.) Specify settings for back detector. Turn filament off to preserve life. Turn reference gas as a precaution: it will prevent filament from being damaged if filament is still hot from being turned on.



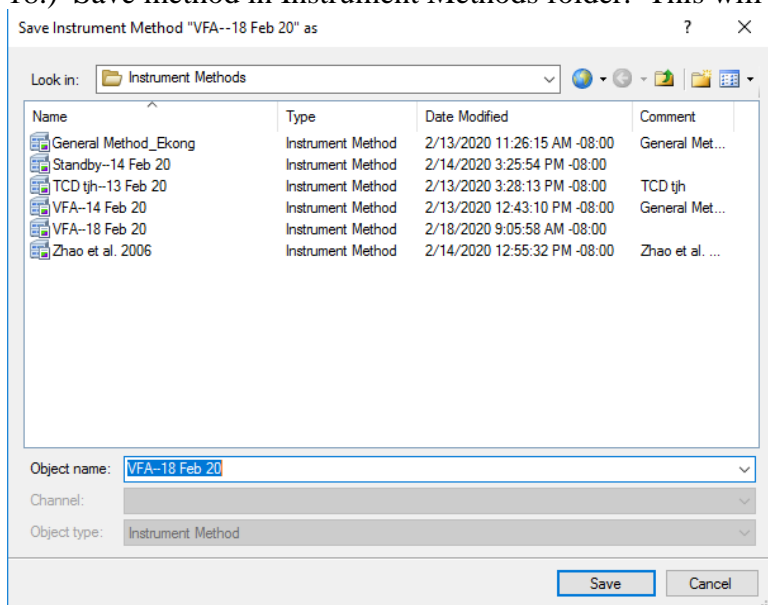
16.) Do not autozero back detector near beginning of program. Back detector will not be used, so autozeroing is unnecessary.



17.) Enter comments for method (optional).



18.) Save method in Instrument Methods folder. This will make it easy to find later.



Notes

- 1.) Samples must always contain 50% of higher methanol, or the column will be damaged. Ethanol or other alcohols may be used in place of methanol.
- 2.) Formic acid is added to samples to clean column. Otherwise, VFA from one sample can be carried over to the next. Formic acid also protonates VFA to make them volatile.
- 3.) Wash samples can be run for additional cleaning of the column. This may be necessary when one sample has high VFA, and the following sample has low VFA. Blank samples are run to verify that the column is clean and there is no carryover.
- 5.) Volumes of supernatant, external, and internal standard are approximate. They can be changed to accommodate samples that have very high or low VFA concentrations.

Measuring hydrogen and methane of culture headspace samples

Preparing samples and standards.

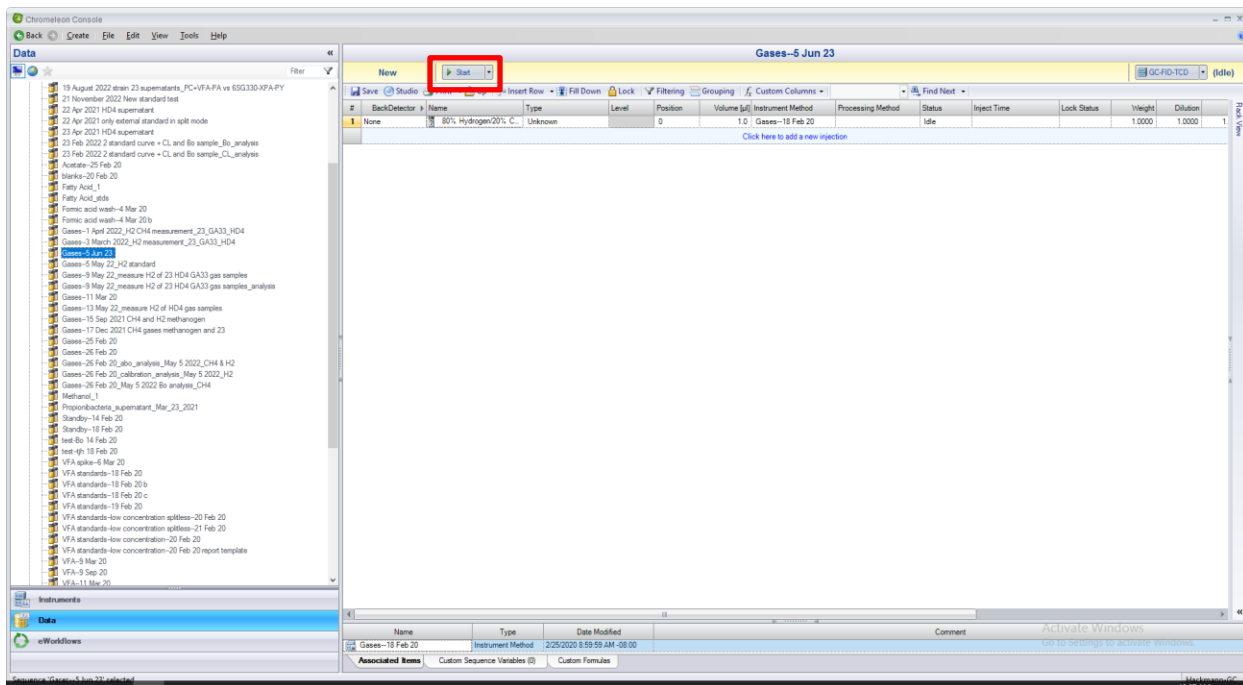
- 1.) Obtain a bacterial culture in a Balch tube (or a similar sample).
- 2.) Prepare a syringe to sample the gas. Attach a needle with a beveled tip (Hamilton 81356) to a 1-mL gas-tight with sample lock valve (Hamilton 81356).
- 3.) Flush the syringe and needle with CO₂. Insert syringe into gassing probe dispensing CO₂, and pull plunger of syringe in and out several times.
- 4.) Obtain the gas sample. Insert needle of syringe through stopper of Balch tube and withdraw 1-mL of gas. After filling, close sample lock valve.
- 5.) Prepare syringe for GC. Remove needle with beveled tip, and attach needle with blunt, cone tip (Hamilton 7784-01). The cone tip is safe for the inlet septum of the GC.
- 6.) Prepare standards similarly, but use gas of known composition. For example, volumes of 0 to 1 mL pure H₂ gas can be used as standard for H₂.

Preparing GC for analysis of samples and standards

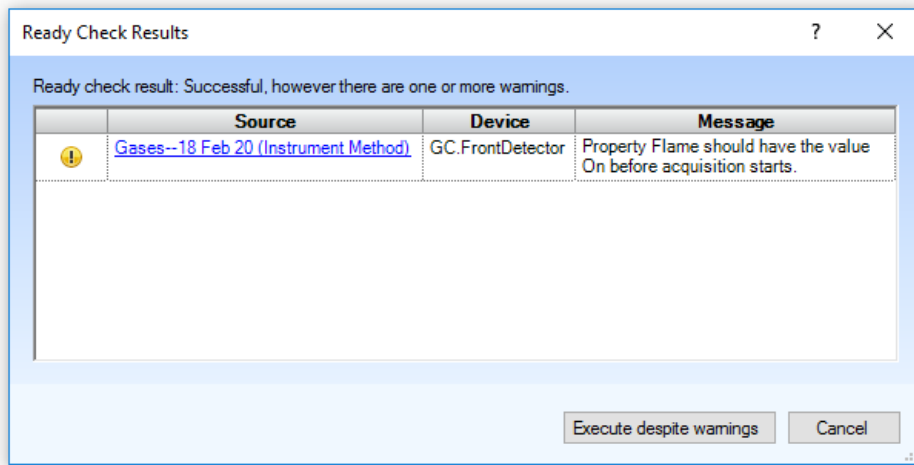
- 1.) Start GC and set up for gas analysis according to *Turning on GC and placing into standby mode*.
- 2.) Turn on TCD detector according to *Turning on detectors*.
- 3.) In Chromeloen software, create a sequence (list of samples to be analyzed). Follow *Setting up a sequence*.

Analyzing samples and standards

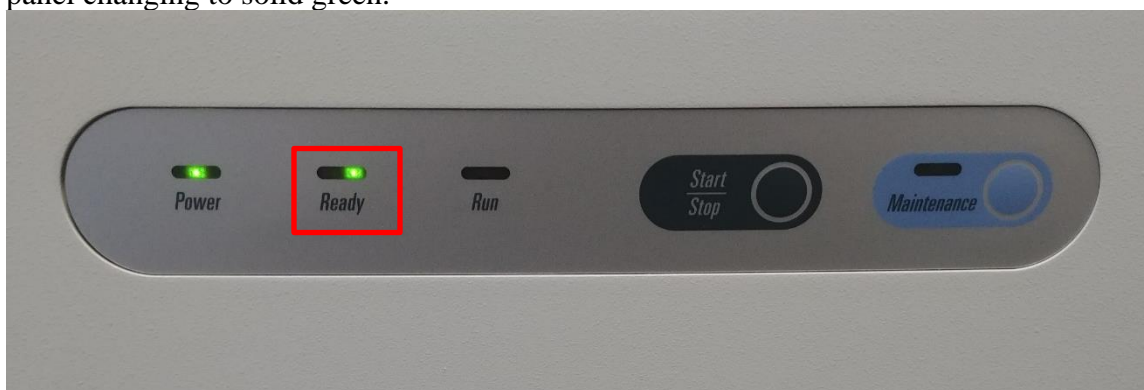
- 1.) Open the sequence.
- 2.) Click Start.



3.) Review any warnings. If they are OK, click “Execute despite warnings”.



4.) Wait for instrument to be ready for injection. This will be indicated by Ready light on front panel changing to solid green.



5.) Inject sample. Insert needle about 1” into back injector, open sample lock valve, and depress plunger quickly.



Inserting the needle about 1” ensures the sample is injected an ideal location (middle of liner). Though 1” is ideal, injector will not be damaged if needle is inserted farther.

6.) While needle is still in injector, press “Start” on front panel. This will communicate to the instrument that the sample has been injected.



7.) Hold the needle in the injector for a total of 4 s, then remove. Holding the needle for 4 s mimics the autosampler.

8.) Allow run to complete, then repeat injection all samples and standards.

Shutting down GC after analysis of samples and standards

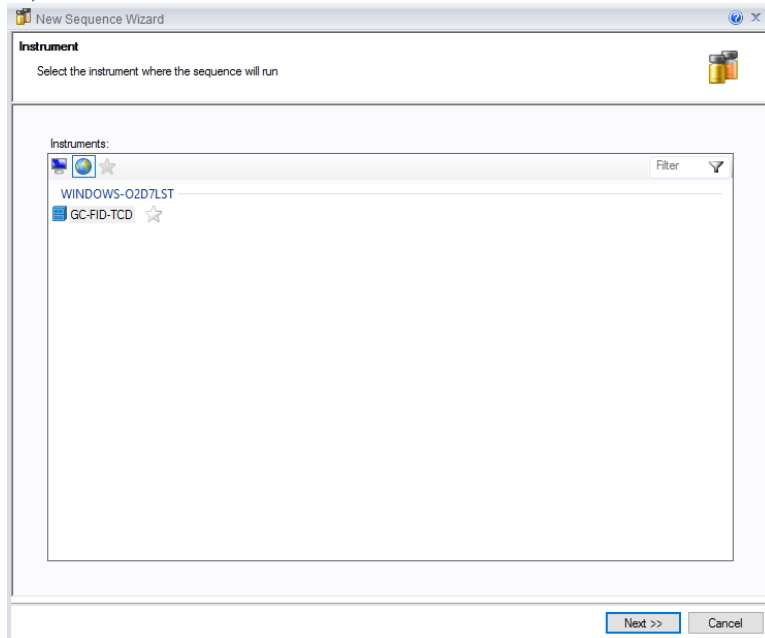
1.) Place the instrument back into standby mode according to *Placing back into standby mode*.

2.) If no samples will be run in the next day or two, proceed to turn off the instrument according to *Turning off*.

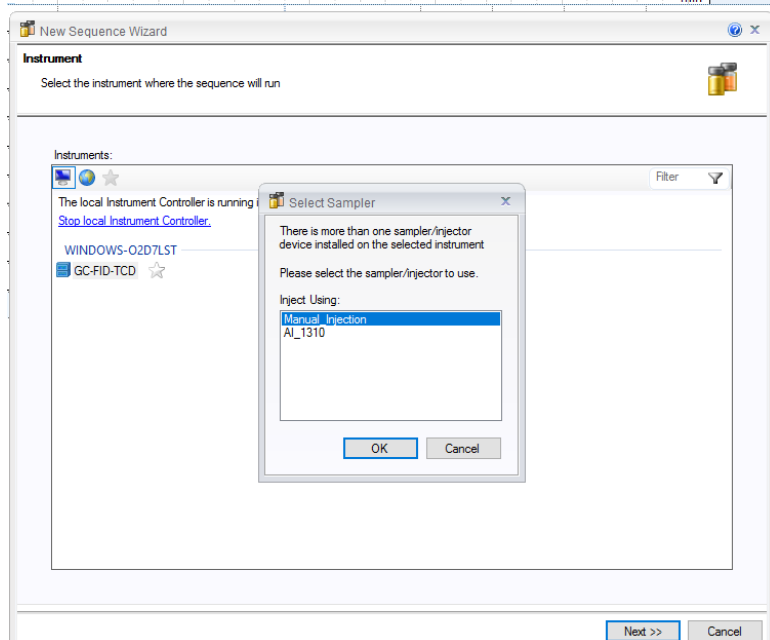
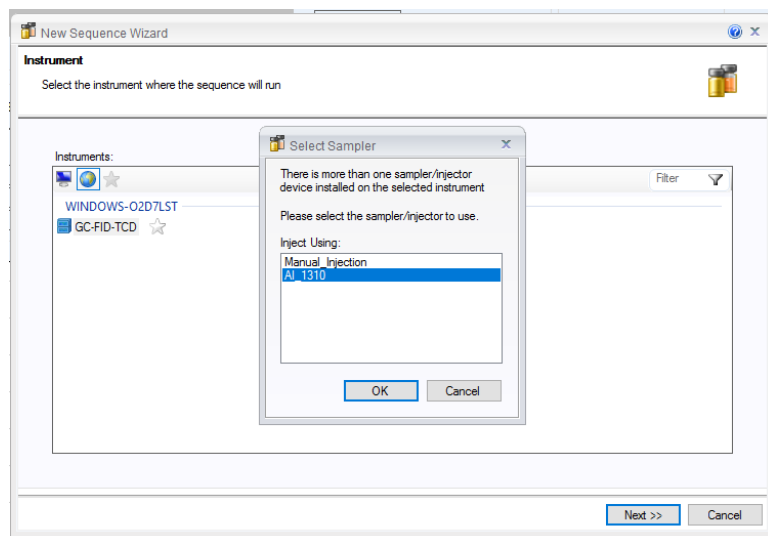
Setting up a sequence

1.) In Chromeleon 7 software, choose Create ->Sequence.

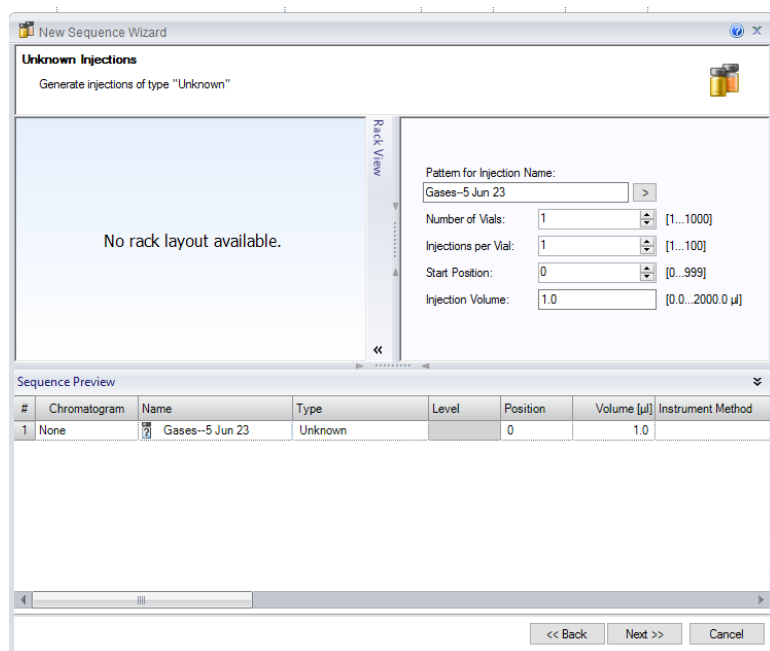
2.) Choose GC-FID-TCD.



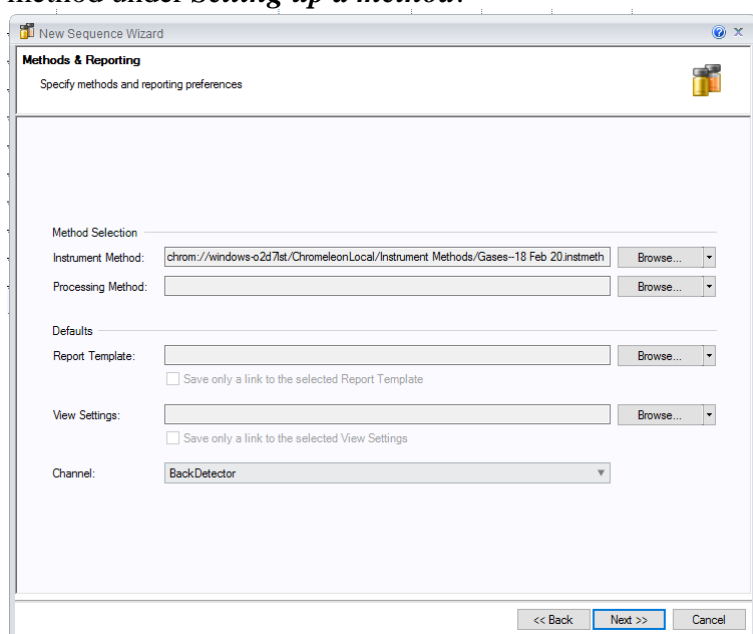
3.) Choose Manual_injection (the autosampler).



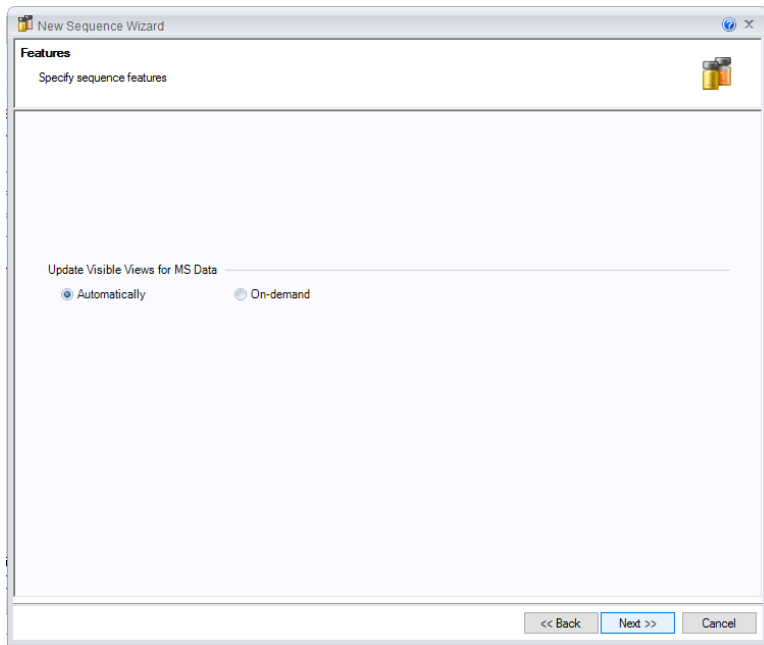
4.) Click Next. (Any information on this screen can be edited later.)



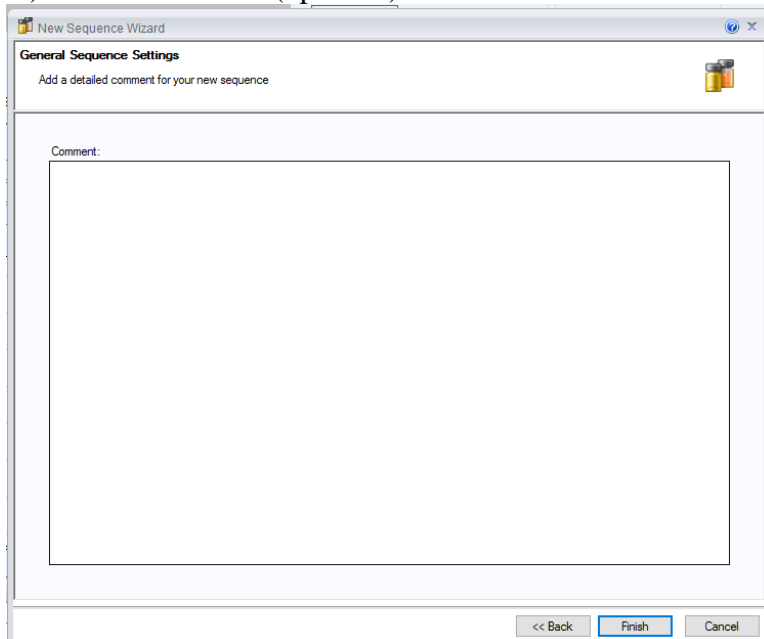
5.) Under Instrument Method, choose “Gases—25 Feb 20.instmeth”. See details for this method under *Setting up a method*.



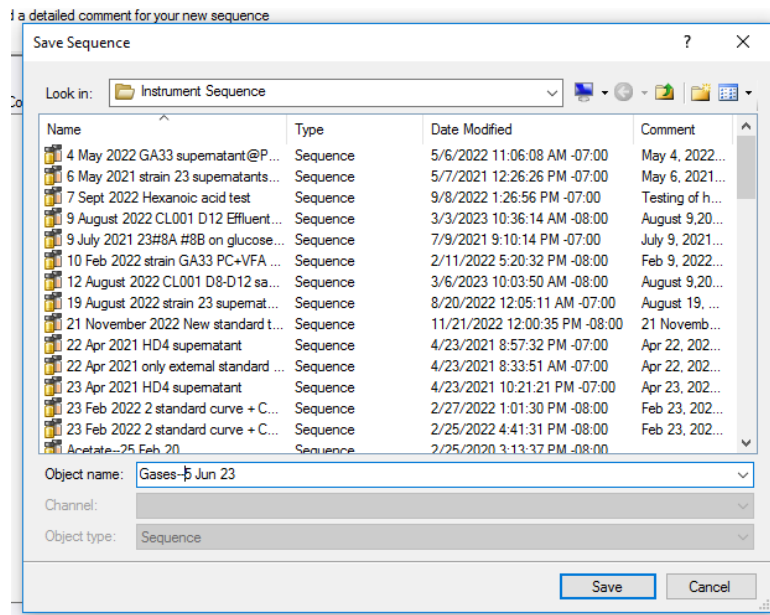
6.) Choose Automatically.



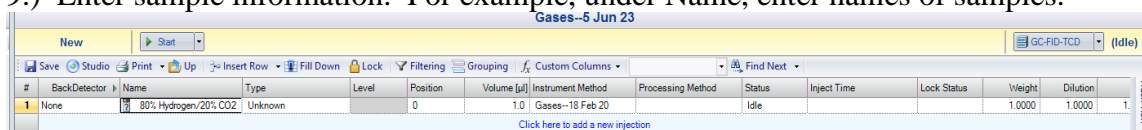
7.) Enter comments (optional).



8.) Save the sequence. Save in Instrument Sequence folder so that it is easy to find.



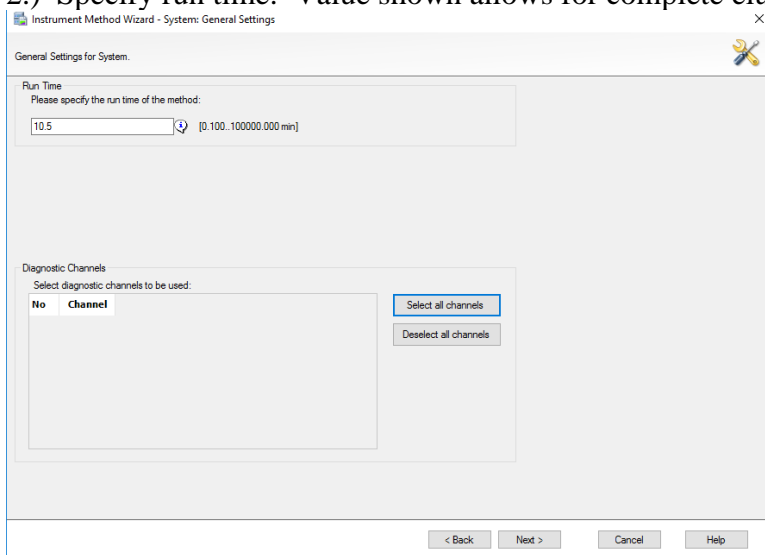
9.) Enter sample information. For example, under Name, enter names of samples.



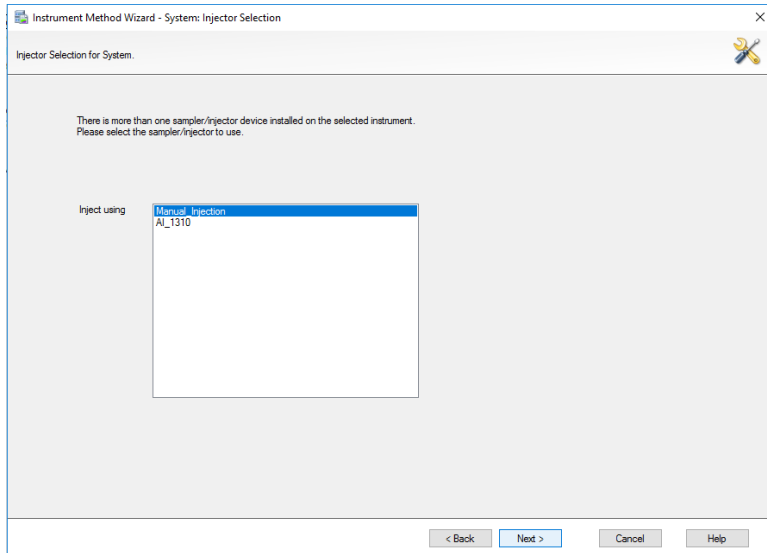
Setting up a method

1.) Go to Create -> Instrument Method.

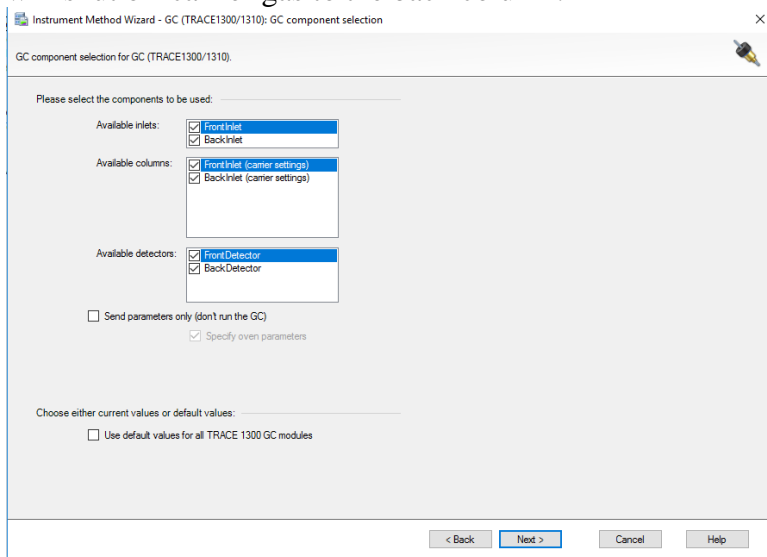
2.) Specify run time. Value shown allows for complete elution of gases.



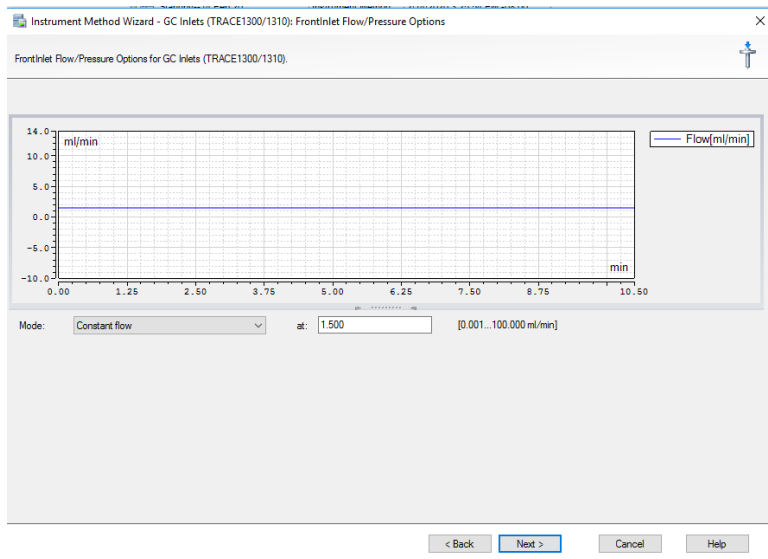
3.) Choose Manual_Injection



4.) Select all GC components. Even though not all components are used for VFA analysis, they still need to be selected to avoid damaging the instrument. Unselecting BackInlet, for example, will shut off carrier gas to the back column.



5.) Specify flow for front inlet. Value shown is minimum needed to purge the back column of oxygen.



6.) Specify settings for front inlet. Values shown are minimum needed to purge the system of oxygen and prevent condensation of water.

Instrument Method Wizard - GC Inlets (TRACE1300/1310): FrontInlet Options

FrontInlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

Enable temperature control

Temperature: 110 [0..400 °C]

Utilities

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

Split flow control

Split flow: 5.0 [5.0..1250.0 ml/min]

Split ratio: 3

Splitless time: 0.80 [0.00..999.99 min]

Purge flow control

Purge flow: 2.000 [0.500..50.000 ml/min]

Constant septum purge

Stop purge for: 0.00 [0.00..999.99 min]

Surge pressure: 5.00 [5.00..1000.00 kPa]

Surge duration: 0.00 [0.00..999.99 min]

Vacuum compensation

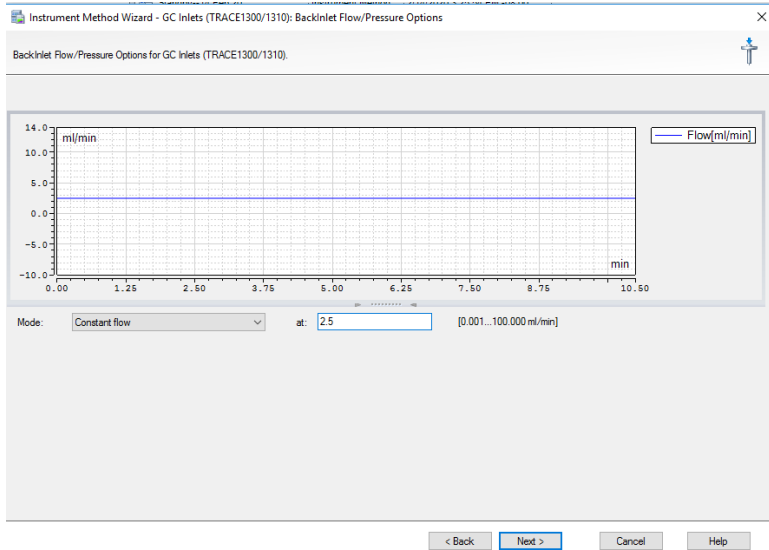
Enable gas saver mode

Gas Saver Flow: 25.0 [5.0..500.0 ml/min]

Gas saver time: 2.00 [0.00..999.99 min]

< Back Next > Cancel Help

7.) Specify flow for back inlet. Value shown is typical.



8.) Specify settings for back inlet. Values shown are typical.

Instrument Method Wizard - GC Inlets (TRACE1300/1310): BackInlet Options

BackInlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

Enable temperature control

Temperature: 150 [0...400 °C]

Utilities

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

Split flow control

Split flow: 75 [5.0...1250.0 ml/min]

Split ratio: 30

Splitless time: 1.00 [0.00...999.99 min]

Purge flow control

Purge flow: 3 [0.500...50.000 ml/min]

Constant septum purge

Stop purge for: 0.00 [0.00...999.99 min]

Surge pressure: 5.00 [5.00...1000.00 kPa]

Surge duration: 0.00 [0.00...999.99 min]

Vacuum compensation

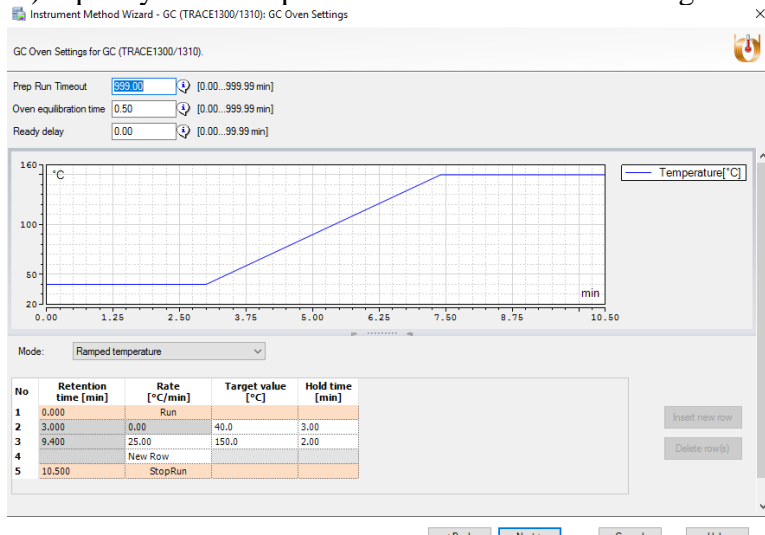
Enable gas saver mode

Gas Saver Flow: 15.0 [5.0...500.0 ml/min]

Gas saver time: 3.00 [0.00...999.99 min]

< Back Next > Cancel Help

9.) Specify oven temperature. Values shown led to good separation of gases.



10.) Specify column settings. These are default values that should not be changed.

Instrument Method Wizard - GC (TRACE1300/1310): Column Configuration Settings

Column Configuration Settings for GC (TRACE1300/1310)

FrontColumn

Validate column configuration

Description: [Empty]

Length: 30.00 [0.10..200.00 m]
 Diameter: 0.320 [0.050..0.999 mm]
 Film thickness: 1.00 [0.00..20.00 µm]

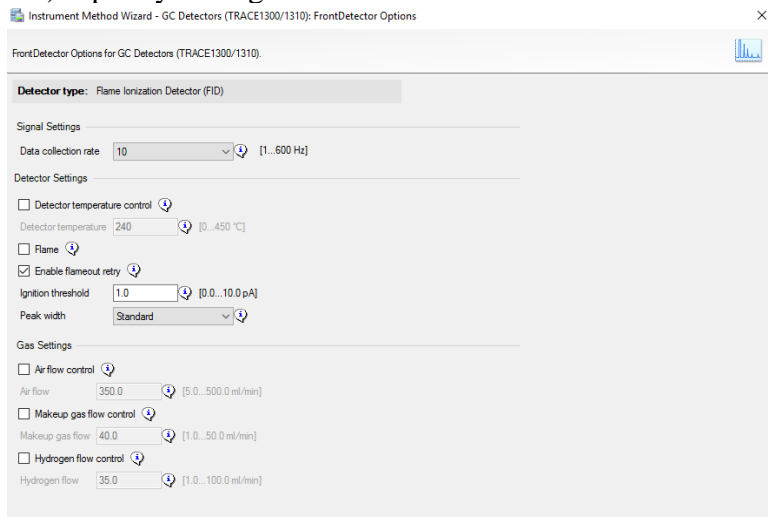
BackColumn

Validate column configuration

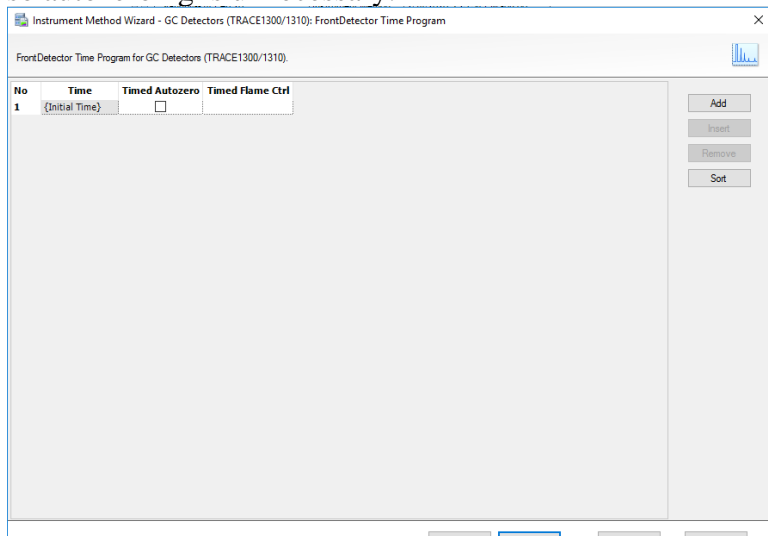
Description: TG-BOND Q

Length: 30.00 [0.10..200.00 m]
 Diameter: 0.530 [0.050..0.999 mm]
 Film thickness: 20.00 [0.00..20.00 µm]

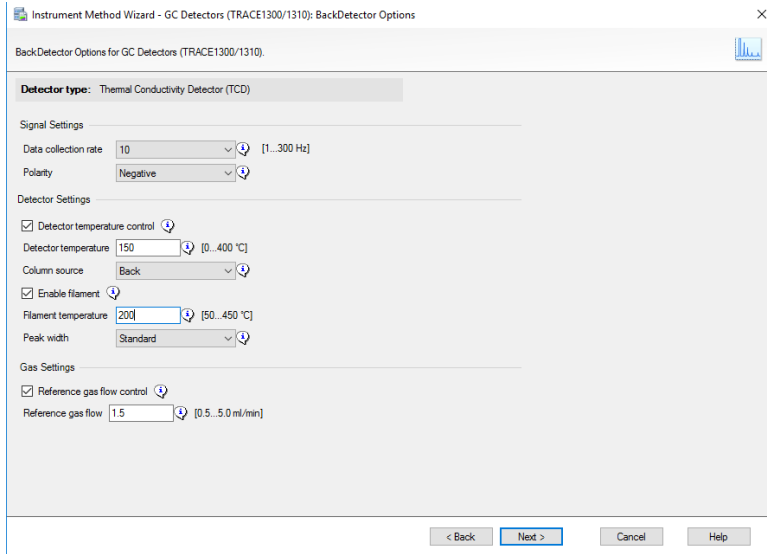
11.) Specify settings for front detector. These values will ensure detector is off.



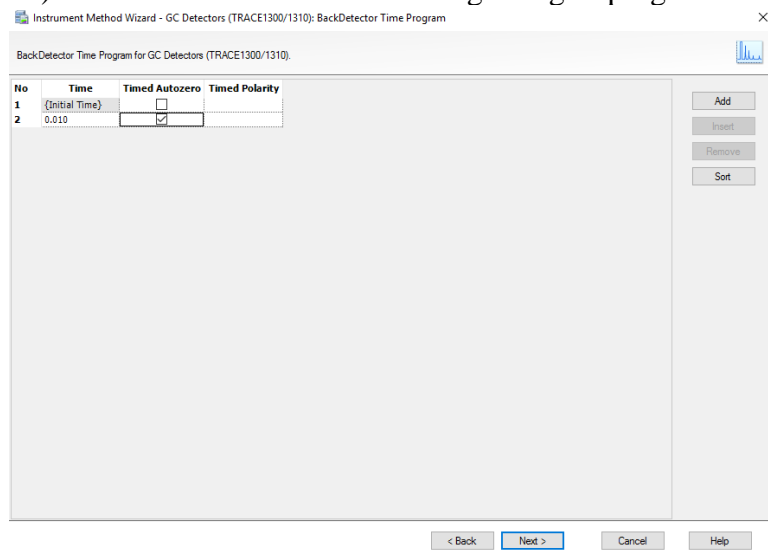
12.) Do not autozero front detector near beginning of program. Front detector will not be used, so autozeroing is unnecessary.



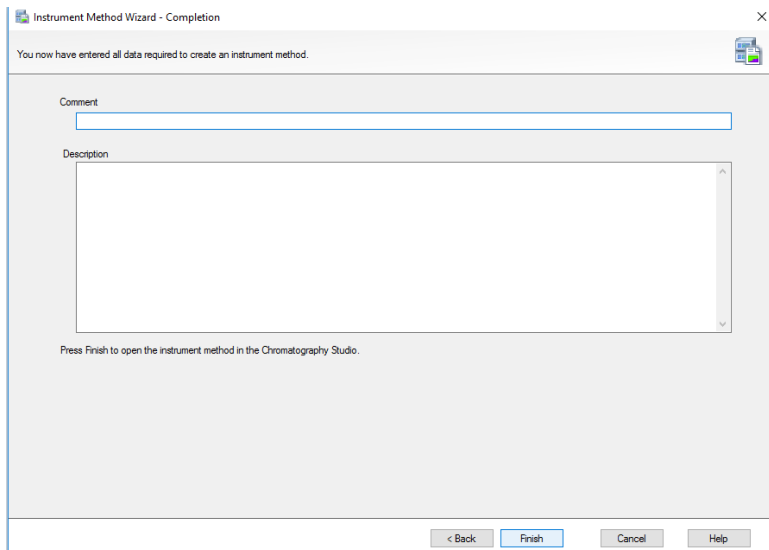
13.) Specify settings for back detector. Values shown are typical



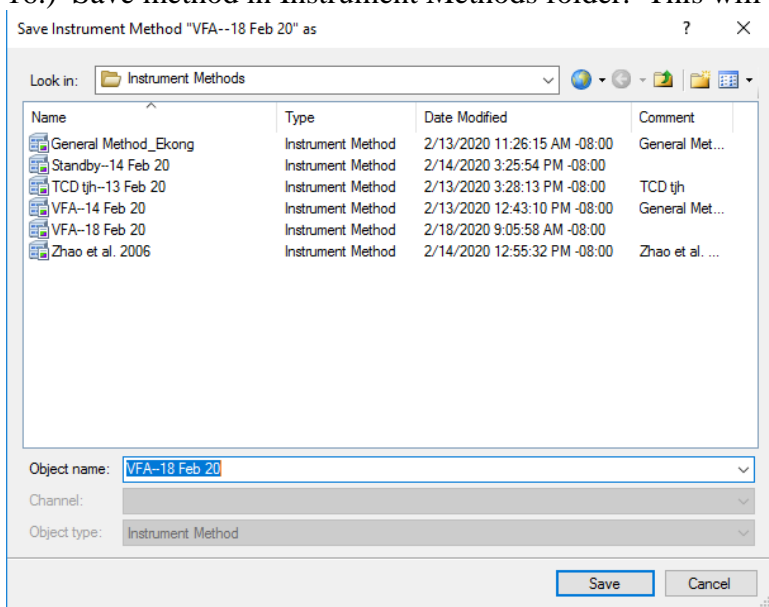
14.) Autozero back detector near beginning of program. This will set the signal to zero.



15.) Enter comments for method (optional).



16.) Save method in Instrument Methods folder. This will make it easy to find later.



SECTION IV: ENZYMATIC ASSAYS

This section shows how to assay enzyme activities of microbes. It also describes measurement of analytes using purified enzymes.

Preparation of cell extracts for enzymatic assays using a French press

Adapted from (67, 68)

See also YouTube videos at

https://www.youtube.com/watch?v=FDMMY4krU_I&list=PLv_wI4iYQb1Azhpo8l0ZW2VBKo_sj9rVGJ

Preparation of cell suspension

- 1.) Inoculate cultures. Inoculate 4 tubes containing 9-mL media with 0.1 mL bacteria. Record OD₆₀₀ over time.
- 2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD₆₀₀ = 1 and requires 3 to 4.5 h from inoculation. Place tubes on ice and transfer to 40-mL screw-cap centrifuge tube.
- 3.) Centrifuge at 4°C, and wash twice with buffer. For *S. bovis*, 10,000 g for 5 min is sufficient for each centrifugation. Buffer should be the same used that will be used in later enzymatic assays.
- 3.) Resuspend pellet in 3.7-mL buffer. Keep on ice.

Preparation of mini pressure cell

- 1.) Locate parts of mini pressure cell (Glen Mills 5500-FA-004) (Fig. 1). Place in refrigerator until cold (requires several hours).
- 2.) Assemble pressure cell (Fig. 3).
 - (a) Lubricate the rubber gaskets on the piston by applying thin layer of glycerol. Use gauze to spread glycerol and clean up excess.
 - (b) Insert piston into cell body. Place on stand. Piston will be supported by a jackscrew.
 - (c) Using pipette, load sample body with the cell suspension (3.7 mL).
 - (d) Assemble closure plug by screwing in sample outlet tube and flow valve assembly. Lubricate rubber gaskets by applying thin layer of glycerol.
 - (e) Insert closure plug into cell body. Make sure it is secure by pushing down gently, while turning the flow valve assembly counterclockwise to let air bubbles escape.
 - (f) Remove any remaining air from cell body. Raise jackscrew to force piston into cell body. Continue until only liquid (no air bubbles) escapes from sample outlet tube.
 - (g) Turn flow valve assembly clockwise until it is hand tight. Overtightening will damage assembly.

Preparation of the press

- 1.) Inspect cell press (Glen Mills 5500-000011) (Fig. 3). Make sure emergency stop is engaged; turn it clockwise to pop out (if not already). Also, make sure pause/run switch is set to “Pause”, and ratio selector is switched to “Down”.
- 2.) Place mini pressure cell on press. When moving cell, support the bottom to prevent the closure plug from dropping out.

- 3.) Rotate the piston so that the T-handle points towards the user (Fig. 3). If not, it will not raise the correct position, and the press will be damaged.
- 4.) Swing the safety clamp over the pressure cell and tighten with the thumbscrew (Fig. 4).

Operation of the press

- 1.) Turn on power switch on back of unit.
- 2.) Set ratio selector to “Low”. Never set to “High” when using the mini-pressure cell.
- 3.) Set pause/run switch to “Run”
- 4.) Turn pressure increase knob to read 800 psi on the gauge. This corresponds to 16,000 psi in the pressure cell (see pressure conversion table). Never exceed 1,000 psi on the gauge.
- 5.) Watch the pressure cell and lower platen as they rise. Wait until it rises fully and piston makes contact with the top plate (Fig. 5).
- 6.) Hold centrifuge tube by sample outlet tube to collect sample (Fig. 6).
- 7.) Allow sample (cell extract) to flow out by turning valve assembly counterclockwise. Turn until cell extract flows out at rate of 10 to 15 drops per minute.
- 8.) As cell extract flow outs, watch cell body and lower platen. They will continue to rise, forcing piston into cell body.
- 9.) Continue operation until the pressure cell and lower platen stop rising. This will happen after reaching the the “STOP” line on the piston (Fig. 6) (when the piston has been forced ~5 cm into the cell). The unit automatically stops when reaching this point; no action is needed on part of the user.
- 10.) Close the centrifuge tube containing cell extract and place on ice. The yield of cell extract is ~3 mL.

Shutdown of cell press

- 1.) Set ratio selector to “Down”. The pressure cell and lower platen will move down.
- 2.) Wait until the platen moves fully downwards (to its original position). Turn the pressure increase knob to read 0 psi on the gauge.
- 3.) Turn off the power switch

Processing of cell extract

- 1.) Centrifuge cell extract (10,000 g, 4°C, 10 min).
- 2.) Aspirate the supernatant. Discard pellet (containing unbroken cells).

3.) Store the cell extract at -80°C .

Clean up of pressure cell

1.) Disassemble the pressure cell

2.) Wash all parts with distilled water. Flush the closure plug with water.

3.) Dry with towel or compressed air and return parts to refrigerator.

Notes

i) In this example, the ratio of culture (36 mL) to cell suspension (3.7 mL) is ~ 0.1 vol/vol. Ratios between 0.01 and 0.1 should be used.

ii) In this example, 3.7 mL cell suspension is loaded into the pressure cell. A lower volume can also be used. Note that 0.7 mL of cell suspension is lost (retained in the cell body) during operation of the press. Low volumes will thus lead to recovery little cell extract.

iii) This model of cell press (Glen Mills 5500-FA-004) automatically stops when the “STOP” line on the piston is reached. This prevents the piston from being forced too far into the cell body. Older models lack this safety feature, and the cell body or press may be damaged if the user is not careful.

Fig. 1. Overview of mini pressure cell.

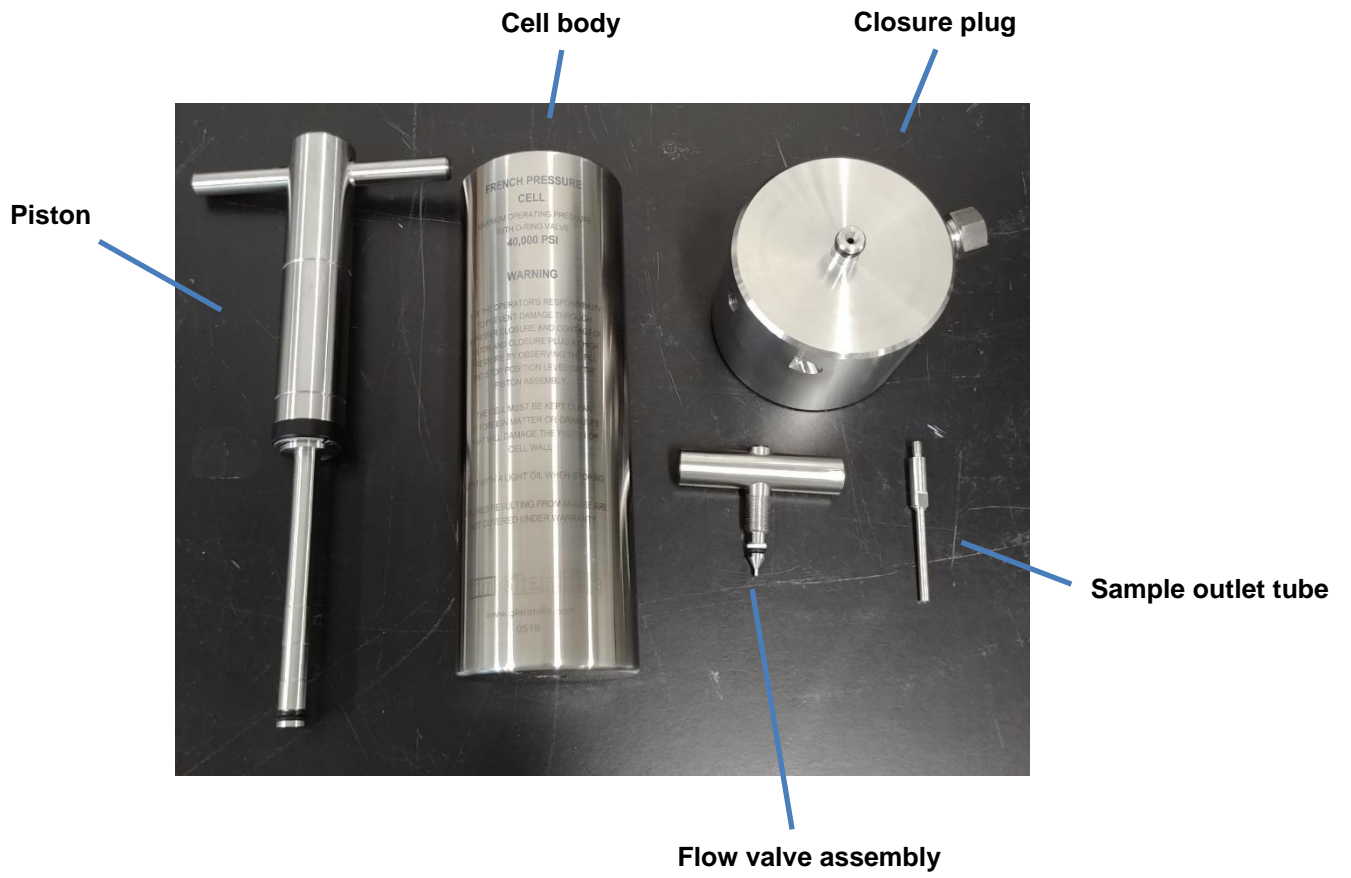


Fig. 2. Pressure cell assembled and loaded on stand



Fig. 3. Overview of cell press.

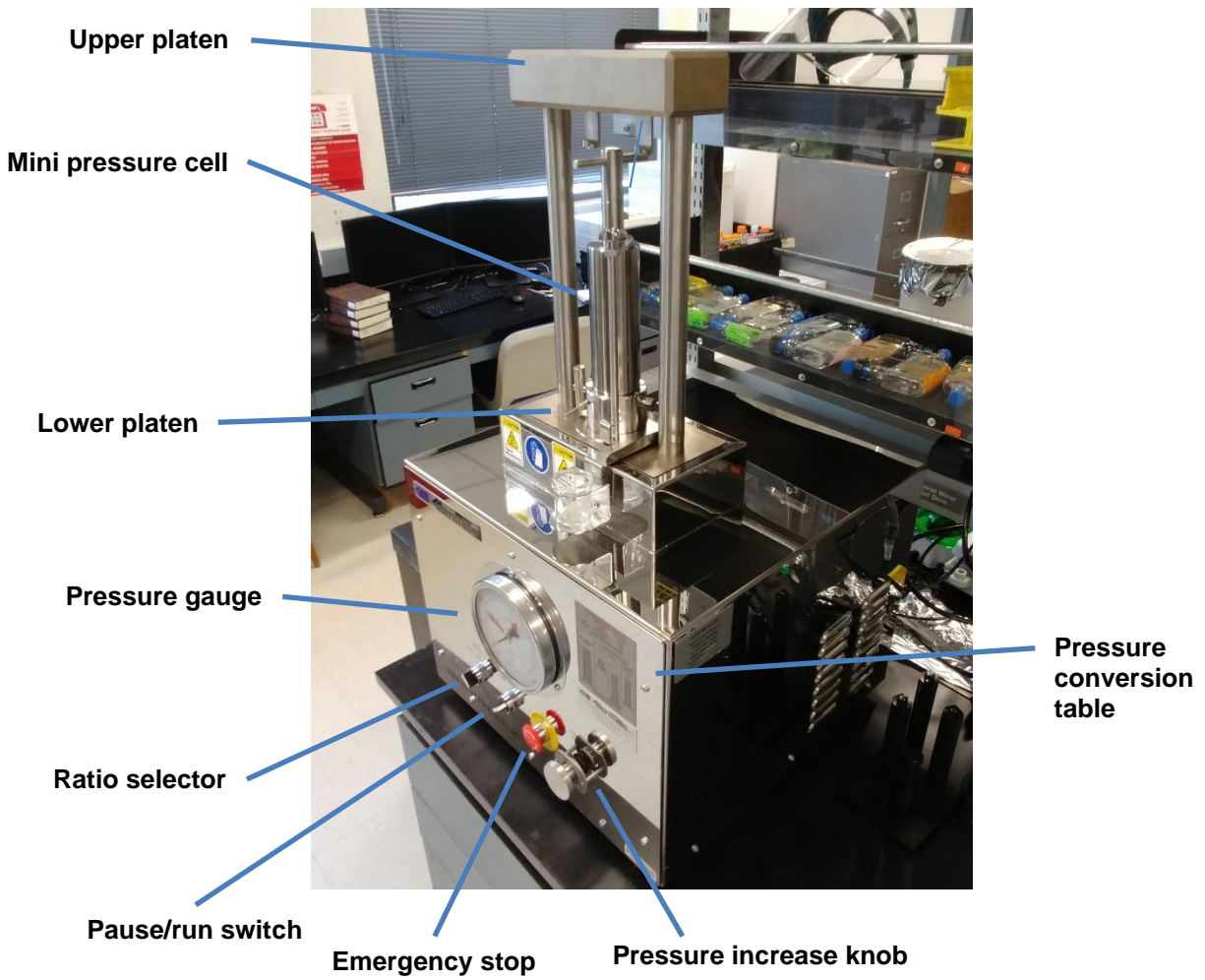


Fig. 4. Safety clamp and thumb screws used to secure cell body

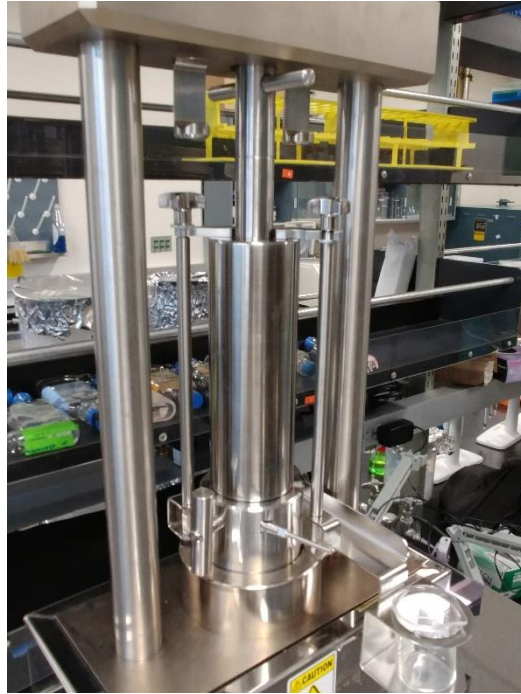


Fig. 5. Operation of cell press. (A) Before operation. Cell body and lower platen are in fully lowered position. (B) During operation. Cell body and lower platen are in intermediate position. Piston has made contact with upper platen. (C) At end of operation. Cell body and lower platen are in fully raised position. The STOP line on piston has been reached. (D) Close up of STOP line in (C).

(A)



(B)



(C)



(D)



Fig. 6. Collection of cell extract in centrifuge tube.



Preparation of cell membrane and cytoplasmic contents by ultracentrifugation

1.) Prepare cell membrane and cytoplasmic contents according to **Fig. 1**. For steps requiring buffer, use the same type of buffer as used in later enzymatic assays. To prepare cell extract, follow *Preparation of cell extracts for enzymatic assays using a French press*. For use performing the 208 000 g centrifugation, follow *Use of Beckman L8-70M ultracentrifuge*.

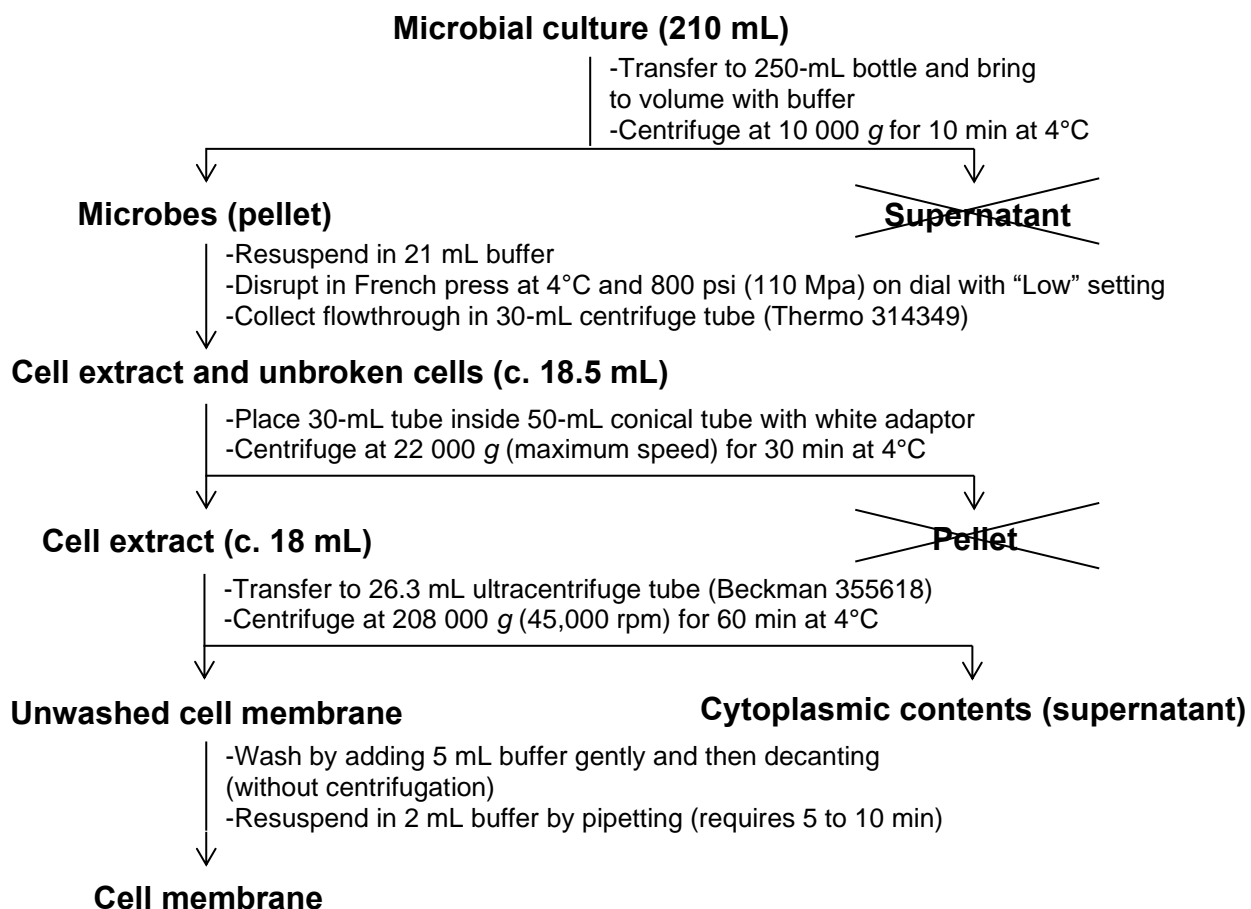
2.) Store cell membrane and cytoplasmic contents at -80°C.

Notes

(i) For samples that need to be prepared anaerobically, flush tubes with N₂ while open and before sealing. Replace the 250-mL bottle with 40-mL centrifuge tubes (Thermo 3139-0050), which are easier to flush with N₂. Use anaerobic buffers and store cytoplasmic contents and cell membrane under N₂.

(ii) Some samples may need to be treated with nuclease. For those, add 25 U/mL Pierce Universal Nuclease after resuspending the microbial pellet in buffer.

Fig. 1. Flowchart for preparing cell membrane and cytoplasmic contents



Use of Beckman L8-70M ultracentrifuge

- 1.) Pre-cool Beckman 70Ti rotor to 4°C by placing in cold room overnight.
- 2.) Turn on Beckman L8-70M ultracentrifuge using switch on front.
- 3.) Open door on top and place rotor on top of the spindle. Unlike with most centrifuges, the rotor just rests on the spindle, and it is not screwed or snapped onto it.
- 3.) Pre-cool ultracentrifuge to 4°C. Press VACUUM button to turn on vacuum and TEMP button to start cooling. Approximately 3 hours (or less) are required to reach 4°C.
- 4.) Fill a centrifuge tube (Beckman 355618) with sample (e.g., cell extract). Fill a second tube with water and balance it to within 0.001 g of the sample tube. The minimum volume for the tubes is reported as 15 mL, but using as little as 2.5 mL has been successful.
- 5.) Place tubes inside rotor. Press VACUUM button to release vacuum, then open door of ultracentrifuge. Remove lid of rotor, place tubes inside, and then replace lid.
- 6.) Confirm final settings, then start run. Using buttons, set TEMP to 4°C, SPEED to 45,000 rpm and TIME to 1:00. PRESS VACUUM, ENTER/DISPLAY, then START. The speed of 45,000 rpm corresponds to 208,000 g and may need to be adjusted.
- 7.) Monitor ultracentrifuge until it reaches full speed (requires 5 min). Speed will increase to approximately 3,000 rpm, then it will hold until vacuum decreases. Once vacuum reaches 200 microns, speed will increase to 45,000 rpm.
- 8.) Allow ultracentrifuge to complete run. Spinning down requires 4 min.
- 9.) Remove tubes from rotor. Press VACUUM button to release vacuum, then open door of ultracentrifuge and remove lid of rotor. Lift tubes with the tip of a 1-mL syringe (placed in hole of tube lid).
- 10.) If planning another run, press replace lid and close door. To keep unit cool, press VACUUM and TEMP buttons.

Reagents for enzymatic assays

Acetate kinase (1,000 U/mL)

Reference: (69)

Dilute 4 μL acetate kinase (available from Megazyme as special order) with 96.0 μL of 3.2 M ammonium sulfate (pH = 6.0). The volume of acetate kinase assumes 25,000 U/mL (concentration indicated by Megazyme technical support). Solution is stable for months at 4°C.

To prepare 3.2 M ammonium sulfate (pH = 6.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 6.0, then bring to 100 mL with dH₂O in a volumetric flask.

Acetyl-CoA (5 mM)

Reference: Product information sheet for Sigma A-2181

Weigh approximately 0.004807 g of acetyl-CoA (Calbiochem 1063-100MG) in a small (10 mL) beaker. Add 1 mL dH₂O per 0.004807 g acetyl-CoA (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH₂O assumes acetyl-CoA has purity of 84.2%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly. Here, purity refers to (purity by enzymatic assay – water - lithium) %. The molecular weight is 809.57 g/mol (free acid basis).

Solution is stable for 6 months at -80°C. Before freezing, store in aliquots (0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Acetyl-CoA synthetase (125 U/mL)

Order acetyl-CoA synthetase from *Bacillus subtilis* (Megazyme E-ACSBS).

To avoid contaminating stock, prepare aliquots 0.5 mL sterile microcentrifuge tube before use.

Acetyl phosphate (50 mM)

Reference: (70)

Dissolve 0.0092 g of acetyl phosphate lithium potassium salt (Sigma 01409-500MG) in 1 mL dH₂O.

The volume of dH₂O assumes acetyl phosphate A has purity of ~100%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly. Here, purity refers to (purity - water) %. The molecular weight is 184.06 mol/g (lithium potassium salt basis).

Solution is stable for months at -20°C. Before freezing, store in aliquots (0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

ADP (100 mM)

Reference: (71)

Add 0.2136 g ADP disodium salt (Sigma A2754-1G) to 15-mL centrifuge tube and dissolve in ~4 mL dH₂O. Adjust pH to a value between 7 to 9 with NaOH (e.g., 4 N). Bring to 5 mL with dH₂O (using the remaining ~1 mL dH₂O to wash the pH probe).

Solution is stable for months at -20°C and several days at 4°C. Before freezing, store in aliquots (0.25 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Aluminum chloride (100 mM)

Dissolve 2.4143 g AlCl₃·6H₂O in ~75 mL ddH₂O, then bring to 100 mL in a volumetric flask. Use AlCl₃·6H₂O, not anhydrous AlCl₃, as the latter reacts violently with water.

Ammonium chloride (1 M)

Dissolve 5.3491 g ammonium chloride ~75 mL dH₂O, then bring to 100 mL in a volumetric flask.

APAD (50 mM)

Reference: (72)

Weigh approximately 0.0020 g of 3-acetylpyridine adenine dinucleotide (Sigma A5251-25MG) in a small (10 mL) beaker. Add 56.2 μL dH₂O per 0.002 g 3-acetylpyridine adenine dinucleotide (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH₂O assumes APAD has purity of 93%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly.

Solution is stable for a few days at 4°C.

ATP (100 mM)

Reference: Product information sheet for Sigma A2383-1G

Dissolve 0.061233 g ATP disodium salt hydrate (Sigma A2383-1G) in 1 mL dH₂O.

The mass of ATP disodium salt hydrate assumes water content of the reagent is 10%; see certificate of analysis for exact content and adjust volume of dH₂O accordingly.

Solution is stable for months at -20°C and one week at 4°C. Before freezing, store in aliquots (0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Blue dextran (1 mg/mL)

Reference: Product information sheet for Sigma D5751

Dissolve 0.01 g blue dextran (MW = 2,000,000; Sigma D5751) in 10 mL of 50 mM Tris (pH = 7.5) containing 100 mM KCl. Filter through 0.22 μm membrane. Store at 4°C.

Citrate synthase (90 U/mL)

Reference: (73)

Dilute 2.9 μL citrate synthase from porcine heart (Sigma C3260-200UN) with 97.1 μL of 3.2 M ammonium sulfate (pH = 7.0). The volume of citrate synthase assumes 3090 U/mL; see pack label for exact U/mL and adjust volumes accordingly. Solution is stable for months at 4°C.

To prepare 3.2 M ammonium sulfate (pH = 7.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH₂O in a volumetric flask.

Coenzyme A (5 mM)

Reference: (74) and Calbiochem website for product 234101-100MG

Weigh approximately 0.003904 g of coenzyme A trilithium salt (Calbiochem 234101-100MG) in a small (10 mL) beaker. Add 1 mL of ice-cold dH₂O per 0.003904 g coenzyme A (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH₂O assumes coenzyme A has purity of 98.3%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly. Here, purity refers to (purity by UV - water - lithium) %. The molecular weight is 767.535 g/mol (free acid basis).

Solution is stable for 6 months at -20°C. Before freezing, store in aliquots (0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Coenzyme A (5 mM) (anaerobic)

Prepare coenzyme A (5 mM) following the standard procedure, but dissolve in water (anaerobic) and dispense the final solution in a 0.5 mL screw-top centrifuge tube. Flush tube with N₂ for a few seconds. Store at -20°C.

Development solution

Reference: (75)

Combine equal volumes of ferric chloride-HCl and trichoroacetic acid (30 % w/v). Store room temperature. Prepare fresh each day.

Dithionite (0.1 M)

Reference: (76) and instructions for DSMZ medium 346 (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium346.pdf)

Weigh 1.5362 g sodium dithionite (sodium hyposulfite) into a 160-mL culture bottle. Flush with N₂. Add 75 mL of water (anaerobic) while flushing with N₂ and stopper. Filter sterilize into a sterile serum bottle previously gassed with N₂. Long-term stability is not established.

The mass of sodium dithionite assumes purity of 85% (purity for technical grade).

Dithiothreitol (0.1 M)

Reference: (20) and instructions for DSMZ medium 559 (https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium559.pdf)

Weigh 1.1569 g dithiothreitol into a 160-mL culture bottle. Flush with N₂. Add 75 mL of water (anaerobic) while flushing with N₂ and stopper. Filter sterilize into a sterile culture bottle previously gassed with N₂. Long-term stability is not established.

DTNB (10 mM)

Reference: (77)

Dissolve 0.0396 g of 5,5'-dithiobis(2-nitrobenzoic acid) in 10 mL of 0.1 M KPO₄ (pH = 7). Solution is stable for up to 6 months at 4°C and protected from light.

To prepare 0.1 M KPO₄ (pH = 7), see procedure below for preparing potassium phosphate buffers (pH = 5.8 to 8.0).

EDTA (0.5 M) (pH = 8.0)

Reference: (78)

Add 93.05 g disodium EDTA·2H₂O to 400 mL dH₂O. Stir while adding ~9 g NaOH pellets. Gradually add more NaOH until pH reaches 8.0 and solution is clear. Bring to 500 mL final volume with dH₂O.

α-ketoglutarate (100 mM)

Reference: (79)

Dissolve 0.073 g α-ketoglutaric acid (Sigma 75890-25G) and 0.121 g Tris base to 5 mL dH₂O.

Solution is stable for 4 to 6 weeks at -20°C. Before freezing, store in aliquots (0.25 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

α-ketoglutarate dehydrogenase

Order α-ketoglutarate dehydrogenase (Sigma K1502-20UN) and store at -20°C. To avoid repeated freeze-thawing and contaminating stock, prepare aliquots 0.5 mL sterile microcentrifuge tube before use.

Pyruvate:ferredoxin oxidoreductase (crude)

Grow *Prevotella brevis* GA33 or *Prevotella ruminicola* 23 to mid-exponential phase (OD₆₀₀ = 1.0 to 1.2 for *P. brevis* GA33 and OD₆₀₀ = 2.7 to 3.0 for *P. ruminicola* 23). Prepare cytoplasmic contents according to *Preparation of cell membrane and cytoplasmic contents by ultracentrifugation*. Do all steps of preparation anaerobically. The buffer used is Tris-MgSO₄ (50 mM Tris-Cl [pH 7.6], 20 mM MgSO₄, 4 mM dithiothreitol, 4 μM resazurin). Treat the cell

pellet with nuclease (25 U/mL Pierce Universal Nuclease). The final concentration of protein in pyruvate:ferredoxin oxidoreductase is about 0.5 to 1.7 $\mu\text{g}/\mu\text{L}$.

Crude pyruvate:ferredoxin oxidoreductase from these bacteria is free of pyruvate dehydrogenase and ferredoxin:NAD⁺ oxidoreductase activity.

Ferric chloride-HCl

Reference: (75)

Add 8.1105 g ferric chloride to ~25 mL ddH₂O in a beaker. Add 41.3 mL concentrated (12.1 M) HCl, then bring to volume with ddH₂O in volumetric flask. Final concentration of ferric chloride is 0.5 M and final concentration of HCl is 5 M. Solution is stable at room temperature.

Glucose (1 M)

Dissolve 9.0078 g glucose in ~40 mL ddH₂O. Bring to 50 mL in a volumetric flask. Filter sterilize into a sterile Balch tubes. Solution is stable at room temperature but is routinely stored at 4°C or -20°C in case of contamination.

Glucose-6-phosphate dehydrogenase (100 U/mL)

Dilute 2.28 μL glucose-6-phosphate dehydrogenase from *Saccharomyces cerevisiae* (Sigma G7877-250UN) with 97.72 μL of 3.2 M ammonium sulfate (pH = 6.0). The volume of glucose-6-phosphate dehydrogenase assumes 4383 U/mL; see certificate of analysis for exact U/mL and adjust volumes accordingly. Solution is stable for months at 4°C.

To prepare 3.2 M ammonium sulfate (pH = 6.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 6.0, then bring to 100 mL with dH₂O in a volumetric flask.

Glutamate dehydrogenase

Order L-glutamic dehydrogenase from bovine liver (Sigma G2501-20MG).

To avoid contaminating stock, prepare aliquots 0.5 mL sterile microcentrifuge tube before use.

Hexokinase (270 U/mL)

Reference: (69, 80)

Add approximately 0.001348 g hexokinase from *Saccharomyces cerevisiae* (Sigma H4502-1KU) to 1 mL of 3.2 M ammonium sulfate (pH = 6.0). Adjust the volume of 3.2 M ammonium sulfate (pH = 6.0) for the actual mass of hexokinase. The mass of hexokinase assumes 364 U/mg; see pack label for exact U/mg and adjust mass accordingly.

Dissolve by pipetting up and down for several minutes. Solution is stable for months at 4°C.

To prepare 3.2 M ammonium sulfate (pH = 6.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 6.0, then bring to 100 mL with dH₂O in a volumetric flask.

To avoid contaminating stock, prepare aliquots 0.5 mL sterile microcentrifuge tube before use.

Hydroxylamine-HCl (pH = 7.0) (2 M)

Reference: (75)

Add 13.9 g of hydroxylamine hydrochloride to ~50 mL ddH₂O in a beaker. Adjust pH to 7.0; add ~10 g KOH pellets to neutralize most acid, then make final adjustment with 8 N KOH. Bring to 100 mL with ddH₂O in a volumetric flask. Solution is stable at 4°C for 3 months.

Lactate dehydrogenase (275 U/mL)

Dilute 10 µL lactate dehydrogenase from rabbit muscle (Sigma 10127230001) with 90 µL of 3.2 M ammonium sulfate (pH = 7.0). The volume of pyruvate kinase assumes 2750 U/mL; see pack label for exact U/mL and adjust volumes accordingly. Solution is stable for months at 4°C.

To prepare 3.2 M ammonium sulfate (pH = 7.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH₂O in a volumetric flask.

DL-Lactate (1 M) (pH = 7.0)

Add 10.17853107 g lactic acid (a liquid; Sigma L6661-100ML) to beaker and bring to ~65 mL with dH₂O. Add strong base (e.g., 8 N KOH) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH₂O in a volumetric flask. Filter sterilize into a 160-mL culture bottle.

The mass of lactic acid assumes purity of 88.5%; see certificate of analysis of exact purity and adjust mass accordingly.

Linoleic acid (5 mg/mL in 1% w/v Tween)

Add the following to a 160-mL culture bottle

0.375 g Linoleic acid

0.75 g Tween 80

Components are viscous liquids and should be weighed directly in bottle. Flush bottle with N₂ for 15 min. While continuing the flush, add 73.8 mL water (anaerobic). Stopper and shake to form a foamy, white suspension. Filter sterilize into culture bottle previously gassed with N₂. Store at 4°C. Long term stability not established.

L-Malate (100 mM)

Reference: (73)

Add 1.34 g L-malic acid (Sigma 02288-10G) to 50 mL dH₂O. Slowly add 20 mL 1 M potassium bicarbonate while stirring. Solution will foam. Bring to 100 mL in a volumetric flask and filter sterilize. Solution is stable for months at 4°C. Immediately before use, remove an aliquot and heat to 100°C for 10 min (to remove traces of oxaloacetate).

Malate dehydrogenase (180 U/mL)

Reference: (73)

Dilute 2.1 μL malic dehydrogenase from porcine heart (Sigma M1567-5KU) with 97.9 μL of 2.8 M ammonium sulfate (pH = 6.0). The volume of malate dehydrogenase assumes 8502 U/mL; see certificate of analysis for exact U/mL and adjust volumes accordingly. Solution is stable for months at 4°C.

To prepare 2.8 M ammonium sulfate (pH = 6.0), add 36.999 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong base (e.g., 1 M HCl) drop-by-drop to adjust to pH to 6.0, then bring to 100 mL with dH₂O in a volumetric flask.

Methyl viologen (1 M)

Add 0.0257 g methyl viologen hydrate (Fisher AC227320010) directly to a small (10 mL) beaker, add 0.1 mL dH₂O, and dissolve by pipetting up and down. Long-term stability is not established.

MgCl₂ (1 M)

Dissolve 10.1655 g of MgCl₂·6H₂O in ~40 mL dH₂O, then bring to 50 mL in a volumetric flask.

MgCl₂ (1 M) (anaerobic)

Add 1.6785 g MgCl₂·6 H₂O to a Balch tube. Flush tube with N₂. While continuing the flush, add 10 mL water (anaerobic) and dissolve. Stopper and autoclave.

NaCl (1 M) (anaerobic)

Add 0.58443 g NaCl to a Balch tube. Flush tube with N₂. While continuing the flush, add 10 mL water (anaerobic) and dissolve. Stopper and autoclave.

NAD (40 mM)

Reference: (79) and Product information sheet for Sigma N0632-1G

Add 0.13708 g NAD dinucleotide sodium salt (Sigma N0632-1G) to 15-mL centrifuge tube and dissolve in ~4 mL dH₂O. Check that pH has value between 2 and 6 (applying a 2- μL aliquot to indicator paper suffices). If needed, adjust pH with 1 M Tris. Bring to 5 mL with dH₂O.

Solution is stable for 6 months at -80°C and 2 weeks at 0°C. Before freezing, store in aliquots (0.25 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

NAD (200 mM) (anaerobic)

Prepare NAD (200 mM) following the standard procedure, but dissolve in water (anaerobic) and dispense the final solution in a 0.5 mL screw-top centrifuge tube. Flush tube with N₂ for a few seconds. Store at -80°C.

NADH (40 mM)

Reference: Product information sheet for Sigma N8129-1G

Dissolve 0.02987 g NADH disodium salt hydrate (Sigma N8129-1G) in 1 mL of 0.01 M Tris (pH = 8.5).

The NADH disodium salt hydrate assumes water content of the reagent is 5%; see certificate of analysis for exact content and adjust volume of dH₂O accordingly.

Solution is stable for years at -80°C. Before freezing, store in aliquots (0.05 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

NADP (10 mM)

Reference: (80) and product information sheet for Sigma N5755-100MG

Dissolve 0.02628 g NADP disodium salt (Fisher 48-197-2100MG) in 3 mL dH₂O.

The volume of dH₂O assumes NADP has purity of 89.9%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly. Here, purity refers to (assay – water – methanol) %. The molecular weight is 787.4 g/mol.

Solution is stable for 1 year at -20°C and 1 week at 4°C. Before freezing, store in aliquots (0.25 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Oxaloacetate (10 mM)

Dissolve 0.013207 g oxaloacetate (Sigma 04126-5G) in 10 mL ddH₂O. Store on ice when not in use. Long term stability not established.

Perchloric acid (4 N)

Add 34.19 mL concentrated (11.7 N) perchloric acid to ~75 mL water in a beaker on ice. After cooling, bring to 100 mL with dH₂O in a volumetric flask.

Phosphoenolpyruvate (100 mM)

Reference: (74)

Add 0.0206 g phosphoenolpyruvic acid monopotassium salt (Fisher AAB2035806) to a small (10 mL) beaker. Add 1 mL of dH₂O. Dissolve by pipetting up and down.

Solution is stable for 1 wk at -20°C. Before freezing, store in aliquots (0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Phosphotransacetylase (3000 U/mL)

Order phosphotransacetylase from *Bacillus subtilis* (Megazyme E-PTABS).

To avoid contaminating stock, prepare aliquots 0.5 mL sterile microcentrifuge tube before use.

Phosphotransacetylase (1000 U/mL)

Dilute 33.3 μL of phosphotransacetylase (3000 U/mL) with 66.7 μL of 3.2 M ammonium sulfate (pH 7). Store at 4°C. Long-term stability not established.

Potassium acetate (1 M) (pH = 7.0)

Add 9.814 g potassium acetate to ~75 mL dH₂O in a beaker. Add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH₂O in a volumetric flask.

Potassium bicarbonate (1 M)

Add 10.0115 g of potassium bicarbonate to a beaker. Bring to ~75 mL with dH₂O, then stir to dissolve. To prevent decomposition and loss as CO₂, do not heat. Bring to 100 mL with dH₂O in a volumetric flask. Store in serum bottle and stopper to prevent volatilization as CO₂.

Potassium hydroxide (8 N)

Add 44.88 g of KOH pellets to a beaker. Bring to ~75 mL with dH₂O, then stir to dissolve. After cooling, bring to 100 mL with dH₂O in a volumetric flask.

Potassium phosphate buffers (1 M) (pH = 5.8 to 8.0)

Reference: (78, 81)

Mix 1 M K₂HPO₄ and 1 M KH₂PO₄ in volumes indicated

pH	1 M K ₂ HPO ₄ (mL)	1 M KH ₂ PO ₄ (mL)
5.8	8.5	91.5
6.0	13.2	86.8
6.2	19.2	80.8
6.4	27.8	72.2
6.6	38.1	61.9
6.8	49.7	50.3
7.0	61.5	38.5
7.2	71.7	28.3
7.4	80.2	19.8
7.6	86.6	13.4
7.8	90.8	9.2
8.0	94	6

pH in table corresponds to expected value when the buffer is diluted to concentration 0.1 M. For other concentrations, mix 1 M K₂HPO₄ and 1 M KH₂PO₄ in volumes indicated by ref. (81). If verifying pH with a probe, note that several minutes are required for pH probe to equilibrate.

If final concentration of buffer is much greater or less than 0.1 M, mix 1 M K₂HPO₄ and 1 M KH₂PO₄ in volumes indicated by ref. (81). If verifying pH with a probe, note that several minutes are required for pH probe to equilibrate.

To prepare 1 M K₂HPO₄, bring 87.1 g K₂HPO₄ to 500 mL in a volumetric flask.

To prepare 1 M KH₂PO₄, bring 68.043 g KH₂PO₄ to 500 mL in a volumetric flask.

Potassium phosphate buffer (pH = 7.0) (1 M) (anaerobic)

Add 1.0713 g K_2HPO_4 and 0.5239 g KH_2PO_4 to a Balch tube. Flush tube with N_2 . While continuing the flush, add 10 mL water (anaerobic) and dissolve. Autoclave (optional).

Potassium phosphate buffer (pH = 7.0) (0.1 M) with dithiothreitol (2 mM) (anaerobic)

To 75 mL water (anaerobic), add 8.52 mL of potassium phosphate buffer (pH = 7.0) (1 M) (anaerobic) and 1.70 mL of dithiothreitol (0.1 M). Add aseptically using syringes flushed with N_2 ; added volumes will be approximate.

Propionate (1 M) (pH = 7)

Weigh 7.408 g propionic acid (a liquid; Sigma P1386-1L) in a beaker. Bring to ~60 mL with dH_2O . Add strong base (e.g., 8 N KOH) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH_2O in a volumetric flask.

Propionyl-CoA (5 mM)

Add 1061.2 μL of ice-cold dH_2O directly to 0.005 g propionyl-CoA (Sigma P5397-5MG) in the manufacturer's vial. Dissolve by pipetting up and down.

The volume of dH_2O assumes propionyl-CoA has purity of 87.4%; see certificate of analysis for exact purity and adjust volume of dH_2O accordingly. Here, purity refers to (purity [HPLC] - water - solvent - lithium) %.

Long term stability is not established, but stability of acetyl-CoA is 6 months at $-80^\circ C$. Before freezing, store in aliquots (0.05 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

Pyruvate (100 mM)

Reference: Product information sheet for Sigma P4562

Dissolve 0.1100 g sodium pyruvate (Fisher AC132150250) in 10 mL dH_2O . Filter sterilize. Solution is stable for 24 months at $4^\circ C$.

Pyruvate (1 M) (anaerobic)

Add 1.1004 g sodium pyruvate (Fisher AC132150250) to a Balch tube. Flush tube with N_2 . While continuing the flush, add 10 mL water (anaerobic) and dissolve. Filter sterilize into sterile Balch tube previously gassed with N_2 . Solution is stable for 24 months at $4^\circ C$.

Pyruvate kinase (100 U/mL)

Dilute 2.19 μL pyruvate kinase from rabbit muscle (Sigma C3260-200UN) with 97.81 μL of 3.2 M ammonium sulfate (pH = 6.0). The volume of pyruvate kinase assumes 4576 U/mL; see pack label for exact U/mL and adjust volumes accordingly. Solution is stable for months at $4^\circ C$.

To prepare 3.2 M ammonium sulfate (pH = 6.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH₂O. Stir and gently heat to dissolve, add strong acid (e.g., 1 M HCl) drop-by-drop to adjust to pH to 6.0, then bring to 100 mL with dH₂O in a volumetric flask.

Sodium fluoride (100 mM)

Add 0.4199 g NaF to a screw-cap plastic bottle. Add 100 mL ddH₂O with a volumetric pipette. Dissolve. Solution will etch glass bottles and should not be stored in them.

Sodium vanadate (200 mM) (pH = 10.0)

Reference: (82)

Dissolve 3.6781 g Na₃VO₄ in ~50 mL ddH₂O. Adjust pH to 10.0 with 1 N HCl (or 1 N NaOH). Solution will turn yellow. Boil until solution clears (~10 min). Repeat boiling and pH adjustment until solution remains clear.

Succinate (0.25 M) (pH = 7.0)

Add 2.9522 g succinic acid to beaker and bring to ~75 mL with dH₂O. Stir and heat to dissolve. Add strong base (e.g., 8 N KOH) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with dH₂O in a volumetric flask. Filter sterilize into a 160-mL culture bottle.

Succinyl-CoA (5 mM)

Reference: (83)

Add 907.1 μL of ice-cold 0.01 M H₂SO₄ directly to 0.005 g succinyl-CoA (Sigma S1129-5MG) in the manufacturer's vial. Dissolve by pipetting up and down.

The volume of H₂SO₄ assumes succinyl-CoA has purity of 78.7%; see certificate of analysis for exact purity and adjust volume of dH₂O accordingly. Here, purity refers to (purity [HPLC] - water - solvent - sodium) %.

Solution is stable for 6 months at -20°C. Before freezing, store in aliquots (0.05 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

To make 0.01 M H₂SO₄, add 275.9 μL of concentrated H₂SO₄ to ~125 mL in a beaker. Bring up to 500 mL in a volumetric flask. The volume of H₂SO₄ assumes assay of 96.6 % w/w; see pack label for exact assay and adjust volume of dH₂O accordingly.

Thiamine pyrophosphate (0.1 M) (anaerobic)

Add 0.046077 g thiamin pyrophosphate (Sigma C8754-5G) to a 0.5 mL screw-top centrifuge tube. Add 1 mL of anaerobic water and dissolve. Flush tube with N₂ for several minutes. Store at -20°C.

Trichoroacetic acid (30 % w/v)

Dissolve 30.0 g trichloroacetic acid in ~30 mL ddH₂O, then bring to 50 mL in a volumetric flask.

Triethanolamine buffer (1 M) (pH = 7.3 to 8.4)

Add 18.565 of triethanolamine hydrochloride to ~75 mL dH₂O in a beaker. Stir to dissolve. Add strong base (e.g., 4 N KOH) drop-by-drop to adjust to desired pH (e.g., 7.4), then bring to 100 mL with dH₂O in a volumetric flask.

Tris buffers (1 M) (pH = 7.2 to 9.0)

Reference: Sigma Technical Bulletin 106B

Mix 1 M Tris HCl and 1 M Tris base in volumes indicated

pH	1 M Tris HCl (mL)	1 M Tris base (mL)
7.2	89.1	11.0
7.3	86.9	13.2
7.4	83.9	16.0
7.5	80.6	19.4
7.6	76.9	22.9
7.7	72.6	27.3
7.8	67.5	32.4
7.9	61.9	37.9
8	56.3	43.6
8.1	51.0	48.9
8.2	44.9	55.0
8.3	39.0	60.9

pH in table corresponds to expected value when the buffer is diluted to concentration 0.05 M and when at room temperature.

If final concentration of buffer is much greater or less than 0.05 M, solution must be made by 1) diluting 1 M Tris base to ~75% of final volume of buffer, 2) adjusting pH with HCl, and 3) bringing to final volume with dH₂O. Because pH of Tris is sensitive to temperature, ensure solution is at room temperature before final pH adjustment.

To prepare 1 M Tris HCl, bring 78.8 g Tris HCl to 500 mL in a volumetric flask.

To prepare 1 M Tris base, bring 60.57 g Tris base to 500 mL in a volumetric flask.

Tris (pH 7.6) (1 M) (anaerobic)

Add 7.69 mL Tris HCl (1 M) (anaerobic) and 2.29 mL Tris base (1 M) (anaerobic) to a Balch tube flushed with N₂. Mix and stopper.

To prepare 50 mL anaerobic Tris HCl (1 M), add 7.88 g Tris HCl to a serum bottle flushed with N₂ for 10 min, add 44.32 mL anaerobic dH₂O to the bottle and cap it with butyryl stopper under N₂. Shake it to dissolve. Autoclave at 121°C for 20 min.

To prepare 50 mL anaerobic Tris base (1 M), add 6.057 g Tris base to a serum bottle flushed with for 10 min, add 45.193 mL anaerobic dH₂O to the bottle and cap it with butyryl stopper under N₂. Shake it to dissolve. Autoclave at 121°C for 20 min.

The amount of water for preparing Tris HCl (1 M) and Tris base (1 M) was determined in separate experiments.

Vitamin B₁₂ (1 mg/mL)

Dissolve 0.01 g vitamin B₁₂ in 10 mL ddH₂O. Filter through 0.22 µm membrane. Store at 4°C.

Water (anaerobic)

Boil ~350 mL water under N₂ for 5 to 7 min (timed after first bringing to a boil). Dispense ~75 mL aliquots in 160-mL culture bottles while flushing with N₂ and stopper. Autoclave (optional).

Purification of ferredoxin from *Clostridium pasteurianum*

Adapted from (84-86). Preparation of reagents and solutions guided by Whatman Instructions 29106895 AB for Ion Exchange Celluloses, Cold Spring Harbor protocol doi:10.1101/pdb.rec10471, and ref. (87).

Preparation of reagents and solutions

1 M Tris-base

Add 12.114 g Tris base to a 100 mL volumetric flask. Bring to volume with dH₂O.

1 M Acetic acid

Add 5.72 mL glacial acetic acid to a 100 mL volumetric flask. Bring to volume with dH₂O.

Ammonium sulfate (10% saturated, pH = 6.8)

Add 5.3 g ammonium sulfate and 100 mL dH₂O to beaker then stirring to dissolve. Adding 1 M Tris-base drop-by-drop to adjust to pH to c. 6.8. In the past, final pH has been as high as 7.2, with no effect on purification.

Ammonium sulfate (60% saturated, pH = 6.8)

Add 36.96 g ammonium sulfate and 100 mL dH₂O to beaker then stir to dissolve. Add 1 M Tris-base drop-by-drop to adjust to pH to c. 6.8. In the past, final pH has been as high as 7.3, with no effect on purification.

Ammonium sulfate (90% saturated, pH = 6.8)

Add 61.43 g ammonium sulfate and 100 mL dH₂O to beaker then stir to dissolve. Add 1 M Tris-base drop-by-drop to adjust to pH to c. 6.8. In the past, final pH has been as high as 7.4, with no effect on purification.

Ammonium sulfate (60% and 90% saturated mix, pH = 6.8)

Combine 120 mL of ammonium sulfate (60% saturated, pH = 6.8) and 60 mL ammonium sulfate (90% saturated, pH = 6.80).

Potassium phosphate buffer (pH = 7.2)

Add 12.49 K₂HPO₄ and 3.85 g KH₂PO₄ to 1 L volumetric flask. Bring to volume with dH₂O. Verify correct pH (should require no adjustment).

NaCl (0.9%, w/v)

Add 9 g of NaCl to a 1 L volumetric flask. Bring to volume with dH₂O.

NaCl (2 M)

Add 11.688 g NaCl to ~75 mL dH₂O in beaker and stir to dissolve. Transfer to a 100 mL volumetric flask and bring to volume with dH₂O.

NaCl (100 mM)

Add 2.3376 g NaCl to 250 mL volumetric flask. Bring to volume with dH₂O.

Acetone (chilled)

Pour acetone stock into Pyrex bottle and place in -80°C overnight (or until chilled).

Polymin-P (10% w/v , pH = 6.8)

Pour 5 g of Polymin-P (BASF 50019138), a viscous liquid, into a tared beaker on a scale. Dissolve in 30 mL dH₂O by rapid stirring. Make sure that all Polymin-P is dissolved. Add glacial acetic acid to adjust pH to 6.8. Make sure that pH is stable at 6.8, as pH creeps up slowly after adding acid. Transfer to and bring to volume in 50 mL volumetric flask. Filter-sterilize and store at 4°C. Solution is stable indefinitely.

Universal nuclease

Dispense small (5 µL) aliquots of Pierce universal nuclease (Thermo Scientific 88700) into 0.5 mL centrifuge tube. Store at -20°C.

Tris-Cl (10 mM, pH = 8.0)

Add 1 mL 1 M Tris to ~75 mL dH₂O. Add 10% HCl drop-by-drop to adjust pH to 8. Transfer to a 100 mL volumetric flask and bring to volume with dH₂O.

Lysozyme (50 mg/mL).

In a 2-mL centrifuge tube, add 50 mg lysozyme (stored at -20°C) to 1 mL of 10 mM Tris-Cl (pH 8.0). Keep on ice and prepare immediately before use.

Lysozyme (10 mg/mL)

Prepare this concentration only if cell pellet is less than 20 g. Follow directions for lysozyme (50 mg/mL), except use 10 mg lysozyme. Prepare immediately before use.

HCl (0.5 N)

Add 1.024 mL of concentrated (37.4 % w/w) hydrochloric acid to ~200 mL dH₂O. Transfer to a 250 mL volumetric flask and bring to volume with dH₂O.

NaOH (0.5 N)

Add 4.9996 g of NaOH to ~200 mL dH₂O. Transfer to a 250 mL volumetric flask and bring to volume with dH₂O.

Water (anaerobic)

Boil ~350 mL water under N₂ for 5 to 7 min (timed after first bringing to a boil). Dispense ~75 mL aliquots in 160-mL culture bottles while flushing with N₂ and stopper. Autoclave.

Preparing medium for *C. pasteurianum****C. pasteurianum* minerals A**

Add following to a 1-L volumetric flask

153.29 g K₂HPO₄ to 1 L

Bring to volume to 1 L with dH₂O and store at 4°C.

***C. pasteurianum* minerals B**

Add the following to a 1-L volumetric flask

15 g KH₂PO₄
1 g NaCl
0.9766 g MgSO₄
0.1 g Na₂MoO₄·2H₂O
10 g NH₄Cl

Bring to volume with dH₂O and store at 4°C.

C. pasteurianum iron sulfate

Add 0.5 g FeSO₄·7 H₂O to a 1-L volumetric flask. Bring to volume with dH₂O and store at 4°C.

C. pasteurianum vitamins

Add the following to a 1-L volumetric flask

0.05 g 4-aminobenzoic acid

0.01 g biotin (stored at 4°C)

Bring to volume with dH₂O and store at 4°C.

Glucose (40% w/v, anaerobic)

Add 40 g glucose to a 150-ml serum bottle. Flush the bottle under N₂ for 15 min with occasionally swirling. Add 74.2 mL anaerobic water to dissolve it. The final volume will be 100 mL.

While continuing to flush with N₂, stopper the bottle. Autoclave at 121°C for 15 min.

C. pasteurianum medium (sterilized)

Add following to a 1-L round bottom flask

230 mL dH₂O

30 mL *C. pasteurianum* minerals A

30 mL *C. pasteurianum* minerals B

30 mL *C. pasteurianum* iron sulfate

30 mL *C. pasteurianum* vitamins

Bring to boil under N₂ (requires about 5 min). Cool it down in ice water under N₂ and bubble for 5 min. Approximately 50 mL will be lost, giving 300 mL final volume. pH should be approximately 7.1.

Bubble with CO₂ to decrease pH to 6.8 (requires about 3 min).

Under CO₂, dispense media in Balch tubes in 9.5 mL aliquots or 250-mL Pyrex bottles in 95 mL aliquots. Stopper tubes or bottles and autoclave at 121°C for 15 min. For bottles, stoppers should be wired in place and covered with aluminum foil.

After autoclaving, add glucose (40% w/v, anaerobic) to a final concentration of 2% w/v. The volume of glucose added will be 0.5 mL for 9.5 mL aliquots of media and 5 mL for 95 mL aliquots.

C. pasteurianum medium (non-sterilized)

Add 18 g glucose and c. 400 mL dH₂O to a 1-L Pyrex bottle. Stir to dissolve, then bring to 540 mL with dH₂O. Wire a stopper in place, cover with aluminum foil, and autoclave at 121°C for 15 min. Pressurize the bottle with N₂, cool, then remove stopper and flush with N₂.

Meanwhile, add following to a 500-mL separatory funnel

- 90 mL C. pasteurianum iron sulfate
- 90 mL C. pasteurianum macrominerals B
- 90 mL C. pasteurianum macrominerals A
- 90 mL C. pasteurianum vitamins

Bubble with N₂ for 4 min, then continue bubbling with CO₂ for 4 min. Drain contents of separatory funnel into Pyrex bottle with glucose solution. Bubble with CO₂ until pH decreases from 7.45 to 6.8. Prepare immediately before inoculation (optional: pre-warm the medium for several hours at 37°C before inoculation).

Preparing DEAE-cellulose slurry

1.) Precycle DEAE-cellulose by first treating with 0.5 N HCl.

(a) Add 5 g of DEAE cellulose (Santa Cruz Biotechnology sc-211213) to a 50-mL tube and add 75 mL (15 volumes) of 0.5 N HCl.

(b) Stir briefly with glass stir rod and let stand for 30 to 40 min (or longer). Here and throughout, stir gently and avoid use of stir bar; ion exchanger will be broken up into fines otherwise.

(c) Remove the supernatant using a 10-mL pipette.

(d) Using dH₂O, transfer ion exchanger to Buchner funnel lined with 2 layers of #1 Whatman filter paper.

(e) Wash ion exchanger with dH₂O. Fill a 1 L graduated cylinder with dH₂O, apply small aliquots (25 to 50 mL) of water, and apply gently vacuum to remove water. Repeat until filtrate has pH = 4.0 (requires 280 to 300 mL dH₂O).

(f) Transfer back to beaker by scraping off ion exchanger with spatula. To transfer as much as possible, pool residual ion exchanger in one region with dH₂O, and scrape again.

2.) Continue pre-cycling by treating DEAE-cellulose with 0.5 N NaOH. Follow #1 but using 0.5 N NaOH. Wash until pH = 7.0 (requires about 980 ml dH₂O).

3.) Transfer the wet, precycled ion exchanger to a tared 15-mL or 50-mL centrifuge tube. Yield will be about 4.25 g. Proceed to next step or store at 4°C.

4.) Equilibrate DEAE-cellulose in ammonium sulfate.

(a) Add 6 volumes of ammonium sulfate (60% saturated) (e.g., 30 mL for 5 g ion exchanger in #3). Ammonium sulfate (60% and 90% saturated mix, pH = 6.8) can be used in place of ammonium sulfate (60% saturated).

(b) Vortex for 2 min.

(c) Adjust pH to 6.8 with 1 M acetic acid drop-by-drop.

5.) Remove fines.

(a) Transfer slurry in #4 to tared 2-mL centrifuge tubes.

(b) Centrifuge at 10,000 g for 4 min.

- (c) Aspirate off supernatant, including any fines (usually minimal). Use a 10-mL pipette to remove most supernatant, then a syringe and needle to remove any remainder.
- (d) Weigh to determine mass of equilibrated ion exchanger.
- (e) Resuspend in ammonium sulfate (60% saturated, pH = 6.8) to make 0.25 g/mL slurry.

Cultivation of *C. pasteurianum* and preparation of cell pellets

- 1.) Inoculate a 10-mL aliquot of *C. pasteurianum* medium (sterilized and anoxic) with 0.1 mL *C. pasteurianum* 5 and grow at 37°C overnight.
 - 2.) Inoculate a 100-mL aliquot of *C. pasteurianum* medium (sterilized and anoxic) with 5 mL of the overnight culture. Grow at 37°C until culture reaches pre- or mid-log phase (OD₆₀₀ of 1.5 to 2) and produces a large number of bubbles. Growth may require 1 to 2 d.
 - 3.) Inoculate 900-mL aliquots of *C. pasteurianum* medium (non-sterilized and anoxic) with 25 mL aliquots of culture from #2. Wire stopper in place and cover with aluminum foil. Transfer to 37° incubator.
 - 4.) At intervals, take aliquots of culture with disposable pipette (gas under CO₂). In cuvette (1-cm pathlength), dilute 1→10 with 0.9% NaCl. Measure OD₆₀₀.
 - 5.) Harvest culture when it reaches mid-exponential phase (OD₆₀₀ = 0.4 to 0.6 for 1→10 dilution). Transfer culture to 250-mL bottles and centrifuge at 8,000 g for 3 min at 4°C. Discard the supernatant.
- If culture enters stationary phase (OD₆₀₀ = 0.7 for 1→10 dilution), ferredoxin will be degraded and yield during purification decreased.
- 6.) Resuspend cell pellets from 0.9-L culture in 15 mL of 50 mM Tris-Cl (pH 7.2), 10 mM MgCl₂. Transfer the resuspended cells to 50-mL centrifuge tubes. Before transferring, weigh the empty tubes and write the weight on the tube label.
 - 7.) Centrifuge at 12,000 g for 10 min and 4°C. Discard the supernatant.
 - 8.) Weigh the pellets to determine wet mass. Write the weight (g) on the label of the 50-mL tube. The typical weight per 900-mL culture is 5.4 g. Yield can be estimated more accurately as 0.0015 g wet cells × OD₆₀₀⁻¹ × mL culture⁻¹ (e.g, 5.4 g wet cells for 900 mL of culture grown to OD₆₀₀ = 4.0).
 - 9.) Store tubes at -20°C.
 - 10.) Repeat #1 to 9 to prepare at least 80 g of cells in total. For faster growth, 25-mL culture in #3 can be mixed with 0.4 g cell pellet from #8 to inoculate the 900-mL medium.

Purification of ferredoxin

Cell lysis

Do all subsequent steps aerobically and on ice (unless otherwise noted).

- 1.) Remove 50-mL tubes containing a total of ≥ 80 g cell pellet from -20°C . If each tube contains 5.4 g cell pellet, ~ 15 tubes will be needed.
 - 2.) Resuspend cell pellets in dH_2O at room temperature.
 - (a) To each 50-mL tube, add 1.5 mL dH_2O /g wet cells.
 - (b) Use spatula to loosen pellet from tube wall. Use some of the dH_2O to wash the spatula.
 - (c) Mix thoroughly to homogenize the pellet. Resuspended pellet will be brown and sticky/viscous.
 - (d) Prepare a beaker and a stir bar, record weight, and add the stir bar in the beaker. Transfer the resuspended pellets to a 500-mL beaker with a 25-mL pipette. Record the volume of resuspended cells (200 to 220 mL for 80 g cell pellet).
 - 3.) Add 10 μL of lysozyme (50 mg/mL) and 0.1 μL of universal nuclease (250 U/ μL) per mL of resuspend cells.
 - 4.) Incubate at 37°C in water bath for 40 min. Mix well with a spatula in every 5 min. The lysate will remain brown and turbid, but will no longer be sticky/viscous.
 - 5.) Cool to room temperature ($23\text{-}25^{\circ}\text{C}$) by mixing on a stir plate or by placing on ice for a few minutes. Temperature can be measured with pH or conductivity probe used below.
 - 6.) Measure pH with a pH probe. Initial pH will be ~ 6 .
 - 7.) Adjust pH to 6.8 using 1 M Tris-base (4 to 6 mL for 80 g cells). Adjust pH carefully, as downstream precipitation of ferredoxin will be low otherwise. If pH is too high, lower it with 1 M acetic acid.
 - 8.) Adjust conductivity.
 - (a) Prepare the conductivity probe (Fisher WD-35662-30).
 - (b) Measure conductivity of 100 mL of 100 mM NaCl at room temperature (about 10.7-11.3 mS/cm).
 - (c) Measure conductivity of the lysate at room temperature ($\sim 4\text{-}5$ mS/cm).
 - (d) Add NaCl (2 M) to lysate until the conductivity matches that of 100 mM NaCl (15 to 19 mL for 80 g of cells).
- If conductivity is too high, dilute with dH_2O . Adjust conductivity carefully, as downstream precipitation of ferredoxin will be low otherwise.
- 9.) Cool lysate on ice.)
 - 10.) Weigh the lysate. Typical yield is 210 g from 80 g of cells. Transfer 100 g aliquots to 500-mL beakers.

Precipitation of proteins with acetone and Polymin-P

- 1.) Transfer a 100 g aliquot of lysate to a 500-mL beaker with a stir bar on a stir plate. Keep remaining lysate on ice.
- 2.) Weigh 120 g cold acetone in a separate beaker (1.2 g/g lysate).
- 3.) Slowly pour the acetone into the lysate on a stir plate while mixing. Do all subsequent steps immediately, as ferredoxin slowly denatures in acetone.
- 4.) Transfer acetone-lysate mixture to 50-mL centrifuge tubes. Centrifuge at 24,400 g for 10 min at 4°C. The supernatant will be transparent and deep brownish-yellow. The pellet will be brownish-white.

Acetone will damage the rotor. If a tube breaks during centrifugation, pipette out the acetone-lysate mixture immediately and rinse the rotor with water.
- 5.) Transfer the supernatant to a plastic beaker and record the weight. The typical yield of the acetone supernatant from 100 g lysate is about 205 g (or 430 g per 80 g of cells).
- 6.) Transfer the acetone supernatant to a 500 mL glass beaker containing a stir bar.
- 7.) Precipitate ferredoxin by adding 50 μ L of polymin-P (10% w/v, pH = 6.8) per gram of acetone supernatant (10.25 mL for ? 205 g acetone supernatant). Add polymin-P slowly using a 1-mL pipette. Supernatant will turn milky white.
- 8.) Transfer the supernatant to 50-mL tubes. Centrifuge at 24,400 g for 10 min at 4°C. Keep the beaker and stir bar on ice, as some ferredoxin will remain bound to it and recovered later.
- 9.) Remove supernatant completely by aspiration or pouring. If not all supernatant is removed, final purity will be low. The pellet will be dark brown. Keep all the tubes on ice.
- 10.) Check supernatant to ensure ferredoxin is absent. The color should be very faint yellow. Additionally, measure A_{390} in a microcuvette (Fisher 13-878-121). When measured relative to water, the A_{390} should be <0.1 (ideally 0.001).
- 11.) Repeat steps #1 to 10 for remaining 100 g aliquots of lysate. Process only one aliquot at a time to prevent ferredoxin from being denatured by acetone.
- 12.) Resuspend the ferredoxin pellets in ammonium sulfate (60% saturated, pH = 6.8).
 - (a) Measure out 1 mL ammonium sulfate (60% saturated, pH = 6.8) per gram of original wet cell pellet (e.g., 80 mL for 80 g of cells).
 - (b) Add a small portion of the ammonium sulfate to the 50-mL tubes. Resuspend the pellets with a 1-mL pipette. Transfer the resuspended pellets to a 50-mL centrifuge tube.
 - (c) Add more ammonium sulfate to wash the tubes and pipette. Transfer the wash to a 50-mL centrifuge tube.

(d) Use some ammonium sulfate to wash the beaker and stir bar. Transfer the wash to a 50-mL centrifuge tube.

13.) Centrifuge the 50-mL tube(s) containing the resuspended pellet at 24,400 g for 10 min at 4°C.

14.) Transfer supernatant to a 250 mL glass beaker. Place the beaker on ice.

Purification of ferredoxin with DEAE-cellulose

1.) To the beaker containing crude ferredoxin, add 120 μ L of DEAE-cellulose slurry per gram of original cell pellet (e.g., 9.6 mL for 80 g cell pellet). If slurry is not 0.25 g/mL, adjust the volume proportionately.

2.) Adsorb the ferredoxin onto the slurry by mixing it for 2 to 4 h. Place a stir bar into the beaker, place the beaker in a cooler with ice, and place the cooler on the stir plate.

3.) Transfer the slurry to two 50-mL tubes. Let the contents stand for 30 min (or longer) to allow the DEAE-cellulose slurry to settle. Alternatively, pellet the slurry by centrifuging it at 24,400 for 20 min at 4°C.

4.) Remove supernatant (yellow) without disturbing DEAE-cellulose pellet (deep brown). Keep both the pellet and supernatant.

5.) Wash the DEAE-cellulose pellet. Add 0.5 to 1 mL ammonium sulfate (60% and 90% saturated mix, pH = 6.8) per gram of original wet cell pellet (e.g., 40 to 80 mL per 80 g cell pellet). Centrifuge at 24,400 for 20 min at 4°C. Discard the supernatant (light yellow).

6.) Wash again by repeating #5, but keep a small amount (c. 1 mL) of supernatant. Transfer the pellet and 1 mL supernatant to a 2-mL tube.

7.) Centrifuge the DEAE-cellulose at 21,100 g 15 min at 4°C. Remove the supernatant fully by inserting a 200- μ L pipette into the DEAE-cellulose pellet and pipetting out all liquid. The DEAE-cellulose pellet should look dry.

8.) Elute the ferredoxin from DEAE-cellulose. Add a small volume (e.g., 1.5 mL) of ammonium sulfate (10% saturated, pH = 6.8) to the DEAE-cellulose pellet. Mix by vortexing (2000 to 3000 rpm for 15 s).

9.) Centrifuge the DEAE-cellulose at 21,100 g for 15 min at 4°C. Pipette off most supernatant, but do not disturb the pellet. Transfer the supernatant to a 50-mL tube and label "A1 elute". Both pellet and supernatant will be brown.

10.) The DEAE-cellulose from step #9 still contains ferredoxin, which can be re-eluted by repeating steps #8 and 9. Label the supernatant as "A1 re-elute".

11.) The DEAE-cellulose from step #10 still contains ferredoxin, which can be re-eluted a second time. Label the supernatant “A1 re-re-elute”. After this step, the DEAE-cellulose pellet should contain little ferredoxin and be whitish brown.

12.) The supernatant from #4 still contains ferredoxin, which can be adsorbed onto more DEAE-cellulose. Repeat steps #1 to 11, except use 60 µL of DEAE-cellulose slurry per gram of original cell pellet (e.g., 4.8 mL for 80 g cell pellet). The time for mixing in step #2 can be extended to overnight. Keep the supernatant and label “A2 elute”; “A2 re-elute”, “A2 re-re-elute”.

13.) The supernatant from #12 still contains ferredoxin, which can be adsorbed onto more DEAE-cellulose. Label the supernatant “A3 elute”; “A3 re-elute”, “A3 re-re-elute”. After this step, the supernatant should contain little ferredoxin.

14.) Store the purified ferredoxin at 4°C.

Measurement of yield and purity of ferredoxin

1.) Measure the absorbance of the purified ferredoxin using Molecular Devices M3 plate reader. Pipette 1000 µL of each sample into a UV-cuvette. Alternatively, pipette 200 µL of each sample to a 96-well plate (UV). Measure the values of A_{280} , A_{390} , A_{430} , and perform a spectral scan from $\lambda = 220$ to 750 nm. Include one sample of 10% saturated ammonium sulfate (as a blank).

2.) For each sample, determine the concentration and purity of ferredoxin. The concentration is

$$A_{390} = c * \epsilon_{390} * L$$

where A_{390} is the absorbance at 390 nm, c is the concentration in mM, ϵ_{390} is 30/mM/cm, and L is the pathlength (1 cm for UV-cuvette or 0.57508 cm for 200 µL liquid volume in 96-well plate).

The purity is expressed as A_{390}/A_{280} .

3.) Group samples by purity.

	Purity (A_{390}/A_{280})
Good	0.70-0.75
Marginal	0.65-0.70
Bad	<0.65

4.) Concentrate the “Good” and “Marginal” ferredoxin by ultrafiltration. Transfer up to 10 mL of ferredoxin to a filter with molecular weight cutoff of 3 kDa (Fisher 88525). Centrifuge at 4,500 g at 4°C until the ferredoxin is concentrated to 900 to 1000 µM. For 80 g of cells, this corresponds to a final volume of about 1.5 mL.

Centrifugation will take several hours (2 h if concentration is <30 µM and 4 h if concentration is > 100 µM). Do not transfer more than 10 mL to the filters, or they will leak.

5.) Measure the final concentration and purity. Repeat steps #1 to #2, except dilute samples 1→30. From 80 g of cells, a typical yield is 8 mg in 1.5 mL concentrated sample, with concentration of ~940 μ M (~5300 mg/L) and purity of $A_{390}/A_{280} = 0.73$.

6.) Dispense ~150 μ L aliquots in 500- μ L tubes. Store at -80°C. They can be stored for at least 1 year. For short-term storage, 4°C can also be used.

7.) Optional: test the activity of the ferredoxin by measuring its reduction by hydrogenase of *Clostridium pasteurianum* 5.

Optional: Re-purification of low-quality ferredoxin

If the quality of ferredoxin is low, it may be salvageable by repeating some steps of the purification.

1.) Mix all bad samples together in a beaker. Calculate the volume (mL) and amount of crude ferredoxin (nmol) in them.

2.) Adjust the concentration of ammonium sulfate to 60% saturated (the concentration needed to prior to adsorption on DEAE-cellulose). Mix well for 15 min in a beaker on ice. The initial concentration of ammonium sulfate is 10% saturated. Slowly add solid ammonium sulfate to raise the concentration, minding that the volume will also be increased.

Example: Step #1 produces a 50 mL sample with 10% saturated ammonium sulfate. A total of 15.83 g ammonium sulfate needs to be added, giving a 60% saturated ammonium sulfate solution with final volume of 60 mL.

3.) Transfer the solution to 50-mL tubes and centrifuge at 24,400 g for 10 min at 4°C.

4.) Transfer the supernatant to a beaker on ice. Add 0.25 g/mL DEAE-cellulose slurry to mix with the supernatant until all ferredoxin is bound. If step #1 starts with 920 nmol crude ferredoxin, ~6 mL of 0.25 g/mL slurry can be used for the binding.

5.) Mix well on ice for 2 to 4 h.

6.) Repeat **Purification of ferredoxin with DEAE-cellulose** from step #3 onward, then repeat **Measurement of yield and purity of ferredoxin**. When purifying ferredoxin with DEAE-cellulose, it may not be necessary to perform multiple bindings, as all ferredoxin should be bound the first time.

Assay of lactate dehydrogenase activity

Modified from (68)

Assay mixture

Prepare and add the assay mixture to cuvettes (Fisher 14-955-127).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
KPO ₄ (pH = 6.2) (1 M)	35	70
NADH (40 mM)	2.5	0.2
ddH ₂ O	425	NA
Cell extract	25	NA
Pyruvate (100 mM) or ddH ₂ O	12.5	25
Final volume	500	

Some components are added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
KPO ₄ (pH = 6.2) (1 M)	A
NADH (40 mM)	A

The volume of mastermix A is 37.5 μL per sample. The volume of mastermix B is 16.09 μL per sample. Prepare the mastermix in a 2-mL centrifuge tube. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Addition	Cuvette	
	1	2
1	Mastermix A	
1	ddH ₂ O	
1	Cell extract	
2	Pyruvate	ddH ₂ O

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize (1 to 3 min) before making next addition.

After addition 2, record absorbance at short (0.5 min) intervals for 5 to 10 min.

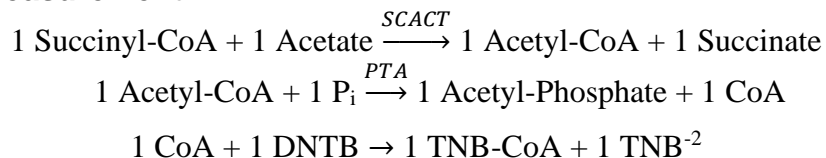
Calculation of activity

- 1.) Denote the absorbance after addition 1 as A_1 . A_2 and other absorbances are defined similarly.
- 2.) Plot A_2 vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.
- 3.) Calculate the reaction rate
$$\text{Rate} = (\text{slope}/6\ 220)$$
where $6\ 220\ \text{M}^{-1}\ \text{cm}^{-1}$ is the extinction coefficient of NADH.
- 4.) Calculate the activity ($\text{mol L}^{-1}\ \text{min}^{-1}$) by correcting the reaction rate with control cuvettes.
$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}}$$

Assay of succinyl-CoA:acetate CoA-transferase activity with phosphotransacetylase and DTNB

Modified from (88)

Principle of measurement



where SCACT is succinyl-CoA:acetate CoA-transferase and PTA is phosphotransacetylase. TNB²⁻ has a yellow color and is measured with a spectrophotometer.

Preparation of enzyme samples

Prepare cell extract according to *Preparation of cell extracts for enzymatic assays using a French press*. The final concentration of protein should be about 750 ng/ μ L.

The concentrations assume activity of 0.085 U/mg protein for cell extract and 15 U/mg protein for recombinant protein. Adjust concentrations according to actual activity.

Preparation of assay mixture

Prepare and add the assay mixture to wells of a 96-well plate (UV transparent; Corning 3635)

For each well, the assay mixture will contain

Component	Volume (μ L)	Final concentration (mM or U/mL)
ddH ₂ O	3	NA
KPO ₄ (1 M) (pH = 8.0)	5	50
KCl (1 M)	10	100
DTNB (10 mM)	15	1.5
Phosphotransacetylase (3000 U/mL)	1	30
Potassium acetate (1 M) (pH =7) or ddH ₂ O	30	300
Enzyme sample or ddH ₂ O	6	NA
Succinyl-CoA (5 mM) or ddH ₂ O	30	1.5
Final volume	100	

Some components are added as master mixes, not individually, for convenience and uniformity.

Component	Master mix
ddH ₂ O	A
KPO ₄ (pH = 8.0) (1 M)	A
KCl (1 M)	A
DTNB (10 mM)	A
Phosphotransacetylase (3000 U/mL)	A

Prepare the master mix in a 1.5-mL centrifuge tube or larger volume tube. Make extra (equivalent of 1/2 sample) to ensure sufficient volume.

Measurement of activity

Add components to wells in the following order:

Addition	Well			
	1	2	3	4
1	Mastermix A			
1 Potassium acetate	ddH ₂ O	Potassium acetate	Potassium acetate	Potassium acetate
1 Enzyme sample	Enzyme sample	ddH ₂ O	Enzyme sample	Enzyme sample
2 SucCoA	SucCoA	SucCoA	ddH ₂ O	ddH ₂ O

After making addition 1, mix contents of wells and record absorbance (412 nm) on Molecular Devices M3 spectrophotometer. Take readings at 20 s intervals for 5 min.

Make addition 2, then record absorbance for 30 to 45 min. Because succinyl-CoA is unstable in solution, keep it in the freezer until shortly (5 min) before this addition. Thaw succinyl-CoA by flicking the tube and vortexing, then keep on ice until use.

Calculation of activity

- 1.) Denote the absorbance after addition 1 as A_1 . A_2 is defined similarly.
- 2.) Plot A_2 vs. time, find the linear part of the curve, and then calculate the slope. Do this for each well.
- 3.) Calculate the reaction rate

$$\text{Rate} = (\text{slope}/14\ 150) / \text{path length}$$
 where $14\ 150\ \text{M}^{-1}\ \text{cm}^{-1}$ is the extinction coefficient of TNB^{2-} (89). The path length is expressed in cm and measured according to *Measurement of path length of 96-well plates*. A typical value of path length is 0.288 cm.
- 4.) Calculate the activity ($\text{mol L}^{-1}\ \text{min}^{-1}$) by correcting the reaction rate with control cuvettes.

$$\text{Activity} = \text{Rate}_{\text{well 1}} - \text{Rate}_{\text{well 3}}$$

Though the above is one way to correct the reaction rate, another way is

$$\text{Activity} = \text{Rate}_{\text{well 1}} - \text{Rate}_{\text{well 2}} - \text{Rate}_{\text{well 4}}$$

Notes

i) If A_2 is not linear with time, then reduce the volume of enzyme sample added. If volume is not reduced, then activity will be underestimated.

Assay of succinyl-CoA:acetate CoA-transferase activity with citrate synthase and malate dehydrogenase

Modified from (90)

Assay mixture

Prepare and add the assay mixture to ultramicrocuvettes (Brandtech 759200).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
ddH ₂ O	38.37	NA
Tris (1 M) (pH = 8.0)	10	100
L-malate (100 mM)	0.4	0.4
NAD (40 mM)	2.5	1
KPO ₄ (1 M) (pH = 6.8)	0.284	2.84
Malate dehydrogenase (180 U/mL)	4.89	8.8
Citrate synthase (90 U/mL)	1.56	1.4
Cell extract	24	NA
Potassium acetate (1 M) (pH = 7.0)	6	60
Succinyl-CoA (5 mM)	12	0.6
Final volume	100	

Some components are added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
Tris (1 M) (pH = 8.0)	A
L-malate (100 mM)	A
NAD (40 mM)	A
KPO ₄ (1 M) (pH = 6.8)	B
Malate dehydrogenase (180 U/mL)	B
Citrate synthase (90 U/mL)	B

The components are added to cuvettes in the following order:

Addition	Cuvette			
	1	2	3	4
1			ddH ₂ O	
1			Mastermix A	
1			Mastermix B	
1	Cell extract	Cell extract	Cell extract	ddH ₂ O
2	Acetate	Acetate	ddH ₂ O	Acetate
3	SucCoA	ddH ₂ O	SucCoA	SucCoA

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize (1 to 3 min) before making next addition. Repeat for addition 2.

After addition 3, record absorbance at short (0.5 min) intervals for 5 to 10 min.

Because the reaction occurs quickly, combine components and record absorbance for only one cuvette at a time; it is not possible to handle all 4 cuvettes at once.

Calculation of activity

- 1.) Denote the absorbance after addition 1 as A_1 . A_2 and other absorbances are defined similarly.
- 2.) Plot A_3 vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.
- 3.) Calculate the reaction rate

$$\text{Rate} = (\text{slope}/6220)$$
 where $6220 \text{ M}^{-1} \text{ cm}^{-1}$ is the extinction coefficient of NADH.
- 4.) Calculate the activity ($\text{mol L}^{-1} \text{ min}^{-1}$) by correcting the reaction rate with control cuvettes.

$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}} - \text{Rate}_{\text{cuvette 3}}$$

Another plausible way to correct the reaction rate and calculate activity is

$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 4}}$$

It is up to the experimenter to decide which measure of activity is more appropriate.

Assay of succinyl-CoA synthetase activity with hydroxylamine

Modified from (75, 91)

Assay mixture

Prepare and add the assay mixture to pairs of 0.5 mL screw-top centrifuge tubes. Include at least 4 pairs.

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
ddH ₂ O	20.8	NA
Tris (pH = 7.2) (1 M)	5	50
MgCl ₂ (1 M)	1	10
Succinate (pH = 7.0) (0.25 M)	4	10
ATP (100 mM)	1.2	1.2
Coenzyme A (5 mM)	8	0.4
Hydroxylamine (pH = 7.0) (2 M)	10	200
Cell extract	50	NA
Final volume	100	

Some components are added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
Tris (pH = 7.2) (1 M)	A
MgCl ₂ (1 M)	A
ATP (100 mM)	A
Coenzyme A (5 mM)	A
Hydroxylamine (pH = 7.0) (2 M)	A

The components are added to tubes in the following order:

Component	Volume (μL)	Addition
ddH ₂ O	20.8	1
Mastermix A	25.2	1
Succinate or ddH ₂ O*	4	1
Cell extract	50	1
Development solution	28.57	2

*One half of the pair of tubes should have succinate, and the other half should have ddH₂O

Conditions for the additions are:

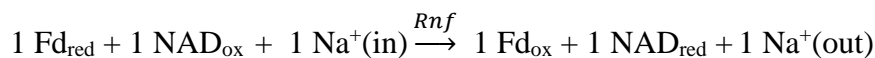
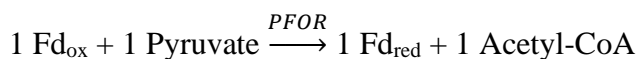
Addition	Conditions
1	Incubate at room temperature for variable time
2	Incubate at room temperature for at least 5 min, centrifuge at 18,000 g, then read A_{540} of supernatant (110 μL) in microcuvettes

For addition 1, stop incubation at intervals (e.g., 0, 5, 15, 30, and 60 min). For the 0 min incubation, add development solution before cell extract. For addition 2, incubation at room temperature can be done for up to 30 min.

Assay of ferredoxin:NAD⁺ oxidoreductase (Rnf) activity

Modified from (88)

Principle of measurement



where Fd_{ox} is oxidized ferredoxin, Fd_{red} is reduced ferredoxin, NAD_{ox} is oxidized NAD, NAD_{red} is reduced NAD, and PFOR is pyruvate:ferredoxin oxidoreductase. NAD_{red} absorbs at 340 nm and is measured with a spectrophotometer.

Preparation of enzyme samples

Prepare cell membrane of test organism according to *Preparation of cell membrane and cytoplasmic contents by ultracentrifugation*. Prepare it anaerobically. The buffer used is Tris-MgSO₄ (50 mM Tris-Cl [pH 7.6], 20 mM MgSO₄, 4 mM dithiothreitol, 4 μM resazurin). Add nuclease (25 U/mL Pierce Universal Nuclease) before disruption by French press. The final concentration of protein in the assay should be about 40 μg/mL.

Preparation of assay mixture

Prepare and add the assay mixture to a 1.4 mL-glass cuvette (Hellma HL114-10-20) capped with chlorobutyl stopper (DWK Life Sciences W224100-081).

For each cuvette, the assay mixture contains

Component	Volume (μL)	Final concentration (mM)
dH ₂ O (anaerobic)	630	NA
Tris (pH 7.6) (1 M) (anaerobic)	50	50
MgCl ₂ (1 M) (anaerobic)	10	10
Dithiothreitol (100 mM) (anaerobic)	40	4
NaCl (1 M) (anaerobic)	10	10
Cell membrane or dH ₂ O (anaerobic)	100	40 ^a
Coenzyme A (40 mM) (anaerobic)	5	0.2
Ferredoxin (c. 1 mM)	30	0.03
Thiamine pyrophosphate (0.1 M) (anaerobic)	1	0.1
Phosphotransacetylase (1000 U/mL)	4	4 ^b
Crude pyruvate:ferredoxin oxidoreductase (anaerobic)	100	36 ^a
Pyruvate (1 M) anaerobic)	10	10
NAD (200 mM) (anaerobic)	10	2
Final volume	1000	

^aμg protein/mL

^bU/mL

Add components to the cuvette anaerobically. Transfer small aliquots of components to 0.5 mL centrifuge tubes flushed with N₂. While flushing the cuvette with N₂, transfer the component to the cuvette with a micropipette. Cap the cuvette once done. Tap the cuvette to remove any bubbles.

Measurement of activity

Add components to cuvettes in the following order:

Addition	Cuvette	
	1	2
1		dH ₂ O
1		Tris
1		MgCl ₂
1		Dithiothreitol
1		NaCl
1	Cell membrane	dH ₂ O
1		Coenzyme A
1		Ferredoxin
1		Thiamine pyrophosphate
1		Phosphotransacetylase
1	Crude pyruvate:ferredoxin oxidoreductase	
2		Pyruvate
3		NAD

Make addition 1, then incubate for 5 to 10 min. Make addition 2, then record absorbance at 340 and 430 nm with a Molecular Devices M3. Wait until absorbance at 430 nm reaches plateau (requires 5 to 10 min). Make addition 3 and continue to record absorbance.

Perform the incubations and absorbance recordings at 37°C. To configure the Molecular Devices M3 to do this, choose “Temperature ON, 37°C” with parameters “Absorbance, Kinetics, 2 wavelengths (340 and 430), time length 15 min and 10 second interval”. When absorbance is not being recorded, incubation can be done in a water bath.

Example results are shown in Fig. 1.

Calculation of activity

- 1.) Denote the absorbance after addition 3 as A₃.
- 2.) Plot A₃ vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.
- 3.) Calculate the reaction rate

$$\text{Rate} = (\text{slope}/6\ 200)$$

where 6 200 M⁻¹ cm⁻¹ is the extinction coefficient of NADH.

4.) Calculate the activity ($\text{mol L}^{-1} \text{min}^{-1}$) by correcting the reaction rate with control cuvettes.
$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}}$$

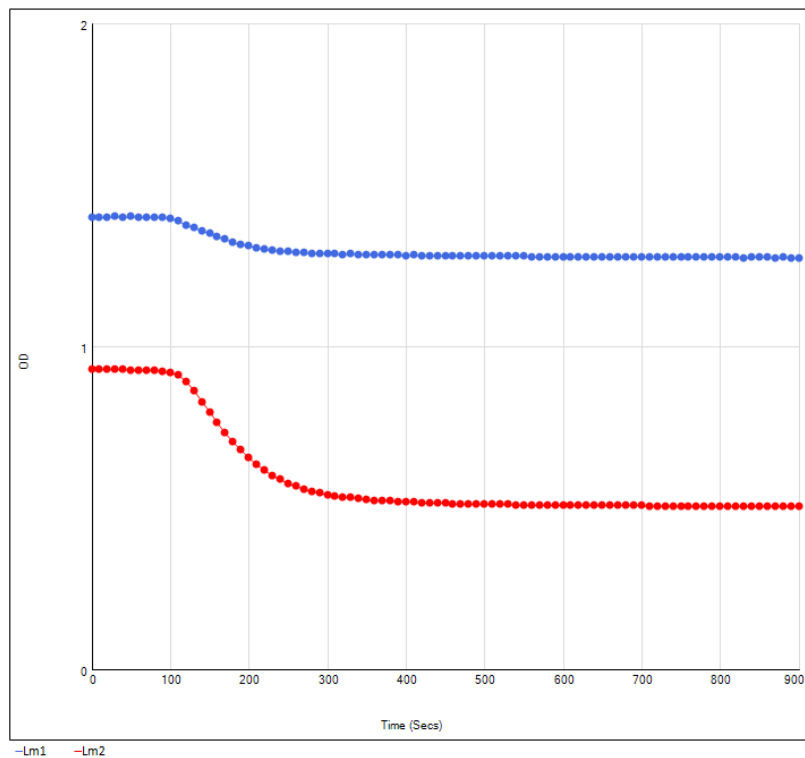
5.) Measure the protein of the membrane and express activity as $\text{mol mg protein}^{-1} \text{min}^{-1}$.

Notes

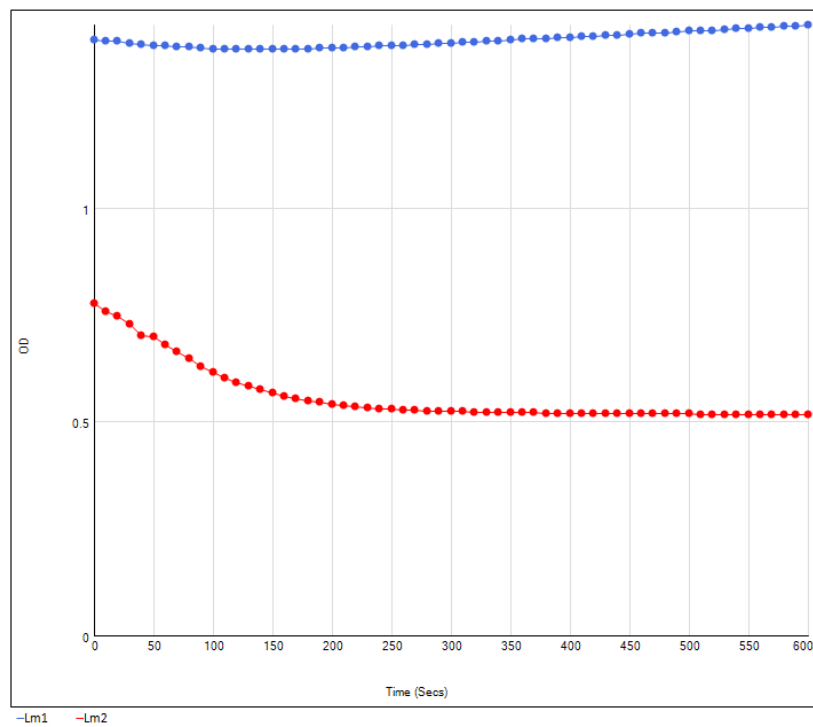
i) Low activity may be observed and be due to oxidation of NADH (by a NADH dehydrogenase). To correct for this oxidation, include a control cuvette containing NADH (in place of NAD).

Fig. 1. Change in absorbance over time. (A) After addition 2. (B) After addition 3. Results are for cuvette 1. Blue = absorbance at 340 nm; red = absorbance at 430 nm.

(A)



(B)



Assay of acetate with lactate dehydrogenase, pyruvate kinase, phosphotransacetylase, and acetate kinase

Modified from (92) and Megazyme acetic acid kit (K-ACETM)

Preparation of samples

Prepare samples to contain up to 0.1 mM acetate. To inactivate any enzymes, samples can be heated in 100°C water bath for 10 min then cooled on ice, with no loss of acetate.

Assay mixture

Prepare and add the assay mixture to cuvettes (Fisher 14-955-127).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
Triethanolamine (pH = 7.4) (1 M)	21.1	42.2
MgCl ₂ (1 M)	4.2	8.4
EDTA (pH = 8) (100 mM)	4.2	4.2
ATP (100 mM)	1.1	0.22
Phosphoenolpyruvate (100 mM)	5.5	1.1
Coenzyme A (5 mM)	11	0.11
NADH (40 mM)	2.5	0.2
Sample	435.91	NA
Lactate dehydrogenase (275 U/mL)	3.82	2.10
Pyruvate kinase (100 U/mL)	3.15	0.63
Phosphotransacetylase (3,000 U/mL)	3.76	22.56
Acetate kinase (25,000 U/mL)	3.76	188
Final volume	500	

Some components are made added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
Triethanolamine (pH = 7.4) (1 M)	A
MgCl ₂ (1 M)	A
EDTA (pH = 8) (100 mM)	A
ATP (100 mM)	A
Phosphoenolpyruvate (100 mM)	A
Coenzyme A (5 mM)	A
NADH (40 mM)	A
Lactate dehydrogenase (275 U/mL)	B

Pyruvate kinase (100 U/mL)	B
Phosphotransacetylase (3,000 U/mL)	C
Acetate kinase (25,000 U/mL)	C

The volume of mastermix A is 49.6 μL per sample. The volume of mastermix B is 6.97 μL per sample. The volume of mastermix C is 6.67 μL per sample. Prepare each mastermix in 2-mL centrifuge tube. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
Mastermix A	1
Mastermix B	1
Sample	1
Mastermix C	2

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least one blank (e.g., buffer).

Calculation of analyte concentration

1.) Denote the absorbance after the first addition as A_1 . A_2 and other absorbances are defined similarly.

2.) Calculate analyte concentration for cuvette as

$$(A_2^* - A_1) / 6220 \times (500/435.91)$$

where $6220 \text{ M}^{-1} \text{ cm}^{-1}$ is the extinction coefficient of NADH and $(500/435.91)$ is the ratio of the final volume to sample volume.

A_2^* is the absorbance after addition 2, extrapolated to the time of addition 2. To find it, plot A_2 vs. time after addition 2. Fit a line to the linear portion of the curve. The y-intercept of the curve is A_2^* .

3.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the supernatant (done before heating in 2-mL screw-top tube).

Assay of acetyl-CoA with citrate synthase and DTNB

Modified from (68, 73)

Preparation of samples

Prepare samples to contain up to 50 μM acetyl-CoA. To inactivate any enzymes, samples can be heated in 100°C water bath for 10 min then cooled on ice, with no loss of acetyl-CoA.

Assay mixture

Prepare and add the assay mixture to cuvettes (Fisher 14-955-127).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
KPO ₄ (pH = 7.0) (1 M)	50	100
Oxaloacetate (10 mM)	50	1
DTNB (10 mM)	50	1
Sample	345.83	NA
Citrate synthase (90 U/mL)	4.17	0.75
Final volume	500	

Some components are made added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
KPO ₄ (pH = 7) (1 M)	A
Oxaloacetate (10 mM)	A
DTNB (10 mM)	A

The volume of mastermix A is 150 μL per sample. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
Mastermix A	1
Sample	1
Citrate synthase (90 U/mL)	2

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making

next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least blank (e.g., water).

Calculation of analyte concentration

4.) Denote the absorbance after the first addition as A_1 . A_2 and other absorbances are defined similarly.

5.) Calculate analyte concentration for cuvette as

$$(A_2 - A_1) / 14\,150$$

where $14\,150 \text{ M}^{-1} \text{ cm}^{-1}$ is the extinction coefficient of TNB^{2-} .

6.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the sample.

Assay of acetyl-CoA with malate dehydrogenase and citrate synthase

Modified from (68, 73)

Preparation of samples

Prepare samples to contain up to 0.1 mM acetyl-CoA. To inactivate any enzymes, samples can be heated in 100°C water bath for 10 min then cooled on ice, with no loss of acetyl-CoA.

Assay mixture

Prepare and add the assay mixture to cuvettes (Fisher 14-955-127).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
KPO ₄ (pH = 7) (1 M)	50	100
L-malate (100 mM)	25	5
NAD (40 mM)	18.75	1.5
Sample	399.58	NA
Malate dehydrogenase (180 U/mL)	2.5	0.9
Citrate synthase (90 U/mL)	4.17	0.75
Final volume	500	

Some components are made added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
KPO ₄ (pH = 7) (1 M)	A
L-malate (100 mM)	A
NAD (40 mM)	A

The volume of mastermix A is 93.75 μL per sample. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
Mastermix A	1
Sample	1
Malate dehydrogenase (180 U/mL)	2
Citrate synthase (90 U/mL)	3

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least blank (e.g., water).

Calculation of analyte concentration

7.) Denote the absorbance after the first addition as A_1 . A_2 and other absorbances are defined similarly.

8.) Calculate analyte concentration for cuvette as

$$(A_3 - A_2)/6220 \times \{1 + (A_2 - A_1)/[(A_2 - A_1) + (A_3 - A_2)]\} \times (500/401.25)$$

where $6220 \text{ M}^{-1} \text{ cm}^{-1}$ is the extinction coefficient of NADH and $(500/401.25)$ is the ratio of the final volume to sample volume.

9.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the sample.

Assay of acetyl phosphate with malate dehydrogenase, citrate synthase, and phosphotransacetylase

Modified from (73, 93)

Preparation of samples

Prepare samples to contain up to 0.1 mM acetyl-P.

Assay mixture

Prepare and add the assay mixture to cuvettes (Fisher 14-955-127).

The assay mixture consists of

Component	Volume (μL)	Final concentration (mM or U/mL)
Triethanolamine (pH = 7.5) (1 M)	75	150
MgCl ₂ (1 M)	1.5	3
L-malate (100 mM)	50	10
Coenzyme A (5 mM)	17	0.17
NAD (40 mM)	12.5	1
Sample	333.57	NA
Malate dehydrogenase (180 U/mL)	2.5	0.9
Citrate synthase (90 U/mL)	4.17	0.75
Phosphotransacetylase (3000 U/mL)	3.76	22.56
Final volume	500	

Some components are made added as mastermixes, not individually, for convenience and uniformity.

Component	Mastermix
Triethanolamine (pH = 8.4) (1 M)	A
MgCl ₂ (1 M)	A
L-malate (100 mM)	A
ATP (100 mM)	A
Coenzyme A (5 mM)	A
NAD (40 mM)	A

The volume of mastermix A is 156 μL per sample. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
-----------	----------

Mastermix A	1
Sample	1
Malate dehydrogenase (180 U/mL)	2
Citrate synthase (90 U/mL)	3
Phosphotransacetylase (3000 U/mL)	4

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least blank (e.g., water).

Calculation of analyte concentration

10.) Denote the absorbance after the first addition as A_1 . A_2 and other absorbances are defined similarly.

11.) Calculate analyte concentration for cuvette as

$$(A_4^* - A_3) / 6220 \times \{ 1 + (A_3 - A_1) / [(A_4^* - A_3) + (A_3 - A_1)] \} \times (333.57 / 401.25)$$

where $6220 \text{ M}^{-1} \text{ cm}^{-1}$ is the extinction coefficient of NADH and $(500/333.57)$ is the ratio of the final volume to sample volume.

A_4^* is the absorbance after addition 4, extrapolated to the time of addition 4. To find it, plot A_4 vs. time after addition 4. Fit a line to the linear portion of the curve. The y-intercept of the curve is A_4^* .

12.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the sample (if any).

Measurement of path length of 96-well plates

Cuvettes have a fixed path length (usually 1 cm), but 96-well plates do not. Path length can be measured empirically using a NADH standard solution.

NADH standard solution

Prepare the NADH standard solution

Component	Volume (μL)	Final concentration (mM or U/mL)
ddH ₂ O	997.7	NA
NADH (40 mM)	2.5	0.1
	Final volume	1000

Absorbance in cuvettes

Add NADH standard solution to cuvettes (Fisher 14-955-127).

Addition	Cuvette	
	1	2
1	ddH ₂ O	ddH ₂ O
2	NADH	NADH

After making addition 2, record absorbance (340 nm) using cuvette port of Molecular Devices M3 spectrophotometer

Absorbance in well plates

Add contents to cuvettes well plates (e.g., Corning 3635).

Addition	Well	
	1	2
1	Cuvette 1	Cuvette 2

Do not add the full 1000 μL volume; instead, add the volume required by a specific assay (often 100 to 200 μL).

After making addition 1, record absorbance (340 nm) using plate reader of Molecular Devices M3 spectrophotometer.

Calculation of path length

1.) Denote the absorbance of cuvettes as $A_{\text{cuvette 1}}$ and $A_{\text{cuvette 2}}$. $A_{\text{well 1}}$ and $A_{\text{well 2}}$ are the absorbances of well plates.

2.) Calculate path length (cm) as

$$\text{Path length} = (A_{\text{well 1}} - A_{\text{well 2}}) / (A_{\text{cuvette 1}} - A_{\text{cuvette 2}})$$

For 200 μL in Corning 3635 plate, path length is c. 0.58 cm. For 100 μL , it is c. 0.288. Path length must be determined for each plate and volume.

SECTION V: MOLECULAR TECHNIQUES AND BIOINFORMATICS

Molecular techniques are wide-ranging, and this manual focuses mostly on nucleotide sequencing, which has a renewed importance with the genomes of hundreds of rumen bacteria now available. This manual describes how to PCR-amplify and sequence a gene, as well as look up sequences of genes catalogued in the IMG database.

Preventing contamination in molecular biology experiments

For molecular biology experiments, aseptic technique is not necessary (as when handling bacteria), but certain practices should still be observed.

- 1.) Before any work, clean bench with 70% ethanol.
- 2.) Use pipettes, tips, and other materials designated for molecular biology use.
- 3.) Before working with molecular biology H₂O, pour aliquots in 15- or 50-mL centrifuge tubes. This avoids contaminating the stock bottle.
- 4.) When handling any tube with a screw cap, carefully remove cap and either (a) hold cap in hand or (b) place on lab with the screw threads facing up.
- 5.) Sterilize microtubes by autoclaving in a glass or plastic container covered with aluminum foil. When accessing tubes, remove aluminum foil, place aluminum foil (sterile side up) on lab bench, and “pour” tubes onto foil. Carefully pick up tubes by cap. Do not put tubes back in container.
- 6.) For sensitive work, clean bench and supplies with 10% bleach (made fresh daily). Use only microtubes that are PCR-certified, and do not autoclave.
- 7.) For very sensitive work, perform all procedures in a biological safety cabinet.

Colony PCR

Adapted from NEB Q5 High-Fidelity DNA Polymerase online protocol (<https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491>) and Zymo D4003 protocol

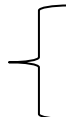
- 1.) Order PCR primers from Life Technologies. See other procedures for primers to use, or Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) to design primers.
- 2.) Prepare 100 μM stock concentration of primers. Look up concentration of primers in manufacturer datasheet accompanying primers. Add 10 μL molecular biology H_2O per nmol of primer (e.g., 185 μL H_2O for tube containing 18.5 nmol) directly to primer tube. Store at -20°C until ready for use.
- 3.) Prepare 10 μM working concentration of primers. Add 90 μL molecular biology H_2O to 1.5 mL sterile centrifuge tube, then add 10 μL aliquot of 100 μM stock. Store at -20°C until ready for use.
- 3.) Prepare overnight broth culture of bacteria (see *Growth in broth of Culturing bacteria*).
- 4.) Collect culture for PCR.
 - (a) Set up autoclaved gassing probe on clamp. Flame sterilize.
 - (b) Flame sterilize rubber bung of tubes using ethanol. Keep under a lit Bunsen burner.
 - (c) Flush 22-gauge needle of 1-mL syringe with CO_2 by inserting it into gassing probe (see *Making buffer and media*). Withdraw ~ 0.1 mL CO_2 (same volume as culture removed in next step).
 - (d) Withdraw ~ 0.1 mL culture from stock tube after injecting 0.1 mL CO_2 .
 - (e) Inject 1 drop (~ 20 μL) into sterile 1.5 mL microcentrifuge tube containing 200 μL molecular biology H_2O .
- 5.) Prepare PCR mastermix. See **Table 1**. Prepare at least one reaction per sample, 1 reaction for negative control, 1 reaction for positive control, and 0.5 reaction extra for waste. Keep DNA polymerase at -20°C until ready for use, and add last. All other components can be kept on ice until ready for use.
- 6.) Aliquot 49 μL PCR mastermix into PCR tubes. Add 1 μL of DNA template (diluted culture prepared in #4) to each of 2 tubes. Add 1 μL of molecular biology H_2O to another tube (negative control). Add 1 μL of DNA from previous experiment (one proven to give PCR amplification) to another tube (positive control).
- 7.) Run PCR using conditions listed in individual procedures, or general conditions given in **Table 2**.
- 8.) Run a 1% agarose electrophoresis gel to determine if amplification was successful (see *Agarose gel electrophoresis*).

9.) Clean up PCR products using Zymo D4003 kit and Fig. 1.

Table 1. Components of PCR mastermix

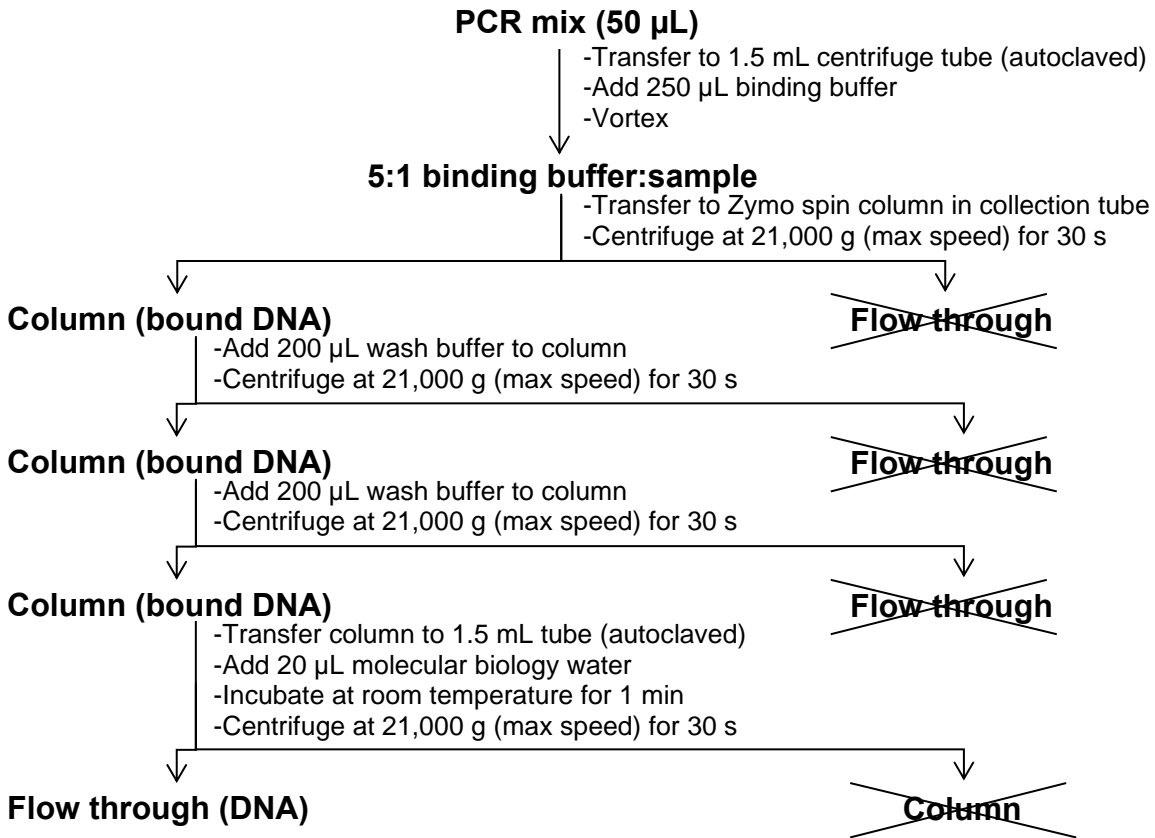
Component	μL per 50 μL reaction
5x Q5 reaction buffer (from NEB M04935)	10
10 mM dNTP (NEB N0447S)	1
Forward primer working concentration	2.5
Reverse primer working concentration	2.5
Molecular biology H ₂ O	32.5
Q5 DNA polymerase fidelity (from NEB M04935)	0.5

Table 2. PCR cycling conditions

	Temp ($^{\circ}\text{C}$)	Length	
	98	30 s to 3 min	
25 to 35 cycles		98	5 to 10 s
		*	10 to 30 s
		72	20 to 30 s per kb of amplicon
	72	2 min	
	4	∞	

*Use NEB T_m calculator (<http://tmcalculator.neb.com/#!/main>); typically 3 $^{\circ}\text{C}$ above T_m of lower T_m primer

Fig. 1. Clean up of PCR products with Zymo D4003 kit.



Sequencing

Primers

- 1.) Order sequencing primers from Life Technologies. See other procedures for primers to use, or design primers using Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). Choose primers so that all base pairs of the amplicon will be sequenced at least twice; each sequence read covers between 200 to 800 bp typically.
- 2.) Prepare 100 μM stock concentration of primers. Look up concentration of primers in manufacturer datasheet accompanying primers. Add 10 μL molecular biology H_2O per nanogram of primer (e.g., 185 μL H_2O for tube containing 18.5 nmol) directly to primer tube. Store at -20°C until ready for use.
- 3.) Prepare 10 μM working concentration of primers. Add 90 μL molecular biology H_2O to 1.5 mL sterile centrifuge tube, then add 10 μL aliquot of 100 μM stock. Store at -20°C until ready for use.

PCR and sample submission

- 1.) Perform PCR according to *Colony PCR*. One 50 μL reaction per sample is usually sufficient.
- 2.) Purify the PCR product according to *Clean up of PCR products*.
- 3.) Confirm presence of product by running 1% agarose electrophoresis gel (see *Agarose gel electrophoresis*).
- 4.) Check DNA concentration with NanoDrop. A concentration of 50 to 100 $\text{ng}/\mu\text{L}$ is typical.
- 5.) Prepare PCR product for submission to the UC Davis DNA Sequencing Center. For each sequencing reaction, the center requires 6 μL template at a concentration of 2 $\text{ng}/\mu\text{L}$ per 100 bp of PCR product. Pipette enough PCR product and molecular biology H_2O at the bottom of a 0.5 mL tube to meet requirements.

Example: A PCR product of 1,500 bp has a concentration of 60 $\text{ng}/\mu\text{L}$, and it needs to be sequenced using two primers. Pipette 6 μL of product and 6 μL of molecular biology H_2O at bottom of tube. This will meet the volume (12 μL) and concentration (30 $\text{ng}/\mu\text{L}$) requirements.

- 6.) Prepare the primers for submission. For each sequencing reaction, the sequencing center requires 4 μL of primer at concentration of 3 μM . Pipette enough primer and molecular biology H_2O at the bottom of a 0.5 mL tube to meet the requirement.

Example: Products A, B, and C need to be sequenced using primers 1 and 2. The working concentration of primers is 10 μM . Pipette 3.6 μL of primer 1 and 8.4 μL molecular biology H_2O at the bottom of one tube. Repeat for primer 2 using a second tube. This will meet the volume (12 μL) and concentration (3 μM) requirements.

7.) Fill out a sample submission form at the website of the UC Davis DNA Sequencing Center.

8.) Place tubes in a Ziploc bag and walk them over to the center.

Sequence alignment and assembly

1.) Download sequence files (**Fig. 1**).

2.) Generate a consensus sequence. Use the R script in Appendix 1.

Appendix 1: R script for generating consensus sequence

```
#Install and load packages
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
#BiocManager::install()
#BiocManager::install("ShortRead")
#BiocManager::install("Biostrings")
#BiocManager::install("msa")
#BiocManager::install("sangerseqR")

library(ShortRead)
library(Biostrings)
library(msa)
library(sangerseqR)
library(stringr)

#Set inputs
#Set directory for sequences
setwd("C:\\My Directory")

#Set primer names
forward_primer_name = "27f"
reverse_primer_name = "1492r"

#Set cutoff for quality scores (bases low with scores lower than cutoff will be trimmed from
sequence)
quality_cutoff = 30

#Set window for calculating average quality score
#Moving average of quality scores calculated over this window
moving_average_window = 5

#Set file paths and names
#Get all file names in directory
file_names=list.files()

#Get file names for forward reads
forward_file_names = file_names[grepl(pattern=paste0(forward_primer_name,".*ab1"),
x=file_names)]

#Get file names for reverse reads
reverse_file_names = file_names[grepl(pattern=paste0(reverse_primer_name,".*ab1"),
x=file_names)]

#Get sequencing reaction number for forward reads
forward_sequencing_reaction_number=str_extract(string=forward_file_names, pattern=paste0("[0-
9]*\\+",forward_primer_name))
forward_sequencing_reaction_number=str_extract(string=forward_sequencing_reaction_number,
pattern="[0-9]*")
forward_sequencing_reaction_number=as.numeric(forward_sequencing_reaction_number)

#Get sequencing reaction number for reverse reads
reverse_sequencing_reaction_number=str_extract(string=reverse_file_names, pattern=paste0("[0-
9]*\\+",reverse_primer_name))
reverse_sequencing_reaction_number=str_extract(string=reverse_sequencing_reaction_number,
pattern="[0-9]*")
reverse_sequencing_reaction_number=as.numeric(reverse_sequencing_reaction_number)

#Place file names in order of sequencing reaction
forward_file_names=forward_file_names[order(forward_sequencing_reaction_number)]
reverse_file_names=reverse_file_names[order(reverse_sequencing_reaction_number)]

#Create consensus sequence
for(i in 1:length(forward_file_names))
{
  #Get file name
  forward_file_name=forward_file_names[i]
  reverse_file_name=reverse_file_names[i]
```

```

#Convert ab1 to fastq
#Set file names for conversion function
#File path
forward_file_path_abi=file.path=(forward_file_name)
reverse_file_path_abi=file.path=(reverse_file_name)

#File name without extension
forward_file_name_no_extension = gsub("\\\\.\\.*", "", forward_file_name)
reverse_file_name_no_extension = gsub("\\\\.\\.*", "", reverse_file_name)

#File name with fastq extension
forward_file_name_fastq= paste(forward_file_name_no_extension, ".fastq", sep="")
reverse_file_name_fastq= paste(reverse_file_name_no_extension, ".fastq", sep="")

#Perform conversion
abifToFastq(seqname = forward_file_name_no_extension, fname = forward_file_path_abi, outfile = forward_file_name_fastq)
abifToFastq(seqname = reverse_file_name_no_extension, fname = reverse_file_path_abi, outfile = reverse_file_name_fastq)

#Load fastq files and put reverse read in correct orientation
#File path
forward_file_path_fastq=file.path=(forward_file_name_fastq)
reverse_file_path_fastq=file.path=(reverse_file_name_fastq)

#Load fastq files
forward_shortreadq=readFastq(forward_file_path_fastq)
reverse_shortreadq=readFastq(reverse_file_path_fastq)

#Perform reverse complement on reverse read
reverse_shortreadq =
ShortReadQ(reverseComplement(sread(reverse_shortreadq)),FastqQuality(reverse(quality(quality(reverse_shortreadq))))),id(reverse_shortreadq))

#Trim ends with low quality scores
#Get quality scores
#Forward read
forward_quality = (as(quality(forward_shortreadq[1]),"matrix"))
forward_quality = as.vector(forward_quality)

#Reverse read
reverse_quality = (as(quality(reverse_shortreadq[1]),"matrix"))
reverse_quality = as.vector(reverse_quality)

#Average quality scores over a window of bases
#Forward read
forward_quality_moving_average = forward_quality

for(j in 1:length(forward_quality))
{
  k = (j):(j+moving_average_window)
  forward_quality_moving_average[j] = sum(forward_quality[k])/moving_average_window

  if(is.na(forward_quality_moving_average[j]))
  {
    forward_quality_moving_average[j]=0
  }
}

#Reverse read
reverse_quality_moving_average = reverse_quality

for(j in 1:length(reverse_quality))
{
  k = (j):(j+moving_average_window)
  reverse_quality_moving_average[j] = sum(reverse_quality[k])/moving_average_window

  if(is.na(reverse_quality_moving_average[j]))
  {
    reverse_quality_moving_average[j]=0
  }
}

```

```

}

#Identify base where to start trimming (where average quality score falls below cutoff)
#Forward read
forward_region_high_quality = which(forward_quality_moving_average>=quality_cutoff)
forward_run = rle(diff(forward_region_high_quality))$lengths

forward_trim_right = sum(forward_run[1:which(forward_run==max(forward_run))]) -
max(forward_run) + 1
forward_trim_left = forward_trim_right + max(forward_run) - 1

forward_region_high_quality=forward_region_high_quality[forward_trim_right:forward_trim_left]

#Reverse read
reverse_region_high_quality = which(reverse_quality_moving_average>=quality_cutoff)
reverse_run = rle(diff(reverse_region_high_quality))$lengths

reverse_trim_right = sum(reverse_run[1:which(reverse_run==max(reverse_run))]) -
max(reverse_run) + 1
reverse_trim_left = reverse_trim_right + max(reverse_run) - 1

reverse_region_high_quality=reverse_region_high_quality[reverse_trim_right:reverse_trim_left]

#Perform trimming
forward_shortreadq = narrow(forward_shortreadq, start=min(forward_region_high_quality),
end=max(forward_region_high_quality))
reverse_shortreadq = narrow(reverse_shortreadq, start=min(reverse_region_high_quality),
end=max(reverse_region_high_quality))

forward_file_name_trimmed = paste(forward_file_name_no_extension, "_trimmed.fasta",
sep="")
reverse_file_name_trimmed = paste(reverse_file_name_no_extension, "_trimmed.fasta",
sep="")

writeFasta(forward_shortreadq, forward_file_name_trimmed)
writeFasta(reverse_shortreadq, reverse_file_name_trimmed)

#Align reads
#Include only first sequence in read (ab1 files have multiple copies of same sequence)
forward_shortreadq = forward_shortreadq[1]
reverse_shortreadq = reverse_shortreadq[1]

#Perform alignment
alignment = pairwiseAlignment(pattern=sread(forward_shortreadq),
subject=sread(reverse_shortreadq), type="local")

#Create consensus sequence
#Get sequence of aligned region
aligned_region = toString(alignment)

#Add region of forward read that comes before aligned region
#Position where aligned region begins
aligned_region_start = attr(attr(pattern(alignment), "range"), "start")

#Sequence of forward region before aligned region
forward_region = narrow(forward_shortreadq, start=1, end=aligned_region_start-1)
forward_region = as(sread(forward_region),"character")

#Add region of reverse read that comes after aligned region
#Position where aligned region ends
aligned_region_end = attr(attr(subject(alignment), "range"), "width")
#Sequence of reverse region after aligned region
reverse_region = narrow(reverse_shortreadq, start=aligned_region_end+1)
reverse_region = as(sread(reverse_region),"character")

#Combine regions into consensus sequence
consensus_sequence = paste(forward_region, aligned_region, reverse_region, sep="")

```

```
#Export consensus sequence as fasta
consensus_file_name = paste0("sequencing_reaction_",i,"_consensus.fasta", sep="")
consensus_sequence = DNASTringSet(consensus_sequence)
writeFasta(consensus_sequence, consensus_file_name)
}
```

Agarose gel electrophoresis

1.) Make 0.5 M EDTA (pH = 8.0). Add 93.05 g disodium EDTA·2H₂O to 400 mL dH₂O. Stir while adding ~9 g NaOH. Gradually add more NaOH until pH reaches 8.0, then continue to stir until solution is clear. If solution does not clear within 15 min, gently heat and add more dH₂O. Bring to 500 mL final volume with dH₂O.

Make 5x TBE buffer. Add to 300 mL dH₂O

27 g Tris base

13.75 g boric acid

10 mL 0.5 M EDTA pH = 8.0

Stir until dissolved. Bring to 500 mL final volume.

Dilute TBE to 0.5x.

2.) Prepare 1% agarose. Add 0.3 g agarose (Sigma A7431-100G) to 30 mL 0.5x TBE in 250 mL Erlenmeyer flask. Heat in microwave until dissolved (requires 30 s). Add 3 µL SYBR Safe (Thermo S33102), then swirl to mix

3.) When cool (c. 60°C), pour agarose into gel mold (7 × 10 cm; Bio-Rad 1704435). Insert comb and allow to solidify. Store at 4°C in ziploc bag until use.

4.) Fill gel box with 0.5x TBE. Insert gel.

5.) Load first well with 7 µL 1 kB Plus ladder (Invitrogen) and 1.75 µL loading dye. Load remaining wells with 4 to 7 µL PCR product and 1 to 1.75 µL loading dye (maintaining product and dye in 4:1 ratio).

6.) Run gel at 120 V until loading dye reaches ~¾ of total length of gel. Image.

Example experiment: Sequencing and taxonomic identification of bacteria using 16S rDNA gene

Modified from (101, 102) and NEB Q5 High-Fidelity DNA Polymerase online protocol (<https://www.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491>)

- 1.) Perform PCR on bacterial culture using 27f and 1492r primers (**Table 1**). Use conditions in **Table 2**. See *Colony PCR*.
- 2.) Remove primers following *Clean up of PCR products*.
- 3.) Sequence PCR products according to *Sequencing*. Use primers in **Table 1**; for quick verification, primers 27f and 1492r alone are usually adequate.
- 4.) Generate a consensus sequence after aligning and assembling reads (**Fig. 1**). See *Sequencing*.
- 5.) Perform taxonomic identification with EzTaxon (ezbiocloud.net/eztaxon/identify) (**Fig. 2**). Paste the consensus sequence into the dialog box and click “Identify”. When job is completed, scroll down to see assignment to species level. The top match is shown in the first output screen (**Fig. 2b**). To see additional matches, click the link below “Query”, which will bring up a more detailed output screen (**Fig. 2**). An excel file can be downloaded that shows top match and sequence identities.

Table 1. Primer sequences.

Primer	Sequence
27f	AGAGTTTGATCMTGGCTCAG
530f	GTGCCAGCMGCCGCGG
926f	AAACTYAAAKGAATTGACGG
342r	CTGCTGCSYCCCGTAG
907r	CCGTCAATTCMTTTRAGTTT
1492r	TACGGYTACCTTGTTACGACTT

Table 2. PCR cycling conditions

Temp (°C)	Length (min)	
	98 3	
35 cycles	} 98 0.167	
		63 0.5
		72 1
		72 2
	4 ∞	

Fig. 1. Example consensus sequence of 16S rDNA gene.

GCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTT
GCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGA
TAACTACTGGAAACGGTAGCTAATAACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG
CCTCTTGCCATCGGATGTGCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT
GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATAC
CTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGGTGCAAGCGTTAATCGGAATTAAGCGTAAAGCGCACGCAGGCGGTTTGTAAAG
TCAGATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATCTGATACTGGCAAGCTTGAGTCTCG
TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAAGGTTAAAAC TCAAT
GAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTTTAAATTCGATGCAACGCGAAGAACC
TTACCTGGTCTTGACATCCACGGAAGTTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAAGTCAAAGGAGACTGCCAGTGATAAAC
TGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTA
CAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCCTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAAT
GCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTACACCATGGGAGTGGGTTGCA
AAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTGATTCATGACTGGGGTGA
AGT

Fig. 2. Taxonomic assignment with EzTaxon. (a) Interface for uploading files. (b,c) Output of assignment. Classification is to species *Escherchia coli* with identity of 99.93%.

(a)

The screenshot shows the EzTaxon web interface. The top navigation bar includes 'EZBIOCLOUD', a search bar, and user information 'Tim Hackmann Logout'. The main header has 'EZTaxon' and links for 'How to cite', 'Hierarchy', 'Taxonomic Group', 'Cart', and 'Q&A'. A left sidebar contains navigation options like 'EzGenome', 'EzTaxon', 'Overview', 'Identify', 'CompGen', 'Results', 'Assemble', 'Replace Accession', 'Resource Central', 'App Central', 'Education Central', and 'My Information'. The main content area is titled 'Identifying a Strain' and explains that EzTaxon-e uses BLAST and a pair-wise sequence alignment algorithm. It provides options for file upload and retrieval from GenBank. A large text area displays a 16S rRNA sequence in red text.

(b)

The screenshot shows the 'Analysis Results' page in EzTaxon. The top navigation and sidebar are identical to screenshot (a). The main content area is titled 'Analysis Results' and includes controls for 'Columns to Display' (Completeness, Accession, Taxonomy) and 'Task Icons' (View query, Re-identify, Delete). Below these are buttons for 'Re-identify checked item(s)', 'Delete checked items', and 'Create&Download.CLC'. A 'Download' section offers 'Identify' and 'CompGen' options. A table displays the results for a query, showing a match to 'Escherchia coli KCTC 2441(T)' with a similarity of 99.93% and 100% completeness. The table has columns for 'asks', 'Job Type', 'DB', 'Query (Click to view details)', 'Length (bp)', 'Closest match', 'Similarity (%)', 'Completeness (%)', and 'Accession'.

asks	Job Type	DB	Query (Click to view details)	Length (bp)	Closest match	Similarity (%)	Completeness (%)	Accession
Identify	EzTaxon	Anonymous strain		1475	Escherchia coli KCTC 2441(T)	99.93	100	EU01

(c)

EzGenome

EzTaxon

[Overview](#)[Identify](#)[CompGen](#)**Results**[Assemble](#)[Replace Accession](#)

Resource Central






App Central

Education Central

My Information

Results of Identify Analysis

Tasks Icon

-  [View hit sequence](#)
-  [Perform CompGen Analysis against the genus](#)
-  [View pairwise alignment](#)
-  [Link to J.P. Euzéby's site](#)
-  [Taxonomic group information](#)

Columns to display

- Taxonomy
- Accession
- Pairwise Similarity
- Diff/Totant
- Completeness








[Hide all](#) [Show all](#)

Functions

[CSV file](#) [Excel file](#) [FASTA\(gz\)](#) [Publish](#) [EzEditor file](#)
■ Identification of Anonymous strain [Edit](#)

 Length of sequence :1475bp [View query sequence](#)

 Completeness :100% 1 - 1476

Tasks	Rank	Name	Strain	Authors	Taxonomy
	1	Escherichia coli	KCTC 2441(T)	(Migula 1895) Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact
	2	Escherichia fergusonii	ATCC 35469(T)	Farmer et al. 1985	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact fergusonii;
	3	Shigella flexneri	ATCC 29903(T)	Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact
	4	Shigella sonnei	GTC 781(T)	(Levine 1920) Weldin 1927	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact
	5	Shigella boydii	GTC 779(T)	Ewing 1949	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact
	6	Escherichia coli	O157:EC4115	(Migula 1895) Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact
	7	Escherichia albertii	TW07627(T)	Huys et al. 2003 (Shiga 1898) Castellani and Chalmers	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobact

Example experiment: Sequencing of the mannose phosphotransferase (PTS) operon

Modified from (99)

- 1.) Perform PCR on bacterial culture using 55F and 2808R primers (**Table 1**). Use conditions in **Table 2**. See *Colony PCR*. Repeat with 2439F and 3927R primers.
- 2.) Sequence PCR products according to *Sequencing*. Use primers in **Table 2**. For the amplicon generated with 55F and 2808R, use primers 55F to 2439F and 305R to 2808R. For the amplicon generated with 2439F and 3927R, use the remaining primers.
- 3.) Generate a consensus sequence after aligning and assembling reads. See *Sequencing*. Include a reference sequence (**Fig. 1**) to aid alignment and identification of aberrant base calls.

Notes

- i) Two amplicons are generated (55F and 2808R, 2439F and 3927R) because a single amplicon that covered the entire sequence could not be easily generated.

Table 1. Primer sequences (from Primer3Plus with default settings)

Primer	Sequence
55F	TTTCCCGAACGTTATTTTGC
452F	TCGCACAATTTGATGCTGAT
935F	TCAAACAAGCAGCACCAAAC
1425F	CATTGAAAACTTTATAAACGAAAGGA
1936F	TTGCAGTACCAACTTCAGCAG
2439F	TGCTCTTGAACGTCACCTGG
2938F	TCCCTAAAGGAAACGTAAGTGG
3447F	CGTAACAAAGTCAGCCGTCA
305R	CCGACACCCATTCTTGTCT
787R	CACCGATGACAGTTCCTTCA
1313R	CCTGCTGAAAGCTTAATTCAAA
1785R	AGGAAAAGACCTGCAACAGC
2276R	GCCATGTTTGTACCCCTTTC
2808R	TGGATGATACCACCTGAAAGG
3299R	AGCCGTTAATTCCACGGTAG
3787R	GGTTCATCCAGCTTCACAT
3927R	CAGCAAGTTTTTCAGCAGCA

Table 2. PCR cycling conditions

Temp (°C)	Length (min)	
35 cycles	94	2
	94	0.5
	55	0.5
	68	4
	4	∞

Figure 1. Mannose PTS operon. Sequence is from (100) and annotated using (99).

TTTCCCGAACGTTATTTTGC = first forward primer (55F)

TTGCCG = promoter 1

GATTAT = promoter 2

TTGTTT... = transcription start site

GGAGG = Shine-Dalgarno

ATG...TAA = manX

ATG...TAG = manY

ATG...TAA = manZ

ATG...TAA = manO

GCT...CAGC = terminator

TGCTGCTGAAAACCTGCTG = last reverse primer (3957R)

>2601025965 PTS system, mannose-specific IIB component
[Streptococcus bovis JB1 : Ga0062118_101] (+)strand

ACCTTTTCATGATTCTTTCAAAAAACATAGCGTTTTTCCCGAACGTTATT
TTGCGAATTATACAATTTAATCGCTTTATTTTGTGGGTTTCTGAAAATTT
TTCATGATTTCTGTTATTTTTTCTCAAAAAGCTATTGCATTTTTTGTGAA
AACGTTTTACAATAATATCGTTCTTAAAATTTCTTTGGAACGGAAAAGAA
TTGCCC GACTTGTCCGGAAGGA GATTAT TCTTTT TTGTTT CAACGAAAA
TAATCATAA GGAGGAAGAACAAGA ATGGGTGTCGGTATTATTATTGCCAG
CCATGGTAAATTTGCTGAAGGTATTCATCAATCAGGTTCTATGATCTTTG
GTGACCAAGAGAAAAGTTCAAGTTGTAACTTTCATGCCAAGCGAAGGACCT
GATGATTTATATGCACACTTCAACGATGCTATCGCACAATTTGATGCTGA
TGATGAAATCCTCGTACTAGCTGACCTTTGGAGTGGTTCTCCATTTAACC
AAGCTAGTCGCGTAATGGGTGAAAATCCAGACCGCAAGATGGCTATCATT
ACAGGTCTTAATTTGCCAATGCTTATCCAAGCCTACACTGAGCGTATGAT
GGACGCAAATGCAGGTGTTGAACAAGTTGTCGCAAATATCATTAAGAGT
CTAAAGACGGTGTAAAGCACTTCTGAAGA ACTCAACCCAGCTGAAACA
GCTGCTGCAGCGCCTGCCGCACAAGCTGCTCCTCAAGGTGCTATCCCTGA
AGGAACTGTCATCGGTGATGGTAAACTTAAAATCAACCTCGCTCGTATTG
ACACACGCTTCTTACGGACAAGTTGCAACAACTGGACACCAGCTTCT
AAAGCTGATCGTATCATCGTTGCATCTGACACTGTATCTAAAGATGAATT
GCGTAAAGGCTTGATCAAACAAGCAGCACCAAACGGTGTAAAGCAAACG
TTGTTCCAATCAAGAAATTGATTGAAGCTTCTAAAGACCCTCGTTTTGGT
AACACACACGCACTTATCTTGTGTTGAAACACCTCAAGAAGCTCTTGAAGC
TATCGAAGGCGGTGTGCCAATTAAAGA ACTTAACGTTGGTTCAATGGCTC
ACTCAACAGGTAAAACAATGGTTAACAACGTATTGTCAATGGACAAAGAT
GACGTTGCTACATTTGAAAAATTACGTGACCTTGGCGTAACATTCGATGT
TCGTAAAGTCCCTAATGATTCTAAAAAAGACTTGTTTTGATCTAATCAAAA
AAGCAAACGTTCAATAA TTTTTGAATTAAGCTTTCAGCAGGGTGAAAAAG
AAAGCGTTTCTATGAAGGGCTTTCACCTGTCGTGAGAATCAAAC TTTCT
GTTTTTATCCAAAACACGTAAGCCTTGGTTTTCTGGCTTACAATTGCACAT
TGAAAAC TTTTATAAACGAAAGGA ACTTAGAATA ATGTCAGTTATTTCTAT
GATTTTAGTCGTTGTAGTTGCCTTCTTCGCTGGTCTTGAAGGTATCCTTG
ACGAATTCCAATTCACCAACCACTAGTTGCCTGCACACTTATCGGTCTT
GTTACTGGTAACCTTGAAGCAGGTATCATCCTTGGCGGTTCTCTACAAAT
GATCGCTCTTGGTTGGGCTAACATTGGTGCCGCTGTTGCGCCAGATGCTG
CCCTTGCTTCTGTAGCCGCTGCTATCATCATGGTTAAAGGTGGAGACTTC
ACTTCTAAAGGTATCGCCGTTGCAACAGCAACAGCTATCCCTCTTGCTGT
TGCAGGTCTTTTCCTTACTATGCTTGTTCGTA CTGCTTCTGTTGCCCTTG
TTCACGGTGCTGACGCTGCTGCTAAAGAAGGTAACATCGCTGCAGTTGAA
CGTACTCACTTGGTAGCTCTATTCCCTTCAAGGTCTTCGTATTGCTGTTCC
TGCTGCTCTTCTTCTTGCACTACCAACTTCAGCAGTACAATCTATCCTTA
ACGCTATGCCAGACTGGTTGTCAGGTGGTATGGCTGTCGGTGGTGGTATG
GTTGTTGCCGTAGGTTACGCTATGGTTATCAACATGATGGCTACAAGCGA
AGTATGGCCTTTCTTCGCAATCGTTTTTGCCGTTGCTGCTATCTCTGACC
TTACTCTTATCGCCCTTGGTACAATTGGTGTGCTCTTGCCTTCATCTAC
CTTAACCTTTTCAGAAAAGGTGGAAATGGTGGCGGAACTATCTCAGGTTCC

TGGTGACCCAATCGGCGACATCTTGGAAGACTACTAGAAAGGGGTACAAA
CATGGCTGAAAACTTCAATTATCAAAATCTGATCGTCAAAAAGTTTGGT
GGCCTTCTACTTTCCTACAAGGTTCTTGGAACACTACGAACGTATGCAAAAC
TTAGGTTGGGCATACGCTTTGATCCCTGCTATCAAAAACTTTATACATC
TAAAGAAGACCGAGCTGCTGCTCTTGAACGTCACCTGGAATTCTTCAATA
CTCACCCATACGTTGCTGCTCCAATCATCGGTGTAACCTTTGCCCTTGAA
GAAGAACGCGCAAATGGTGTGAAATTGATGACACAGCTATCCAAGGGGT
TAAAATCGGTATGATGGGACCTCTTGCTGGTGTGGTGACCCAGTCTTCT
GGTTTACAGTTCGTCCTATCCTTGGTGCCCTTGGTGCTTCACTTGCTATG
GCAGGTAACATCGTTGGTCCACTTCTATTCTTCTTTGGATGGAACATCAT
CCGTATGGCATTCTTGTGGTACACTCAAGAACTTGGTTACAAAGCTGGTT
CTGAAATCACTAAAGACCTTTCAGGTGGTATCATCCAAAAATCACTAAA
GGTGCTTCAATCCTTGGTATGTTTCATCTTGGCTGTCTTGGTTGAACGTTG
GGTATCAATTAACCTTCACTGTTGATCTTCCCTTCAACTAACTTTCAGAAG
GTGCCTACATCGAATTCCCTAAAGGAAACGTAACCTGGTACTGAACTTCAA
GGTATTCTTGGTAAAGTGGCTGATGGACTTAGCCTTTCACCAGAAAAAGC
TAACACACTTCAAGGTCAATTGAACTCATTGATTCCTGGTTTGGATGGGAC
TTGCCCTTACATTCTTTGCATGTGGTTGCTTAAGAAAAAAGTTTCTCCA
ATCACAATCATCATCGGATTGTTTCATCGTTGGTATCATCGCTCGTTTCTT
CGGAATCATGTAAATAAAGAAAGGAAGGATTCGGTCCTTCTTTTTTTAT
TATGTTATAATAGACTAAAAGTTTAAATCAGAGGAGATTACATGGCACAAAT
CACTTAACTCTACCGTGGAATTAACGGCTACTGGTGTTCCTTACCTTGGT
ATGGGAGGAAAAGTAGGGAAATTCCTTCTCGGTAACAAAGGTCTTGAATT
TTATAGCGACGCTAACGTCGAAGATTATATCCAAATTCCTTGGGAAAACA
TCGAAAAAATCGGCGCTAACGTTTCTCGTAACAAAGTCAGCCGTCACCTT
GAAATCTTTACAGACAAAGGGAAATTTCTTTTTGCCTCAAAGACTCAGG
AAAAATCTTAAAAGTGGCTCGCCAGCATATCGGAAACGATAAAGTTGTT
GTATGCTTACGCTTGTTCAAGTCCTTATGAAAAAATAACAGGATTTGTC
AAAAAGAAATAAATCTAGTATAATAAATAGTACGGATAAGTAAGAGTGGA
GCTCTTTCAGAAAGTCTGCGGTTGCTGTGAGCAGATAGAGCGACAGGTCT
GAAATTCTACCGTTATATCAATTAATAAATAGAAATTAAGTGCACAGGATG
TGAAAGCTGGATGGAACCGCGGATTGCGCTCCAGCAGTTAACATGCTGTG
CTTTTTGTTTCATCTGGGCACAGCTAACCCCTATGAGCGCTAAAAAACTGG
AGGAACTTATGTTAGACATCAAACGTATTCGTAACGATTTTCGACGAAGT
CGCTAAAAAATTAGCTACACGTGGCGTTGCTGCTGAAAAACTTGCTGAAC
TTAAAGAACTTGACGACAAACGTCGTGAATTGCTT

Example experiment: Finding the sequence of mannose phosphotransferase (PTS) genes with IMG

Modified from (100)

- 1.) Go to <https://img.jgi.doe.gov/cgi-bin/m/main.cgi>. You may be prompted to register and log in first.
- 2.) Go to “Genomes” -> “Genome search”.
- 3.) In “Keyword”, type the genome of interest. Click “Go”.
For a large set of rumen bacterial genomes, enter “Hungate 1000”. Under “Filters”, find “Study Type”.

IMG Home Find Genomes Find Genes Find Functions Compare Genomes Analysis Cart OMICS ABC My IMG Data Marts Using

Home > Find Genomes Loaded

NERSC Quarterly Maintenance: All IMG systems will be down for **two days** from **Tue Feb 3 2015** to **Wed Feb 4 2015**. Sorry for the inconvenience.

Genome Search

by Fields by Metadata Categories by Metadata Category Operation

Genome Search by Fields

Find genomes by keyword or substring.

Keyword:

Filters:

Go Reset

hint: All searches treat the keyword as a substring (a word or part of a word).
The search should contain some alphanumeric characters.
Use an underscore () as a single-character wildcard.
Use % to match zero or more characters.
All matches are case insensitive.

Examples

- "pseudomonas" as Genome Name retrieves all genomes with the substring "pseudomonas" such as "Pseudomonas syringae B728a".
- "62977" for NCBI Taxon ID retrieves "Acinetobacter sp. AD1".
- "NC_008009, NC_008010, NZ_AAKW01000001" as Scaffold External Accession will retrieve genomes that have scaffolds with those accession ID's.
- "arch" for Domain retrieves all Archaea.
- "proteo" for Phylum retrieves all proteobacteria.
- "finished" for Sequencing Status retrieves all finished genomes.
- "draft" for Sequencing Status retrieves all finished genomes.
- "JGI" for Sequencing Center retrieves all genomes sequenced at JGI.
- "2.0" for IMG Release retrieves all genomes with IMG release 2.0

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- 4.) Click “Select All” and “Add Selected to Genome Cart”. Make sure that you are showing all entries on one page; select “All” from the drop down menu.

IMG Home Find Genomes Find Genes Find Functions Compare Genomes Analysis Cart OMICS ABC My IMG Data Marts Using

Home > Find Genomes 3 genomes retrieved

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Genome Field Search Results

hint: Go to [Preferences](#) to show or hide plasmids, GFragment and viruses. Go to [home page statistics](#) under [IMG Genomes](#) to select individual phylogenetic domains or all genomes.

Domains(D): B=Bacteria, A=Archaea, E=Eukarya, P=Plasmids, G=GFragment, V=Viruses.
Genome Completion(C): F=Finished, P=Permanent Draft, D=Draft.

Add Selected to Genome Cart Select All Clear All

3 of 3 rows selected

Filter column: Domain Filter text Apply

Export Page 1 of 1 << first < prev 1 next > last >> All

Select	Domain	Status	Genome Name	Study Name	Sequencing Center	Genome Size	Gene Count
<input checked="" type="checkbox"/>	A	F	Methanospirillum hungatei JF-1	Methanogenic Syntropic Consortium	DOE Joint Genome Institute	3544738	3307
<input checked="" type="checkbox"/>	B	P	Butyrivibrio hungatei NK4A153	The Hungate 1000. A catalogue of reference genomes from the rumen microbiome.	DOE Joint Genome Institute	3372542	3164

5.) In the menu tabs, point to “Find Functions”, then “KEGG”, then “Pathways via KO terms”. Click.

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242 Genomes Quick Genome Search: Go Hi Timothy Hackmann | Logout

img INTEGRATED MICROBIAL GENOMES

IMG Home Find Genomes Find Genes Find Functions Compare Genomes Analysis Cart OMICS ABC My IMG Data Marts Using

Home > Find Genomes 241 genomes retrieved

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Genome Field Search

hint: Go to [Preferences](#) to show or hide plasmids, GFragment and viruses. Go to [home page statistics](#) under [IMG Genomes](#) to select individual phylogenetic domains or all genomes.

Domains(D): B=Bacteria, A=Archaea, E=Eukarya, P=Plasmids, G=GFragment, V=Viruses.
Genome Completion(C): F=Finished, P=Permanent Draft, D=Draft.

Add Selected to Genome Cart Select All Clear All

Filter column: Domain Filter text Apply

Export Page 1 of 3 << first < prev 100 next > last >>

- Function Search
- Search Pathways
 - COG
 - KOG
 - Pfam
 - TIGRFam
 - Transporter Class.
 - KEGG
 - Orthology KO Terms
 - Pathways via KO Terms
- Enzyme
- MetaCyc
- Phenotypes
- InterPro Browser
- Protein Family Comparison

Select	Domain	Status	Genome Name	Study Name	Sequencing Center	Genome Size	Gene Count
<input type="checkbox"/>							

https://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindFunctions&page=ffoAllKeggPathways&view=ko

6.) Search for and click on “Phosphotransferase system (PTS)”

- 03 [Protein export](#) (39)
- 03 [Protein processing in endoplasmic reticulum](#) (140)
- 03 [SNARE interactions in vesicular transport](#) (34)
- 03 [Ubiquitin mediated proteolysis](#) (124)
- 03 [Sulfur relay system](#) (21)
- 03 [Proteasome](#) (48)
- 03 [RNA degradation](#) (75)
- 02 **Replication and repair**
 - 03 [DNA replication](#) (51)
 - 03 [Base excision repair](#) (41)
 - 03 [Nucleotide excision repair](#) (48)
 - 03 [Mismatch repair](#) (45)
 - 03 [Homologous recombination](#) (59)
 - 03 [Non-homologous end-joining](#) (19)
 - 03 [Fanconi anemia pathway](#) (54)
- 01 **Environmental Information Processing**
 - 02 **Membrane transport**
 - 03 [ABC transporters](#) (433)
 - 03 [Phosphotransferase system \(PTS\)](#) (79)
 - 03 [Bacterial secretion system](#) (74)
 - 02 **Signal transduction**
 - 03 [Two-component system](#) (408)
 - 03 [Ras signaling pathway](#) (161)
 - 03 [Rap1 signaling pathway](#) (151)
 - 03 [MAPK signaling pathway](#) (188)
 - 03 [MAPK signaling pathway - fly](#) (17)
 - 03 [MAPK signaling pathway - yeast](#) (41)
 - 03 [ErkB signaling pathway](#) (64)
 - 03 [Wnt signaling pathway](#) (98)
 - 03 [Notch signaling pathway](#) (28)
 - 03 [Hedgehog signaling pathway](#) (40)
 - 03 [TGF-beta signaling pathway](#) (61)
 - 03 [Hippo signaling pathway](#) (104)
 - 03 [Hippo signaling pathway - fly](#) (52)
 - 03 [VEGF signaling pathway](#) (37)
 - 03 [Jak-STAT signaling pathway](#) (123)
 - 03 [NF-kappa B signaling pathway](#) (86)

7.) Click tab for “View Map for Selected Genomes”. Click “Add All>>”, then click “View Map”.

KEGG Pathway Details

Details for Pathway: *Phosphotransferase system (PTS)*
 *Showing counts for genomes in genome cart only

KO Terms in Pathway | Save to My Workspace | **View Map for Selected Genomes**

KEGG Map for Selected Genomes

Sequencing Status: All Finished, Permanent Draft and Draft | Domain: Genome Cart | Selected: 242

Search for: <enter a genome name to search>

Streptococcus epidermidis AG42 (B) [P]
 Streptococcus bovis 2B (B) [P]
 Streptococcus bovis AG45 (B) [P]
 Streptococcus bovis B315 (B) [P]
 Streptococcus bovis JB1 (B) [P]
 Streptococcus bovis JN033 (B) [P]
 Streptococcus equinus GA-1 (B) [P]
 Streptococcus bovis SN033 (B) [P]
 Streptococcus equinus GA-1 (B) [P]
 Streptococcus gallolyticus VTM1R27 (B) [P]
 Streptococcus henryi A-4 (B) [P]
 Succinibacillus dextrinosolvens ACN-10 (B) [P]
 Succinibacillus dextrinosolvens H5 (B) [P]
 Treponema bryantii NK4A124 (B) [P]
 Treponema sp. C6A8 (B) [P]

Selected Genomes

Please select genomes to highlight on KEGG Pathway Map: 242 selected

Streptococcus bovis B315 (B) [P]
 Streptococcus bovis JB1 (B) [P]
 Streptococcus bovis JN033 (B) [P]
 Streptococcus equinus GA-1 (B) [P]
 Streptococcus gallolyticus VTM1R27 (B) [P]
 Streptococcus henryi A-4 (B) [P]
 Succinibacillus dextrinosolvens ACN-10 (B) [P]
 Succinibacillus dextrinosolvens H5 (B) [P]
 Treponema bryantii NK4A124 (B) [P]
 Treponema sp. C6A8 (B) [P]

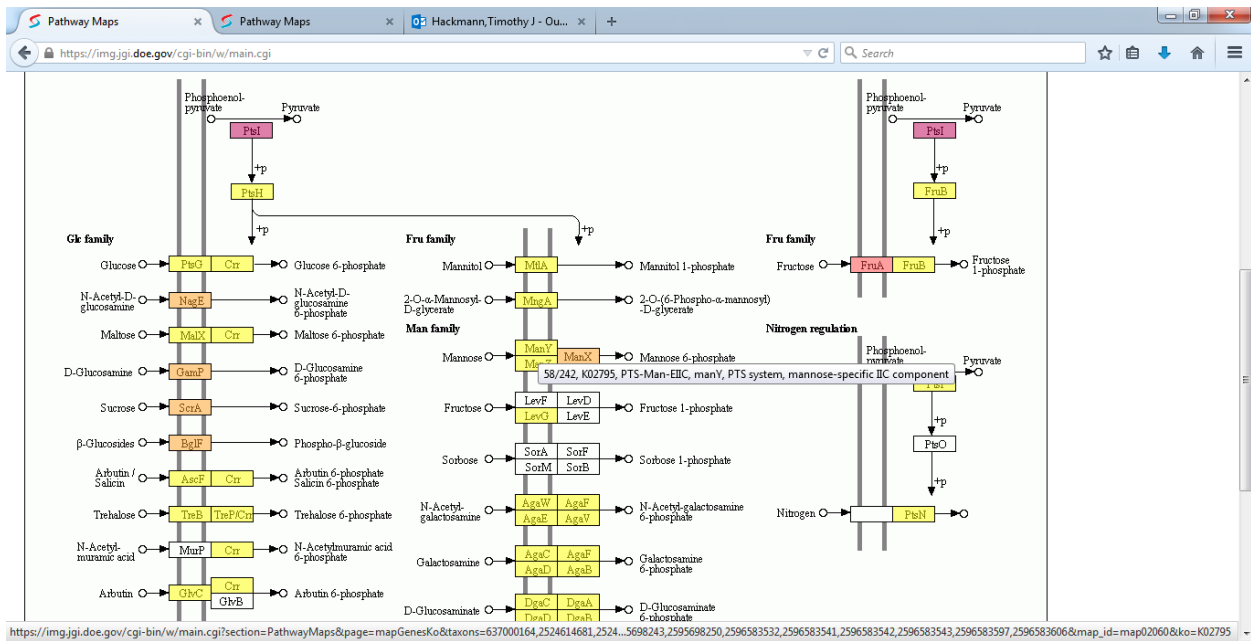
Find missing enzymes. (For single genome selection)

View Map | Reset

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8.) Find the mannose PTS transporter in the figure. Click on the ManX, ManY, or ManZ subunit.



9.) The screen that appears shows all gene IDs for ManX, ManY, or ManZ. Click on a gene ID of interest.

Filter column: Gene ID Filter: text Apply

Export Page 1 of 2 << first < prev 1 2 next > last >> 100

Column Selector Select Page Deselect Page

Select	Gene ID	Locus Tag	Gene Product Name	Genome	KO
<input type="checkbox"/>	2525402220	G598DRAFT_00618	PTS system, mannose/fructose/sorbose family, IIC component	Selenomonas ruminantium ATCC 12561	K02795
<input type="checkbox"/>	2525403206	G598DRAFT_01611	PTS system D-mannose-specific IIC component, Man family (TC 4 A.6.1.1)	Selenomonas ruminantium ATCC 12561	K02795
<input type="checkbox"/>	2525447621	G605DRAFT_00459	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium cadaveris AGR2141	K02795
<input type="checkbox"/>	2525447876	G605DRAFT_00716	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium cadaveris AGR2141	K02795
<input type="checkbox"/>	2525454837	G614DRAFT_04236	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium clostridioforme AGR2157	K02795
<input type="checkbox"/>	2525456699	G594DRAFT_00068	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
<input type="checkbox"/>	2525457711	G594DRAFT_02100	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
<input type="checkbox"/>	2525458478	G594DRAFT_02880	PTS system, fructose-specific, IIB component/PTS system, fructose subfamily, IIA component/PTS system, fructose subfamily, IIC component	Clostridium paraputrificum AGR2156	K02795
<input type="checkbox"/>	2525458728	G594DRAFT_03133	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
<input type="checkbox"/>	2525459007	G594DRAFT_03415	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
<input type="checkbox"/>	2525469216	G619DRAFT_02853	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Lachnospiraceae bacterium NK4A144	K02795
<input type="checkbox"/>	2525473359	G607DRAFT_00212	PTS system D-glucose-specific IIC component, Man family (TC 4 A.6.1.6)	Clostridium butrificum AGR2140	K02795
<input type="checkbox"/>	2525474413	G607DRAFT_01284	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-	Clostridium butrificum	K02795

10.) Scroll down and click “Add to Gene Cart.”

External Links	
Fused Gene	No
Protein Information	
Amino Acid Sequence Length	303aa
COG	COG3716 - Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IID
KOG	
Families	- IPR004704 Phosphotransferase system, mannose/fructose/sorbose family IID component (ProSiteProfiles PS51108) - TIGR00828 PTS system, mannose/fructose/sorbose family, IID component
Transmembrane Helices	Yes
Signal Peptide	No
Statistics	peptide
Pfam	pfam03613 - EIID-AGA
Pathway Information	
Enzymes	
Transport Classification	TC:4.A.6 - The PTS Mannose-Fructose-Sorbose (Man) Family
KEGG Orthology (KO) Term	KO:K02798 PTS-Man-EIID, manZ PTS system, mannose-specific IID component
KEGG Pathway	Amino sugar and nucleotide sugar metabolism Fructose and mannose metabolism Phosphotransferase system (PTS)
KEGG Orthology (KO) Modules	PTS system, mannose-specific IID component
MetaCyc Pathway	
IMG Pathways	
IMG Clusters	
Chromosomal Cassette	Details By -- Select Protein Cluster --

11.) Click “Upload & Export” tab, select the “FASTA Nucleic Acid format” radio button, and click “Show in Export Format”.

Upload Gene Cart

The Gene Cart is used for genes already in IMG. Only previously exported IMG genes can be uploaded. [To upload private data](#), you must submit it to IMG through the [submission site](#).

You may upload a Gene Cart from a tab-delimited file. The file should have a column header 'gene_oid' (or 'Gene ID', or 'Gene Object ID'). (This file can be created using the [Export Genes](#) section below with the format 'Gene information in tab delimited format to Excel')

File to upload:
 No file selected.

Export Genes

hint: Export large number of genes will be very slow. You will be notified for the result via email if exporting over 1000 genes.

You may select genes to export in one of the following export formats.

FASTA Amino Acid format
 FASTA Nucleic Acid format -0 bp upstream +0 bp downstream
 Gene data in tab-delimited format to Excel (Gene Cart uploadable format)

My Email:
 (Results will be mailed to you if selection is over 1000 genes.)

12.) Examine the sequence that is displayed. Copy and paste into Notebook and save as a *.fasta file.

Thurs Mar 17 2016 IMG will be down all day for database maintenance. Sorry for the inconvenience. Get updates from our [IMG Forum](#)

Export Gene Fasta Nucleic Acid Sequence

Red = start or stop codon, Green = upstream or downstream padding.

```
>2601025967 FTS system IID component, Man family [Streptococcus bovis JB1 : Ga0062116_101] (+)strand
ATGGCTGAAAACCTCAATTATCAAAATCTGATCGTCAAAAGTTTGGT
CGGTTCATCTTCTCACAAGGTTCTGGAACACTACGACGTATGCAAACT
TAGGTTGGGCATACGCTTGTATCCCTGCTATCAAAAACCTTATACATCT
AAGAAGACCGAGCTGCTCTTGAACGTCACCTTGAATCTTCAATAC
TCACCCATACGTTGCTGCTCCAATCATCGGTGTAACCTTGGCCCTGAAG
AAGAAGCGCAATGGTGTGAATGATGACACAGCTATCCAGGGGTT
AAATCGGATATGAGGACCTTGTGCTGTTGGTGAACCCAGCTTCTG
GTTTACAGTTGCTATCTTGGTGGCTTGGGCTGCTCACTTCTATGG
CAGTAACATCGTTGGTCCACTTCTATCTTCTTGGATGGAACATCATC
CGTATGGCATTCTTGGGTACACTCAAGAACTTGGTACAAAAGCTGGTTC
TGAATCACTAAAGACCTTTCAGTGGTATCATCCAAAAAACAATAAG
GTGCTTCAATCCTTGGTATGTTCACTTGGCTGTCTTGGTGAACGTTGG
GTATCAATTAACCTCACTGTTGATCTCTTCACTAACTTTCAGGAGG
TGCTCATCGAATCCCTAAGGAACTGTAAGTACTGACTGACTGACTCAAG
GTATCTTGGTAAAGTGGCTGATGAACTTAACTTCACTCACTCACTCACT
AACACACTCAAGGTCATTAACCTATTGATCTCTGGTGTGATGGGACT
TGCCCTTACATCTTGTGATGTGGTGGTAAAGAAAAAGTTTCTCGAA
TCACAAATCATCGGATTGTTCACTGTTGGTATCATCGCTGTTTCTTC
GGAATCATGTA
```

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Version 4.540 Oct. 2015
gpweb04 gemini_shared 5.016000 2016-03-10-14.33.48 128.227.227.195

13.) As an alternative to the above, in step #5, point to “Find Functions”, then “Function Search”, then “Pathways via KO terms”. Click. Under “Keyword”, enter the KO IDs for the genes of interest (separated by commas and spaces), then click “Go”. Continue with step #9.

This approach is faster when the KO IDs are known and many IDs need to be searched. COGs and pfam IDs can be searched analogously.

Function Search

Function search allows you to find functions in selected genomes by keyword.

hint: Search term marked by * indicates that it supports metagenomes. You must add your selections into Selected Genomes.

Keyword: K02795, K02796, K02797
Filters: KEGG Orthology ID (list) *

Search for functions in: Selected Genomes Selected Domain All Isolates

Sequencing Status: All Finished, Permanent Draft and Draft
Domain: Genome Cart
 List Tree Selected: 72

- Search for: <enter a genome name to search>
- Butyrivibrio fibrisolvens AB2020 (B) [P]
 - Butyrivibrio fibrisolvens AR40 (B) [D]
 - Butyrivibrio fibrisolvens DSM 3071 (B) [D]
 - Butyrivibrio fibrisolvens FE2007 (B) [P]
 - Butyrivibrio fibrisolvens MD2001 (B) [P]
 - Butyrivibrio fibrisolvens MD3005 (B) [P]
 - Butyrivibrio fibrisolvens WTE3004 (B) [P]
 - Butyrivibrio fibrisolvens YR82005 (B) [P]
 - Butyrivibrio hungatei DSM 14810 (B) [D]
 - Butyrivibrio hungatei NK4A153 (B) [P]
 - Butyrivibrio hungatei XBD2006 (B) [D]
 - Butyrivibrio proteoclasticus B316 (B) [P]

Selected Genomes: 72 selected

- Butyrivibrio fibrisolvens AB2020 (B) [P]
- Butyrivibrio fibrisolvens AR40 (B) [D]
- Butyrivibrio fibrisolvens DSM 3071 (B) [D]
- Butyrivibrio fibrisolvens FE2007 (B) [P]
- Butyrivibrio fibrisolvens MD2001 (B) [P]
- Butyrivibrio fibrisolvens MD3005 (B) [P]
- Butyrivibrio fibrisolvens WTE3004 (B) [P]
- Butyrivibrio fibrisolvens YR82005 (B) [P]
- Butyrivibrio hungatei DSM 14810 (B) [D]
- Butyrivibrio hungatei NK4A153 (B) [P]

MER-FS Metagenome: Assembled

hint: All searches treat the keyword as a substring (a word or part of a word). The search should contain some alphanumeric characters.

Amplification of DNA from small numbers of rumen bacteria cells

Adapted from (94-97)

Preparation of equipment and other materials

- 1.) Gather 10 μ l pipette tips (Phenix TS-060BR); 30 μ l pipette tips (Diamond F171303); 300 μ l pipette tips (Eppendorf 0030 077.636); 1000 μ l pipette tips (Fisher 02707404); 1.5 mL centrifuge tubes (SARSTEDT 72.706.700); and 0.2 mL strip tubes (Biorad TLS 0801) and caps (Biorad TCS0803). Designate these materials for this work, and do not use for other procedures.
- 2.) Wipe down biological safety cabinet with 70% ethanol and then 10% bleach. Bleach must be made fresh on day of use.
- 3.) Similarly wipe down pipette tip boxes, pipettes, vortex mixer, and mini centrifuge.
- 4.) Place all equipment and materials in biological safety cabinet. Turn on the UV lamp of the cabinet for 60 min.

Preparation of solutions and reagents

Do all procedures (except #4) in the biological safety cabinet.

- 1.) Prepare buffers from the Qiagen Repli-g Single Cell kit (Qiagen 150343).
 - (a) Prepare buffer DLB by adding 500 μ l H₂O sc to the tube provided. Mix well by vortexing and centrifuging. Buffer DLB is stable for 6 months at -20°C.
 - (b) Prepare buffer D2 by mixing DTT (1M) and buffer DLB with a 1:11 ratio. Buffer D2 Buffer DLB is stable at -20°C for no more than 3 months.
- 2.) Prepare 50X SYBR Green. Dilute 1 μ L 10,000X SYBR Green (Fisher S7563) into 199 μ L molecular biology water. Dispense 50 μ L aliquots into 1.5-mL PCR microcentrifuge tubes. Store at -20°C.
- 3.) Order primers (Table 1). Prepare 10 μ M working concentrations according to *Sequencing*.
- 4.) Prepare irradiated anaerobic dilution solution (ADS). Prepare ADS solution in 9-mL aliquots in Balch tubes according to *Buffers and media recipes*. Irradiate tubes in UVC-515 UV multilinker for 80 min. Lay tubes on floor of multilinker to maximize surface area.

Preparation of rumen bacteria

- 1.) Collect rumen fluid. See *Collecting rumen fluid* for more details.
- 2.) Using 40-mL of fluid, prepare the mixed prokaryotes according to *Preparing and culturing mixed prokaryotes from rumen fluid*, except omit flocculation and centrifuge at 4°C. Resuspend the pellet to 20 ml.

- 3.) Prepare serial dilutions of mixed bacteria with irradiated ADS. Add 1 mL of resuspended pellet into 9-mL irradiated ADS solution in Balch tubes using a 1-mL syringe, and mix by inverting the Balch tube at least three times. This gives a 10^{-1} dilution. Repeat to give 10^{-2} to 10^{-4} dilutions.
- 4.) Using a 1-mL syringe, remove an aliquot (~1 mL) of each serial dilution and transfer to a 1.5-mL PCR-certified microcentrifuge tube. Prepare aliquots of a pure culture of rumen bacteria as positive control (e.g. strain C1A1) and molecular biology water as negative control.

Amplification of genomic DNA

Do all procedures with open tubes (e.g., pipetting) in the biological safety cabinet. Buffers and solutions referenced below are from the Qiagen Repli-g Single Cell kit.

- 1.) Add 2 μ l of each sample (serial dilution of mixed bacteria, positive controls, negative controls) to strip tubes in duplicate.
- 2.) Add 1.5 μ l of buffer D2 to each sample. Mix by briefly centrifuging in a mini centrifuge.
- 3.) Incubate strip tubes for 10 min at 65°C (using plate thermocycler).
- 4.) Add 1.5 μ l stop solution to each sample. Mix by briefly centrifuging in a mini centrifuge.
- 5.) Transfer tubes to ice. Store for no longer than 1 h.
- 6.) Thaw polymerase and other component on ice. Mix by vortexing and centrifuging in a mini centrifuge.
- 7.) Prepare a master mix according to Table 2. Add components in order shown. Mix by briefly centrifuging in a mini centrifuge.
- 8.) Add 20.25 μ l mastermix to each sample. Mix by briefly centrifuging in a mini centrifuge. Transfer to ice, and use immediately for next step.
- 9.) Start amplification of DNA with Bio-Rad CFX96 Real-Time system.
 - (a) Turn the system and the computer.
 - (b) Open the Bio-Rad CFX Manager using the icon on the computer desktop.
 - (c) In the “Startup Wizard” window that appears, select the run type as “User-defined” (Fig. 1). Alternatively, “Repeat run” can be chosen if conditions are the same as the last run.
 - (d) In the “Run Setup” window that appears, select “Create New...” (Fig. 2).
 - (e) In the “Protocol Editor” window (Fig. 3), insert conditions shown in Table 3. Click “Insert Step” to insert a step, and then enter the temperature and length. Click “Add Plate Read to Step” to include a plate read at the end of each cycle. Click “Insert GOTO” in order to repeat the cycle. To hold a step forever (an infinite hold), enter zero (0.00) for the time. Under “Settings”, set the temperature of heated lid at 70°C. Click “OK” to finish editing.
 - (f) Go back to “Run Setup” and click “Plate” tab (Fig. 2).

- (g) Edit the plate template in the “Plate Editor” window that appears (Fig. 4). Click on a well to edit, then select the sample type (e.g., “Unknown” for samples). Under “Load”, click SYBR. Enter sample name under “Sample name”. Repeat for all remaining wells. Click “OK”.
- (h) Go back to “Run Setup” and click on “Start Run” tab (Fig. 5). Click “Open Lid”. Lid with automatically open (do not force open).
- (i) Insert strip tubes with samples. Include a second strip tub for balancing the heated lid (Fig. 6). Click “Close Lid”.
- (j) Click “Start Run”. Enter location to save file when prompted, then click “OK”.

10.) Monitor amplification. In the “Run Details” window that appears, click the “Real-time Status tab” (Fig. 7).

11.) After the instrument has entered the final hold step, click “Stop” to terminate the run. Click “Open Lid” and retrieve the strip tubes. Turn off the system and computer.

12.) Store the amplified DNA at 4°C. Under these conditions, DNA is usable (16S rDNA is amplifiable) for at least two weeks. Long term storage can be done at -20°C.

Amplification of 16S rDNA

Do all procedures with open tubes (e.g., pipetting) in the biological safety cabinet. Buffers and solutions referenced below are from the SsoAdvance SYBR Green supermix (Biorad-172-5270).

- 1.) Mix the amplified DNA. Pipette up and down 15 times.
- 2.) Dilute the mixed and amplified DNA 1:20 with molecular biology water. Add 1 µL well mixed DNA product to 19 µL of molecular biology water in 1.5-mL PCR-certified microcentrifuge tubes. Mix well by vortexing and centrifuging in mini-centrifuge.
- 3.) Pipette 1 µL of amplified DNA (1:20 dilution) into strip tube in duplicate.
- 4.) Prepare a master mix according to Table 4.
- 5.) Add 9 µL mastermix to each sample of amplified DNA (1:20 dilution). Mix by briefly centrifuging in a mini centrifuge. Transfer to ice, and use immediately for next step.
- 6.) Start amplification of DNA with Bio-Rad CFX96 Real-Time system. Do steps (a) to (j) in #9 in **Amplification of genomic DNA** above, but follow conditions in Table 5. To include the melt curve analysis, click “Insert Melt Curve” (Fig. 3). Omit setting the lid temperature to 70°C, Click “Insert Melt Curve” .
- 7.) Monitor amplification and then terminate the run following #10 and 11 in **Amplification of genomic DNA** above. To view results for the melt curve, click “Melt Curve” tab. Ensure that curve shows one sharp peak (Fig. 8).
- 8.) Store the amplified DNA at 4°C for short term storage or -20°C for long-term storage.

Notes

i) Samples containing 100 to 100,000 bacterial cells are amplifiable for whole genome amplification using REPLI-g single cell kit (Fig. 9).

ii) DNA yield will be presented in negative controls because DNA is generated by random extensions of primer dimers, generating high molecular weight product.

iii) Irradiation time (80 min) for ADS is based on ref. (98). That reference showed that 75 min irradiation was sufficient to render DNA non-amplifiable when spiked in 15 ml liquid. The radiant exposure in that reference was 4 mW/cm^2 , whereas the exposure of our multilinker only 3.75 mW/cm^2 . Thus, $75 \times 4 / 3.75 = 80$ min irradiation time is required for our multilinker.

Table 1. Primer sequences

Primer	Sequence
926wF	GAAACTYAAAKGAATTGRCGG
1392R	ACGGGCGGTGTGTRC

Table 2. Components of the Repli-g sc DNA Polymerase mastermix

Component	μL per 25.25 μL reaction
H ₂ O sc	4.5
REGLI-g reaction buffer	14.5
50X SYBR green	0.25
REGLI-g DNA polymerase	1
Total	20.25

Table 3. PCR cycling conditions for Repli-g sc DNA Polymerase

	Step	Temp	Length
80 cycles	Amplification	30°C	6 min
	Plate read	30°C	NA
	Heat inactivation	65°C	3 min
	Hold	4°C	∞

Table 4. Components of the SsoAdvanced Universal SYBR Green mastermix

Component	μL per 10 μL reaction
SsoAdvance SYBR Green supermix 2X	5
Forward primer (926wF)	0.2
Reverse primer (1392R)	0.2
Molecular biology water	3.6
Total (does not include 1 μL amplified DNA)	9

Table 5. PCR cycling conditions for SsoAdvanced Universal SYBR Green Supermix

	Step	Temp	Length
40 cycles	Initial denaturation	98°C	3 min
	Denaturation	98°C	15 s
	Annealing and extension	60°C	1 min
	Plate read	60°C	NA
	Melt curve analysis	65 to 95°C ^a	5 s/increment
	Hold	4°C	∞

^aUse 0.5°C increments

Fig. 1. Startup Wizard of Bio-Rad CFX Manager.

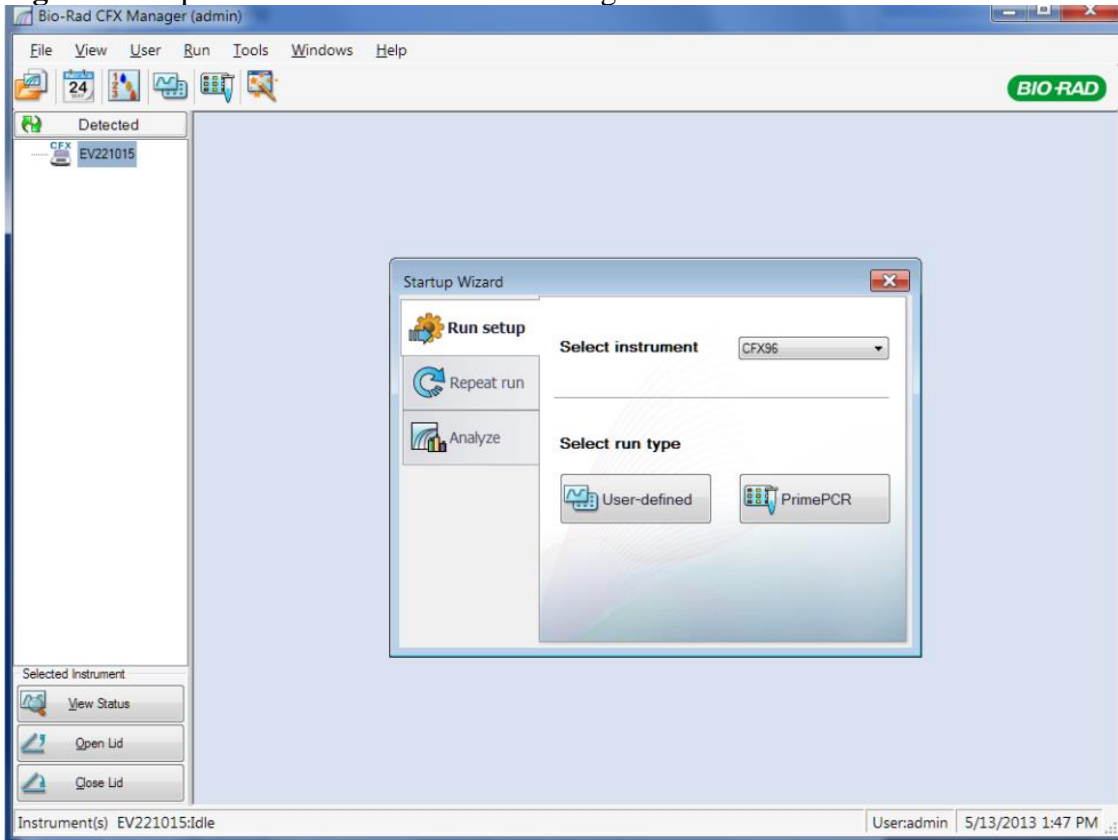


Fig. 2. Run Setup.

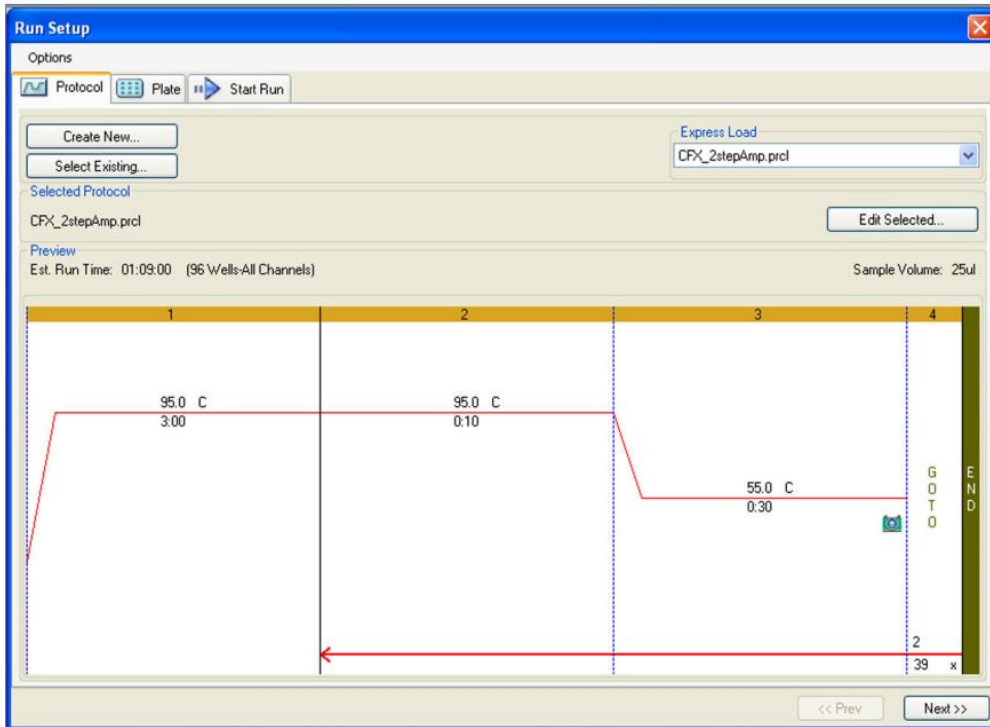


Fig. 3. Protocol Editor window

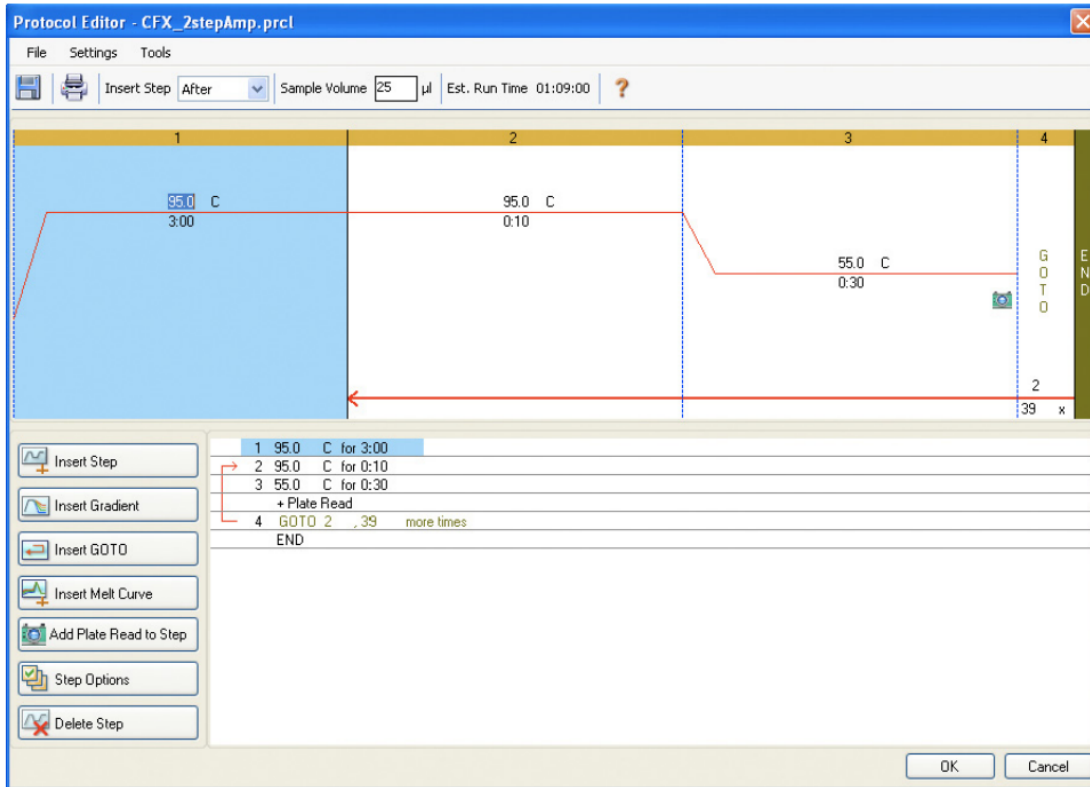


Fig. 4. Plate Editor window

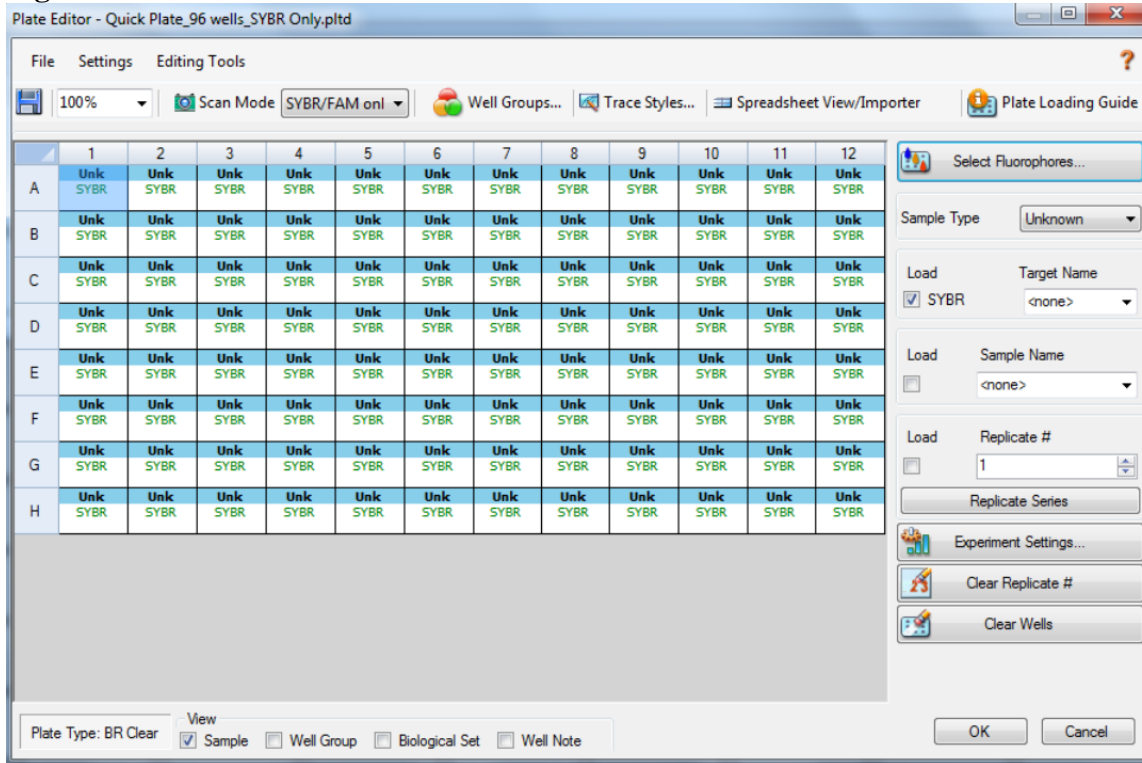


Fig. 5. Start Run tab in Run Setup.

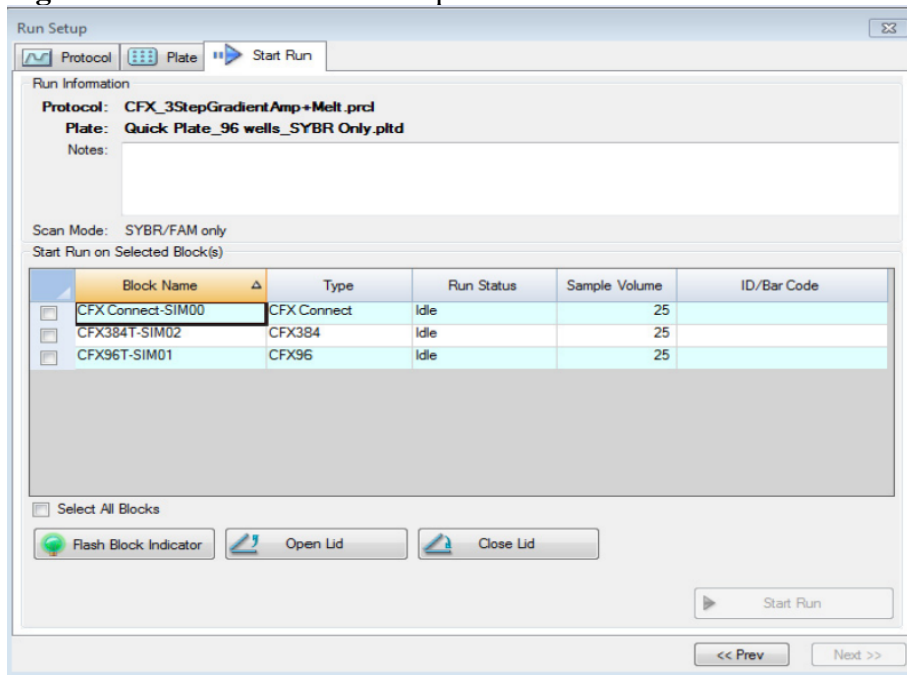


Fig. 6. Balancing of strip tubes.

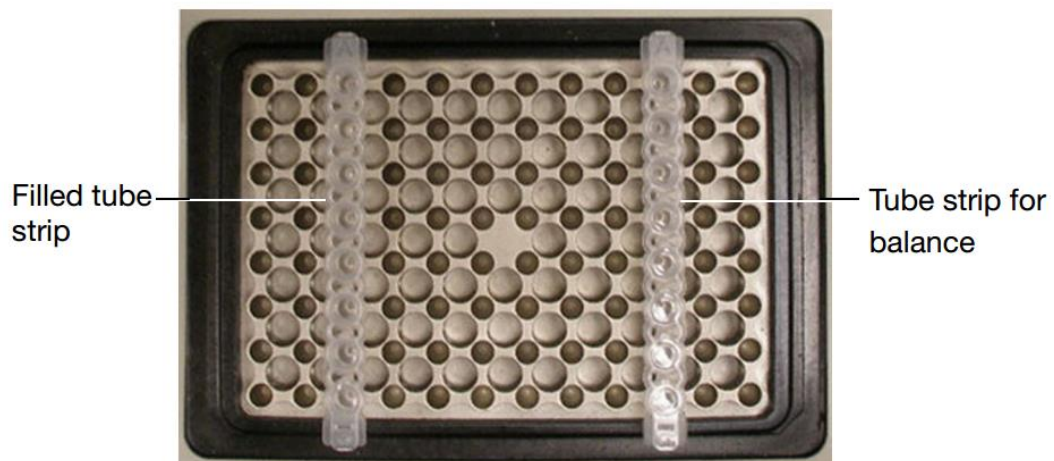


Fig. 7. Run Details window with Real-time Status tab.

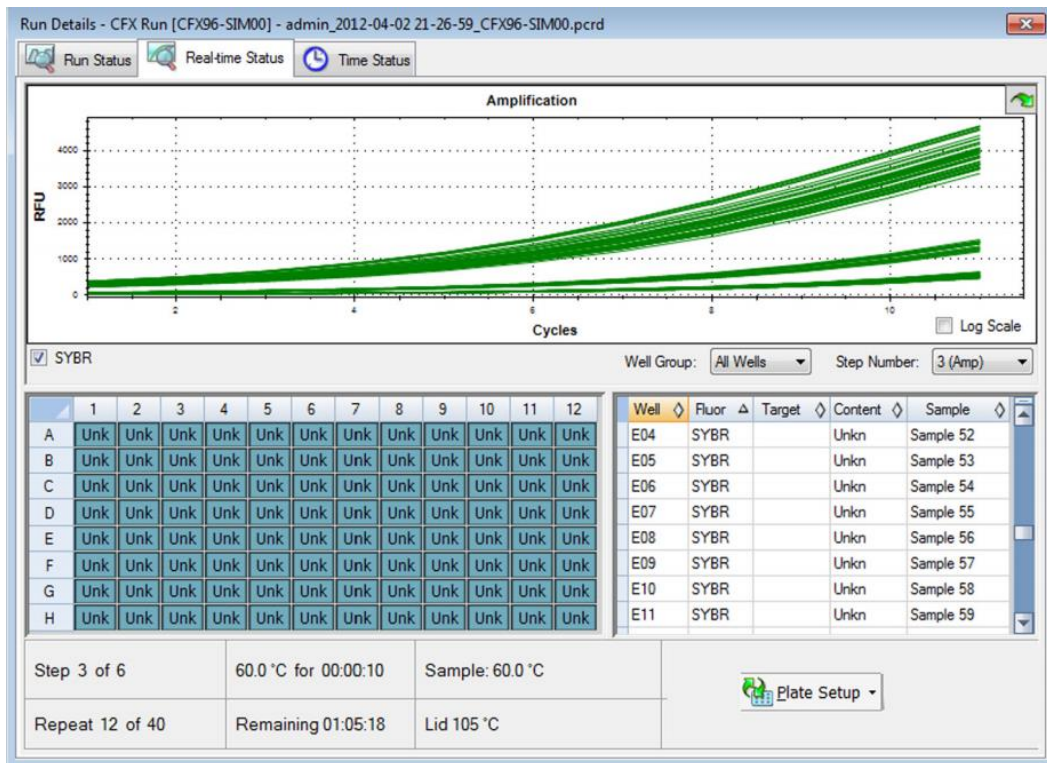


Fig. 8. Melt Curve tab, showing melt curve with sharp peak.

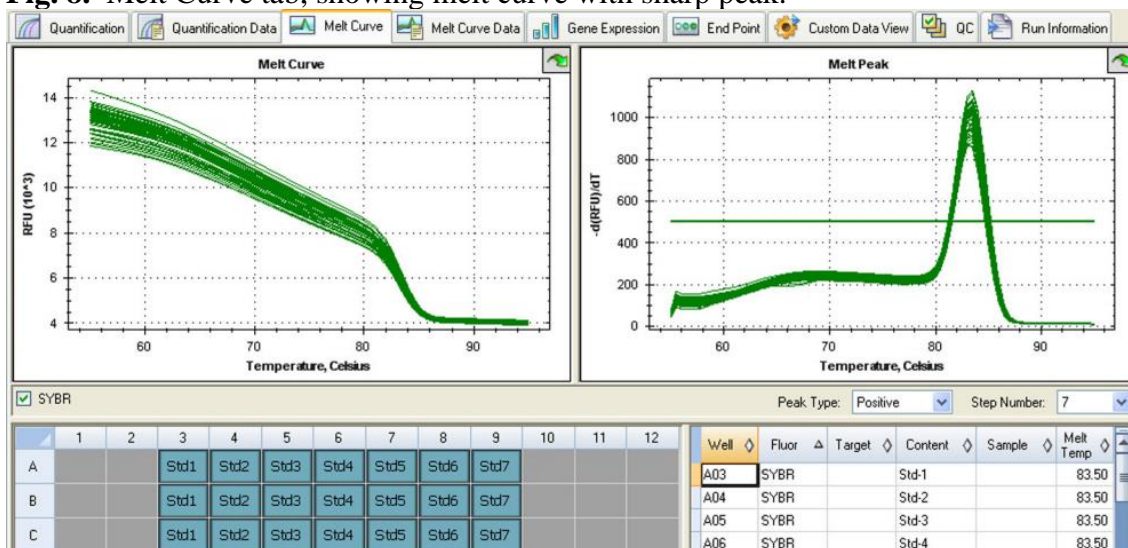
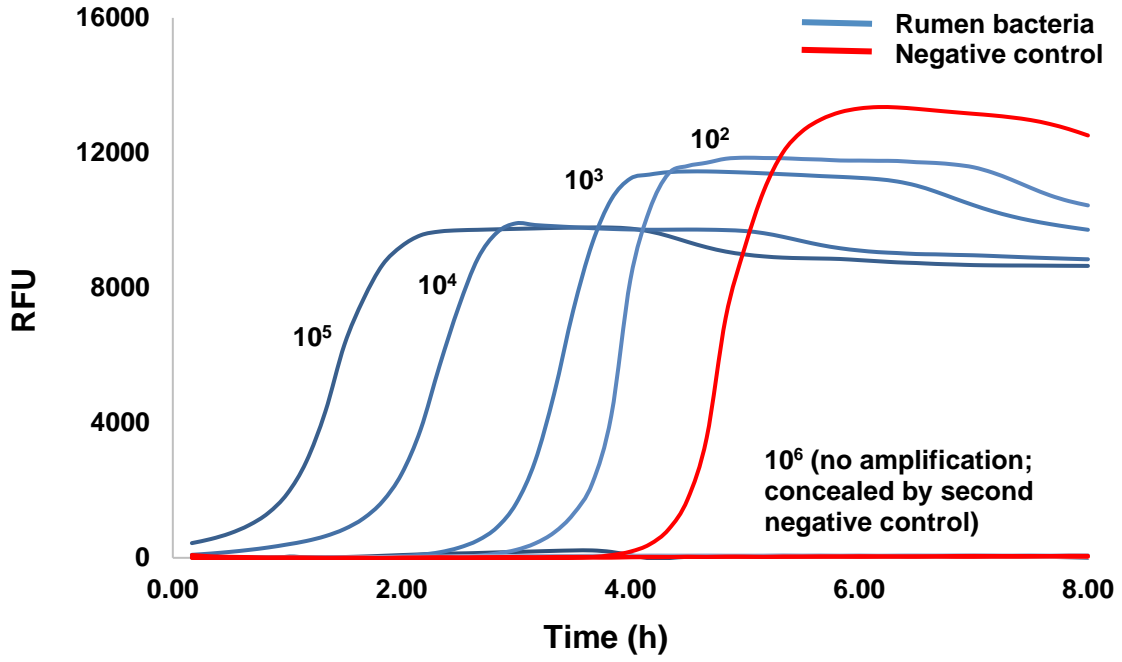


Fig. 9. Amplification of genomic DNA from rumen bacteria. Number of bacteria in each sample is shown alongside curves. All amplifications were run in duplicate, but only one replicate is shown for brevity. Both replicates are shown for the negative control because one amplified while the other did not.



Untargeted proteomics by LC-MS/MS

Preparing reagents

50 mM ammonium bicarbonate (AMBIC)

Add 3.953 g ammonia bicarbonate to a beaker. Bring to ~80 mL with LC-MS water (Fisher W6-4), then stir to dissolve. Bring to 100 mL with LC-MS water in a volumetric flask.

15% (w/v) TCA and 0.2% (w/v) DTT in acetone

Add 0.15 g trichloroacetic acid (TCA, store at 4°C) and 0.002 g dithioerythritol (DTT) in a 2-mL tube. Dissolve in 1 mL acetone and chill at -20°C. Make immediately before use.

50 mM AMBIC

Add 3.953 g ammonia bicarbonate (AMBIC) to a beaker. Bring to ~80 mL with LC-MS water (Fisher W6-4), then stir to dissolve. Bring to 100 mL with LC-MS water in a volumetric flask.

6 M urea in AMBIC

Add 3.6036 g urea to 15 mL tube. Add ~6 mL of 50 mM AMBIC and dissolve. Bring to 10 mL with more AMBIC.

200 mM DTT in AMBIC

Add 0.03 g dithioerythritol (DTT) to 2-mL tube. Dissolve in 1 mL of 50 mM AMBIC. Store at 4°C.

194.6 mM IAM in AMBIC

Add 0.036 g of iodoacetamide (IAM) 2-mL tube. Dissolve in 1 mL of 50 mM AMBIC. Cover the tube with aluminum film and store at 4°C in the dark.

Trypsin/Lys-C

Order trypsin/Lys-C (Promega V5073). Thaw resuspension buffer (50 mM acetic acid) on ice. Add 20 µL of the resuspension buffer to one vial of trypsin/Lys-C. Transfer contents to a 0.5 mL tube. Wash vial with 20 µL resuspension buffer, then transfer wash to 0.5 mL tube. Final volume will be approximately 40 µL, giving 0.5 µg/µL enzyme. Dispense 10 µL aliquots into PCR tubes and store at -80°C.

4% (v/v) TFA

Add 20 mL of LC-MS water (Fisher W6-4) to a 25-mL volumetric flask. Break open a 1-mL vial of trifluoroacetic acid (Fisher A116-1 AMP) and add to water. Rinse vial and bring solution to 25 mL with LC-MS water. Cover the bottle with aluminum film and store in the dark at room temperature.

Equilibration solution (0.1% [v/v] TFA in water)

Add 975 µL of LC-MS water (Fisher W6-4) and 25 µL of 4% (v/v) TFA to a 2-mL tube.

Rinse solution (0.1% [v/v] TFA in 5% [v/v] acetonitrile)

Add 925 µL of LC-MS water (Fisher W6-4), 50 µL acetonitrile (Fisher A955-4), and 25 µL of 4% (v/v) TFA to a 2-mL tube.

10% (v/v) acetic acid

Add 900 μL of LC-MS water (Fisher W6-4) and 100 μL glacial acetic acid (Fisher AX0074-2) to a 2-ml tube.

Elution solution (0.1% [v/v] acetic acid in 60% [v/v] acetonitrile)

Add 390 μL of LC-MS water (Fisher W6-4), 600 μL acetonitrile (Fisher A955-4), and 10 μL of 10% (v/v) acetic acid to a 2-mL tube.

Preparing protein samples

- 1) Obtain protein sample (e.g., cell extract prepared according to *Preparation of cell extracts for enzymatic assays using a French press*).
- 2) Pipette 200 μg of protein sample into a 2-mL tube. The volume of protein sample should be 250 μL if the concentration of protein is to 0.8 g/L (800 $\mu\text{g}/\mu\text{L}$).
- 3) The volume of protein sample can be adjusted to make total protein to be 200 μg if concentration of protein is lower or higher than 0.8 g/L. For example, the volume of protein sample should be 200 μL if the concentration of protein is 1.0 g/L; the volume of protein sample should be 400 μL if the concentration of protein is 0.5 g/L.
- 4) If concentration of protein is low than 0.5 g/L, then concentrate with a 3-kDa ultra centrifugal filter unit (Fisher UFC900324), otherwise the precipitate efficiency would be low. Or, prepare the protein sample in less amount of buffer, ensure the concentration of protein is higher than 0.5 g/L.

Precipitating protein with trichloroacetic acid/acetone

From this point on, do all procedures with open tubes in a biological safety cabinet.

- 1) To 250 μL of protein sample, add 1000 μL of chilled 15% (w/v) TCA and 0.2% (w/v) DTT in acetone. Mix by inverting tube.
- 2) Incubate 20 min at -80°C .
- 3) Thaw at room temperature (requires approximately 3 min).
- 4) Continue incubation overnight at -20°C .
- 5) Centrifuge at 21,100 g for 20 min at 4°C . Remove tubes immediately after centrifuge stops.
- 6) Use a pipette to remove most of the supernatant. Keep a thin layer of supernatant on top of the pellet.
- 7) Remove the remaining supernatant by air drying. Keep in safety cabinet and allow to dry at room temperature. Do not use speed vac, or pellet cannot be re-solubilized.
- 8) Store pellet at -80°C or continue to next step.

Digesting with trypsin/Lys-C

- 1) Resuspend pellet in 10 μL of 6 M urea in AMBIC. Pipette up and down (will create foaming).

- 2) If pellet dissolves, centrifuge at 2,000 g for 2 min at room temperature and transfer the dissolved sample to a 1.5-mL tube (Sarstedt 72.706.700). Measure the actual volume with a pipette.
- 3) If pellet does not dissolve, slowly add more 6 M urea in AMBIC and repeat pipetting. After centrifugation at 2,000g for 2 min, transfer the dissolved sample to a 1.5-mL tube. Measure the actual volume with a pipette. The total volume should be as less as possible.
- 4) In this protocol, we assume that volume of the dissolved sample is 100 μ L. If the volume is not 100 μ L, adjust the volumes of the DTT, IAM, and AMBIC in the following steps accordingly.
- 5) Add 2.5 μ l of 200 mM DTT in AMBIC to the 100 μ L of dissolved sample in the 1.5 mL tube. Incubate at 37°C for 30 min.
- 6) Add 7.7 μ l of 194.6 mM IAM in AMBIC. Incubate at room temperature in dark for 30 min.
- 7) Quench IAM by adding 20 μ l of 200 mM DTT in AMBIC. Incubate at room temperature for 10 min.
- 8) Add trypsin/Lys-C in a 1:25 (enzyme:protein, w/w) ratio and incubate at 37°C for 4 h. The amount of sample protein is assumed to be equal to protein used for TCA/acetone precipitation (i.e. 200 μ g).
- 9) Activate trypsin by adding 550 μ l of 50 mM AMBIC. Use pH paper to verify pH > 7.
- 10) Incubate at 37°C for 14 to 16 h (or overnight).
- 11) Terminate digestion by adding 4% (w/v) TFA to a final concentration of 1% TFA. Use pH paper to verify pH < 4.
- 12) Centrifuge at 16,000 g for 10 min at room temperature.
- 13) Transfer supernatant to new 1.5 mL tube (Sarstedt 72.706.700). If amount of protein in TCA/acetone precipitation step is high, split supernatant into multiple tubes so that each has about 50 μ g.

Desalting with ZipTips

- 1) Attach a Millipore C18 ZipTips (ZTC18S096) to a 10 μ L-pipette. Make sure that tip is well-seated. Set pipette to 10 μ L.
- 2) Wet tip by aspirating 10 μ L of LC-MS acetonitrile (Fisher A955-4), and then discard solvent. Repeat once. Here and throughout, do not introduce air into the tip (or recovery of peptides will be lower).
- 3) Equilibrate tip by aspirating 10 μ L of equilibration solution, then discard solvent. Repeat once. After discarding solvent the second time, immediately proceed with steps below (do not let tip dry).

- 4) Aspirate up to 10 μL of sample. Slowly aspirate it in and out for 7 to 10 cycles for maximum binding. This step takes about 2 to 3 hours if the sample is about 120 μL and repeat binding for 10 cycles.
- 5) Rinse the tip by slowly aspirating 10 μL of rinse solution and then discarding solvent. Repeat once.
- 6) Elute the sample with 100 μL of elution solution. Slowly aspirate in solvent, then dispense flow through into new 1.5 mL tube (Sarstedt 72.706.700).
- 7) Use the 3 μl aliquot to measure peptides concentration. Use the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo 23290) according to instructions. Store sample at -80°C .

Drying samples

- 1) Prepare the SpeedVac system (Savant RT-400A condensation trap and SpeedVac concentrator)
 - (A) Check the level of coolant in condensation trap. Lift up white foam disc and see if level of liquid is near top of glass insert. If not, add coolant (100% ethanol). Trap should contain 800 mL coolant in total.
 - (B) Check vacuum tubing is properly connected between condensation trap and concentrator. Make connections as shown below (lines indicate direction of vacuum)

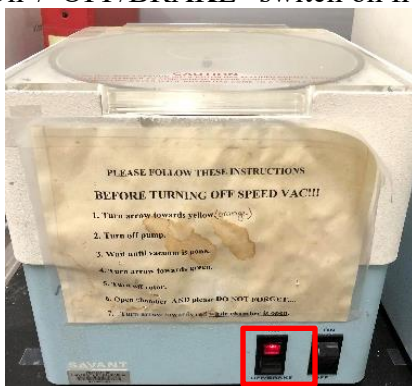


- (C) Turn on trap and wait for 1 hour to allow the temperature to decrease to about -50°C .
- 2) Load samples in SpeedVac concentrator.
 - (A) Take out samples from -80°C and thaw at room temperature. Samples should be in 1.5-ml tubes.

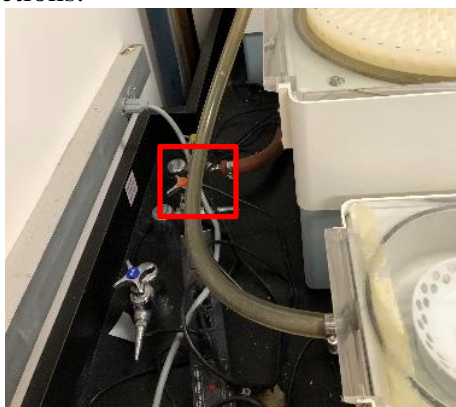
- (B) Place samples in rotor of the concentrator. Arrange the tubes so the rotor is balanced (just as with a centrifuge rotor). If needed, use 1.5-mL tubes filled with solvent to balance.
- (C) Locate blue valve on vacuum tubing, then turn so that vacuum can be applied to trap.



- (D)
- (E) Turn on concentrator using "On"/"OFF/BRAKE" switch on front.



- (F)
- (G) Turn on house vacuum (yellow, labelled "LV"). Lid of concentrator should seal shut. If it does not, check tubing and connections.



- (H)
- (I) Solvent will begin to evaporate. Monitor level of solvent frequently. Continue evaporation until solvent is about to disappear.

(J) Remove samples from rotor. Locate blue valve on vacuum tubing, then turn to release vacuum. Turn off rotor. Open lid and remove samples.

(K) Turn off house vacuum and turn off trap.

(L) Empty glass insert of solvent. Wait until trap to warm up (several hours or overnight), disconnect vacuum tubing from glass insert, then lift insert and white foam out of trap. Pour out contents. Handle glass insert with care because it is fragile and expensive. Handle glass insert with care because it is fragile and expensive.



(M)

(N) Place insert back into trap for next use.

3) Resuspend dried sample in about 40 μL of 0.1% TFA.

4) For measuring peptides, remove a 3 μL aliquot and mix with 9 μL of 0.1% TFA in a 1.5 mL tube. Store the remaining sample at -80°C for up to 3 months.

5) Measure peptides using the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo 23290) according to instructions. For cell extract, a typical peptides concentration is 220 to 270 $\text{ng}/\mu\text{L}$, about 35 to 37 μL , giving a yield of 8 to 9 μg from 200 μg protein in 250 μL original extract if desalt with two 10- μL C18 Tip.

Submitting samples for LC-MS/MS analysis

Contact UC Davis Proteomic Core in advance to coordinate sample submission. Minimum sample needed is 1.5 to 2.0 μg total peptide (for recombinant proteins), or 2.5 to 5 μg (for cell extract) per injection.

Identifying proteins from LC-MS/MS files

1.) Install following software

- MSConvert
- X!Tandem pipeline

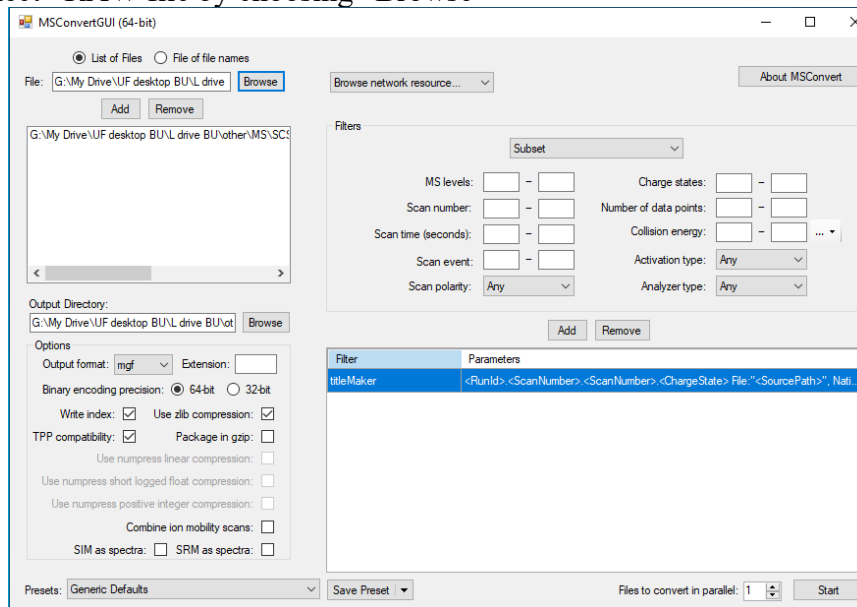
- Skyline (optional)

2.) Obtain the Thermo RAW file from UC Davis Proteomics core. File will be sent to email in the form of a Bioshare link. This file contains the chromatograms from the LC-MS/MS instrument.

3.) Convert Thermo RAW file (*.RAW) to Mascot Generic format (*.mgf).

(A) Open MSConvert.

(B) Select *.RAW file by choosing “Browse”



Click “Add.” Click “Start.”

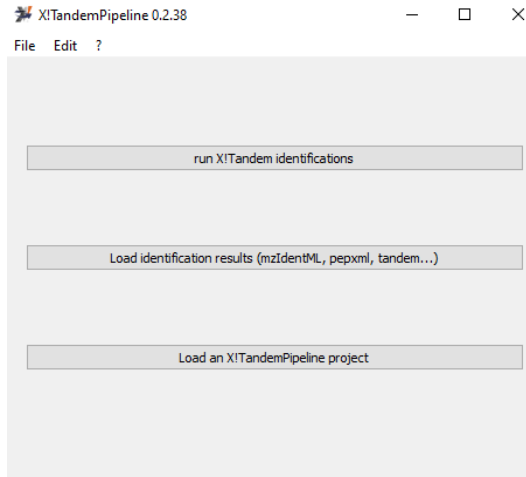
4.) Obtain a *.faa or *.fasta file containing the sequence of the protein. The file should contain sequences of possible contaminating proteins, also. For cell extracts, it would include all proteins predicted in the genome. For *Cutibacterium granulosum* VPI 0507, protein sequences can be downloaded from

https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2814123075

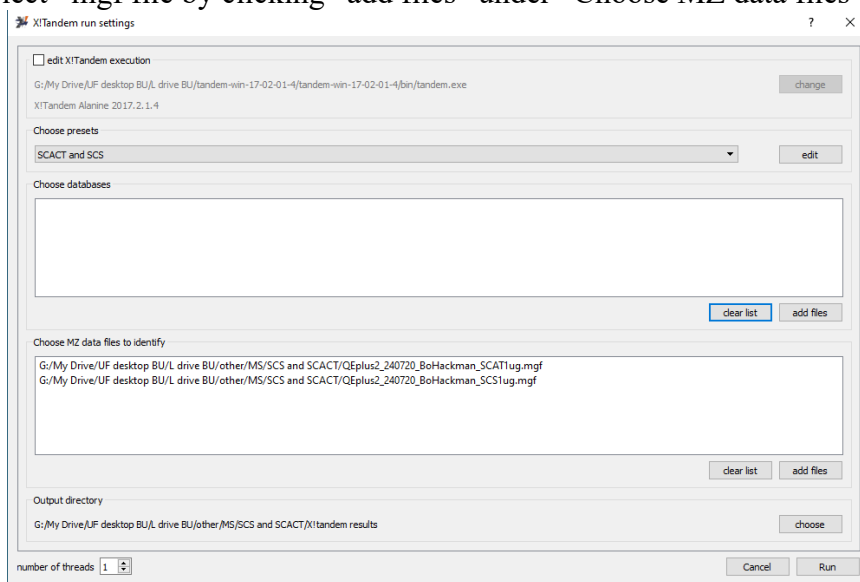
5.) Identify proteins in *.mgf file.

(A) Open X!Tandem pipeline (xtcpp.exe).

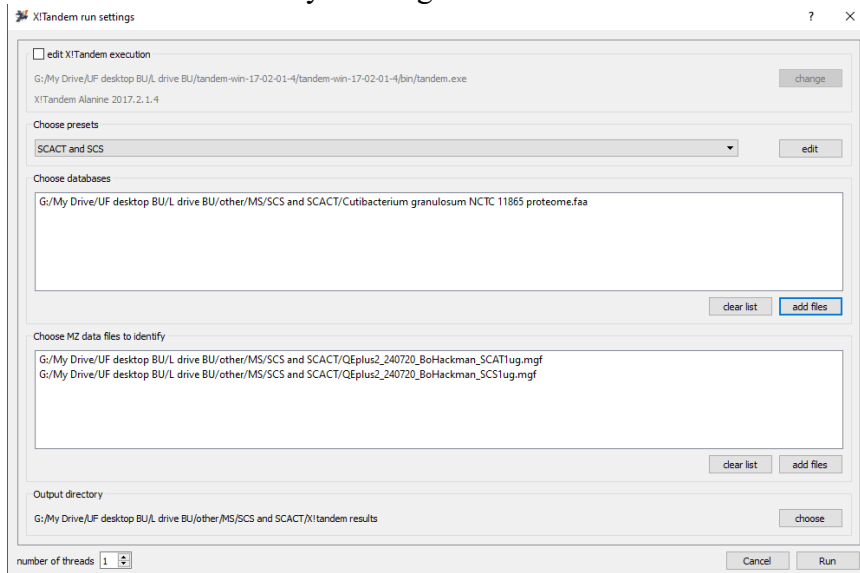
(B) Click “run X!Tandem identifications”



(C) Select *.mgf file by clicking “add files” under “Choose MZ data files to identify”.



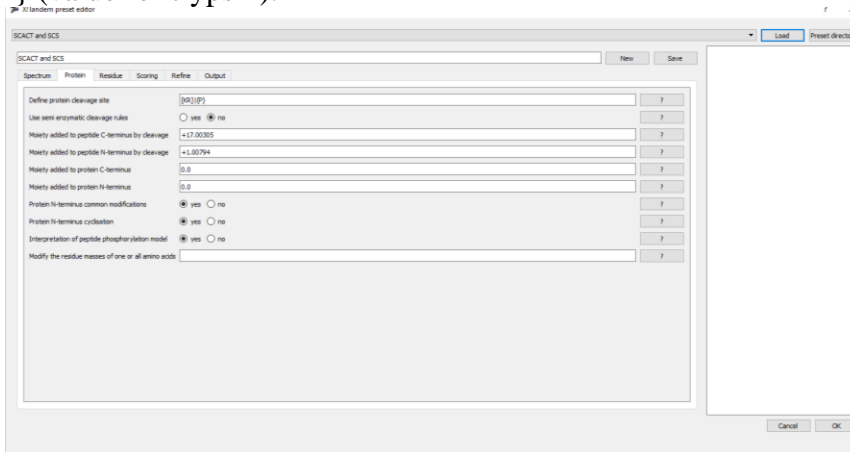
(D) Select *.faa or *.fasta file by clicking “add files” under “Choose database”.



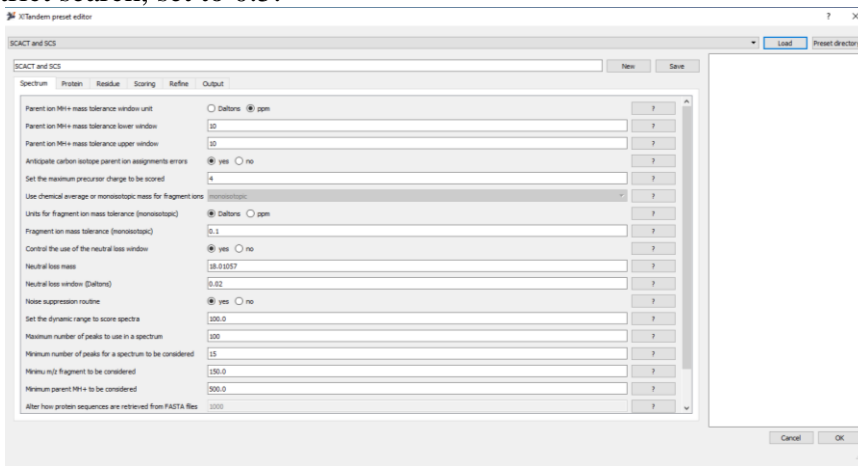
(E) Under “Choose database”, click “add files” (fasta)

(F) Set the parameters by clicking “edit” under “Choose presets”.

a. In the window that appears, click “Protein” tab. Set “Define protein cleavage site” to [KR][P] (value for trypsin).



b. Click the “Spectrum” tab. Set the “Fragment ion mass tolerance (monoisotopic)” to 0.1. For a less strict search, set to 0.5.

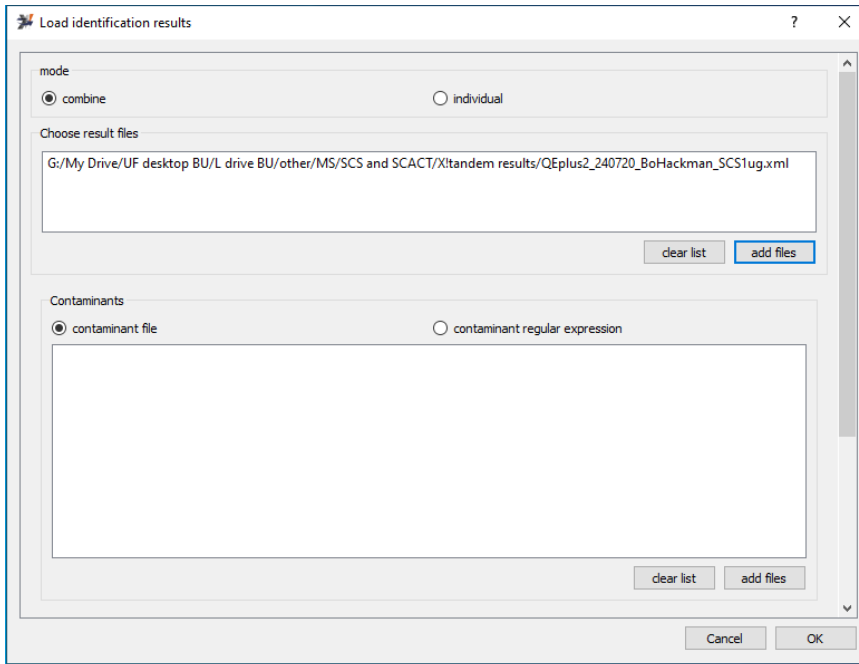


c. Inspect other parameters. They can be left at default values if appropriate.

(G) Click “Run”

6.) View proteins identified above.

(A) In X!Tandem pipeline, click “Load identification results (mzIdentML, pepxml, tandem...)”
Choose identification file (*.xml) using “add files”.



Click OK.

(B) Inspect all entries in output. For more details, click on entry.

Protein list

export show only

checked	group	accession	description	log(Evalue)	Evalue	spectra	specific spectra
<input checked="" type="checkbox"/>	a1.a.1.a1	2814727080	Ga0226301_111405 succinyl-CoA synthetase (ADP-forming) alpha subuni...	-1444.88	0	1407	1407
<input checked="" type="checkbox"/>	a2.a.1.a1	2814727081	Ga0226301_111406 succinyl-CoA synthetase (ADP-forming) beta subunit ...	-1045.62	0	565	565
<input checked="" type="checkbox"/>	a3.a.1.a1	2814727080:reversed	Ga0226301_111405 succinyl-CoA synthetase (ADP-forming) alpha subuni...	-14.9032	1.24964e-15	9	9
<input checked="" type="checkbox"/>	a6.a.1.a1	2814727021:reversed	Ga0226301_111345 DNA polymerase I [Cutibacterium granulosum NCTC ...	-2.84073	0.001443	2	2
<input checked="" type="checkbox"/>	a5.a.1.a1	2814726808	Ga0226301_111132 putative endonuclease [Cutibacterium granulosum N...	-3.5391	0.000289	2	2
<input checked="" type="checkbox"/>	a4.a.1.a1	2814727130	Ga0226301_111457 energy-coupling factor transport system permease pr...	-5.75048	1.77633e-06	3	3

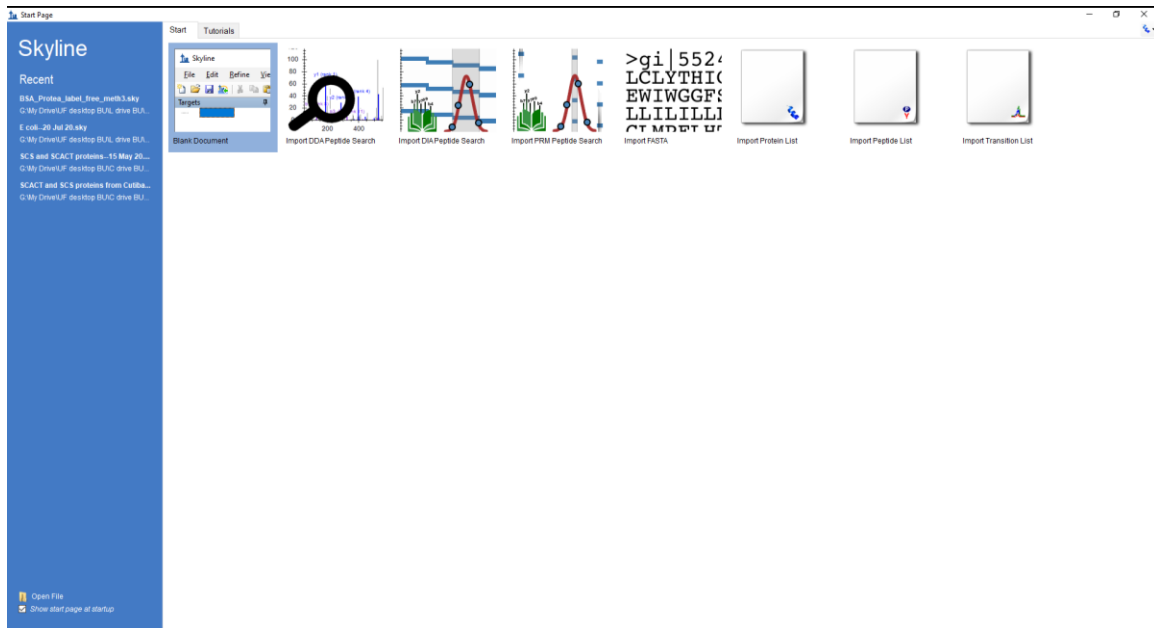
search accession

proteins all:64 valid:6 valid&checked:6 grouped:6 displayed:6

7.) Optional: view chromatograms and proteins in Skyline.

(A) Open Skyline.

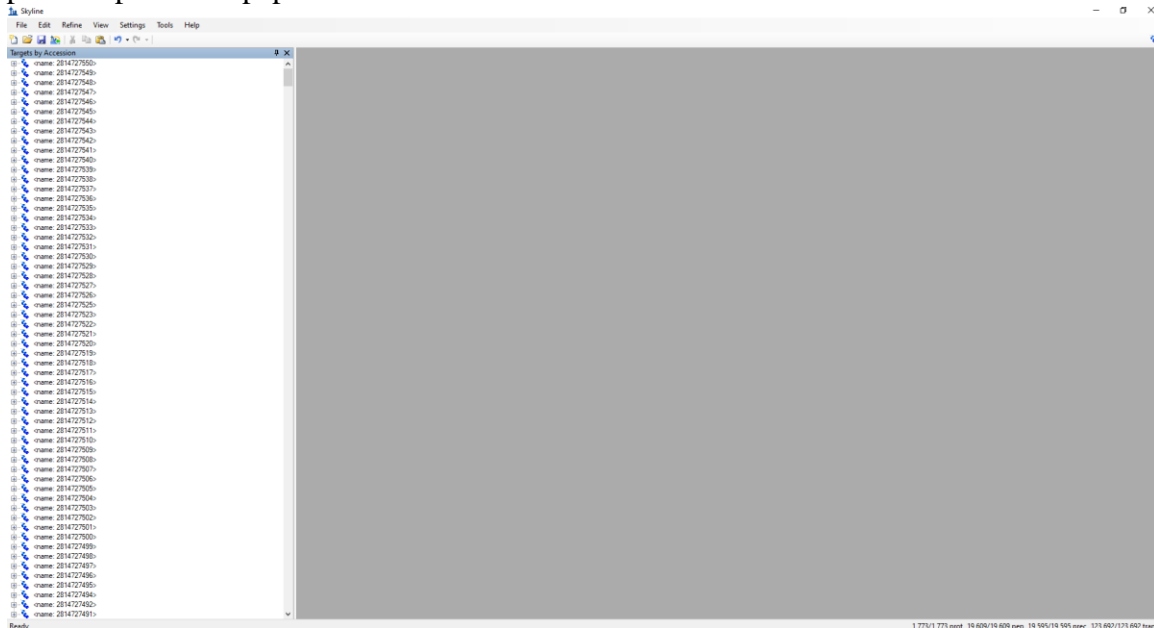
(B) Click “Blank Document”



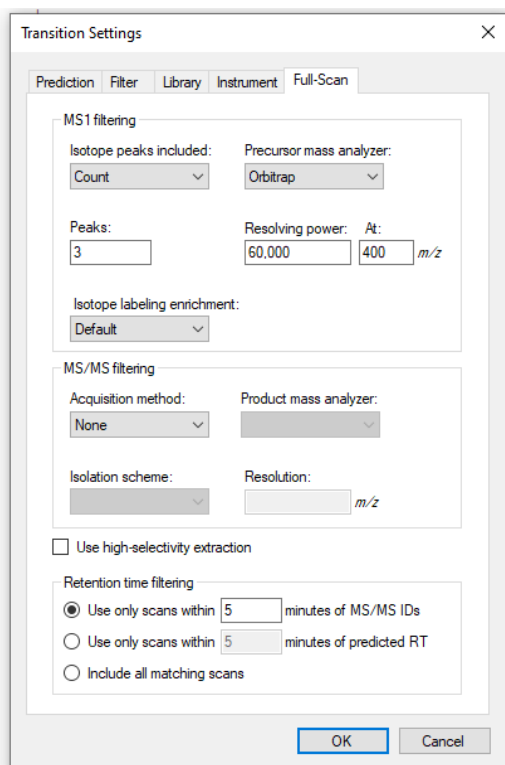
(C) Save file via File -> Save As... (must do this to proceed).

(D) Load the *faa or *fasta file via File -> Import -> Fasta...

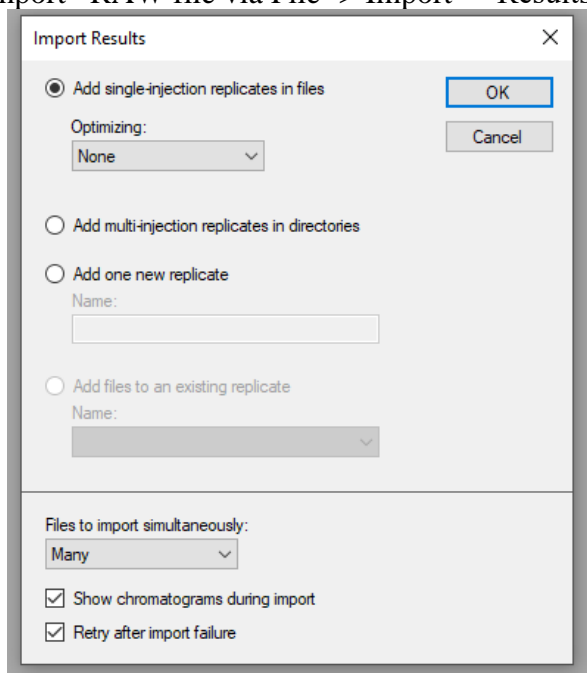
(E) Inspect the predicted peptides.



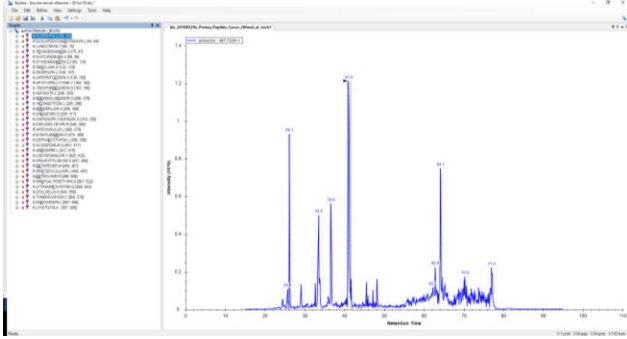
(F) Specify settings for chromatogram via Settings -> Transition Settings.... See example below.



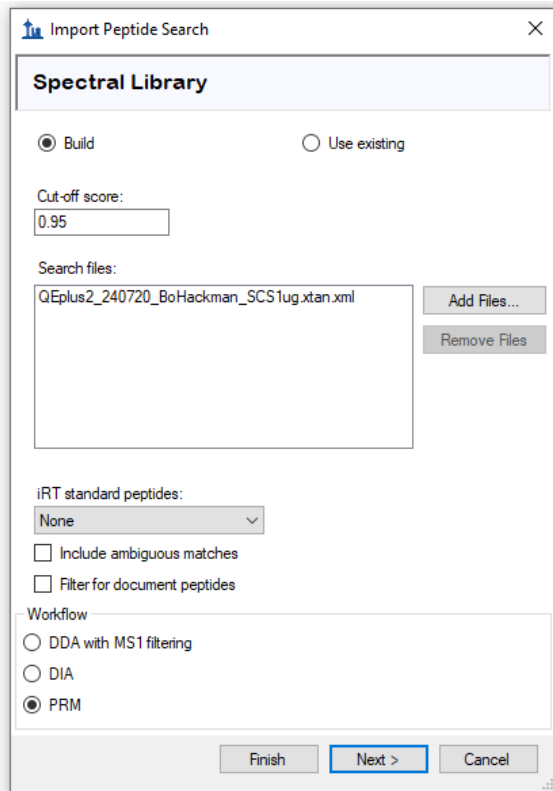
(G) Import *RAW file via File -> Import -> Results... When prompted, click OK.



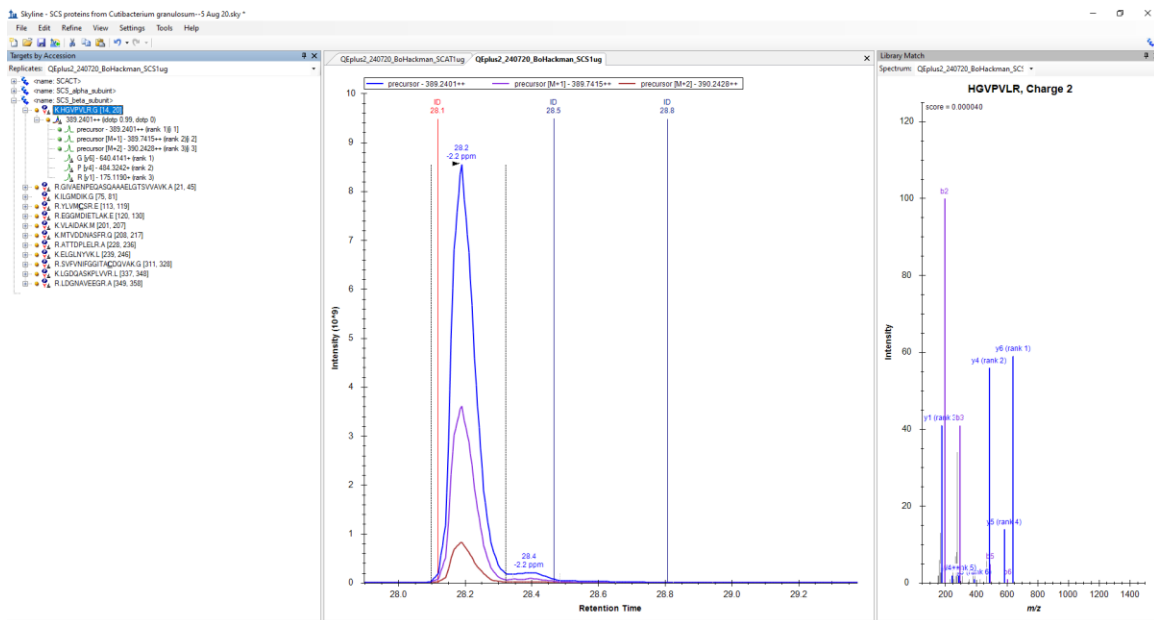
(H) Inspect the chromatograms.



(I) Import search results using File -> Import -> Peptide Search... Before proceeding, rename the file extension for the *.xml file to *.xtan.xml (or error will result). Click “Add Files”. Choose “PRM” under “Workflow. Click “Finish”.



(J) Inspect the chromatograms. The transitions are now shown (see far right window).



Notes

1.) According to manufacturer of condensation trap, the glass insert should be thawed in cold water after use. Following advice from others, we thaw in the trap instead. Thawing in the trap carries the risk that the solvent will expand and crack the insert. However, there is greater risk in leaving out the insert out of the trap for extended periods—other users can (and have) broken the insert when left outside the trap.

SECTION VI: CALORIMETRY

Calorimetry is a common tool for investigating energy metabolism. Calorimeters are often used with higher animals, but small and sensitive calorimeters (microcalorimeters) can be used in microbiology. Microcalorimeters are useful in metabolism studies because they can monitor the heat released by metabolism of any substrate, in real-time, with sensitivity not approached by other technologies. For glucose, fermentation of as little as $2.5 \cdot 10^{-11}$ mol/s can be detected [assuming 200 kJ/mol glucose released during fermentation (103) and $5 \cdot 10^{-6}$ J/s detection limit]. For an experiment with 10^{10} cells, this corresponds to only ~ 1500 glucose molecules/cell/s. This manual describes the installation, calibration, and use of one calorimeter.

The manual culminates in describing an experiment to determine metabolic response of rumen microbes to pulse dose of glucose (20 mM). It combines many of the procedures listed earlier in the manual.

Heat production of microbial cells by calorimetry

Adapted from Thermal Hazard Technology μ RC manual and (104, 105)

Use

- 1.) Turn on calorimeter (Thermal Hazard Technology μ RC) using switch on back (**Fig. 1**) if calorimeter is installed and calibrated. If not already, install and calibrate first, following instructions below.
- 2.) Load uRC.exe.
- 3.) Apply experiment settings in “Experiment” tab of urc.exe. **Fig. 2** shows settings for typical experiments. Click “Update” button.
- 4.) Prepare sample and reference vials (**Fig. 3**). Place stirbars into two vials. Pipette 1 mL of sample (e.g., rumen microbes) into one vial (sample vial). Flush sample with CO₂ while screwing on cap with rubber septum. Pipette 1 mL of dH₂O into the other vial (reference vial).
- 5.) Insert vials and metal stoppers into calorimeter. Remove plastic cap over reference cell (**Fig. 4**). Grasp the top of the vial with long forceps (**Fig. 3**). Lower vial down reference cell hole as far as possible (**Fig. 5**). Gently drop vial into metal holder below. Next, hold the metal stopper with forceps and lower it into the reference cell until it stops. (**Fig. 6**) Replace plastic cap.

Remove syringe tower. Insert syringe into metal stopper after first loading the syringe with the solution to be injected. Screw onto syringe tower (**Fig. 7**). If no injection will be made, omit insertion of syringe. Insert sample vial and replace syringe tower. Syringe needle will pierce the rubber septum of the sample vial.

- 5.) Click on “Monitor” tab. Allow heat production to reach a steady level (equilibrate), which usually requires 15 to 90 min.
- 6.) Run experiment by clicking “Start” button. Name and save file.
- 7.) Run experiment. To stop and save experiment early, click “Stop” button. To run another experiment with different settings, restart uRC.exe.
- 8.) Measure background heat production. In the absence of heat production, the instrument does not register 0 mW, but rather some background value which must be measured. After the main experiment, run another experiment with 1 mL water in both the sample and reference vials. Heat production so measured after equilibration is the background. Subtract this background from heat production measured in the main experiment. For precise work, background heat production can be measured before the experiment, too.

If the syringe was used in the main experiment, use it during measurement of background heat production, too. Background heat production changes by piercing the rubber septum of the sample vial with the syringe needle.

For particularly precise work, measure background heat production by using a formalin injection instead. After the main experiment, keep sample vial containing microbes in the sample vial holder. With a titration experiment, inject 1% formalin (final concentration) into the sample vial. Heat production measured after equilibration is the background. This method is precise because it uses the same pair of vials for the main experiment and for measuring background heat production.

9.) Open saved *.urc file with Excel or Notepad. First column of data (unlabeled) gives the time in s, and second column (“Heat step 1”) gives heat production in mW. Convert mW to J by integration.

Calibration

1.) The calorimeter has an electrical heating device that can be used for calibration. Click the “Calibration” radio button from the “Experiment” tab. **Fig. 8** shows typical calibration settings. Run experiment according to *Use* above.

2.) Alternatively, calibrate using heat of neutralizing HCl by excess NaOH. Prepare and standardize a c. 0.1 M HCl solution. Run a titration experiment and inject 15 μL of this solution into 1 mL 0.01 M NaOH. To correct for heat of diluting HCl, run a second experiment and inject 15 μL 0.1 M HCl into 1 mL dH_2O . For each injection, determine heat released (J) by integration. Subtract heat released from second injection (HCl into dH_2O) from heat released by first injection (HCl into NaOH).

Expected heat production at 39°C is 79.05 mJ (-52.70 kJ/mol HCl). Calculation of this expected heat production assumes infinitely dilute HCl and NaOH, for which heat released is -55.84 kJ/mol at 25°C (106). To correct to 39°C, the calculation also assumes $\Delta_r C_p = 0.22 \text{ kJ}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ (106).

Installation

1.) Place unit in protective enclosure (**Fig. 9**). Connect power supply to calorimeter and plug in power supply. Connect calorimeter to computer using USB cord. (**Fig. 1**). Insert syringe tower into sample vial holder and plug cord into jack on top of calorimeter (**Fig. 4**). Insert plastic cap over reference vial holder (**Fig. 4**).

2.) Install software for calorimeter using supplied CD. Computer must run Windows XP or 2000 for software to install correctly. Sometimes drivers often do not install correctly; seek advice of IT personnel in this case. The executable file installed is uRC.exe.

Notes

(i) To bring the instrument to equilibration faster, run it overnight. At the very least, preheat vials, metal stoppers, and reference vial containing 1 mL dH_2O in 39°C incubator overnight.

(ii) Under current setup, background heat production is usually 0.15 mW but can range from 0.12 to 0.19 mW. For titration experiments, background tends to be lower (0.07 to 0.15 mW) due to syringe piercing the sample vial.

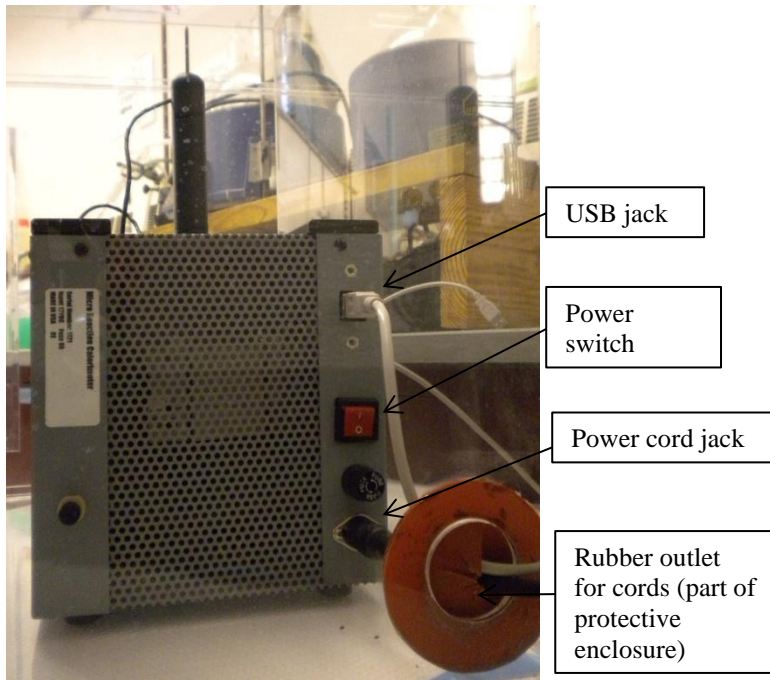
(iii) Handle all parts using gloves and, when possible, forceps to avoid transferring heat and oil from hands. Wipe calorimetry vials, metal stoppers, and syringe needle with Kimwipe before insertion to remove residual moisture.

(iv) Stirring is routinely done at 60 rpm because this rate maximizes heat production after dose of glucose. Slower mixing presumably limits rate of glucose utilization, lowering heat production. Mixing faster than 60 rpm causes no further increase in heat production.

(v) One can move the syringe up or down from the “Experiment” tab. Moving the syringe down this way can purge gas bubbles from the syringe.

(vi) Washed cells produce approximately 0.02 mW/mg protein.

Fig. 1. Rear view of calorimeter.



USB jack

Power switch

Power cord jack

Rubber outlet for cords (part of protective enclosure)

Fig. 2. Settings for typical experiments. (A) Titration. Titration setting is selected to make an injection, with injection number (1) and size (20 μL) indicated. Lead time (1200 s) is time before injection. Injection interval (25 000 s) is total length of experiment or, were multiple injections made, time between each injection. Stirrer is on (ON), and stirbars are spinning at 60 rpm. Rate of syringe injection (1 μL), syringe size (250 μL), and experiment temperature (39°C) are indicated. Sample rate (1) refers to interval at which measurements are recorded. (B) Collection. No injection will be made.

(A)

Experiment Type

- Collection
- Calibration
- Titration
- Scan
- cp Measurement

Settings

Stirrer ON/OFF: **ON**

Stir Speed (rpm): 60

Syringe Speed (ul/sec): 1

Syringe Size (ul): 250

Temperature (C): 39

Sample Rate (sec): 1

Titration

Number of Injections: 1

Injection Size (ul): 20

Injection Interval (sec): 25000

Lead Time (sec): 1200

Experiment

Method Name: mixed rumen microbes + 20 mM glucose

Operator: []

Titrant: 20 uL, 1 M glucose

Sample: mixed rumen microbes

Comments: []

Syringe

Move (0-100%): [] Move UP Move DOWN

(B)

Experiment Type

- Collection
- Calibration
- Titration
- Scan
- cp Measurement

Settings

Stirrer ON/OFF: **ON**

Stir Speed (rpm): 60

Syringe Speed (ul/sec): 1

Syringe Size (ul): 250

Temperature (C): 39

Sample Rate (sec): 1

Collect

Experiment Duration (sec): 75000

Experiment

Method Name: mixed rumen microbes + 20 mM glucose

Operator: []

Titrant: []

Sample: mixed rumen microbes

Comments: []

Syringe

Move (0-100%): [] Move UP Move DOWN

Fig. 3. Reference vial fully prepared. Grasped by long forceps, it is ready to be lowered into reference cell.

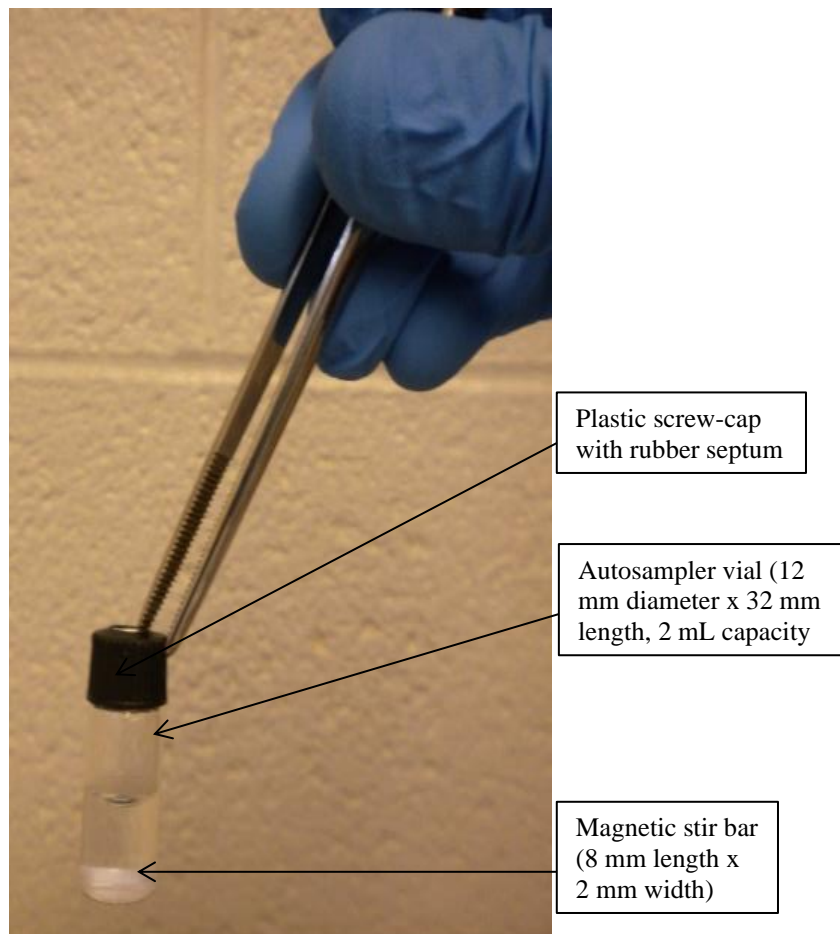


Fig. 4. Front view of calorimeter.

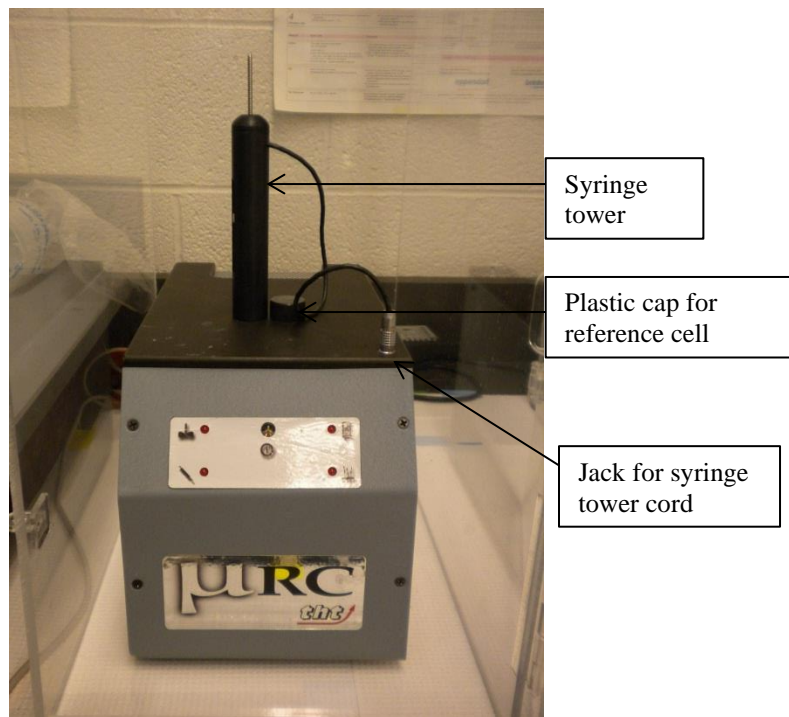


Fig. 5. Top view of calorimeter, with sample and reference cells exposed by removing syringe tower and plastic cap for reference cell.

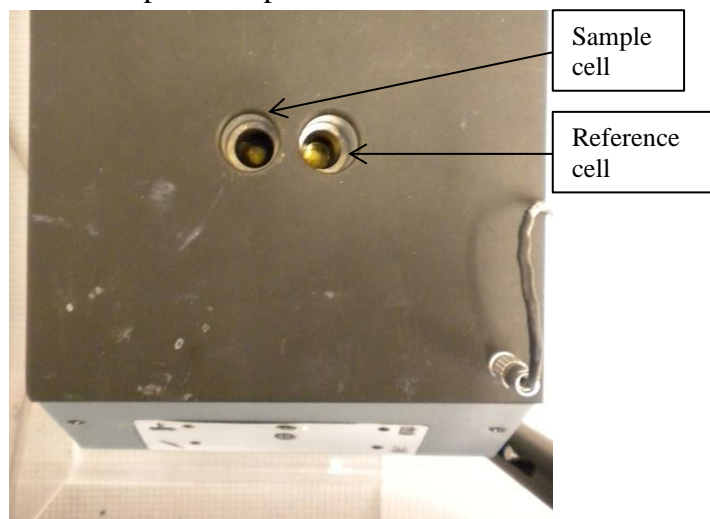


Fig. 6. Lowering metal stopper into reference vessel.

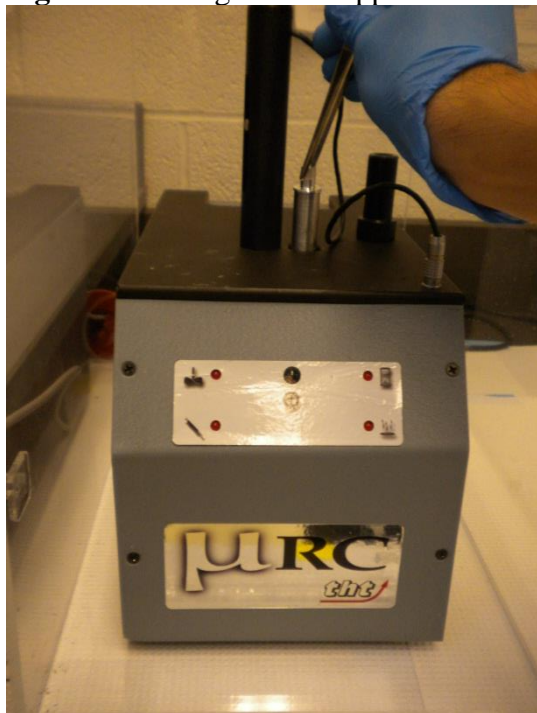
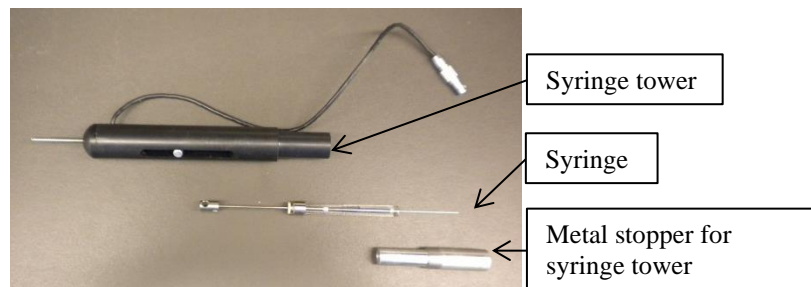


Fig. 7. Syringe tower. (A) Disassembled. (B) Insertion of syringe into metal stopper for sample vial holder. (C) Assembled.
(A)



(B)



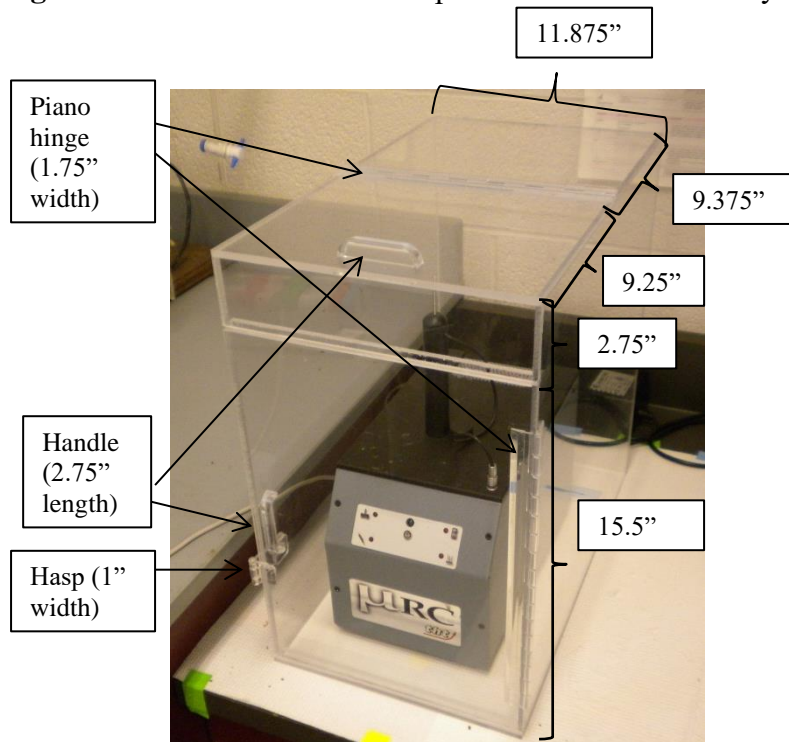
(C)



Fig. 8. Typical settings for calibration

Experiment Type <input type="radio"/> Collection <input checked="" type="radio"/> Calibration <input type="radio"/> Titration <input type="radio"/> Scan <input type="radio"/> cp Measurement	Settings Stirrer ON/OFF <input checked="" type="checkbox"/> ON Stir Speed (rpm) <input type="text" value="200"/> Syringe Speed (ul/sec) <input type="text" value="1"/> Syringe Size (ul) <input type="text" value="250"/> Temperature (C) <input type="text" value="39"/> Sample Rate (sec) <input type="text" value="1"/>	Experiment Method Name: <input type="text" value="electrical calibration"/> Operator: <input type="text"/> Titrant: <input type="text"/> Sample: <input type="text" value="water-water blank"/> Comments: <input type="text"/>
Calibration Number of pulses <input type="text" value="5"/> Pulse Size (mJ) <input type="text" value="50"/> Pulse Interval (sec) <input type="text" value="300"/> Lead Time (sec) <input type="text" value="20"/>	<input type="button" value="Cancel"/> <input type="button" value="Update"/>	Syringe Move (0-100%): <input type="text"/> <input checked="" type="radio"/> Move UP <input type="radio"/> Move DOWN <input type="button" value="Move"/>

Fig. 9. Protective enclosure. All panels are 0.25" thick acrylic, welded together using solvent.



Example experiment: Measuring response of rumen microbes to 20 mM glucose

Results from this experiment and a similar one with 5 mM glucose are shown in (103).

Day 1

- 1.) Label 2 mL microcentrifuge tubes P1 to P12 and S1 to S12 (24 tubes total). Label 2 mL autosampler vials V1 to V12 (12 vials total).
- 2.) Warm up microcalorimeter. Turn on microcalorimeter, then insert vials, metal stoppers, and 250 μ L GC syringe (with dH₂O). Run at 39°C overnight.
- 3.) Pre-warm centrifuge rotor (if preparing mixed rumen microbes or bacteria), Simplex buffer, and separatory funnel to 39°C in incubator.
- 4.) Pack supplies for collecting rumen fluid according to *Collecting rumen fluid*.
- 5.) Prepare 1 M sterile glucose solution. Sterilize in Hungate tubes by autoclaving and store at -20°C (for further protection against microbial degradation). Solution is stable indefinitely.

Day 2

- 1.) Collect fluid in the morning (e.g., 8 a.m.). Begin flocculating rumen fluid to prepare sample.
- 2.) While flocculating, fill GC syringe with 1 M glucose and re-insert calorimeter. Withdraw 1 mL, 1 M glucose from Hungate tube using 1 mL syringe according to *Making and dispensing media of Feeding and transferring protozoa in culture tubes*. Fill 250 μ L GC syringe with an aliquot of this glucose, and store an aliquot at -20°C for analysis.
- 3.) Prepare sample (mixed rumen microbes, protozoa, or bacteria) by following earlier procedures. Transfer 20 mL of sample to 60 mL culture bottle using glass graduated pipette. Gas and cap with rubber bung.
- 3.) Remove 1 mL sample and place in autosampler vial for measurement of heat production with microcalorimeter. Here and throughout, mix contents of culture bottle well by swirling and pipetting contents vigorously before sampling.
- 4.) Allow heat production of calorimeter to equilibrate. Start titration experiment. Inject 20 μ L, 1 M glucose (giving 20 mM final concentration) with 1200 s lead time.
- 5.) Near start of titration experiment (-1200 s relative to glucose injection), remove 1 mL from culture bottle sample and place in P1. Place on ice and record time. Repeat again at c. -600 s and -60 s with P2 and P3.
- 6.) Wait for calorimeter to inject glucose. At same time, inject 320 μ L 1 M glucose into culture bottle (now containing 16 mL culture). Collect sample and place in P4.

- 7.) Centrifuge P1 to P4 at 4°C at 10 000 g for 10 min (use cold room centrifuge in ZT's lab and keep on ice during transport). Although P1 to P3 were collected long before, do not attempt to centrifuge them before this point; sampling, monitoring the calorimeter, and making the glucose injection are more important.
- 8.) Return to main lab. At ~600 s after P4 was collected, collect another sample and place in P5.
- 9.) Remove supernatant from P1 to P4 and place in S1 to S4. Resuspend P1 to P4 in 1 mL 0.9% NaCl. Centrifuge P1 to P5 together at 4°C at 10 000 g for 10 min.
- 10.) Return to main lab. At ~600 s after P5 was collected, collect another sample and place in P6.
- 11.) Remove supernatant from P1 to P4 and compile with supernatant already in S1 to S4. Place P1 to P4 and S1 to S4 on ice and prepare for transport. Remove supernatant from P5, place in S5, and resuspend P5 in 1 mL 0.9% NaCl. Centrifuge P4 to P5 together at 4°C at 10 000 g for 10 min.
- 12.) Return to main lab and place P1 to P4 and S1 to S4 in -20°C as soon as possible. Repeat collecting samples at 600 to 3600 s intervals and centrifuging until P12 and S12 are complete.
- 13.) Prepare VFA samples from S1 to S12 according to *Determination of volatile fatty acid (VFA) content of culture supernatant*. Use V1 to V12 to hold VFA samples. Thaw S1 to S12 if needed. Store at -20°C.
- 14.) Measure background heat production according to *Heat production of microbial cells*. For precise work, measure background heat production prior to running experiment (overnight between Day 1 and 2).

Day 3 and beyond

Perform chemical analysis according to procedures listed earlier.

- 1.) Analyze P1 to P12 for total hexoses according to *Total hexoses of microbial cells using the anthrone reaction*. Dilute the resuspended cell pellet 1→8 by adding 200 µL pellet to 1400 µL dH₂O in a separate 2-mL boil proof tube.

Analyze P1 to P12 for protein according to *Protein content of microbial cells*. Expected protein is 3000 to 5000 µg.

If both analyses cannot be done of the same day, give priority to total hexoses. Multiple freeze-thaw cycles lower content of hexoses, but they do not affect protein.

- 2.) Analyze S1 to S12 and 1 M glucose for glucose according to *Free glucose content of culture supernatant*. For glucose, add 91 μL *N*-ethylmaleimide to 200 μL supernatant, then add 582 μL dH_2O . For 1 M glucose, add 20 μL 1 M glucose to 10 mL dH_2O .
- 3.) Analyze S1 to S12 for lactic acid according to *Lactic acid content of culture supernatant*.
- 4.) Analyze V1 to V12 for VFA according to *Volatile fatty acid (VFA) content of culture supernatant*. Also analyze a blank sample of Simplex buffer alone.

SECTION VII: KINETICS OF SUGAR TRANSPORT

Sugar transport is important for cell growth, reserve carbohydrate synthesis, and energy spilling. Measurement has traditionally used radiolabeled sugars, which require caution during use. The following procedures explain how to use radiolabeled sugars safely in measuring sugar transport, and they describe use of fluorescently-labeled sugars as an alternative.

Example experiment: Transport kinetics of 2-NBDG and radiolabeled sugars by bacteria

Apparatus and reagents

- 1.) Construct apparatus for incubating tubes and adding stop solution. Follow **Fig. 1**.
- 2.) Prepare stop solution. Add 40 mL 2.5 M glucose, 20 mL 10x Simplex buffer, and 140 mL ddH₂O. Mix and store in bottle for bottle-top dispenser. Chill bottle on ice-salt mixture in cooler (Fig. 1). Make 2.5 M glucose and 10x Simplex buffer in advance.

Make 2.5 M glucose by adding 45.04 g glucose to ~50 mL dH₂O. Dissolve by stirring and heating. Cool on ice and bring to volume in 100 mL volumetric flask. Sterilize through filter (0.22 μm attached to 60 mL syringe) into a previously-autoclaved culture bottle with rubber bung. Store at 4°C.

Make 10x Simplex buffer by dissolving components for Simplex buffer (omitting CaCl₂) into 30 mL dH₂O (instead of 300 mL) and omitting CaCl₂. Prepare aerobically. Store at 4°C.

Make ice-salt mixtures. To 1.5 kg ice, add sufficient table salt (30 g or more) to lower temperature to at least -5°C (but not colder than the freezing point of -10°C). Using reagent-grade NaCl (74.8 g NaCl per kilogram of ice) will lead to more accurate temperature control but is usually not necessary.

- 3.) Prepare 2-NBDG and radiolabeled sugar stocks (see *Stocks for 2-NBDG transport experiments* and *Stocks for radiolabeled sugar transport experiments*). Here and throughout, follow *Safe use of radioactive materials*.
- 4.) Add 2-mL NMP aliquots to sample bottles (30 x 40 mm; Wheaton 225536) containing 2-mL NMP. Seal bottles with snap cap. Prepare as many bottles as 2-NBDG samples to be filtered.
- 5.) Record fluorescence of empty, chemically-resistant cuvettes (Brandtech #759150) using Biorad VersaFluor fluorometer (480/20 nm excitation, 520/10 nm emission). Read as many cuvettes as 2-NBDG samples and standards.
- 6.) Place 7 mL scintillation vials in blue rack. Include as many vials as the number of radiolabeled sugar samples. Counting the vials is not necessary (background is minimal).

Inoculation of cultures

- 1.) Inoculate cultures. Inoculate four tubes containing 9-mL media with 0.1 mL bacteria. Record optical density at 600 nm (OD₆₀₀) over time.
- 2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD₆₀₀ = 1 and requires 3 to 4.5 h from inoculation. Place tubes on ice, transfer to 40-mL screw-cap centrifuge tube under CO₂. Centrifuge at 4°C, and wash twice with ice-cold

Simplex buffer under CO₂. For *S. bovis*, 10 000 g for 5 min is sufficient for each centrifugation, whereas 10 min should be used for most strains.

3.) Resuspend pellet in Simplex buffer to give c. 0.4 mg protein/mL. For *S. bovis*, required resuspension volume is 17 mL. Transfer to culture bottle. Keep on ice and under CO₂.

Incubation in 2-NBDG and radiolabeled sugars

1.) Prepare aliquots of culture and pre-incubate. Under CO₂, add 0.25 mL aliquots of resuspended culture to 12x75 mm culture tubes with 00 size stoppers. Pre-incubate in 39°C water bath for 10 min. To avoid “losing” culture due to adhesion to tube walls, add directly to bottom of tube and avoid shaking tube. To avoid long-term starvation at 39°C, which can decrease transport, prepare only small number of aliquots (≤ 7) at one time, and keep remainder of culture on ice. Record time at which pre-incubation was started.

2.) Immediately after pre-incubation, position tube for adding 2-NBDG or radiolabeled sugar (Fig. 1). Place tube in dry incubator at 39°C, remove stopper, and insert needle of gassing probe in upper lip of tube. Insert needle from bottle top dispenser. Record time of incubation.

3.) Add 5 μ L of 2-NBDG or radiolabeled sugar stock. Lift tube slightly from incubator, add and mix stock by pipetting up and down, then place tube back into incubator. Record (mentally) the second when 2-NBDG is added; a stopwatch counting upwards is helpful.

4.) At end of incubation, stop reaction by quickly dispensing 2-mL stop solution with bottle top dispenser. To avoid contact between tube and warm incubator, lift the tube up slightly before adding stop solution. With practice, incubation can be timed to nearest second, and total incubation times as short as 2 s can be achieved.

5.) Using transfer pipette, quickly transfer contents of tube to filter holder with pre-wetted Nuclepore filter (shiny side up; 0.4 μ m, 25 mm diameter; Whatman 110607). To draw up contents all at once, squeeze bulb firmly and completely, then release while holding tip at bottom of tube. Add contents directly on top of filter, avoiding splashing the walls of the holder.

6.) Add 2-mL additional stop solution to tube and transfer to filter holder with same pipette. This step washes the tube, pipette, filter tower, and cells on filter. With practice, transferring contents of tube to filter holder and washing can be done in <10 s.

7.) Wait for liquid to drain from filter, then immediately disassemble holder, remove vacuum tubing, and remove filter with forceps.

8.) For 2-NBDG samples, transfer the filter to bottle containing 2 mL NMP. Try to drop filter directly in NMP, avoiding contact with walls of bottle. Swirl contents of bottle vigorously to dissolve filter. Mix with transfer pipette to complete dissolution. If filter adheres to bottle walls at any point, wash walls thoroughly with pipette. Transfer ~1 mL with transfer pipette to cuvette. Seal with cap, and read fluorescence.

9.) For radiolabeled sugar samples, transfer filter to scintillation vial, add 5 mL scintillation cocktail, shake the vial vigorously and determine DPM following *Liquid scintillation counting*.

10.) Repeat experiment for full range of concentrations and incubation lengths. Include an experiment with incubation length of 0 s, in which stop solution is added before 2-NBDG or radiolabeled sugar stocks. With practice and 2 people splitting duties, each experiment will take 3 min.

11.) Harvest 1-mL aliquots of bacteria for protein analysis (see *Protein content of microbial cells*).

Notes

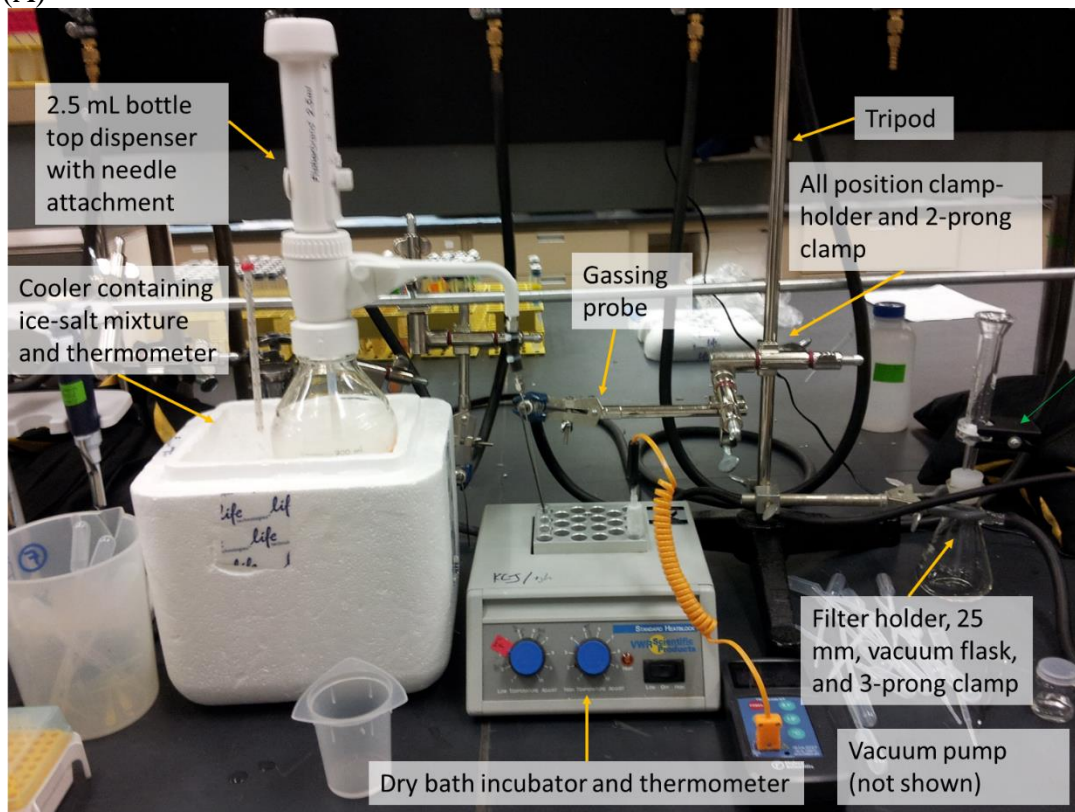
(i) For #3 of *Inoculation of cultures*, cell protein can be roughly estimated by measuring optical OD₆₀₀ and referring to past experiments that have measured both OD₆₀₀ and protein. This rough estimate facilitates adjustment of protein to 0.4 mg/L.

(ii) For some bacteria, 0.4 mg protein/mL is too concentrated and will lead to unacceptably long filtration times in step #7 of *Incubation of 2-NBDG and radiolabeled sugars*. For these bacteria, determine with a preliminary experiment the maximum concentration that will lead to filtration in <15 s.

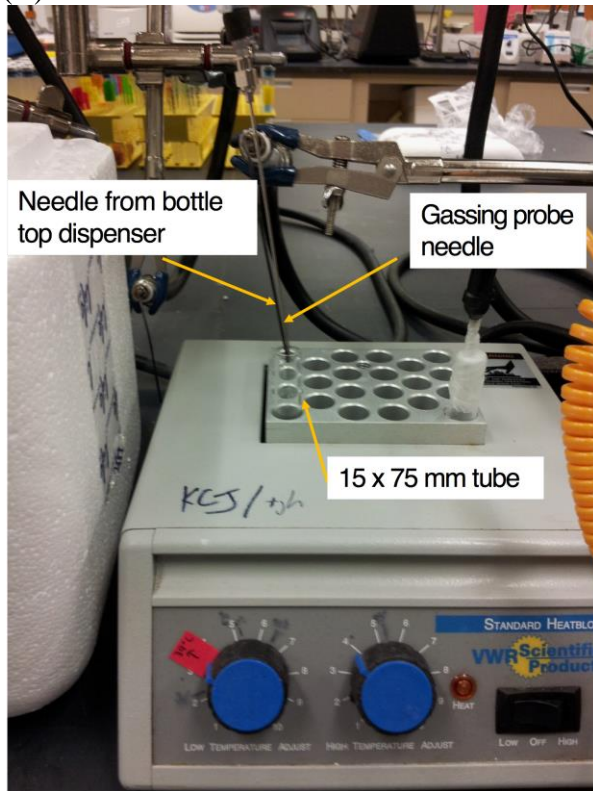
(iii) To avoid spread of radioactive contamination, radiolabeled sugar samples should be done only after all 2-NBDG samples.

Fig. 1. Apparatus for incubating tubes and stopping reaction. For radiolabeled sugar samples, items used should be those designated for radioactive use. (A) Overview. (B) Close up of tube with gassing probe and bottle-top dispenser in lip. (C) Bottle top dispenser with needle attachment.

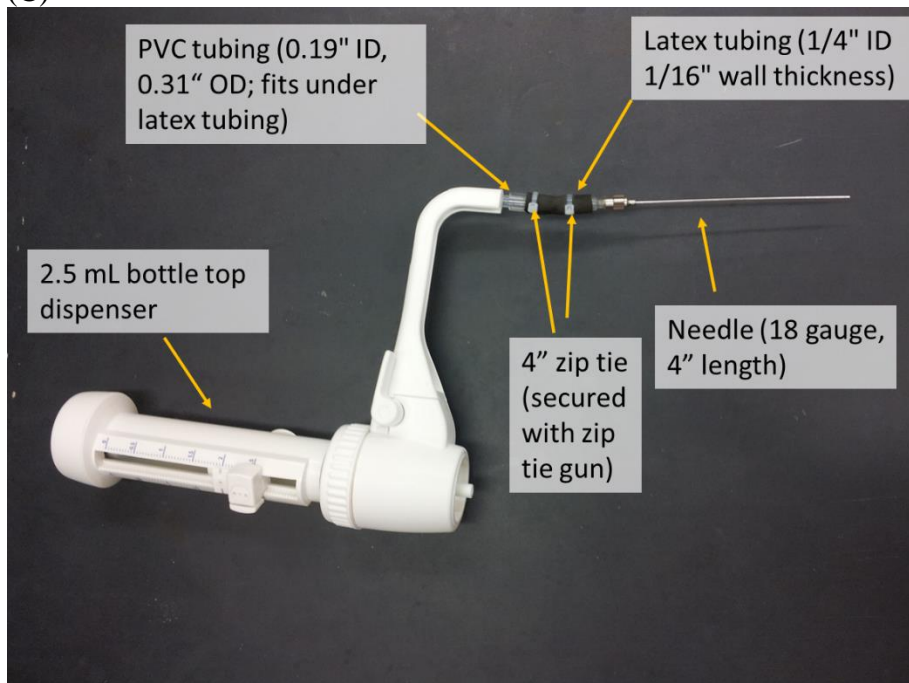
(A)



(B)



(C)



Stocks for radiolabeled sugar transport experiments

1.) Prepare 10 mM glucose. Add 0.18016 g glucose to 100 mL volumetric flask and bring to volume with ddH₂O. Sterilize by filtering through 0.2 μm membrane into autoclaved Balch tube and store at -20°C.

Prepare 10 mM mannose. Repeat procedure for 10 mM glucose but use 0.18016 g mannose.

Prepare 10 mM 2-deoxyglucose. Repeat procedure for 10 mM glucose but use 0.16416 g 2-deoxyglucose.

Prepare sterile water. Filter-sterilize or autoclave water in Balch tube.

2.) Set up lab for radioactive use. Follow *Safe use of radioactive materials*.

3.) Prepare stocks following Tables 1 to 3.

(a) Add ddH₂O and 10 mM sugar to 0.5 mL sterile microcentrifuge tube in volumes indicated in tables. Do not yet add radiolabeled sugar. Here and throughout, work under flame and otherwise maintain aseptic conditions.

(b) Move radioactive materials from hood to bench and finalize bench for radioactive use.

(c) Take stock vial of a radiolabeled sugars out of refrigerator.

(d) Withdraw an aliquot of radiolabeled sugar from the vial (volume sufficient for preparing stocks). Sterile vial septum with 70% ethanol and Kimwipe (attempting to flame will melt plastic of vial). Use 1-mL syringe with 22-gauge needle to retrieve sugar. Place aliquot in sterile 0.5 mL, screw-cap centrifuge tube.

(e) Place stock vial back into refrigerator.

(f) With pipette labelled for radioactive use, add radiolabeled sugar to 0.5 mL centrifuge tube in volume indicated. Mix well. Keep at -20°C for storage.

4.) Verify stocks were prepared correctly by determining radioactivity. Pipette 10 μL of each stock and undiluted radiolabeled sugar into scintillation vial containing 5 mL scintillation cocktail. Count with Beckman LS 6000 IC β-counter using “Auto DPM” card. Compare actual DPM with expected.

Table 1. ¹⁴C-glucose stocks (presuming 0.1 mCi/mL and 289 mCi/mmol ¹⁴C-glucose)

Total glucose	Activity	Sterile ddH ₂ O	10 mM glucose	¹⁴ C-glucose
μM	μCi/5 μL	μL	μL	μL
5000	0.25	4.33	120.7	125
2500	0.20	91.0	59.0	100
500	0.10	189.2	10.8	50
250	0.08	205.1	4.87	40
125	0.08	208.3	1.74	40
50	0.072	213.9	0	36.1

Table 2. ^{14}C -deoxyglucose stocks (presuming 0.1 mCi/mL and 274.6 mCi/mmol ^{14}C -deoxyglucose)

Total deoxyglucose	Activity	Sterile ddH ₂ O	10 mM deoxyglucose	^{14}C -deoxyglucose
μM	$\mu\text{Ci}/5 \mu\text{L}$	μL	μL	μL
5000	0.25	4.55	120.5	125
2500	0.20	91.1	58.9	100
500	0.10	189.3	10.7	50
250	0.08	205.2	4.79	40
125	0.08	208.3	1.67	40
50	0.072	213.9	0	36.13

Table 3. ^3H -mannose stocks (presuming 1 mCi/mL and 19.3 Ci/mol ^3H -mannose)

Total mannose	Activity	Sterile ddH ₂ O	10 mM mannose	^3H -mannose
μM	$\mu\text{Ci}/5 \mu\text{L}$	μL	μL	μL
5000	1	75.3	124.7	50
2500	0.80	147.7	62.3	40
500	0.40	217.6	12.4	20
250	0.32	227.8	6.17	16
125	0.32	231.0	3.04	16
50	0.16	240.8	1.21	8

Stocks for 2-NBDG transport experiments

1.) Prepare 5 mM 2-NBDG by adding 584 μL ddH₂O to vial containing 1 mg of 2-NBDG. Wash walls and lid of vial to ensure full amount is dissolved. Solution is stable at 4°C for several weeks.

2.) Prepare 2-NBDG stocks in 2-mL centrifuge tubes as follows

Stock concentration (μM)	Source of 2-NBDG	Volume of source (μL)	Volume of dH ₂ O (μL)
5000	5 mM solution	100	0
500	5 mM solution	40	360
50	500 μM stock	40	360
12.5	500 μM stock	10	390
5	50 μM stock	40	360
2.5	50 μM stock	20	380

Stocks are stable at 4°C for several weeks.

3.) Prepare standards in 12x75 mm culture tubes covered with parafilm

Standard concentration (μM)	Source of 2-NBDG	Volume of source (μL)	Volume of NMP (mL)
10	500 μM stock	40	2
1	50 μM stock	40	2
0.25	12.5 μM stock	40	2
0.1	5 μM stock	40	2
0.05	2.5 μM stock	40	2
0	ddH ₂ O	40	2

1-methyl-2-pyrrolidone (NMP) should be spectroscopy grade. Store on ice and use fresh.

4.) Read fluorescence of standards. With VersaFluor fluorometer (480/20 nm excitation, 520/10 nm emission), record fluorescence of empty, chemically-resistant cuvettes (Brandtech #759150). Pipette 1 mL standard into cuvettes. Record fluorescence and subtract value of empty cuvette.

Safe use of radioactive materials

- 1.) Put on 2 layers of gloves before handling radioisotopes or any items designated for radioactive use. Change gloves frequently and when contamination is suspected.
- 2.) Tape down bench paper (absorptive side up) on area where radioisotopes will be handled. Clear area of materials not designated for radioactive use.
- 3.) Retrieve items designated for radioactive use from the radioactive cabinet. Items in the radioactive cabinet include pipettes, tips, filter holders, beakers, forceps, vacuum flasks, pens, gassing probe, heat block, water bottle, and polystyrene cooler. If an item is not in cabinet, take one from general stock and label with yellow radioactive tape to designate it for radioactive use. Disposable items do not have to be labeled if they will be discarded at the end of the experiment.
- 4.) Retrieve radioisotopes from refrigerator or freezer designated for radioactive use. Keep the refrigerator and freezer locked when not in use.
- 5.) Conduct experiment on top of bench paper and with items designated for radioactive use. Do not touch your person or items not designated for radioactive use. If non-radioactive items must be handled, change gloves or ask labmate for assistance.
- 6.) If samples in microcentrifuge tubes need to be kept on ice, fill a 1-L plastic beaker with ice, place rack in beaker, and place microcentrifuge tubes in rack. Discard ice down drain only if swipe test shows it is not contaminated.
- 7.) Record notes using pen designated for radioactive use and disposable paper (not a lab book). Transfer notes to lab book after experiment.
- 8.) After completing experiment, decontaminate non-disposable radioactive items with NoCount, ddH₂O, and paper towels. Collect contaminated liquids in liquid waste container. Discard disposable, incinerable materials in polypropylene barrel for Type A waste; discard disposable, non-incinerable materials in Type B waste; and discard liquid scintillation vials (full, not poured out) in designated waste.
- 8.) Follow *Swipe assay* for doing a swipe test.

Liquid scintillation counting

Adapted from Beckman LS6000 Series manual

- 1.) Place sample (e.g., membrane filter or liquid) to be counted in 7 mL scintillation vial (Fisher 03-337-26).
- 2.) Add 5 mL Scintiverse BD scintillation cocktail (Fisher SX18-250) with designated bottle-top dispenser.
- 3.) Mix sample by vortexing or shaking vial.
- 4.) Load vials in a blue scintillation rack. Insert “AutoDPM” card into rack.
- 5.) Load racks in Beckman LS 6000 IC β -counter. Open black lid and place rack on right side. Place the blue rack in front. Place a red rack with “HALT” card immediately behind.
- 6.) From the main menu of beta counter, select “Automatic Counting”, and press “START”.
- 7.) From print out, record results from “auto DPM” column.
- 8.) Discard scintillation vials in designated waste area. Dispose full (do not pour out contents).

Notes

- (i) The program used above is for automatic counting. This suffices for most biological samples containing either ^3H or ^{14}C , but the user can make custom programs. See Beckman LS6000 Series manual for further details. These programs are accessed by using numbered cards (e.g., “1”), instead of the AutoDPM card.
- (ii) The exact volume of scintillation cocktail added (and the resultant dilution of the original sample) is not critical. Unlike most assays, the dilution of the sample does not impact results, as the counter detects photon emissions in the entire sample.
- (iii) For less critical samples (e.g., those from swipe tests), 3 mL cocktail can be used.
- (iv) Periodically, the accuracy of the counter should be checked with unquenched standards (Perking Elmer 6008400).
- (v) Corrections for quenching are done automatically by using a ^{137}Cs source.

Swipe assays

After (107) and Beckman LS 6000 Series manual

- 1.) Cut filter paper into small pieces to serve as wipes. Other paper, glass filter, cotton, or plastic can be used, also.
- 2.) Place 14 scintillation vials (7 mL, borosilicate screw-top vials) in scintillation rack (corresponding to the 12 mapped areas in Fig. 1 and 2 and blanks).
- 3.) With filter paper, wipe a 100 cm² area (approximately 4 × 4 in). Place directly in vial. Do not touch side of filter paper that was used in wiping (to avoid spread of potential contamination).
- 4.) Repeat for all areas in survey map. Include 2 background samples (filter that was not used in wiping).
- 5.) Determine CPM according to *Liquid Scintillation counting*, with the following changes:
 - (i) Add 3, not 5 mL, of cocktail
 - (ii) Use the number “1”, not “AutoDPM” card. This will call user program 1 (settings are Table 1).
- 9.) Record CPM on survey map. To calculate net CPM for 3H and 14C, subtract the average of background from the gross CPM on print out. Once net CPM for each isotope is calculated, add the net CPMs together to get total CPM for all 12 areas. To calculate net DPM, divide 14C net CPM by efficiency for ¹⁴C detection (95.6%, as measured with standards), divide 3H net CPM by efficiency for 3H detection (60% as measured with standards) and add the obtained values together. Save print out.
- 10.) If any areas have >100 DPM, decontaminate the area with No Count, perform a new swipe, and record new DPM in Comments on the survey map.

Notes

- (i) When choosing areas to swipe, focus on those areas most likely contaminated (e.g., refrigerator door) and those receiving high traffic (e.g., floor in front of door).
- (ii) Do not record DPM on print out from β-counter. Because of low CPM, efficiencies are not reliable (and sometimes cannot even be calculated).

Fig. 1. Survey map.

RADIATION/CONTAMINATION SURVEY

PRINCIPAL INVESTIGATOR: HACKMANN, TIMOTHY

SURVEYOR: TAO, JUNYI

ROOM NO: 201

RADIONUCLIDES USED: 3H, 14C

SURVEY NO: 1

SURVEY TYPE: WEEKLY SPECIAL

DATE: 6/22/ 15

	Yes	No	N/A
Caution Signs Posted	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Waste Facilities Posted & Neat	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Inventory up to Date	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Work Areas Clean	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Survey Meter Calibrated	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Survey Meter Operation Verified with Dedicated Check Source	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>

Survey Meter Model & Serial #

NA

Work Area NA mR/hr

Waste Facilities NA mR/hr

Storage Areas NA mR/hr

CONTAMINATION SURVEY

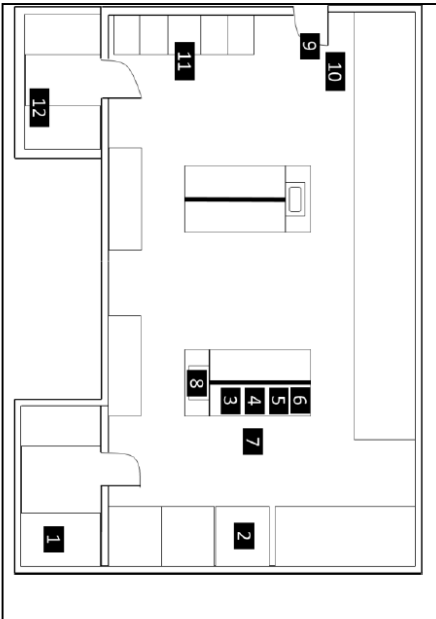
RESULTS

Counting Instrument Model & Serial #

Beckman LS 6000 IC

Detection Efficiency 60% for 3H and 96% for 14C

DETAILED LABORATORY LAYOUT



#	Swipe Identification	Net Cpm	Net dpm*	Post Decon dpm
1	Outside LSC	7	11.05	
2	Lip and base of hood	3	4.07	
3	Metal tripod	11	16.78	
4	Bottle top dispenser	18	27.52	
5	Dry block	5	6.47	
6	Bench	4	5.74	
7	Floor by bench	-0.5	-1.76	
8	Sink	11.5	16.68	
9	Door knob	7.5	9.71	
10	Floor by door	6.5	8.97	
11	Refrigerator door	3	5.00	
12	Freezer door	0	0.93	

*Areas of greater than 100 dpm/swipe must be resurveyed and documented.

DPM = Gross CPM minus background divided by the efficiency of the counting instrument for the particular radioisotope

RADIATION READINGS:

Table 1. Settings for swipe survey for Beckman LS 6000 IC beta counter

Menu/submenu	Option	Setting
Review/Edit	Counting time	2 min
	Scintillator	Liquid
	Isotope 1	14C
	Isotope 2	3H
	Isotope 3	NONE
Data calculation	Calculation mode	CPM
	Count sample	1
	Replicates	1
	Count sample set	1
	Factor for 14C	1.000000
	Factor for 3H	1.000000
Background/blank	Count blanks	No
	Background 14C	0.00
	Background 3H	0.00
Quench/LUM-EX/2 Phase	Quench monitor	IC#
Low Bkg/Low count reject	Low count reject threshold	0 CPM

Phosphotransferase assay of rumen bacteria

Adapted from (34, 35, 108)

Reagents

- 1.) Prepare 20 mM NADH. Weigh out 0.025 g NADH (Sigma 43420-100MG) in a sterile 2-mL centrifuge tube and add 1762 μ L molecular-biology ddH₂O. Before storing at -20°C, make aliquots (17, 0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.
- 2.) Prepare 0.5 M phosphoenolpyruvate (PEP) by adding 970.2 μ L molecular-biology ddH₂O to 0.1 g PEP monopotassium salt. Do this by adding 500 μ L molecular-biology ddH₂O directly to the 0.1 g PEP (Sigma P7127-100MG) in manufacturer vial. Pipette up and down to form suspension (which will be turbid). Transfer to 1.5 mL centrifuge tube. Save the pipette tip. With a second pipette tip, add remaining 470.2 molecular-biology ddH₂O to manufacturer vial, and with first pipette tip, transfer back-and-forth between vial and centrifuge tube (to wash and mix). Final solution will be clear. Store at -20°C in 0.2 mL aliquots.
- 3.) Order L-lactate dehydrogenase (LDH; Roche 1012802300) and store at 4°C.
- 4.) Prepare 1:9 toluene-ethanol. Add 1 mL toluene to 9 mL ethanol and mix.
- 5.) Prepare phosphate buffer (see *Buffer and media recipes* and *Preparing small volumes of buffer/media*). Prepare phosphate buffer + 2 mM DTT by adding 0.18 mL 0.1 M DTT to 9-mL aliquots of buffer. Add DTT after autoclaving. Make sure to prepare buffer without DTT, also (see below).
- 6.) Prepare 5 mM glucose.
- 7.) Prepare 5 mM 2-NBDG. Add 584 μ L ddH₂O to vial containing 1 mg of 2-NBDG. Wash walls and lid of vial to ensure full amount is dissolved. Solution is stable for several weeks at 4°C.

Inoculation of cultures

- 1.) Inoculate cultures. Inoculate 1 tube containing 9-mL media with 0.1 mL bacteria. Record OD₆₀₀ over time.
- 2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD₆₀₀ = 1 and requires 3 to 4.5 h from inoculation. Place tubes on ice, transfer to 10-mL screw-cap centrifuge tube under N₂. Centrifuge at 4°C, and wash twice with ice-cold phosphate buffer + 2 mM DTT under N₂. For *S. bovis*, 10 000 g for 5 min is sufficient for each centrifugation.
- 3.) Resuspend pellet in 3-mL phosphate buffer + 2 mM DTT (or that volume which achieves 0.1 to 1 mg protein/mL). Transfer to 12x75 mm culture tube. Keep on ice and under N₂.

Preparation of toluene-treated cells

- 1.) Transfer 0.5 mL aliquot of re-suspended culture to a 12x75 mm culture tube.
- 2.) While under N₂, vortex tube and add 15 μL toluene-ethanol. Continue vortexing for an additional 1 min. Return to ice.

Measurement of phosphotransferase activity

- 1.) Add the following to a 12x75 mm culture tube under N₂
 - 2855.7 μL phosphate buffer (without DTT)
 - 15.5 μL NADH
 - 6.2 μL PEP
 - 5.64 μL LDH (equivalent to ~2 U for 1100 U/mL LDH)
 - 155 μL toluene-treated cells

Transfer 980 μL aliquots to 3 semi-micro disposable cuvettes (Brandtech #759150). Keep under N₂, stopper, and pre-incubate in 39°C air incubator for 10 min.

- 2.) Initiate the reaction. Add 20 μL 5 mM glucose to one cuvette, 20 μL 5 mM 2-NBDG a second cuvette, and 20 μL phosphate buffer (without DTT) to a third cuvette. Immediately mix, flush with N₂, stopper, and measure absorbance at 340 nm with Thermo Scientific 20 spectrophotometer.

- 3.) Incubate cuvette at 39°C. Re-read absorbance at intervals (e.g., 2.5, 5, 7.5, 10, 15, 17.5, 20 min) after first reading.

- 4.) Calculate rate of NADH consumption. For each cuvette, plot absorbance vs. time, calculate the slope, then calculate NADH consumption as

$$\text{NADH consumption} = (\text{slope}/6\ 220) \cdot 10^{-3}$$

where 6 220 M⁻¹ cm⁻¹ is the extinction coefficient of NADH and 10⁻³ L is the volume of the reaction. The cuvette with 20 μL phosphate buffer (without DTT) is a control, and NADH consumption of cuvettes with glucose or 2-NBDG should be corrected by subtracting values of this control. Typically, cell protein is also measured, and values are further expressed as nmol PEP mg protein⁻¹ min⁻¹. Repeat all experiments in triplicate.

Notes

- (i) Most PTS assays include higher concentrations of sugar [e.g., 5 mM; (34, 35, 108)]. The concentration in the present experiment is limited to 100 μM because of the high extinction coefficient of 2-NBDG (7100 M⁻¹ cm⁻¹).

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