HACKMANN LAB

MANUAL *for* RUMEN MICROBIOLOGY

v. 5.0

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17 July 2023

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(in chronological order)

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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.

CONTENTS

SECTION I: ANAEROBIC TECHNIQUE AND CULTURING

Rumen microbes are challenging to work with because they are sensitive to O_2; most are strictly anaerobic. Special equipment must be built for gassing cultures with O_2-free gas, to exclude O_2. This equipment not only dispenses O_2-free gas, but it also scrubs commercial gas of trace impurities of O_2. 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 O_2-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 CO_2 (99.99%), N_2 (99.999%), and H_2 (99.999%) tanks near gassing station and install 50 psi regulators (**Fig. 1**). Install a flash arrestor on the H_2 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 CO_2, 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, ½" OD; Fisher 14-168B).

9.) Plug heat tape into rheostat (**Fig. 14**) and adjust knob until column temperature reaches 350ºC (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 O_2 from CO_2 and N_2 gas used in culturing. Commercial CO_2 and N_2 gas contains trace O_2 that will kill or inhibit rumen microbes if O_2 is not removed.

1.) If needed, reduce copper turnings in column. Reduced copper is red and is effective in removing O_2. Copper turns pale red, colorless, and then black as it is oxidized. It is ineffective in removing O_2 when oxidized.

To reduce, first turn on CO_2 or N_2 and exhaust through a gassing probe for a few minutes (to flush system of most O_2). Turn on H_2 to 0.5 bar. Combust H_2 by holding a lighter up to the gassing probe until a flame is lit. Allow H_2 to circulate until copper is red and reduced (may require several minutes).

2.) Turn on CO_2 or N_2. 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”.

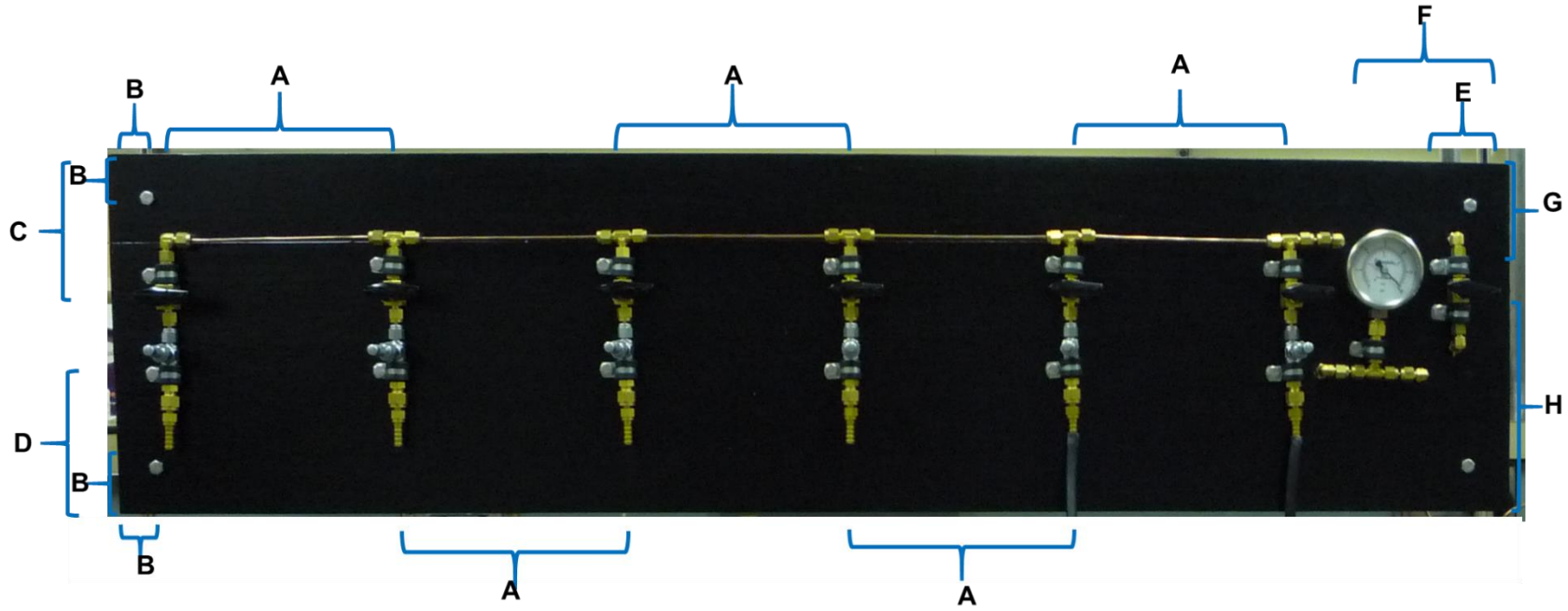


Fig 3. Placement of holes for Swagelok parts. A = 2”, B = 2.75”, C = 6.5”, D = 4.75”, E = 3.25”, F = 6.75”.

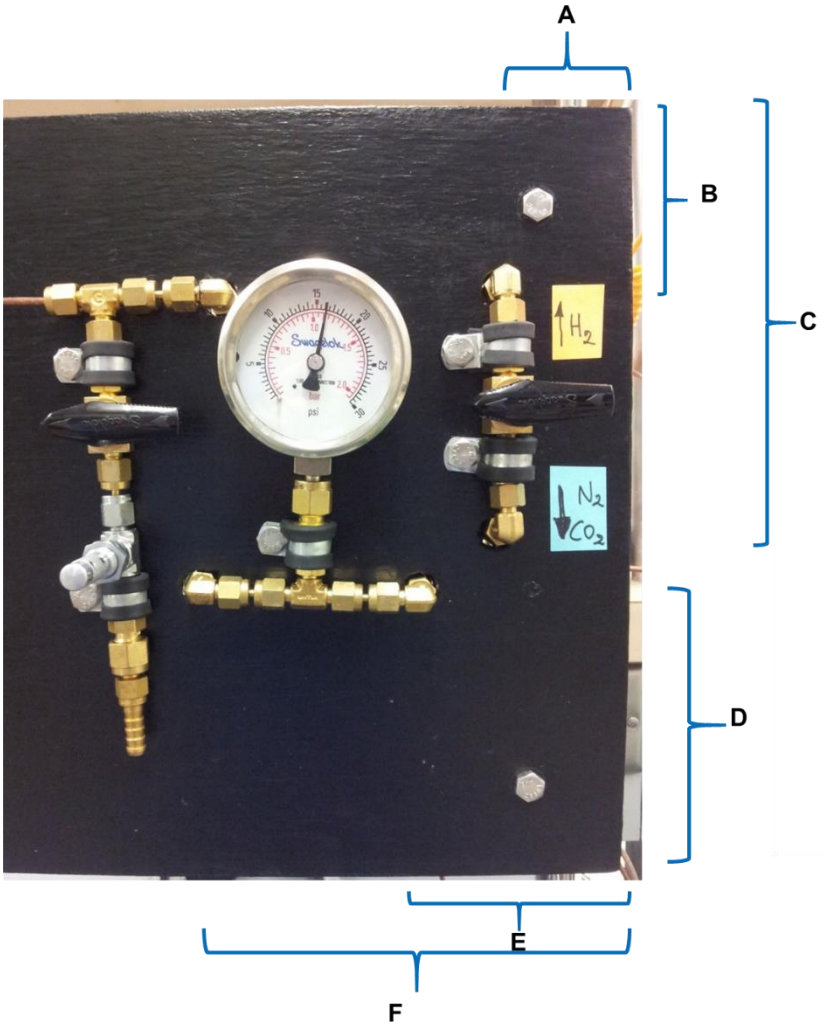
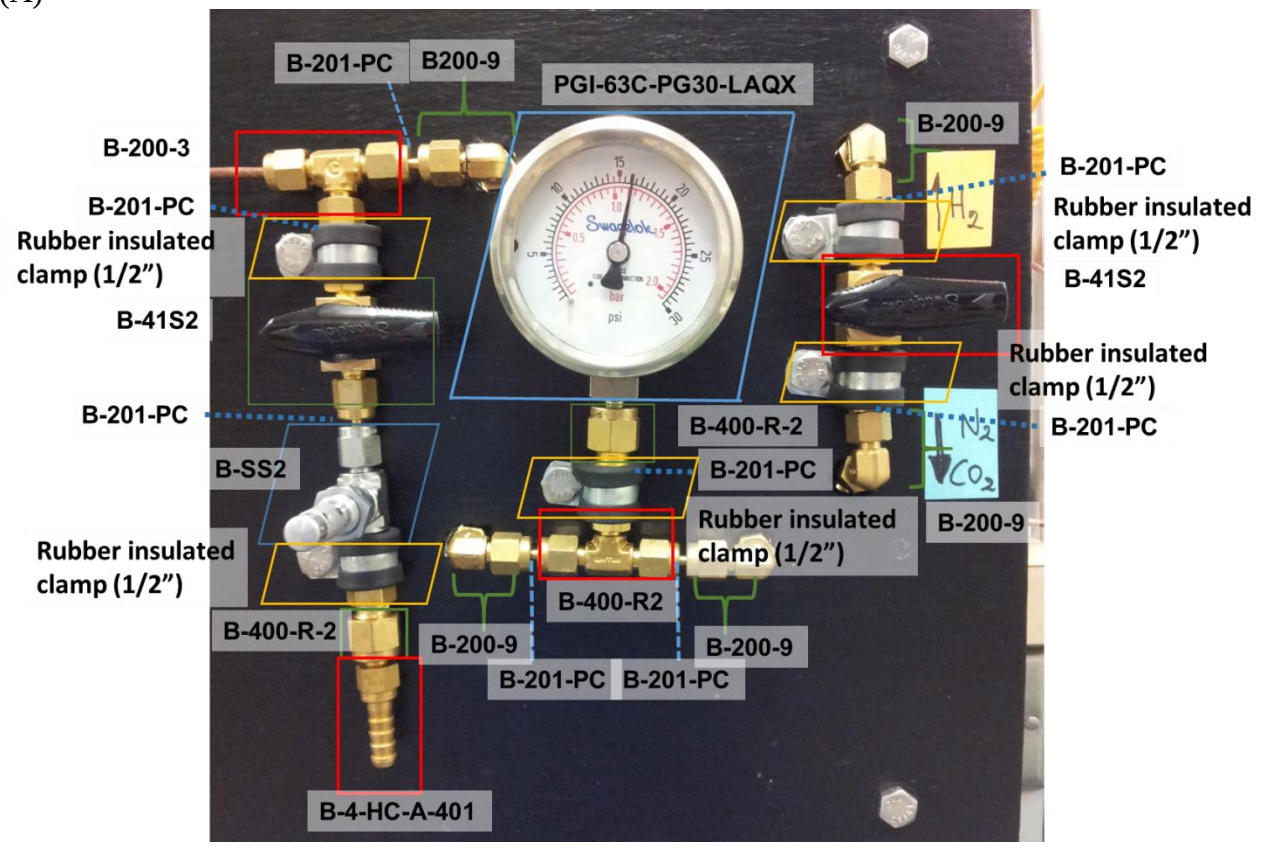


Fig 4. Swagelok and related parts. Unless noted, label refers to catalog number in Swagelok catalog. (A) Front panel. (B) Rear panel.

(A)



(B)

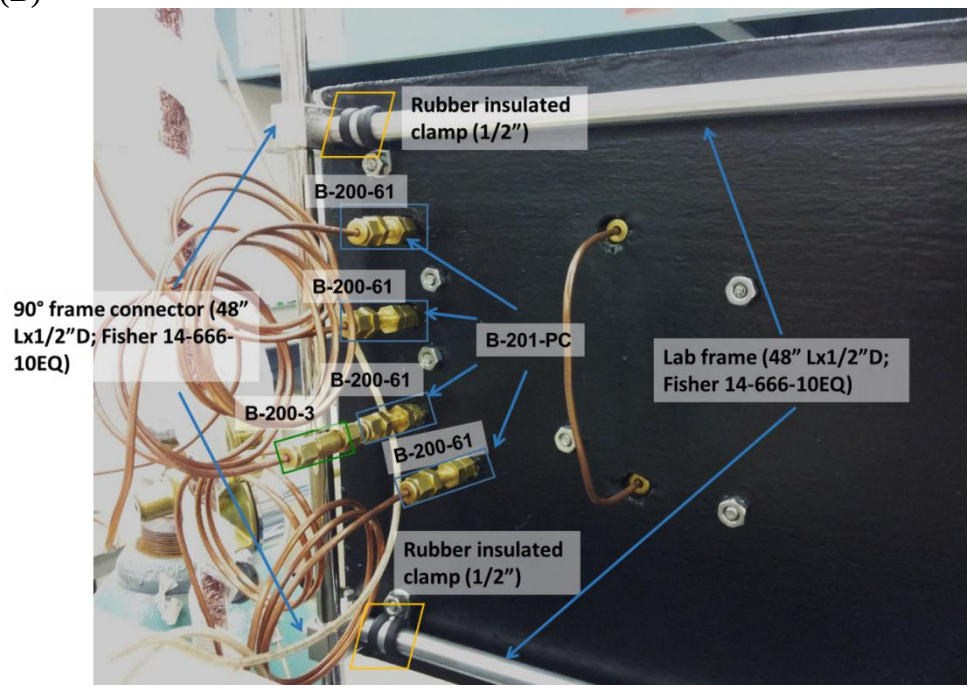
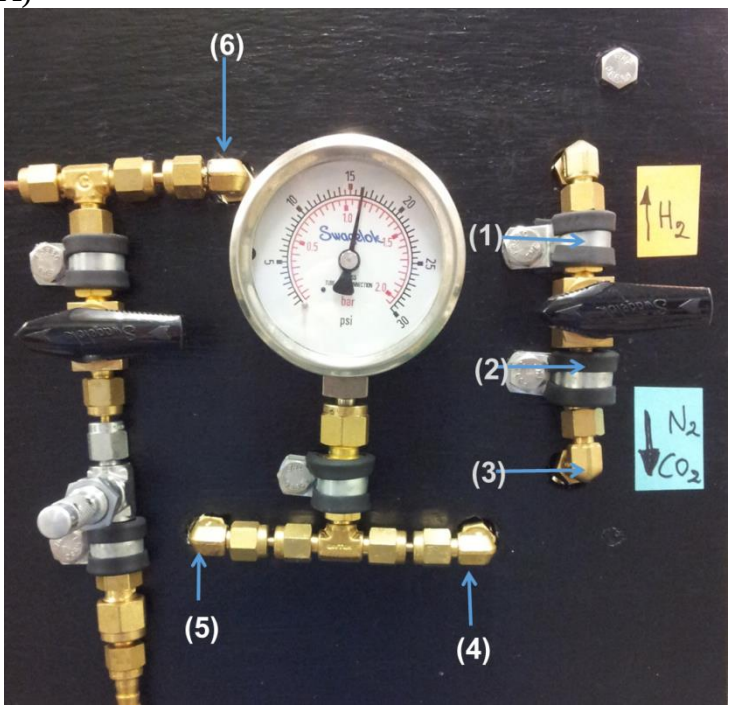


Fig 5. Connections between (A) front and (B) rear panels.

(A)



(B)

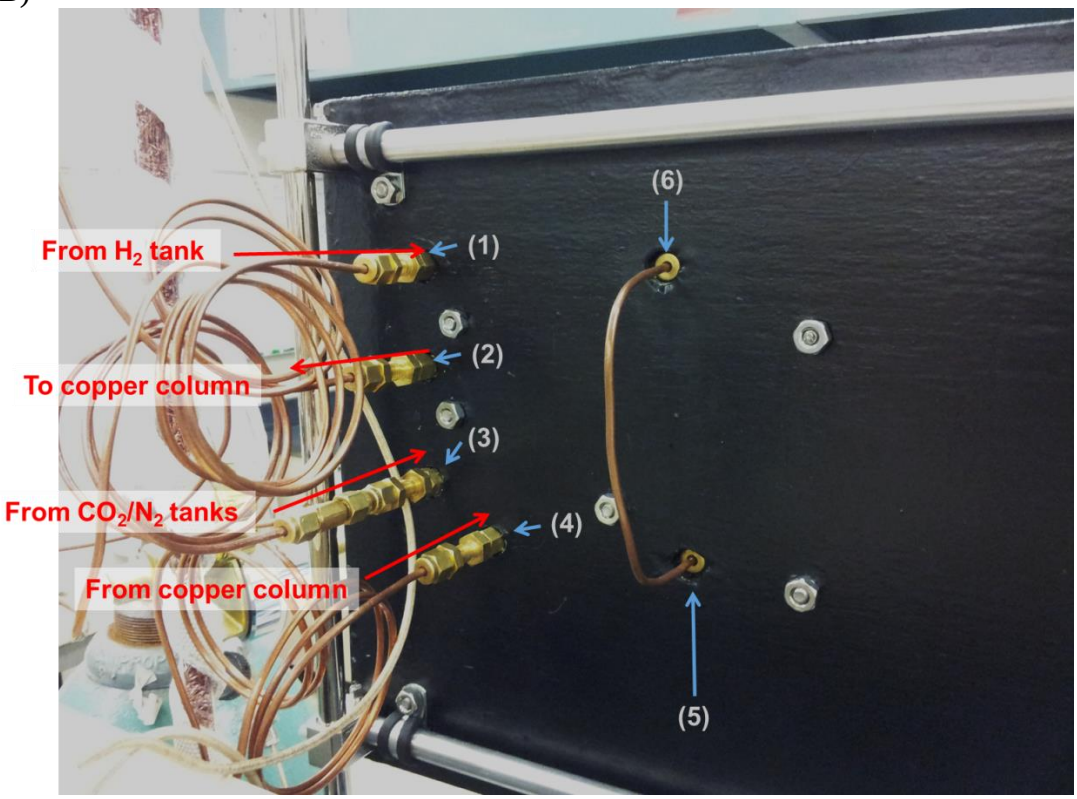


Fig 6. Frame components. For components visible from rear only, see Fig 4B.

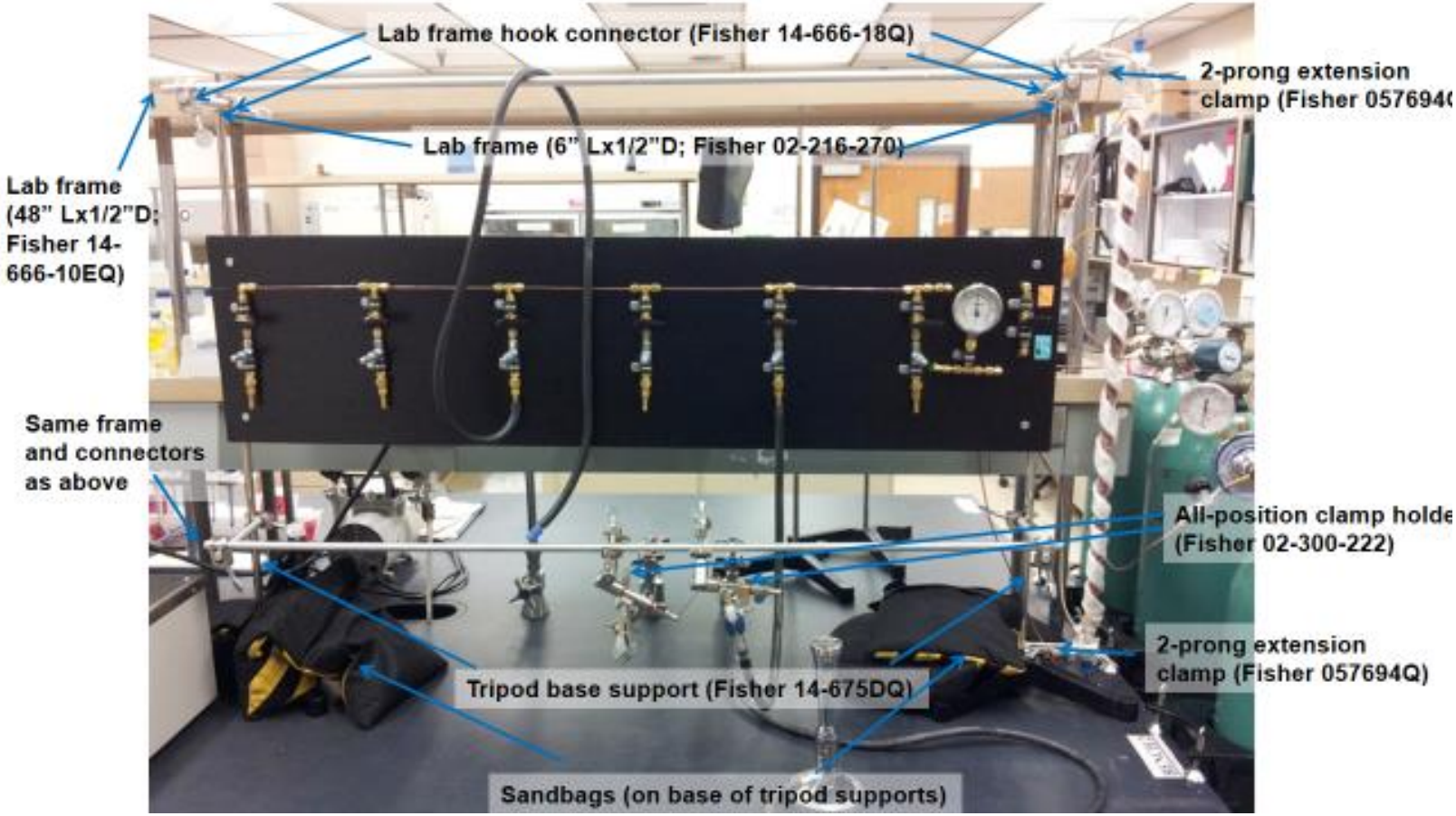


Fig. 7. Close-up of copper column.

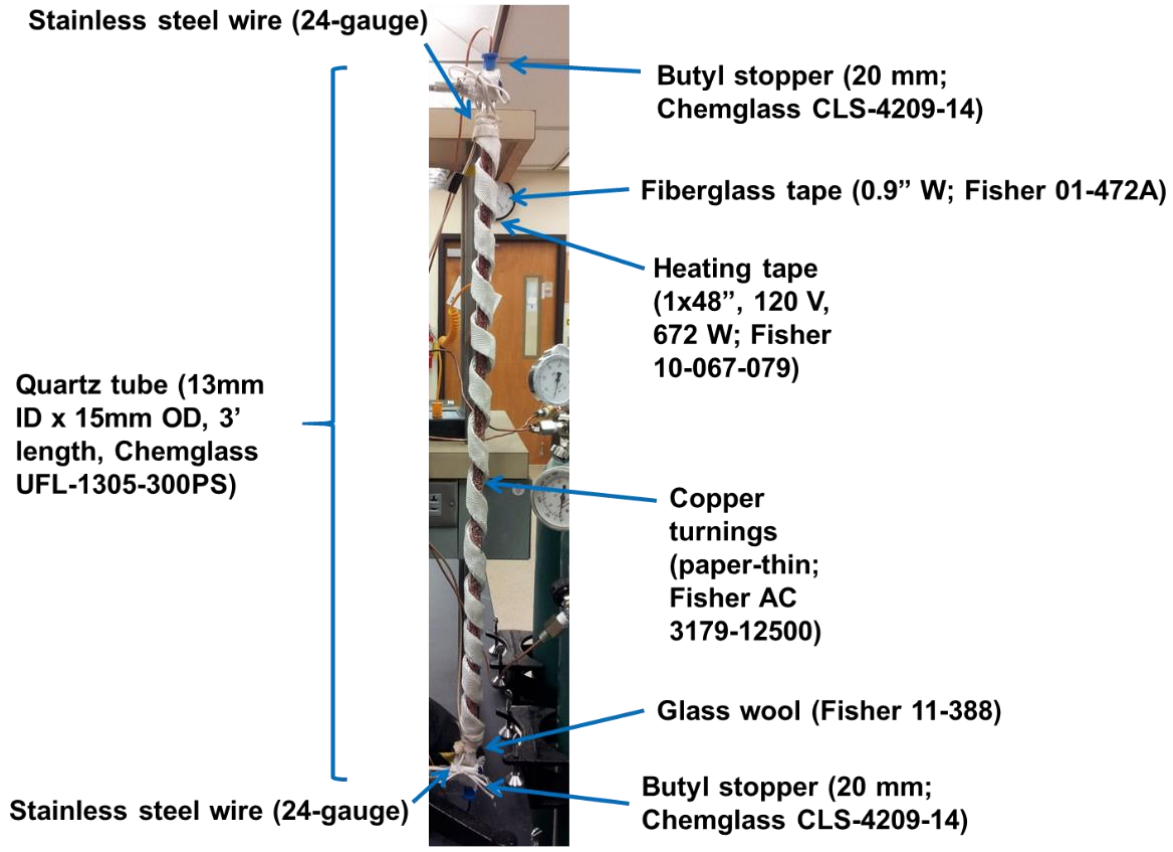


Fig 8. Packing column with copper turnings.

Fig 9. Flash arrestor and compression fitting on H_2 tank.

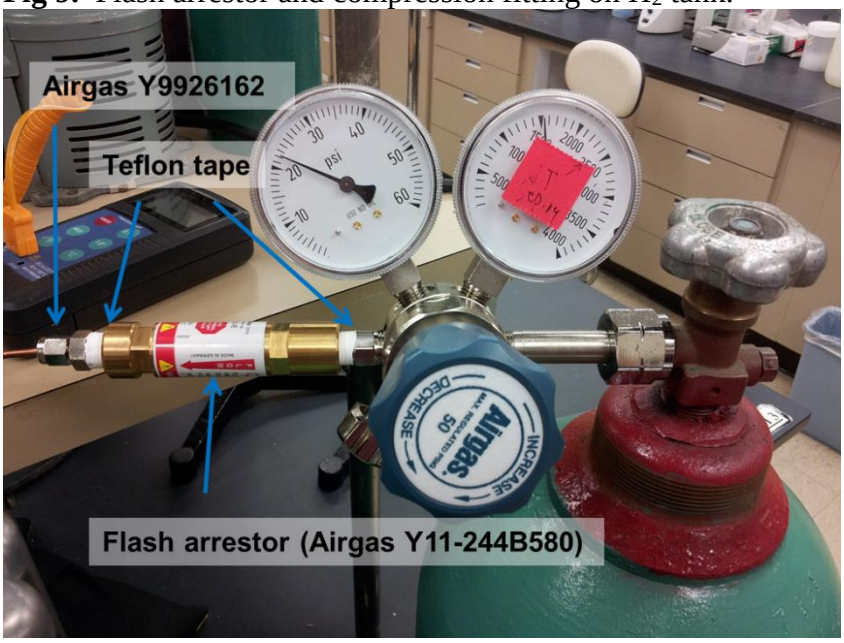


Fig 10. Compression fitting on N_2 tank (identical for CO_2 tank).

Fig 11. Gassing probe.

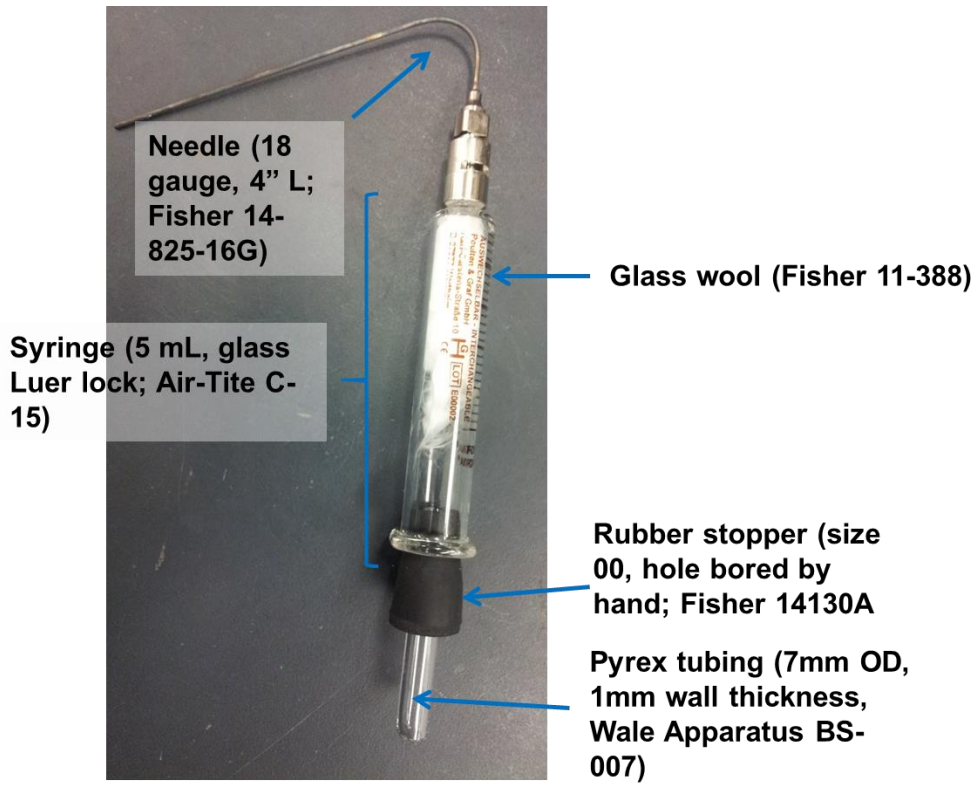


Fig 12. Bending needle of gassing probe. (A) Flaming needle above hub. (B) Bending needle around metal stand.

(A)

(B)

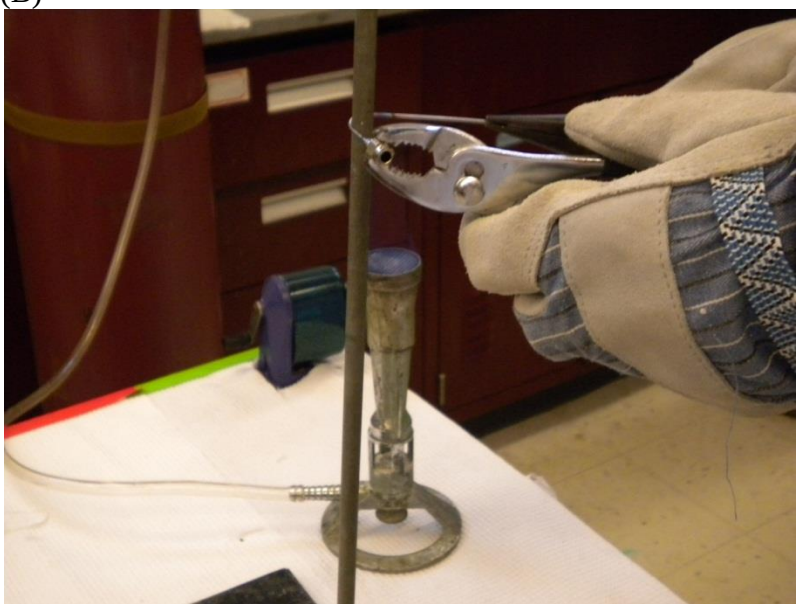


Fig. 13. Airstone bubbler. Airstone will later be inserted into rubber tubing.

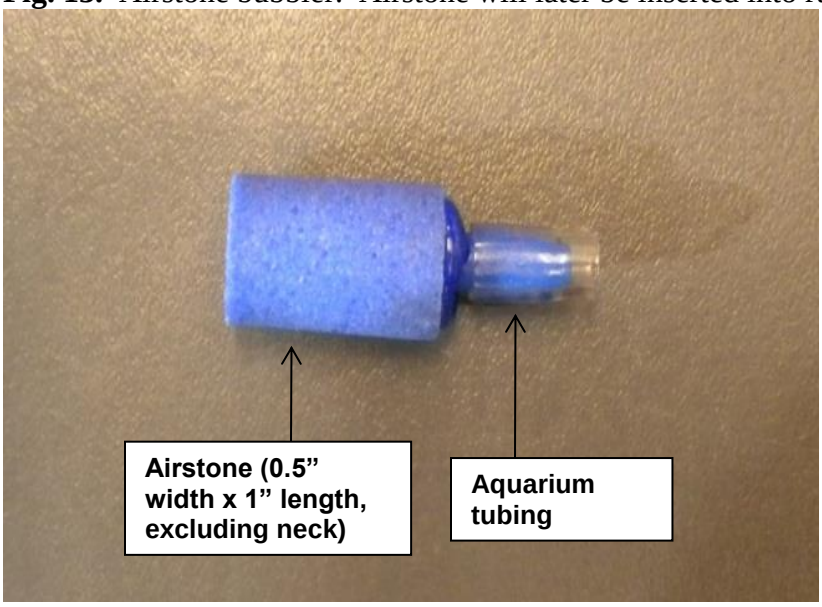


Fig. 14. Rheostat (140 V, 10A), with heat tape plugged in and adjusted to 72.5 V.

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 CO_2 by inserting it into gassing probe (see ***Making buffer and media***). Withdraw 0.3 mL CO_2 (same volume as culture removed in next step).
(d) Withdraw ~0.3 mL culture from stock tube after injecting 0.3 mL CO_2.
(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 CO_2. Agar may be individually weighed in tubes. Seal with rubber bung and aluminum lid then autoclave at 121ºC/15 lb pressure for 20 min. Cool.

2.) Flush bottle plate with CO_2. Insert needle of bottle plate gassing probe (**Fig. 2**) into rubber bung of plate, turn on CO_2 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 121ºC/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 CO_2, 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 CO_2 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. Scape 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 121ºC/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 121ºC/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 CO_2 (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 121ºC/15 lb pressure for 30 min with 10 min dry time.

Fig. 1. Bottle plate and streaking pattern.

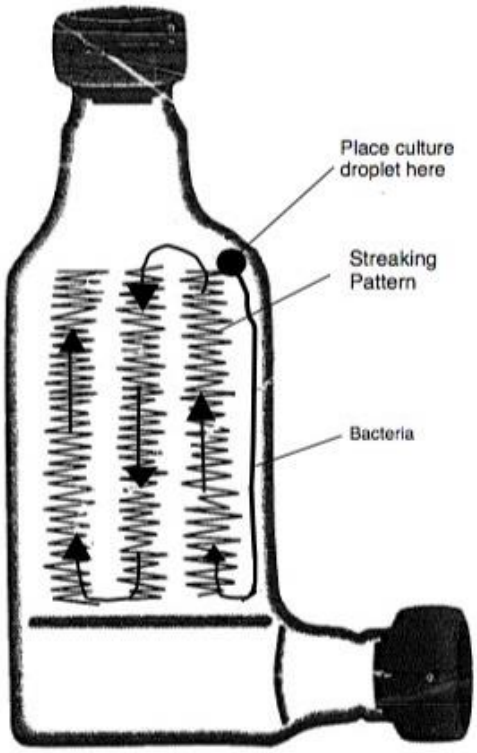


Fig. 2. Bottle plate gassing Probe

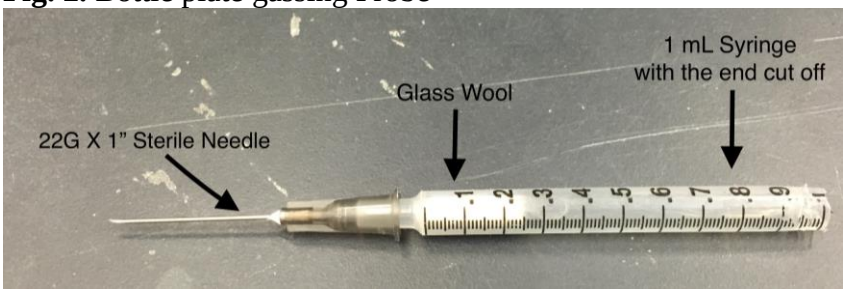


Fig. 3. Colony picker.

Fig. 4. Picking colony from bottle plate

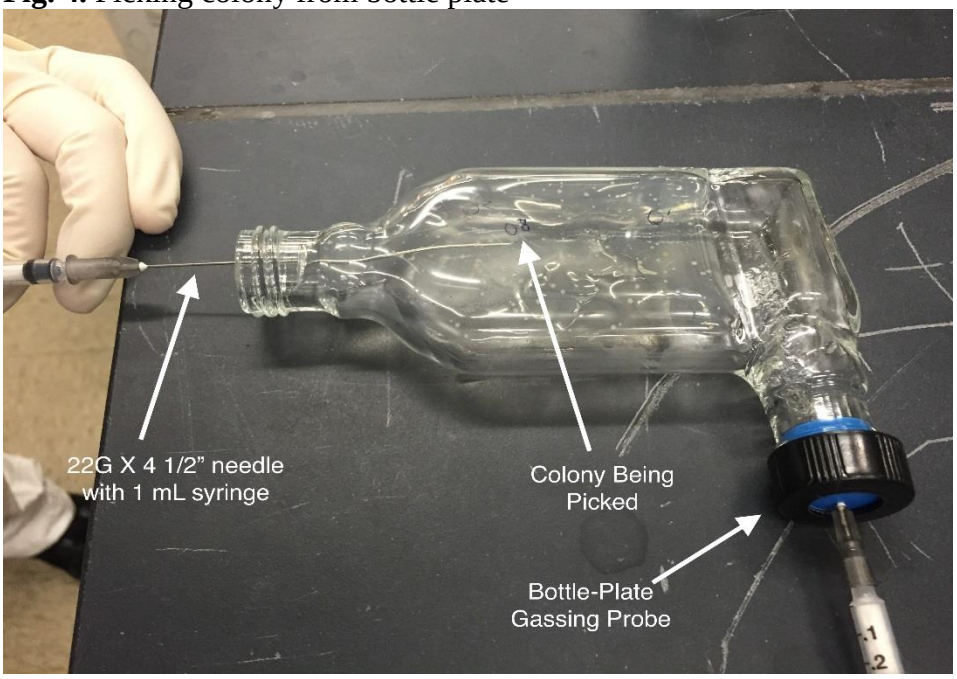


Fig. 5. Roll tubes after incubation for 2 d.

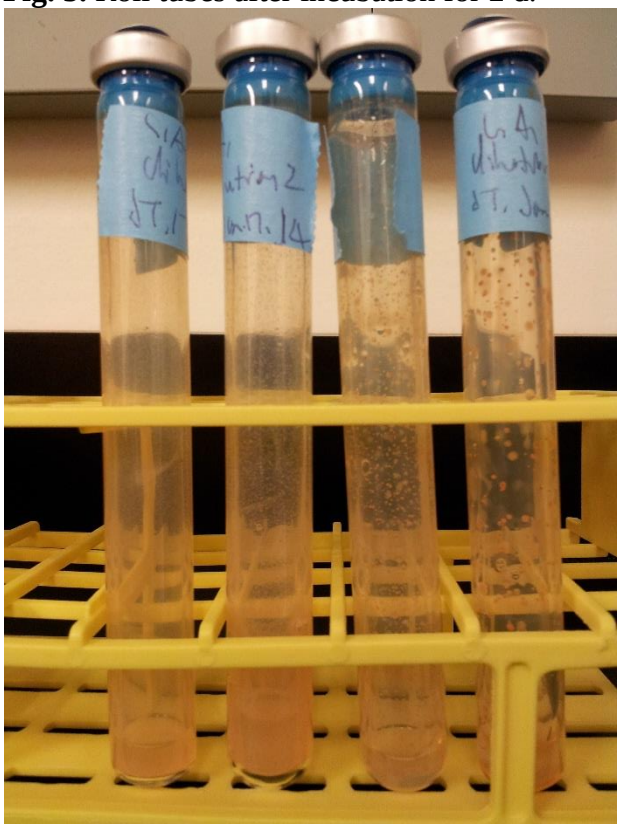


Fig. 6. Colonies of mixed and pure cultures.

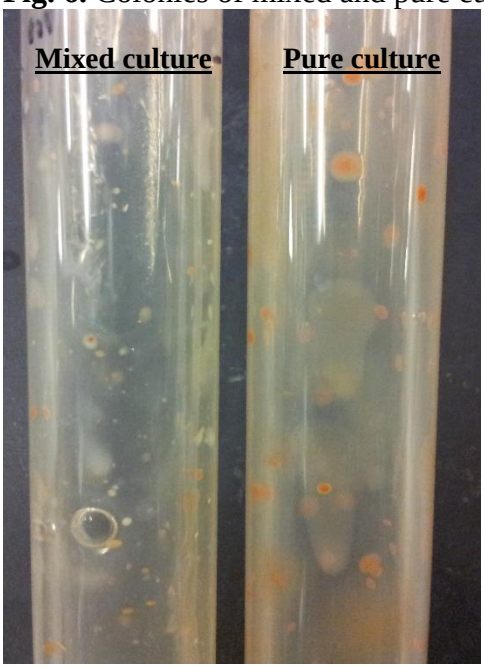


Fig. 7. Preparation of roll tube for picking colony, showing placement of roll tube and gassing probe.

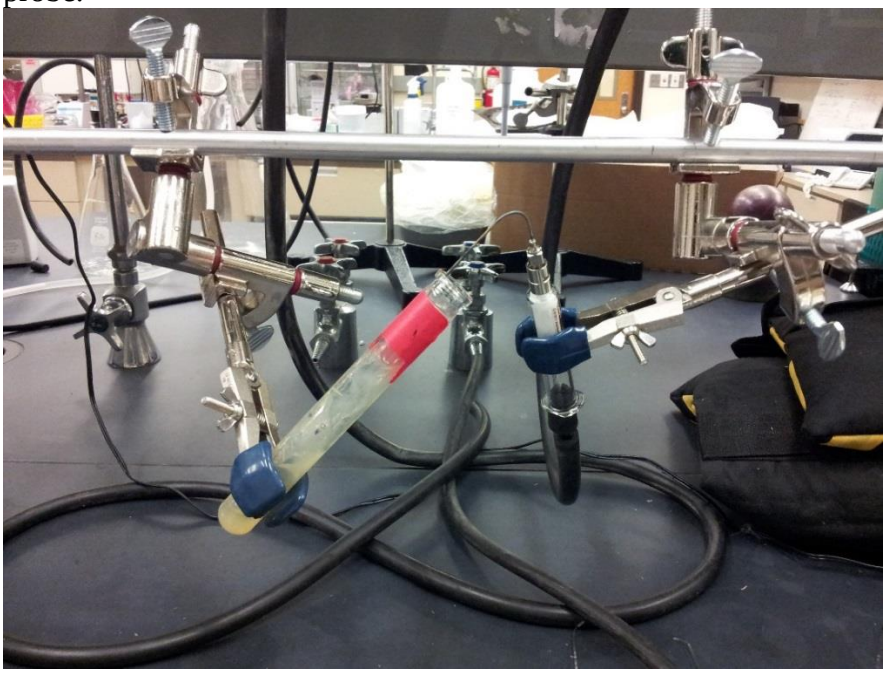


Fig. 8. Picking colony.

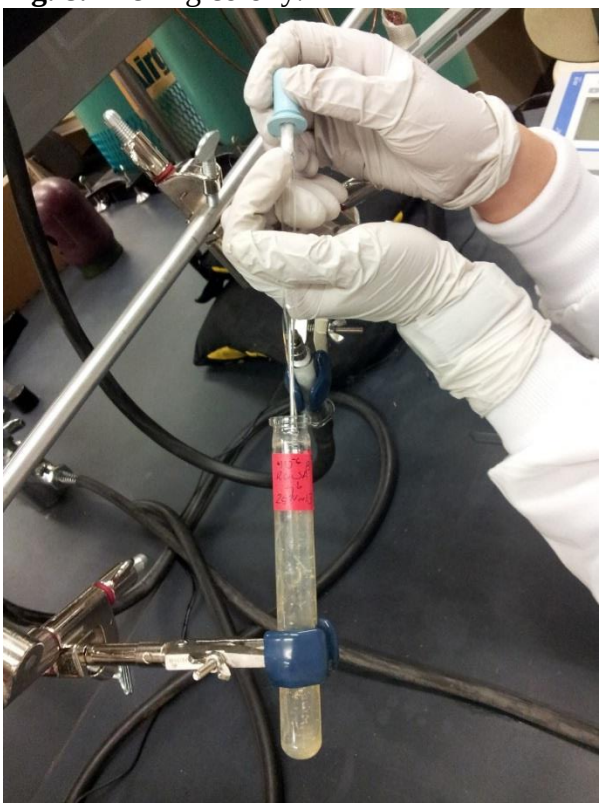


Fig. 9. Inoculated slant bottle, showing appropriate angle of solidified agar.

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 resuspened 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

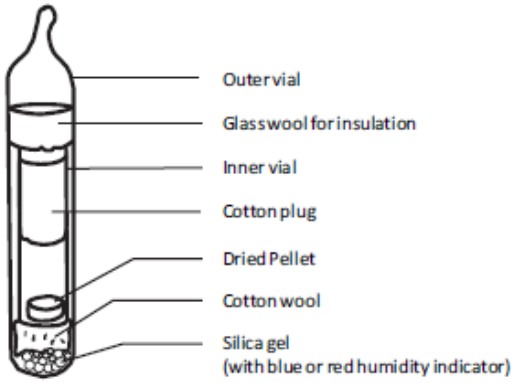
(i) The supplier will not guarantee the viability of the culture if the recommended medium is not used.

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

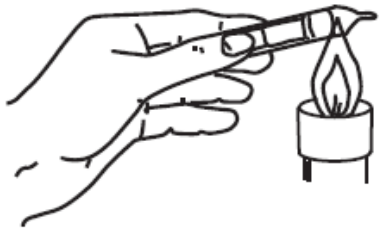
(iii) The first culture that is cryopreserved (usually the primary culture) should be kept but not used unless all subsequent cultures die or are compromised.

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)

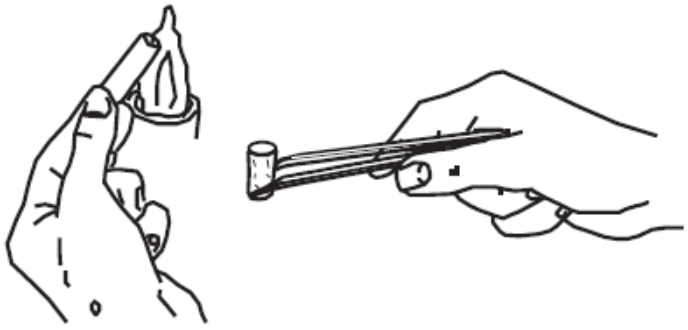


(B)



(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% H_2 /20% CO_2 (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% H_2, not 100% CO_2.

2.) Transfer 0.1 mL from a stock culture tube to 2 new tubes prepared in #1. Do transfer on lab bench and using CO_2 for flushing syringe.

3.) Pressurize tubes with 80% H_2.
(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% H_2 to 1 bar and allow to circulate for a few minutes. To ensure that H_2 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 H_2. 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% H_2.

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% H_2, not 100% CO_2. Bottle plate is still flushed with CO_2.

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% H_2. 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% H_2 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 39ºC water bath and thaw.

2.) Remove protozoal tubes from 39ºC incubator. Place in rack in 39ºC water bath.

3.) After following all steps from ***Constructing and using O_2-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 100ºC oven then cooled. Using pipette bulb, flush pipette with CO_2 by placing the pipette in the upper part of the feeding tube and running CO_2 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 rube, 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 39ºC incubator at a 10º angle (**Fig. 4**). Replace substrate tube in -20ºC 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 100ºC oven then cooled. Flush pipette with CO_2 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 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 dH_2O. Bubble dH_2O with CO_2 for 15 to 20 min to help remove O_2 from dH_2O.

2.) Make a 1.5% w/v ground wheat and1% w/v ground orchardgrass suspension. Add 1.5 g ground wheat and 1 g ground orchardgrass to dH_2O. Continue to bubble with CO_2. Prepare ground wheat and ground orchardgrass by grinding though 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 -20ºC. 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.

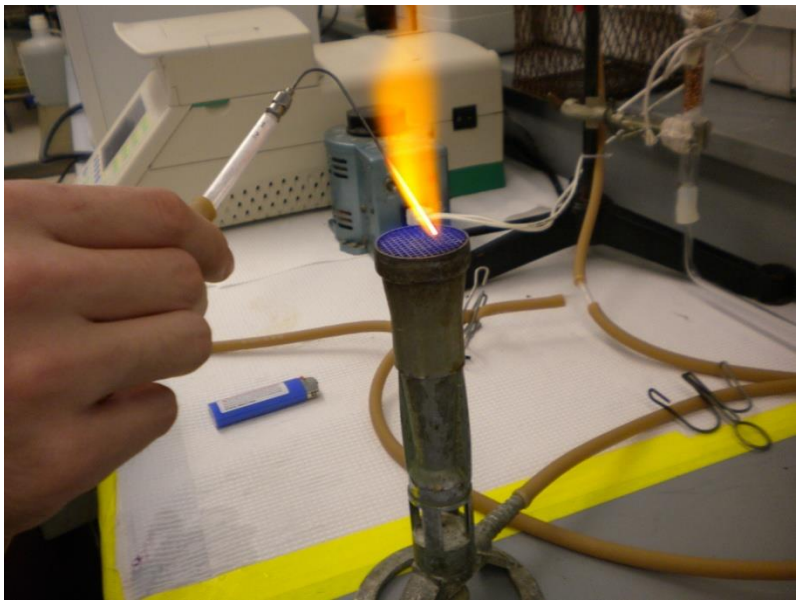
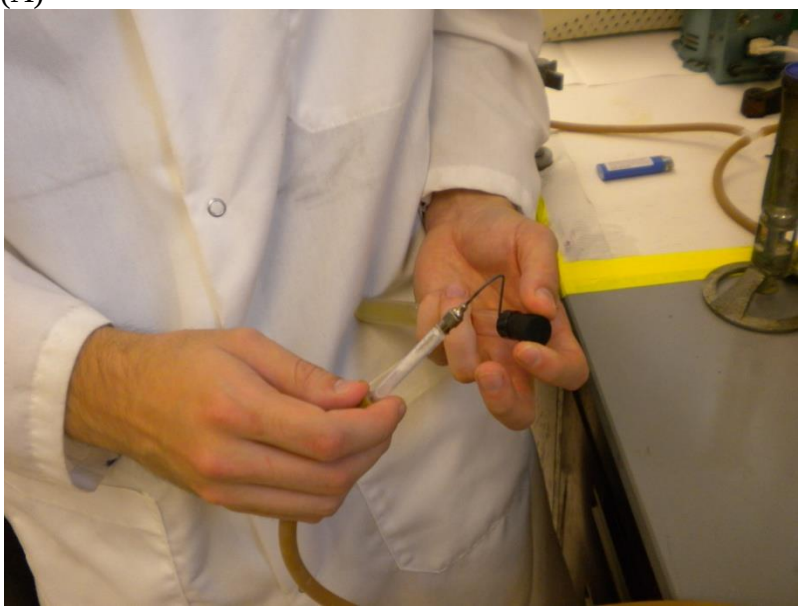


Fig. 2. Method of holding culture tubes, stopper, and gassing probe. (A) Position for opening tube. Needle is held near lip of culture tube for immediate insertion after stopper is removed. (B) Position for holding open tube for feeding and other manipulation. (C) Position for stoppering tube. Needle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

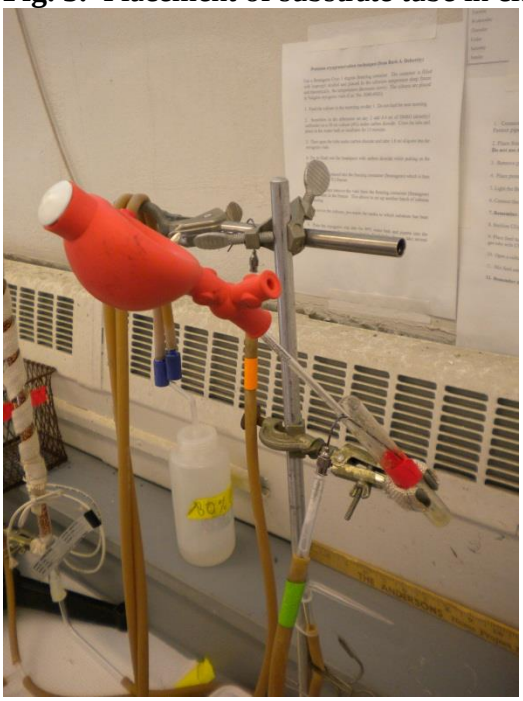
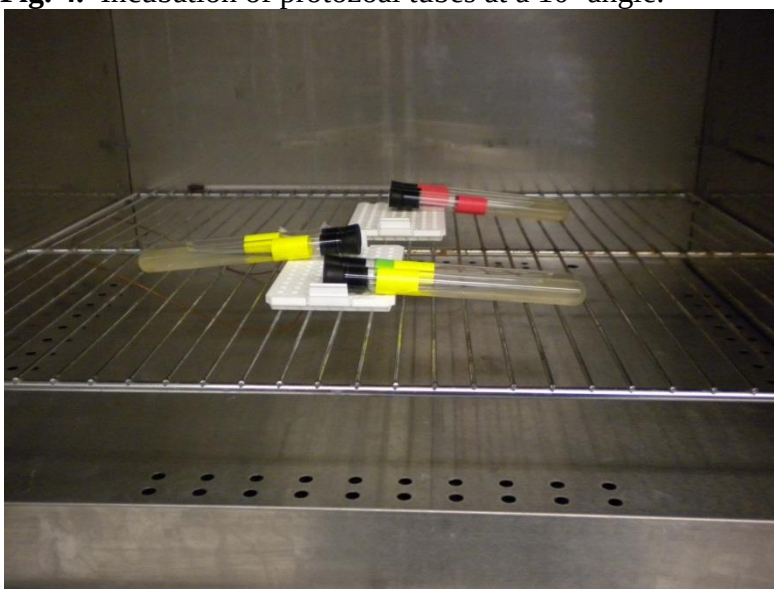


Fig. 4. Incubation of protozoal tubes at a 10º angle.



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 CO_2 and incubate in 39˚C water bath for 15 min.

3.) Fill cryovials with culture. Open 2 mL screw-top cryovial and flush with CO_2 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 CO_2. Repeat for more cryovials.

4.) Place vials in freezing container (Strategene 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 Strategene 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 O_2) 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 CO_2. Add Simplex buffer (to stabilize pH) and continue bubbling with CO_2 (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 CO_2 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 O_2-free CO_2.

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].

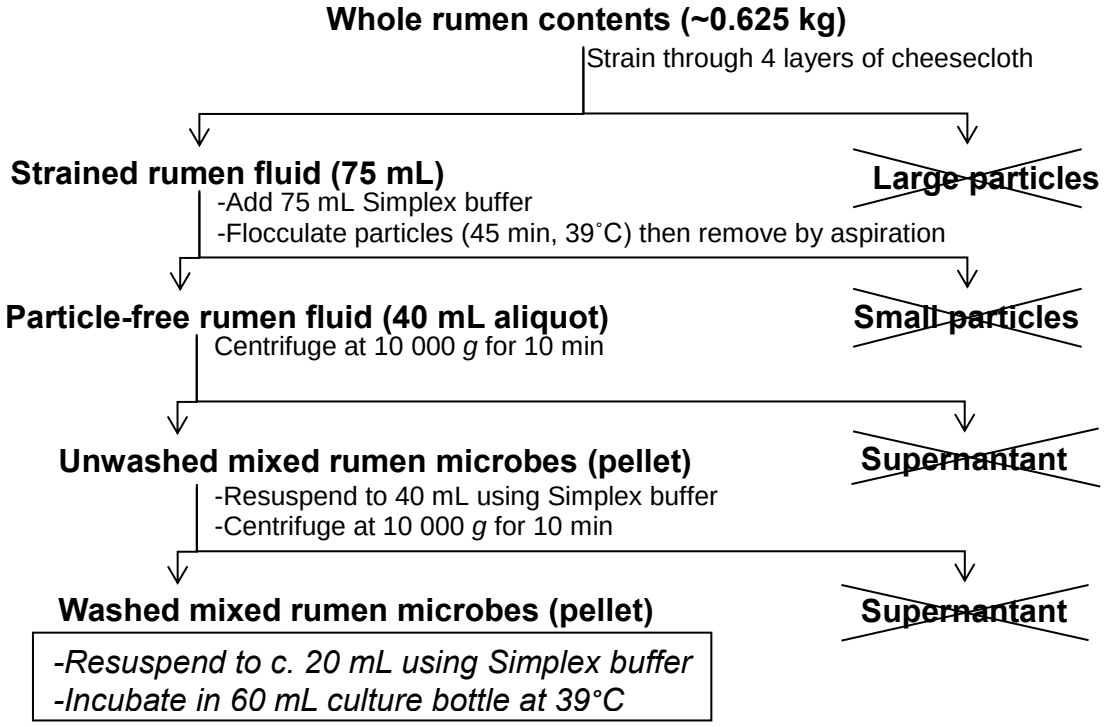
(iv) Metabolic activity of microbes was normal. To verify that centrifugation indeed does not alter metabolic activity of microbes, we compared heat production of (i) particle-free rumen fluid that was not centrifuged and (ii) particle-free rumen fluid that was centrifuged. Fluid that was centrifuged 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.

(A)

(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 O_2-free CO_2. 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 ≤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.

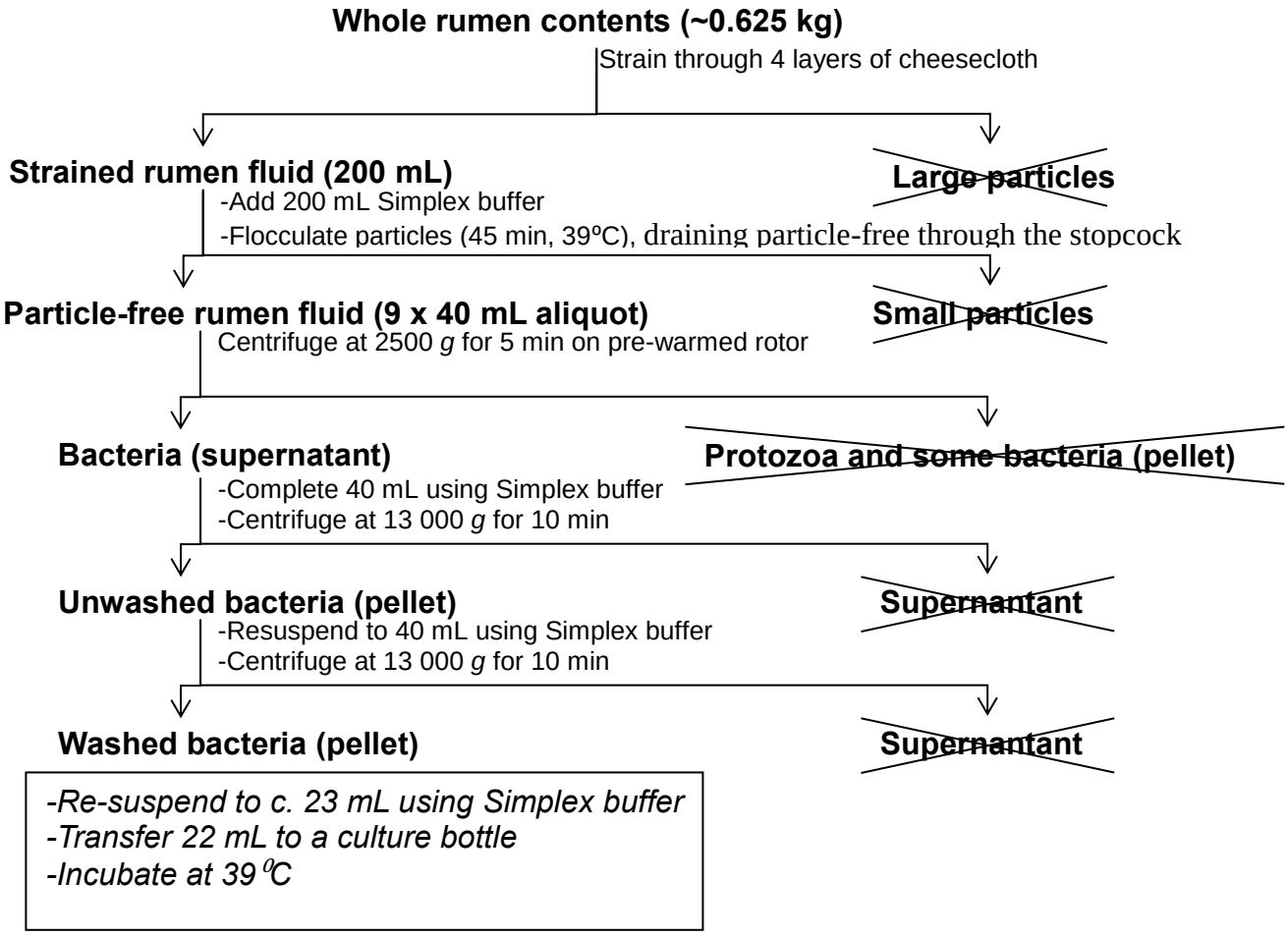
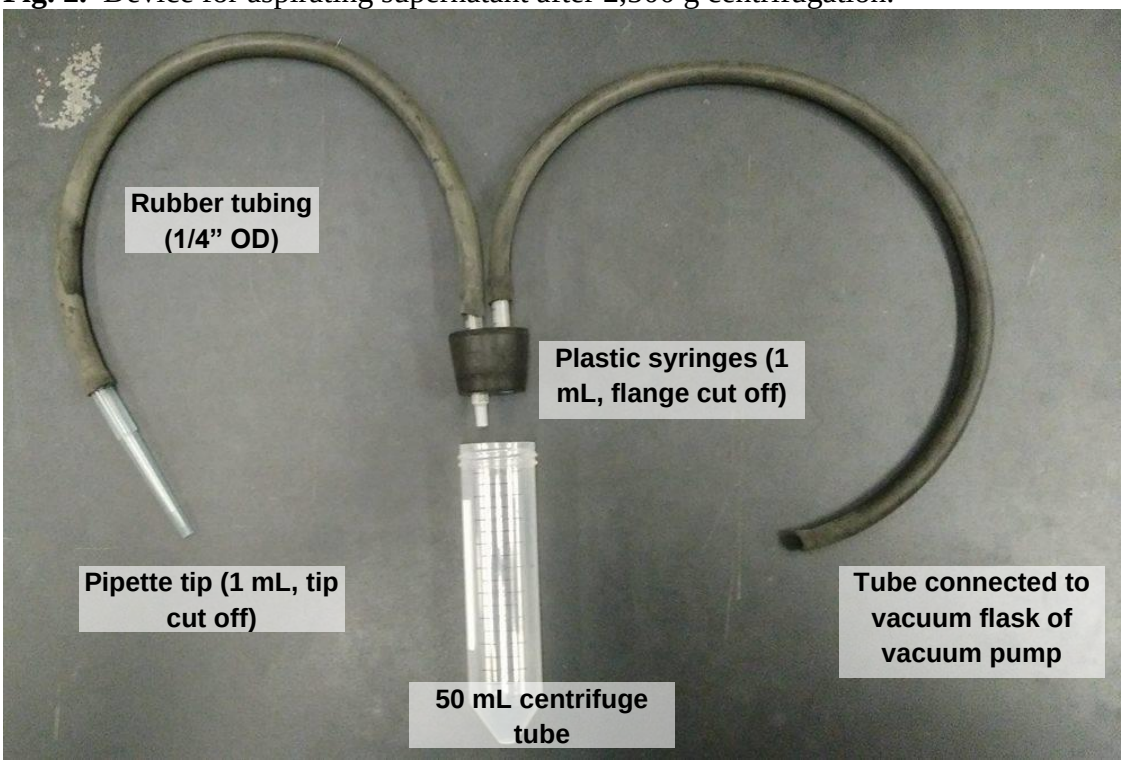

Whole rumen contents (~0.625 kg)
Strain through 4 layers of cheesecloth
Strained rumen fluid (200 mL)
Large particles
-Add 200 mL Simplex buffer
-Flocculate particles (45 min, 39ºC), draining particle-free through the stopcock
Particle-free rumen fluid (9 x 40 mL aliquot)
Small particles
Centrifuge at 2500 g for 5 min on pre-warmed rotor
Bacteria (supernatant)
Protozoa and some bacteria (pellet)
-Complete 40 mL using Simplex buffer
-Centrifuge at 13 000 g for 10 min
Unwashed bacteria (pellet)
Supernantant
-Resuspend to 40 mL using Simplex buffer
-Centrifuge at 13 000 g for 10 min
Washed bacteria (pellet)
Supernantant
-Re-suspend to c. 23 mL using Simplex buffer
-Transfer 22 mL to a culture bottle
-Incubate at 39 °C

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 μm) 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.

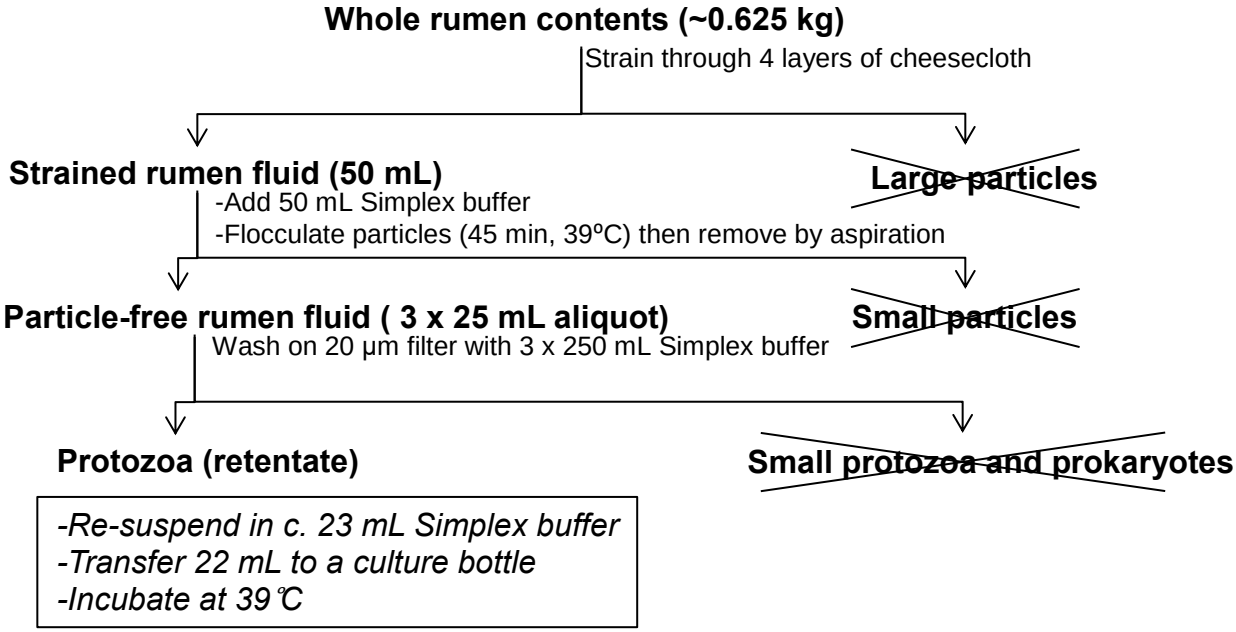


Fig. 2. Filtration apparatus for separation of rumen protozoa. Ten-micron filter is pictured but 20-µm is recommended.

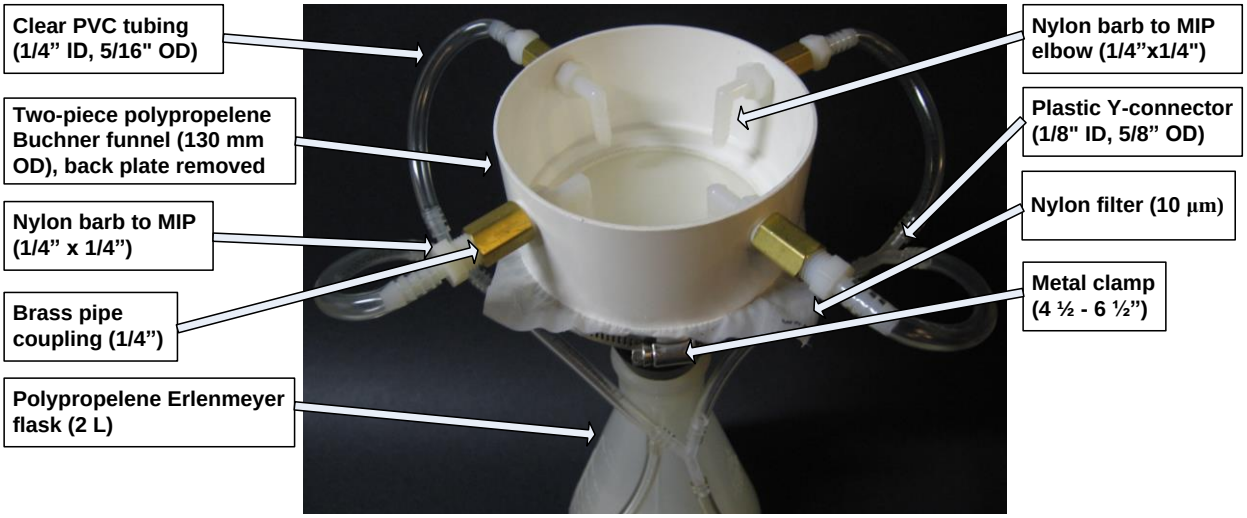


Fig. 3. Rinse bottle assembly for washing protozoa with Simplex buffer.

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) % (n = 3). Contamination of protozoa with bacteria was 1.89 (0.18) 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 $3.2 \cdot 10^{-9}$ g/cell. Not enough data is available for *Epi. caudatum* to give reliable values.

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

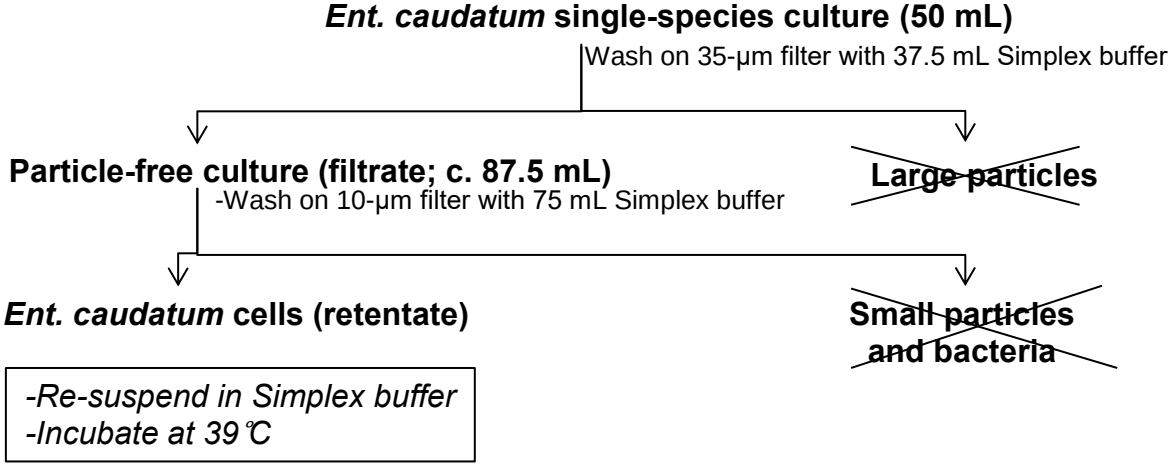
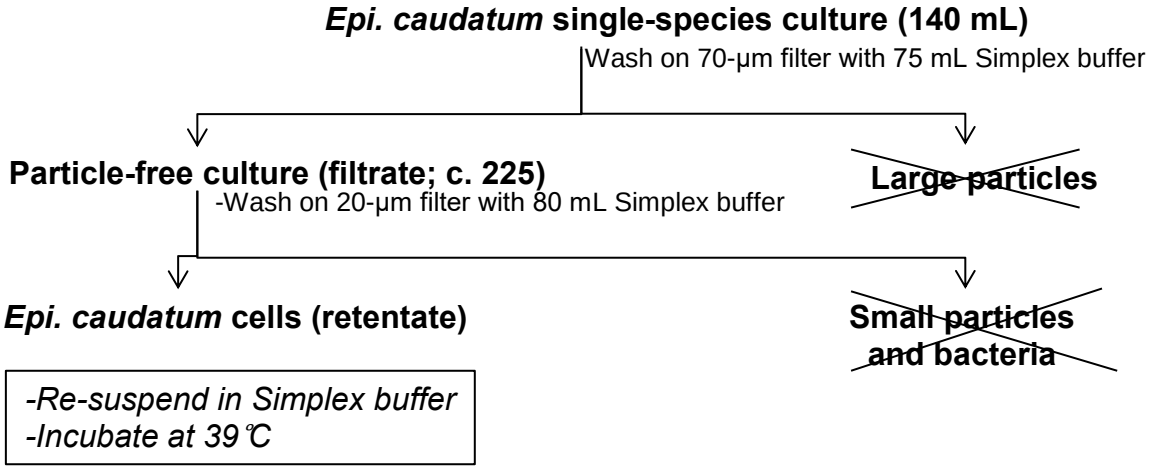


Fig. 2. Flowchart for preparation of *Epidinium caudatum* cells



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 (dH_2O, salts, rumen fluid, resazurin,) to 1-L round bottom flask in quantities directed in ***Buffer and media recipes***. Do not include $NaCHO_3$ or cysteine·HCl at this step.

2.) Boil buffer/media to remove most O_2 (**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 $NaCHO_3$ in quantity directed in individual procedures. Buffer/media must be cool before adding $NaHCO_3$ because $NaHCO_3$ will boil off as CO_2 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 CO_2 by drawing plunger in and out several times, and fill syringe with volume of CO_2 equal to amount of cysteine to be withdrawn. Puncture bung of culture bottle with syringe needle, inject CO_2, 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 CO_2, 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 CO_2 by placing pipette in the upper part of the round-bottom flask tube and drawing CO_2 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 CO_2 by placing it under a gassing probe clamped to a metal stand. Flush volumetric pipette with CO_2 by drawing placing the pipette in the upper part of the round-bottom flask tube and drawing CO_2 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, dH_2O) to 3-L round bottom flask in amounts directed in individual procedures. Do not include $NaCHO_3$ 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 CO_2 tubing and bubble solution with CO_2.

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 $NaCHO_3$ 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 CO_2 for 4 min (**Fig. 7A**).

(ii) Flush dispensing tube (**Fig. 7B**). Raise 3-L tubing assembly so end is above liquid (but CO_2 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 121ºC/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 CO_2 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 CO_2 tubing to gassing station.

Preparing buffer or media by distillation

Procedures above remove O_2 mostly by boiling. To remove even more O_2, water can be distilled under O_2-free CO_2. 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 dH_2O) to the 500-mL Erlenmeyer flask in quantities directed in ***Buffer and media recipes***. $NaCHO_3$ can be included, but do not include cysteine at this step.

3.) Add 600 mL of dH_2O 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 O_2).

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.

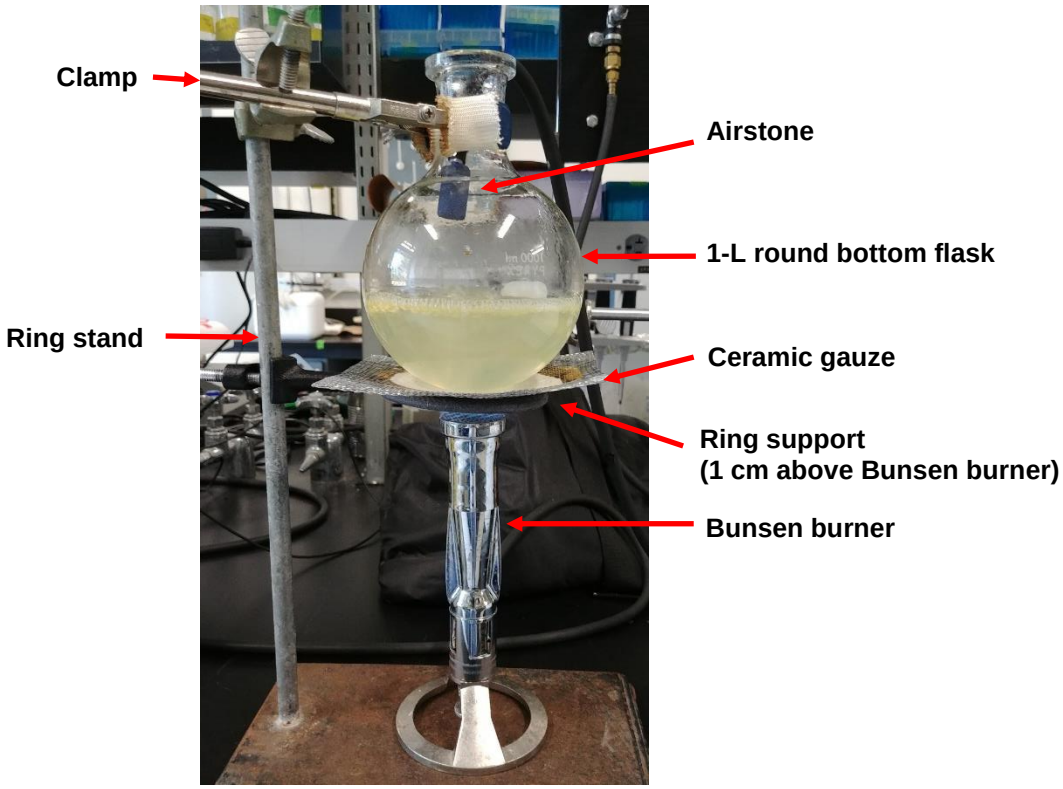
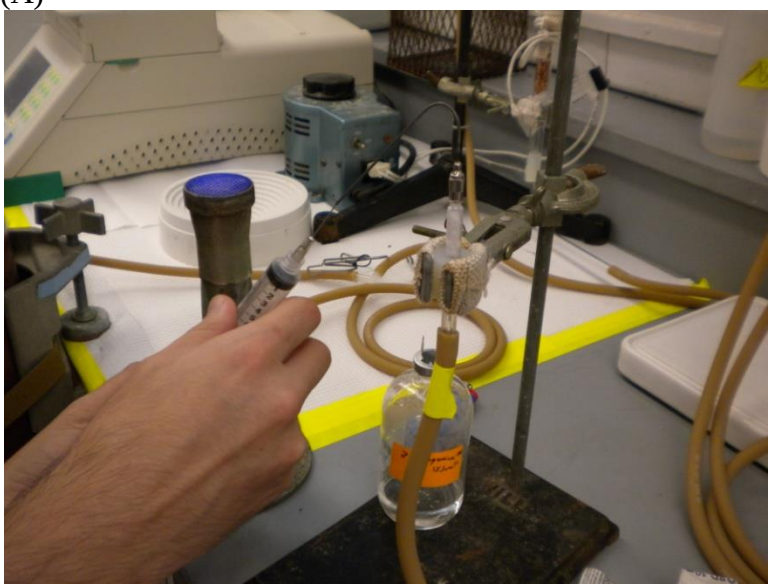


Fig. 2. Technique for withdrawing cysteine from culture bottle. (A) Flushing syringe with CO_2 via gassing probe clamped to stand. (B) Withdrawing cysteine.

(A)



(B)

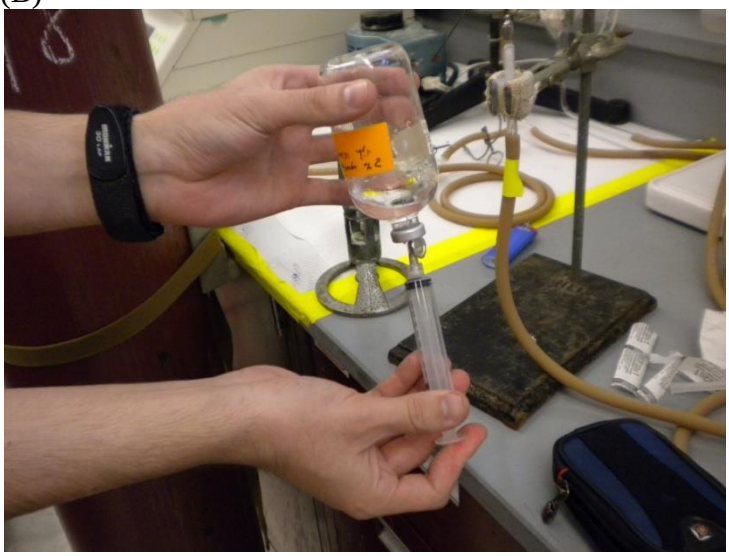


Fig. 3. Rack for autoclaving culture tubes.

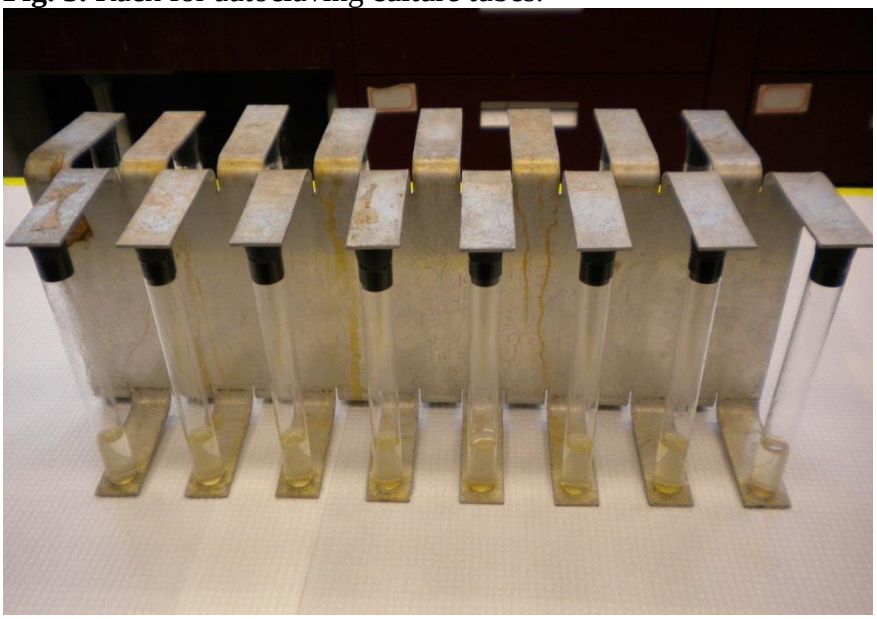
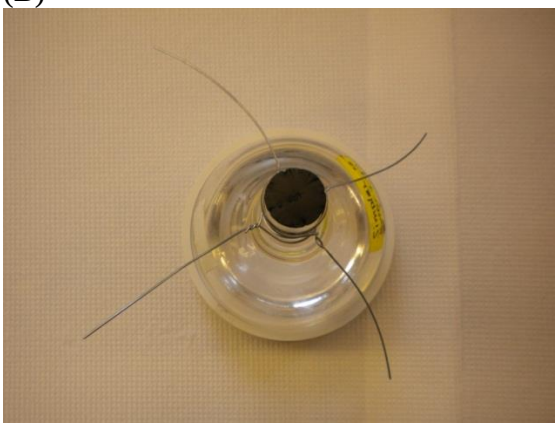


Fig. 4. Wiring stopper in place. (A). Securing 1 wire around neck of flask. (B) Repeating 3 more times to give 4 secured wires. Wires are positioned for securing around stopper; they are at 12, 3, 6, and 9 o'clock. (C) Securing 2 wires around stopper. (D) Securing remaining 2 wires around stopper.

(A)

(B)

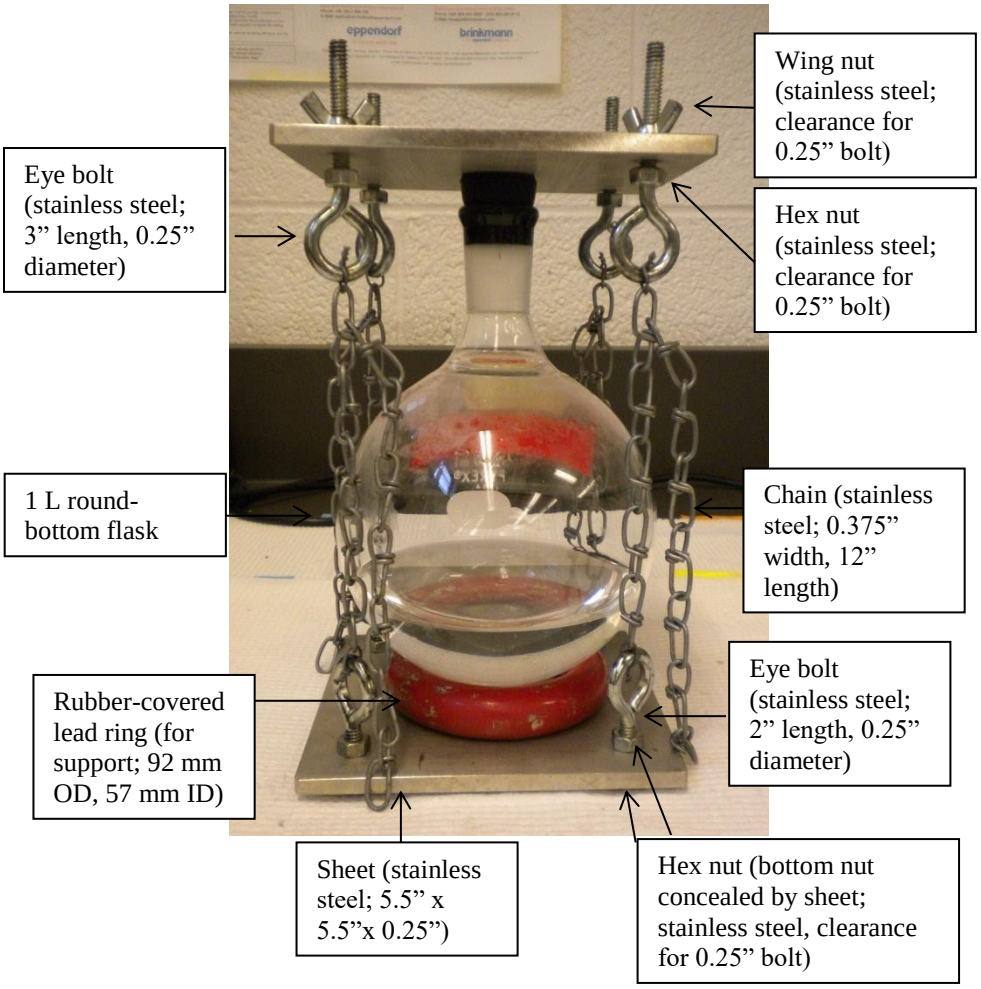


(C)

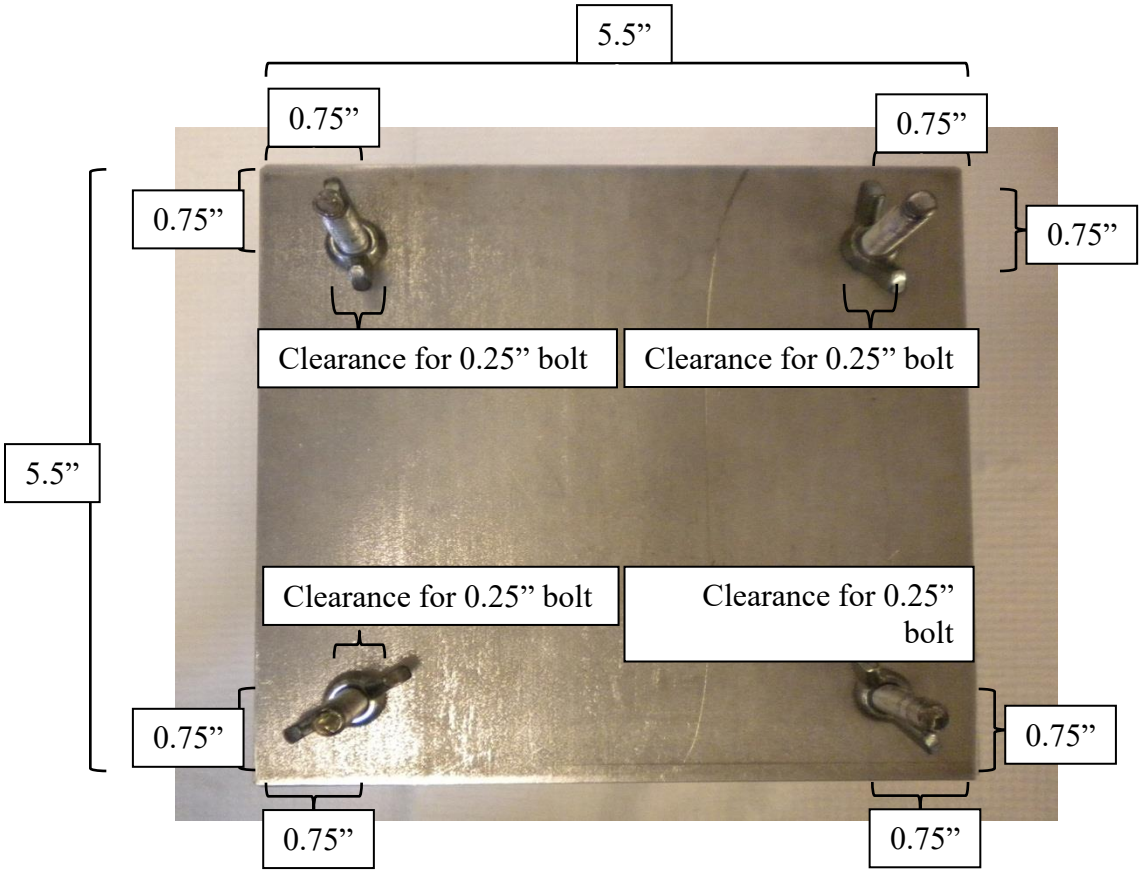
(D)

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

(A)



(B)


5.5”
0.75”
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0.75”
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Clearance for 0.25” bolt
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Fig. 6. Boiling buffer in 3-L flask.

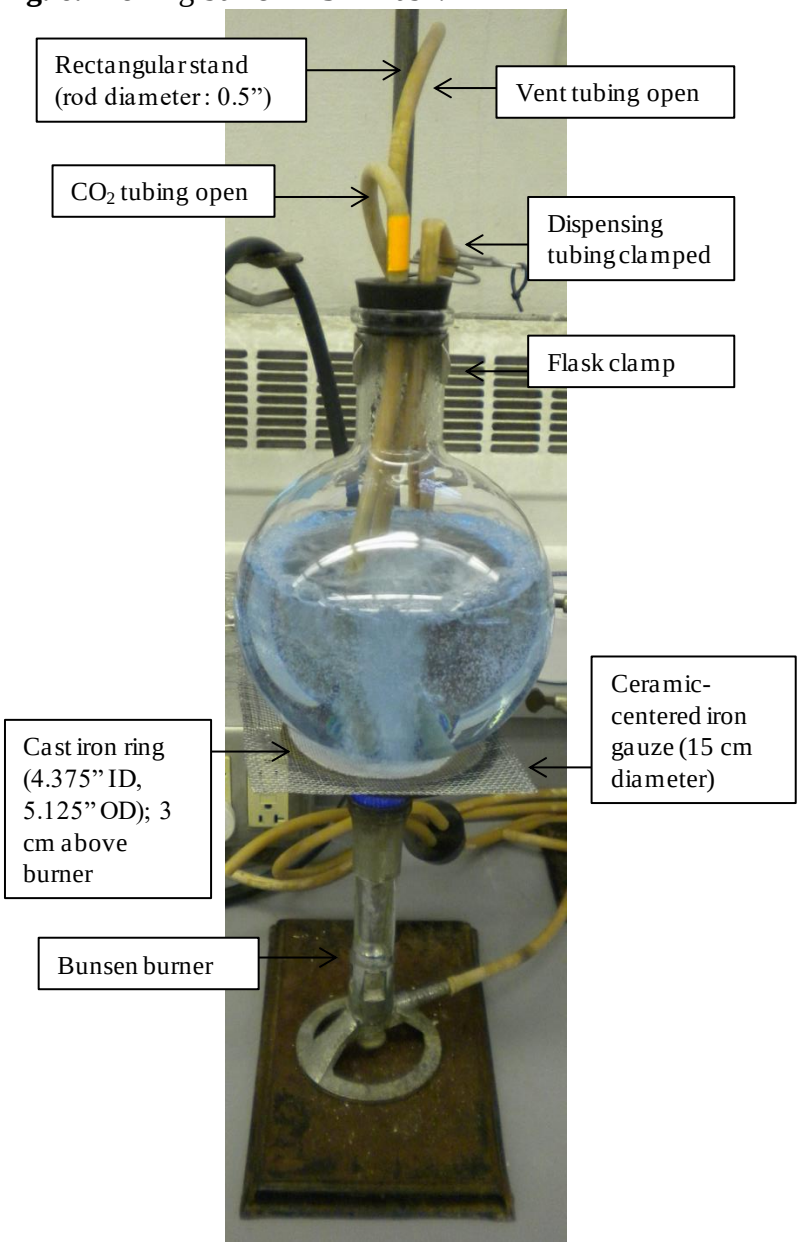
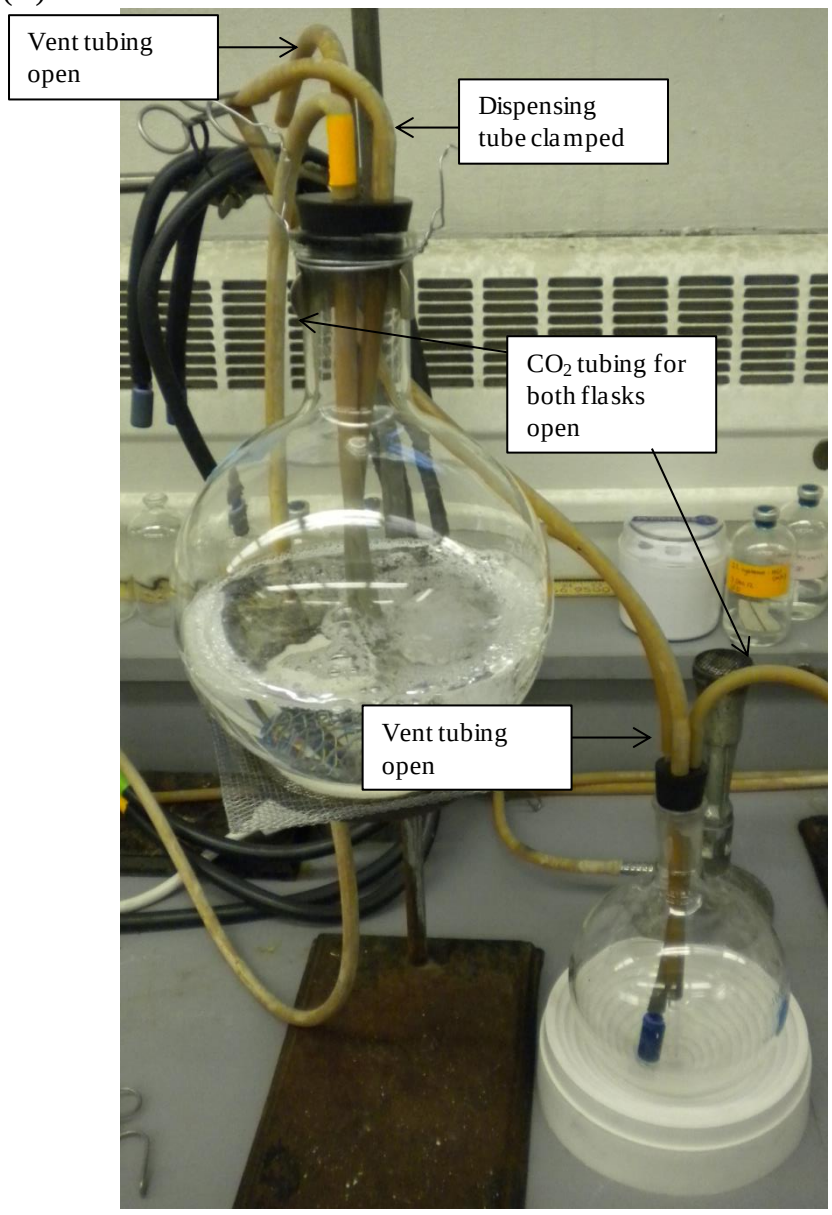
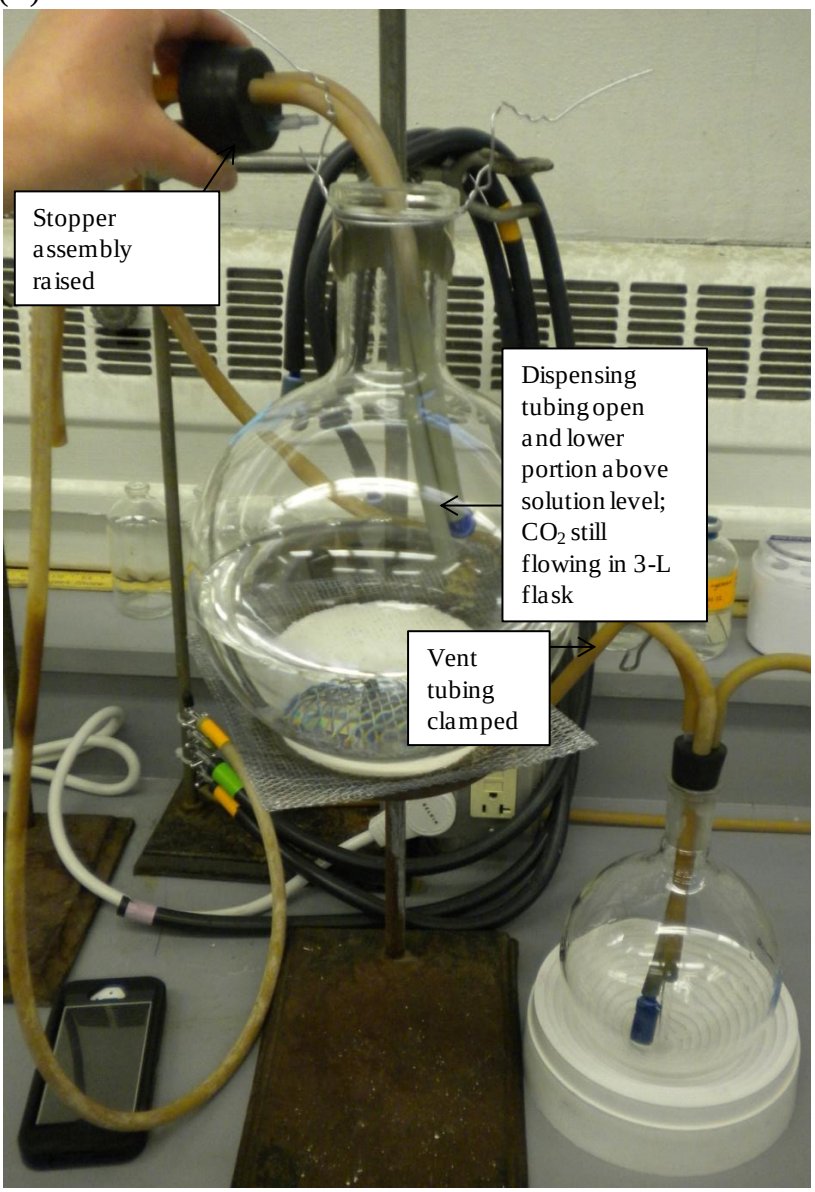


Fig. 7. Dispensing buffer into 1-L flask. (A) Flushing 1-L flask. (B) Flushing dispensing tube. (C) Dispensing buffer.

(A)



(B)


Stopper assembly raised
Dispensing tubing open and lower portion above solution level; CO_2 still flowing in 3-L flask
Vent tubing clamped

(C)

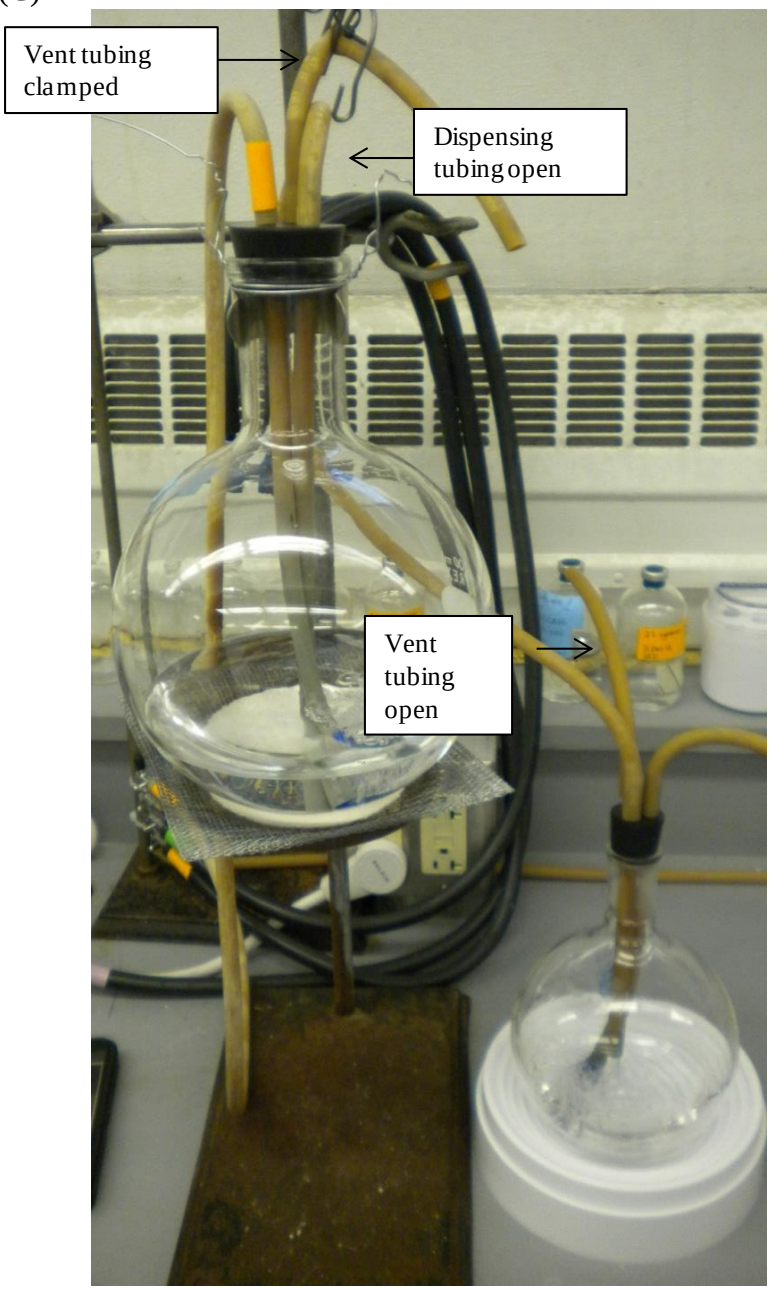

Vent tubing clamped
Dispensing tubing open
Vent tubing open

Fig. 8. Stopper with syringe barrels inserted.

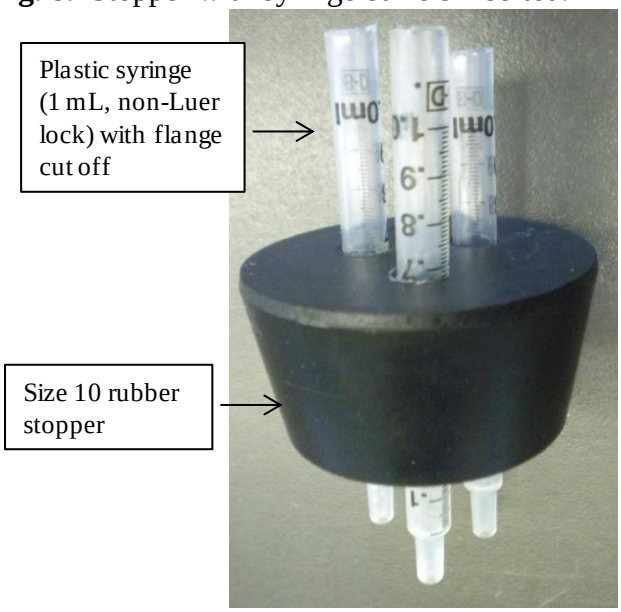


Fig. 9. Tubing assembly for 3-L flask.

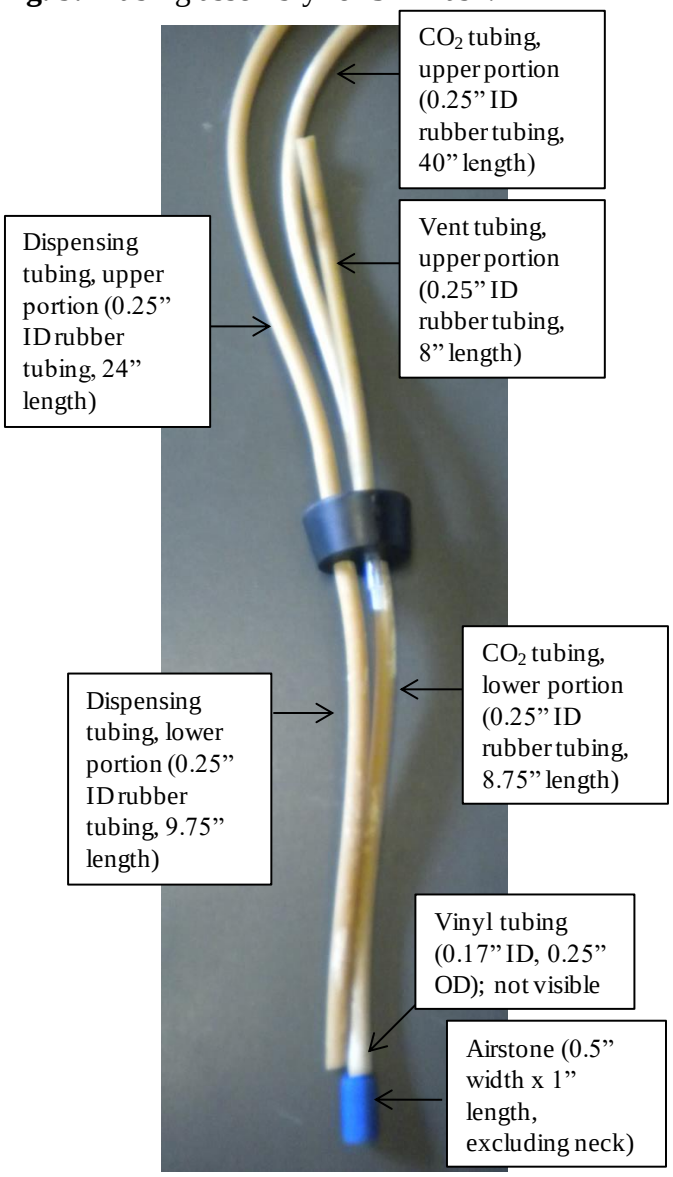


Fig. 10. Tubing assembly for 1-L flask.

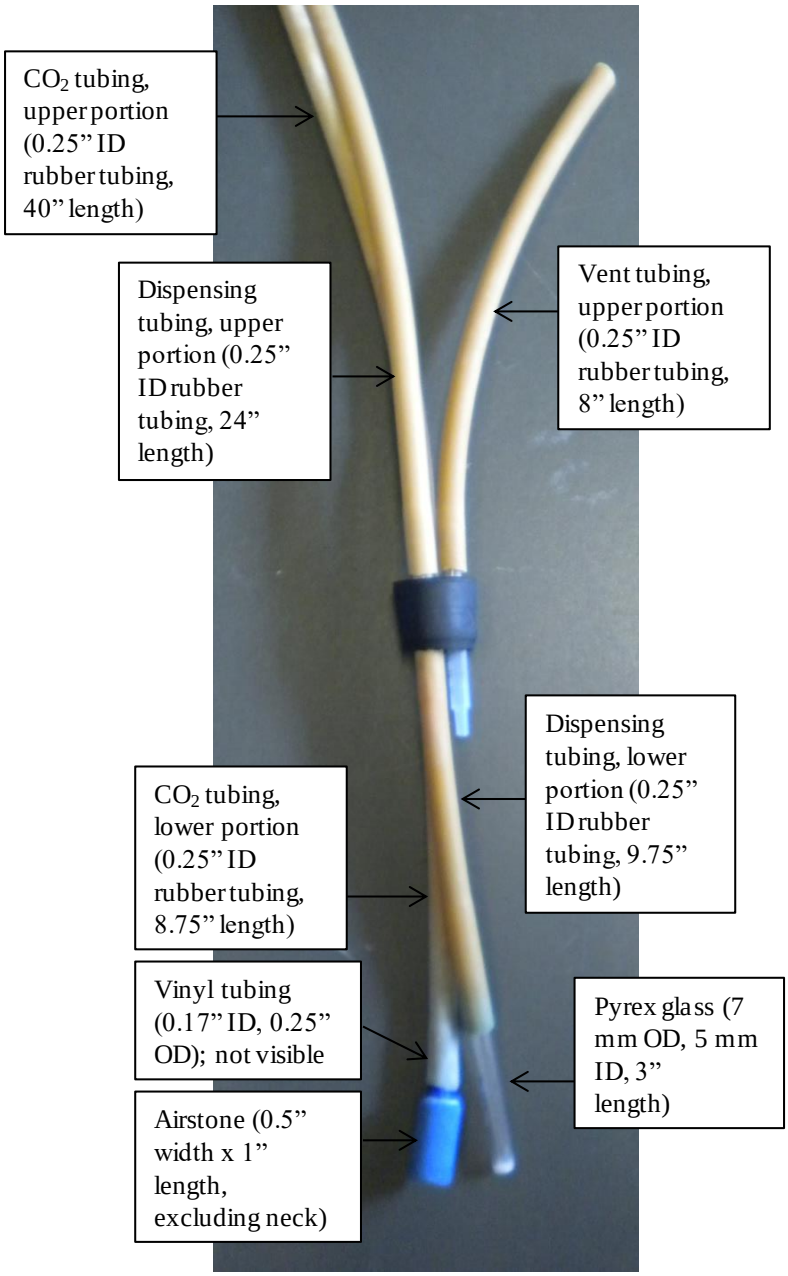
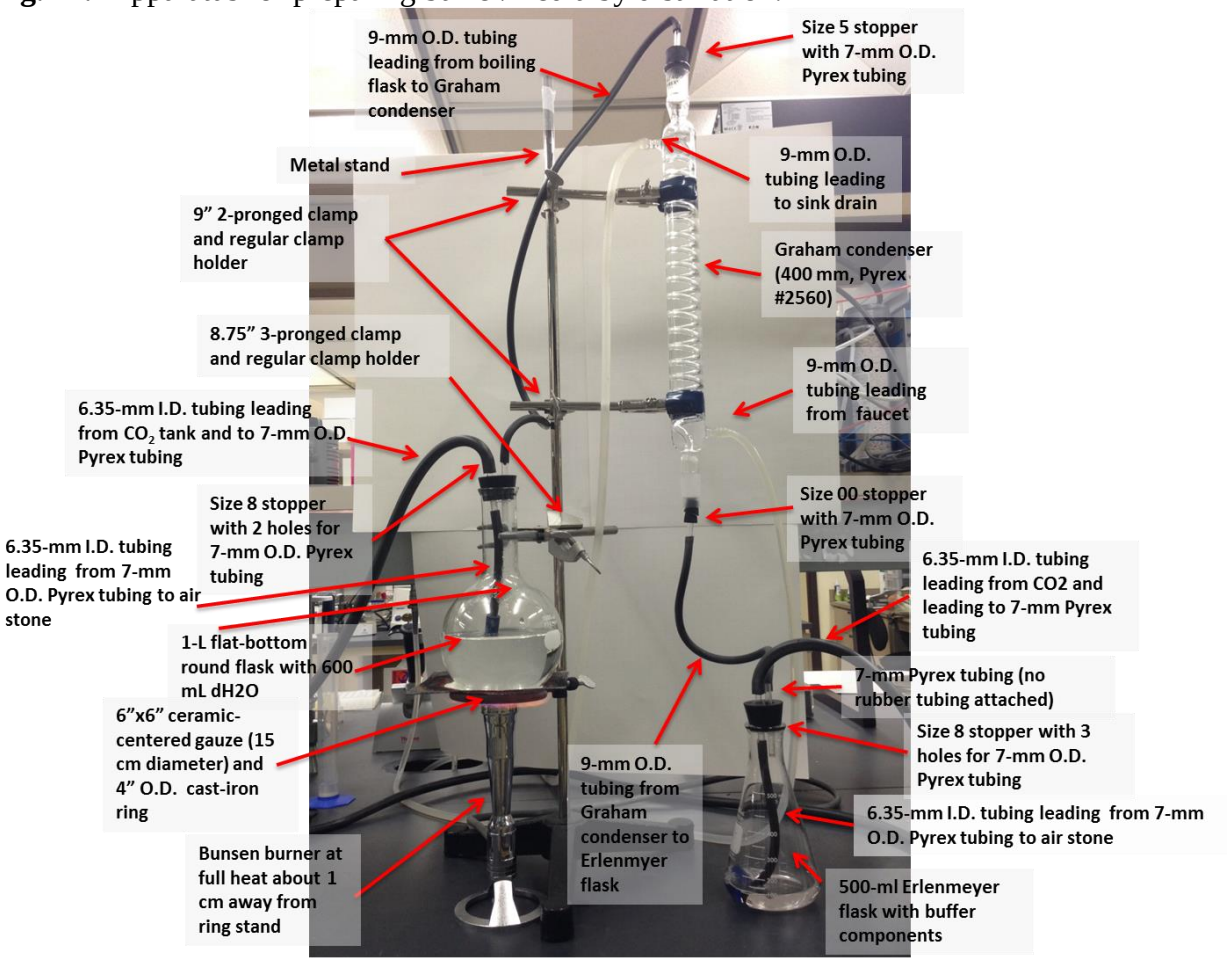


Fig. 11. Apparatus for preparing buffer/media by distillation.



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 dH_2O
- 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 K_2HPO_4
- 0.135 g KH_2PO_4
- 0.135 g $(NH_4)_2SO_4$
- 0.27 g NaCl
- 0.0563 g $MgSO_4$
- 0.036 g $CaCl_2 \cdot 2H_2O$
- 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 dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na_2CO_3

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 $FeCl_2.4H_2O$ 7.5 mM

0.0618 g H_3BO_4 1 mM
0.0682 g $ZnCl_2$ 0.5mM
0.0169 g $CuCl_2.2H_2O$ 0.1mM
0.0989 g $MnCl_2.4H_2O$ 0.5 mM
0.1189 g $CoCl_2.6H_2O$ 0.5mM
0.0238 g $NiCl_2.6H_2O$ 0.1mM
4.13 mL HCl 50mM

Dissolve in dH_2O 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 Na_2SeO_3 0.1mM
0.0255 g Na_2WO_4 0.1 mM
0.0206 g Na_2MoO_4 0.1 mM
0.4 g NaOH 10mM

Dissolve in dH_2O 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 dH_2O
0.135 g K_2HPO_4
0.135 g KH_2PO_4
0.27 g $(NH_4)_2SO_4$
0.27 g NaCl
0.027 g $MgSO_4$
0.0358 g $CaCl_2 \cdot 2H_2O$
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
0.9 g Na_2CO_3

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 dH_2O instead of 350 mL dH_2O. 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 K_2HPO_4
- 0.3 g cellobiose
- 0.3 g maltose
- 0.3 g starch
- 3 drops of 0.1% resazurin

Boil under CO_2 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 dH_2O
- 1.2 g glucose
- 0.0876 g K_2HPO_4
- 0.0876 g KH_2PO_4
- 0.1440 g $(NH_4)_2SO_4$
- 0.1440 g NaCl
- 0.0147 g $MgSO_4$
- 0.0192 g $CaCl_2 \cdot 2H_2O$
- 3 drops of 0.1% resazurin
- 0.3 mL hemin solution
- 3.0 mL Russell vitamin solution

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 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 dH_2O
0.3375 g KH_2PO_4
0.309 g NaCl
0.309 g $(NH_4)_2SO_4$
0.03 g $MgCl_2 \cdot 6H_2O$
0.0225 g $CaCl_2 \cdot 2H_2O$
0.00943 g$MnCl_2$ $4H_2O$
0.00686 g $FeSO_4$ 7 H_2O
0.00324 g $ZnCl_2$
0.00072 g $CoCl_2$ $6H_2O$
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 dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add
1.2 g $NaCHO_3$

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 dH_2O
4.5 g trypticase peptone (BD 211921) or casamino acids (BD 223120)
0.0876 g K_2HPO_4
0.0876 g KH_2PO_4
0.144 g Na_2SO_4
0.144 g NaCl
0.0146 g $MgSO_4$
0.0192 g $CaCl_2·2H_2O$
1.5 mL Pfennigs heavy metal solution
3 mL Russell vitamin solution
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

1.2 g Na_2CO_3

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 Pfennins 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 -20ºC.

3% (w/v) cysteine HCl

Add ~100 mL dH_2O to a 1-L round-bottom flask, bubble with CO_2, and boil for 5 to 7 min. Transfer 75 mL dH_2O to 160-mL culture bottle that is being flushed with gassing probe. Add

2.507 g L-cysteine $HCl \cdot H_2O$

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 dH_2O to a 1-L round-bottom flask, bubble with CO_2, and boil for 5 to 7 min. Transfer 75 mL dH_2O 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 CO_2). Add after autoclaving medium.

Glucose (10% w/v) (anaerobic)

Add 1 g glucose to a Balch tube. Flush with N_2 for 10 min. While continuing to flush, add 9.3 mL water (anaerobic). Stopper tube and shake to dissolve glucose. Filter sterilize into a sterile Balch tube previously flushed with N_2 and autoclaved.

The volume of water (9.3 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 beaker

0.28 g KOH
25 mL 95% ethanol
0.1 g hemin

Dissolve and bring to 100 mL final volume in volumetric flask with dH_2O. 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 dH_2O
10 g sodium lactate (60% w/w)
6 g trypticase peptone (BD 211921)
0.6 g yeast extract (BD 212750)
0.135 g K_2HPO_4
0.135 g KH_2PO_4
0.27 g $(NH_4)_2SO_4$
0.27 g NaCl
0.027 g $MgSO_4$
0.0358 g $CaCl_2 \cdot 2H_2O$
1.5 mL Pfennigs heavy metal solution
6 mL hemin solution
3 mL VFA solution
3 drops of 0.1% resazurin

Adjust pH to 6.7 to 6.8 with NaOH

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

1.911 g $NaHCO_3$

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

0.1705 g L-cysteine $HCl \cdot H_2O$

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

Immediately before inoculation, add 0.205 mL of 2.5% $Na_2S \cdot 9H_2O$ 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 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 $NiCl_2 \cdot 6\ H_2O$), 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 $MgSO_4$
0.26 g $CaCl_2 \cdot 2\ H_2O$
2.0 g KH_2PO_4 g/L

Bring to volume with dH_2O.

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

150 mL mineral mix M

30 mL clarified rumen fluid
15 mL 1.5% CH_3COONa
128 mL dH_2O
2 drops 0.1% resazurin

Approximately 50 mL dH_2O will be lost during boiling, giving 273 mL final volume. After boiling and cooling, add
25 mL of 6% $NaHCO_3$

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 KH_2PO_4
4 g NaCl
0.212 g $CaCl_2 \cdot 2\ H_2O$
0.154 $MgSO_4$
Bring to volume with dH_2O.

Add the following to a 1-L round-bottom flask
75 mL 20 g/L K_2HPO_4
75 mL SP-2
30 mL clarified rumen fluid
128 mL dH_2O
2 drops 0.1% resazurin

Approximately 50 mL dH_2O will be lost during boiling, giving 273 mL final volume. After boiling and cooling, add
25 mL of 6% $NaHCO_3$

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 $CaCl_2 \cdot 2H_2O$
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 HCl·H2O

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 dH_2O
3g trypticase peptone (BD 211921)
3g yeast extract (BD 212750)
1.5. Glucose
0.15g $(NH_4)_2SO_4$
0.012g K_2HPO4
0.012g KH_2PO_4
0.0024g $MgSO_4$
0.00318g $CaCl_2$ $2H_20$
0.024g NaCl
3 drops of 0.1% resazurin
0.93 ml VFA solution
3 mL of Vitamin K_3-Hemin solution

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 $NaHCO_3$

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 dH_2O
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 K_2HPO_4
0.012 g KH_2PO_4
0.00293 g $MgSO_4$
0.003g $CaCl_2·2H_20$
0.024 g NaCl
0.3 ml Tween80
3 ml 0.05% hemin solution
0.06 mL vitamin K1 solution

3 drops of 0.1% resazurin

Adjust pH to 6.7 with HCl.
Approximately 50 mL dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g $NaHCO_3$

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

0.15 g L-cysteine $HCl \cdot H_2O$

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 dH_2O
0.12 g KH_2PO_4
0.159 g Na_2HPO_4
0.09 g NH_4Cl
0.09 g NaCl
0.03 g $MgCl_2.6H_2O$
0.044 g CaCl2.$2H_2O$
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 dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g $NaCHO_3$

After boling, 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% $Na_2S \cdot 9H_2O$ 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 dH_2O

Attach the flask to a metal stand, and boil under CO_2 for 8 to 10 minutes (until meat browns). Stir contents gently while boiling. Approximately 50 mL dH_2O 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 dH_2O 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 CO_2, transfer approximately 1.4 g of meat particles to a Balch tubes also kept under CO_2. 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 dH_2O to a 1-L round-bottom flask, bubble with CO_2, and boil for 5 to 7 min. Transfer 75 mL dH_2O 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 dH_2O
1.2 g glucose
0.0876 g K_2HPO_4
0.0876 g KH_2PO_4
0.1440 g $(NH_4)_2SO_4$
0.1440 g NaCl
0.0147 g $MgSO_4$
0.0192 g $CaCl_2 \cdot 2H_2O$
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 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 for one hour (color turns yellow). Then add

0.2 g cysteine $HCl \cdot H_2O$

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 dH_2O
1.2 g glucose
0.0876 g K_2HPO_4
0.0876 g KH_2PO_4
0.1440 g $(NH_4)_2SO_4$
0.1440 g NaCl
0.0147 g $MgSO_4$
0.0192 g $CaCl_2 \cdot 2H_2O$
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 dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na_2CO_3

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 N_2 before use.

10% (w/v) NaOH

Add ~100 mL dH_2O to a 1-L round-bottom flask, bubble with N_2, and boil for 5 to 7 min. Transfer 75 mL dH_2O 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) $Na_2S \cdot 9\ H_2O$

Adapted from (31, 32)
Add ~100 mL dH_2O to a 1-L round-bottom flask, bubble with N_2, and boil for 5 to 7 min.

In fume hood, hold a crystal of Na2S·9 H_2O with tweezers and use spray bottle to wash with dH_2O (to remove toxic oxidation products). Collect wash water in beaker (for later disposal in chemical waste bottle). Blot crystal dry on paper towel. Crush with mortar and pestle. Weigh approximately

1.875 g $Na_2S \cdot 9\ H_2O$

into culture bottle, and record actual weight. Seal bottle with bung, and remove from hood.

Flush 160-mL culture bottle with N_2 from gassing probe for 1 to 2 min. Do not use CO_2, which will acidify the solution and increase loss of Na_2S as H_2S (toxic). Add

1 drop 10% NaOH
75 mL boiled dH_2O

Add the NaOH first to make solution basic and loss of H_2S. The volume of dH_2O listed is for 1.875 g $Na_2S \cdot 9\ H_2O$; adjust the volume for the actual weight of $Na_2S \cdot 9\ H_2O$.

Immediately seal with bung and aluminum seal (to minimized exposure to any H_2S), 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 dH_2O
1.5 g cellobiose
3.0 g trypticase peptone (BD 211921)
0.75 g yeast extract (BD 212750)
0.135 g K_2HPO_4
0.135 g KH_2PO_4
0.27 g $(NH_4)_2SO_4$
0.27 g NaCl
0.027 g $MgSO_4$
0.027 g $CaCl_2 \cdot 2H_2O$
3 drops of 0.1% resazurin
45 mL rumen fluid (supernatant from 16,000 *g* centrifugation)

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

1.8 g Na_2CO_3

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 dH_2O
1.2 g glucose
0.3 g trypticase peptone (BD 211921)
0.15 g yeast extract (BD 212750)
0.0876 g K_2HPO_4
0.0876 g KH_2PO_4
0.1440 g $(NH_4)_2SO_4$

0.1440 g NaCl
0.0147 g $MgSO_4$
0.0192 g $CaCl_2 \cdot 2H_2O$
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
1.2 g Na_2CO_3

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 dH_2O
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 K_2HPO_4
0.0876 g KH_2PO_4
0.1440 g $(NH_4)_2SO_4$
0.1440 g NaCl
0.0147 g $MgSO_4$
0.0192 g $CaCl_2 \cdot 2H_2O$
0.93 mL VFA solution
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

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 $FeSO_4 \cdot 7\ H_2O$
- 0.2 g $MnCl_2 \cdot 4\ H_2O$
- 0.01 g $ZnSO_4 \cdot 7\ H_2O$
- 0.03 g H_3BO_4
- 0.02 g $CoCl_2 \cdot 6\ H_2O$
- 0.001 g $CuCl_2 \cdot 2\ H_2O$
- 0.002 g $NiCl_2 \cdot 6\ H_2O$
- 0.003 g $NaMoO_4 \cdot 2\ H_2O$

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

Phosphate buffer for phophotransferase 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 $MgCl_2 \cdot 6H_2O$
- 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 $(NH_4)_2SO_4$
- 0.015g $MgSO_4$
- 0.03g$CaCl_2 \cdot 2H_2O$
- 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 HCl·H2O

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 dH_2O
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 K_2HPO_4
0.0684 g KH_2PO_4
0.0684 g $(NH_4)_2SO_4$
0.1368 g NaCl
0.0139 g $MgSO_4$
0.0182 g $CaCl_2·2H_2O$
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 dH_2O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add
1.2 g Na_2CO_3

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% $Na_2S·9H_2O$
to individual tubes.

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

From (3)

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

270 mL dH_2O
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)
0.1 g pyridoxal hydrochloride (stored at -20°C)
0.1 g pyridoxine
0.2 g riboflavin
0.2 g thiamine HCl
0.2 g nicotinamide
0.2 g calcium pantothenate
0.1 g lipoic acid (stored at 4°C)
0.01 g 4-aminobenzoic acid
0.005 g folic acid (stored at 4°C)
0.005 g biotin (stored at 4°C)

0.005 g coenzyme B_{12} (stored at 4°C)

Add 1 L 0.1 M KH_2PO_4 (pH = 6); this is prepared by adding 13.609 g KH_2PO_4 and ~700 mL dH_2O to a beaker, adding 10% NaOH drop-by-drop until pH = 6, then bringing to 1 L with dH_2O in volumetric flask.

Stir solution with stir bar. 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 ddH_2O

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 dH_2O
1.905 g K_2HPO_4
1.5 g KH_2PO_4
0.195 NaCl
0.027 $MgSO_4 \cdot 7H_20$
0.0045 g $CaCl_2 \cdot 2H_2O$
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

2.25 g $NaCHO_3$

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 (K_2HPO_4, KH_2PO_4, NaCl, $MgSO_4 \cdot 7H2O$, $CaCl_2 \cdot 2H_2O$) 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 dH_2O
11.430 g K_2HPO_4
9.0 g KH_2PO_4
1.170 g NaCl
0.162 g $MgSO_4$ $7H_2O$
0.027 g $CaCl_2 \cdot 2H_2O$
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 $NaCHO_3$

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 dH_2O to a 1-L round-bottom flask, bubble with N_2, and boil for 5 to 7 min.

Prepare saturated Na_2CO_3 by adding approximately

20 g Na_2CO_3

to 160-mL culture bottle flushed with N_2 gas from a gassing probe. Transfer 75 mL of boiled dH_2O. Seal with rubber bung and aluminum cap. Autoclave.

Transfer 45 mL dH_2O to a 125-mL Erlenmeyer flask flushed with N_2 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% $TiCl_3$

Keep pH above 2 by adding $TiCl_3$ slowly and alternating with saturated Na_2CO_3 (otherwise precipitate will form). Adjust to pH = 7 with saturated Na_2CO_3. Approximately 3.8 mL Na_2CO_3 will be used in total. Solution begins as green, but it will turn violet-blue as $TiCl_3$ is progressively added.

Adjust to 75 mL volume with boiled dH_2O. Filter through 0.2 μm membrane into 160-mL culture bottle (previously autoclaved and flushed with N_2). 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 $ZnCl_2$
- 0.1 g $MnCl_2 \cdot 4H_2O$
- 0.006 g HBO_3
- 0.19 g $CoCl_2 \cdot 6H_2O$
- 0.002 g $CuCl_2 \cdot 2H_2O$
- 0.024 g $NiCl_2 \cdot 6H_2O$
- 0.036 g $Na_2MoO_4 \cdot 2H_2O$

Some components stick to weigh paper and must be washed into the beaker with a small volume of dH_2O.

Add the following ingredients to another 500 mL beaker

- 10 mL 7.7 *M* HCl
- 1.5 g $FeCl_2 \cdot 4H_2O$

Dissolve $FeCl_2$ completely by gently swirling.

Combine contents of the two beakers, dilute in ~250 mL dH_2O 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 dH_2O
- 90 mL rumen fluid, clarified
- 2 g glucose
- 0.135 g K_2HPO_4
- 0.135 g KH_2PO_4
- 0.27 g $(NH_4)_2SO_4$
- 0.27 g NaCl
- 0.026 g $MgSO_4$
- 0.036 g $CaCl_2 \cdot 2H_2O$
- 3 drops of 0.1% resazurin

Adjust pH to 7.0 with NaOH

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

5 g $NaHCO_3$

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 K_3- Hemin solution

Adapted from ATCC
(https://www.atcc.org/~/media/5A86796703884E068DBDB2B74E1346DB.ashx)

Part A

100 mg menadione (vitamin K_3)
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 B_{12} (stored at 4°C)
0.1 g 4-aminobenzoic acid
0.1 g pantothenic acid

Dissolve in dH_2O 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.

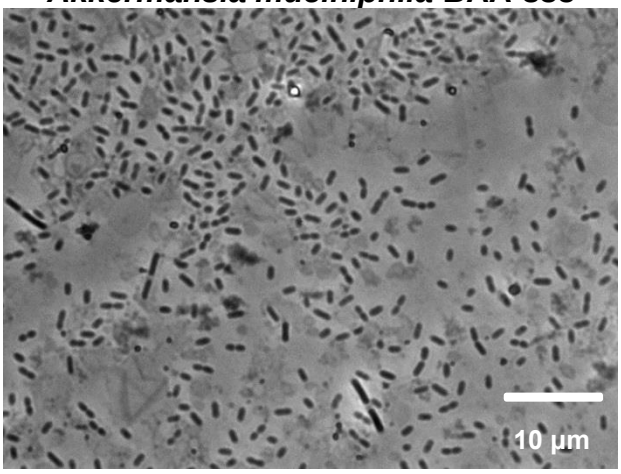
Table 1. Media compatibility

Strain	Medium[1]
Akkermansia muciniphila BAA-835	Mucin medium > PYG (Anaerobe Systems)
Anaerovibrio lipolytica 5S	Prins semi-defined medium > PC + VFA + glycerol
Butyrivibrio fibrisolvens D1	PC+VFA = PC – VFA = minimal media + VFA > rumen bacteria medium
Butyrivibrio proteoclasticus B316	6SG330 > minimal media + VFA = PC + VFA
Clostridium aminophilum F	Chen w/ yeast extract[2] > Chen
Clostridium sticklandii SR	Chen w/ yeast extract[2] > Chen
Fibrobacter succinogenes S85	PC + VFA + cellobiose[2] > PC+VFA[2] > cellulolytic medium
Lachnobacterium bovis YZ87	PC+VFA = PC – VFA > rumen bacteria medium
Lachnospira multipara D32	PC+VFA > PC – VFA
Lactobacillus ruminis RF1	MRS > PYG (DSMZ) > PC+VFA.
Megasphaera elsdenii B159	PC – VFA
Megasphaera elsdenii T81	PC – VFA
Mitsuokella jalaludinii M9	PYG (DSMZ) > PC+VFA = PC – VFA > rumen bacteria medium
Prevotella bryantii B_14	Treponema bryantii medium > PC+VFA = minimal media + VFA > PC – VFA
Prevotella albensis M384	Treponema bryantii medium > PC+VFA = PC – VFA
Pseudobutyrivibrio sp. B1A1	RGCSA
Ruminococcus albus 7	cellulolytic medium = PC+VFA+cellobiose[2]
Ruminococcus albus 8	cellulolytic medium > PC + VFA + cellobiose[2]
Ruminococcus flavefaciens FD-1	cellulolytic medium = PC + VFA + cellobiose[2]
Selenomonas bovis WG	PYG (DSMZ) > PC-VFA > PC +VFA = rumen bacteria medium
Selenomonas ruminantium GA-192	PC + VFA > Selenomonas ruminantium medium > PC – VFA
Selenomonas ruminantium HD4	PC-VFA
Streptococcus bovis JB1	PC - VFA = PC + VFA = RGCSA
Streptococcus sp. C1A1	PC - VFA = PC + VFA = RGCSA
Succinimonas amylolytica 13846	PC + VFA = rumen bacteria medium
Succinivibrio dextrinosolvens 24	PC + VFA > PC – VFA.
Succinivibrio dextrinosolvens 226	PC+VFA
Entodinium caudatum	medium SP
Epidinium caudatum	medium M

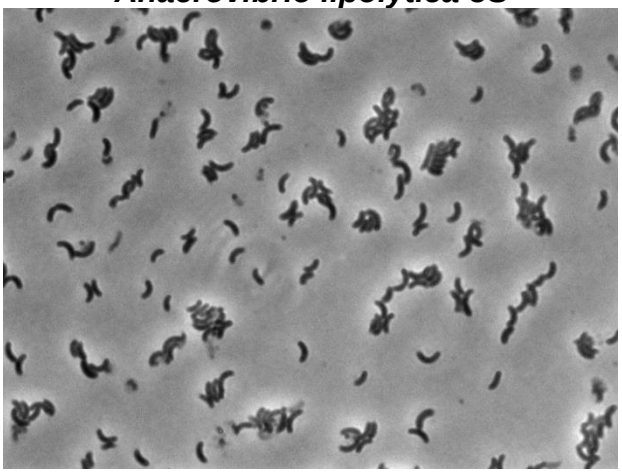
[1]Media are ranked according to ability to support growth; "=" indicates equal performance
[2]These media should be avoided for long-term maintenance because they may cause biosynthetic mutants (loss of the wild-type function)

Images of pure cultures of bacteria and methanogens

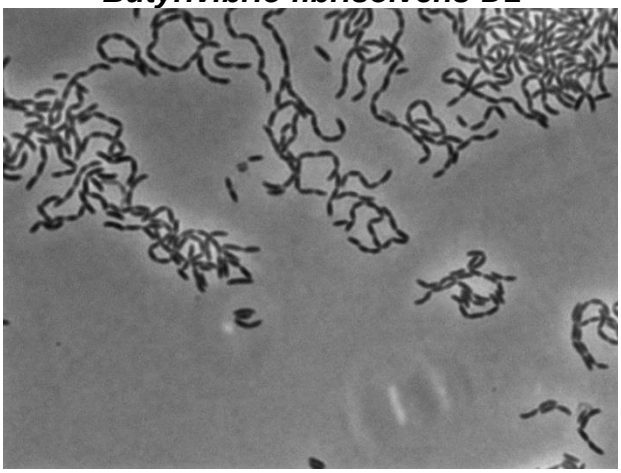
***Akkermansia muciniphila* BAA-835**



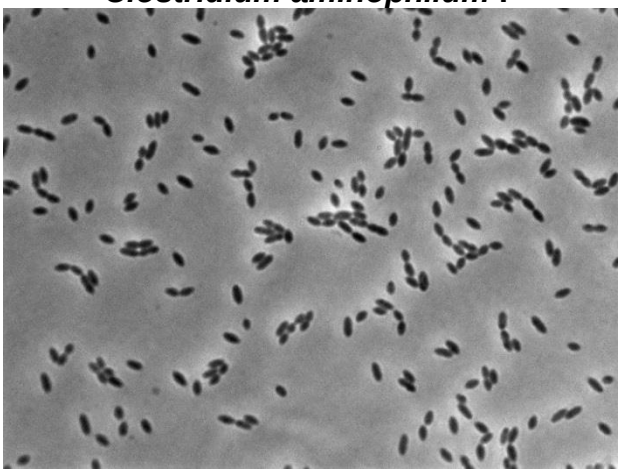
***Anaerovibrio lipolytica* 5S**



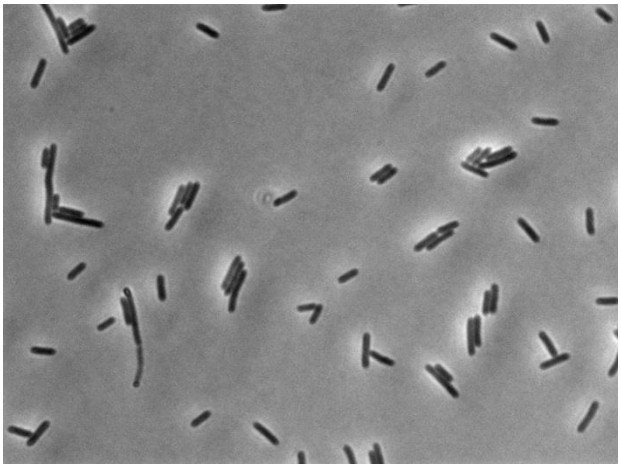
***Butyrivibrio fibrisolvens* D1**



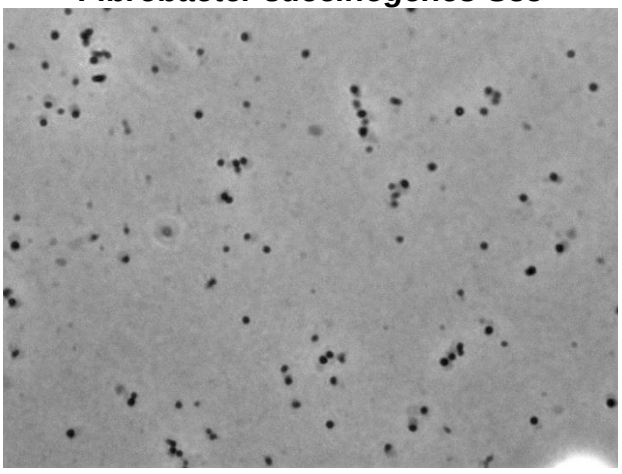
***Clostridium aminophilum* F**



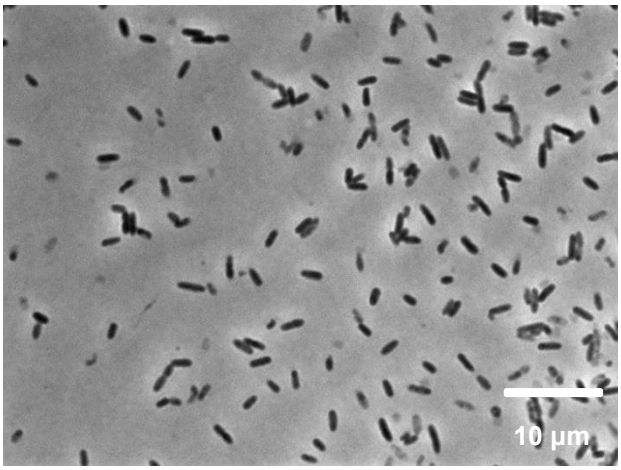
***Clostridium sticklandii* SR**



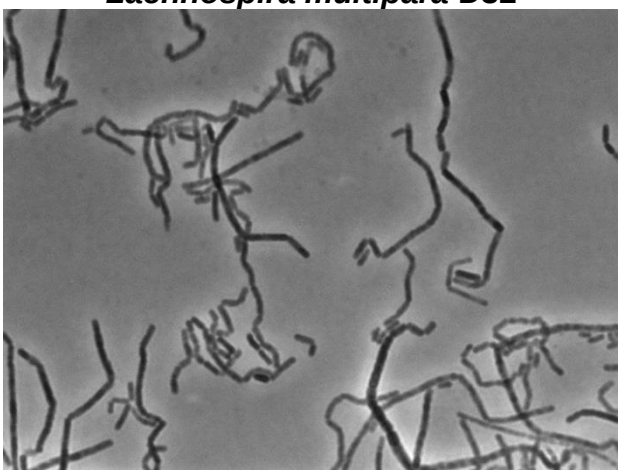
***Fibrobacter succinogenes* S85**



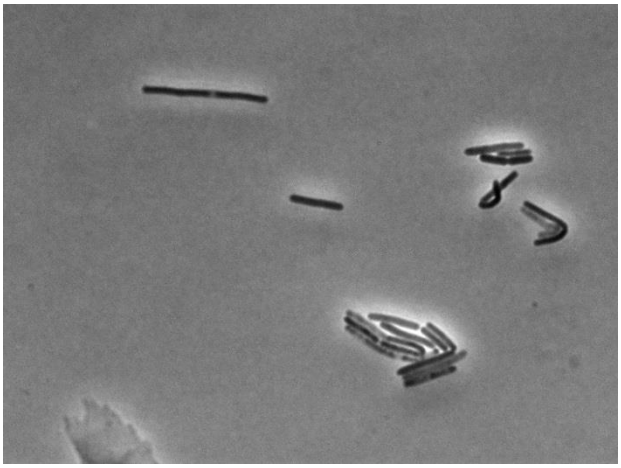
***Lachnobacterium bovis* YZ87**



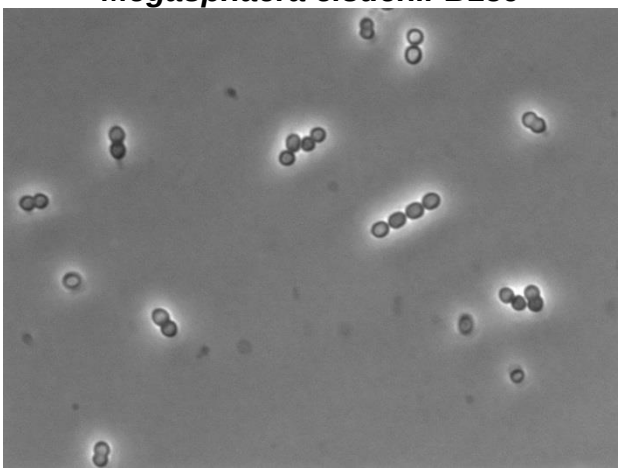
***Lachnospira multipara* D32**



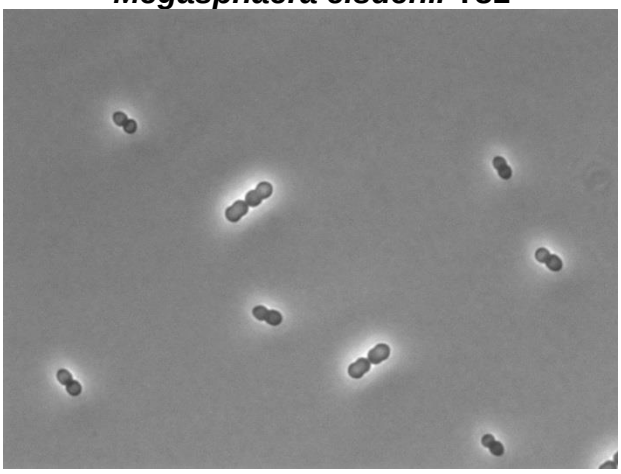
***Lactobacillus ruminis* RF1**



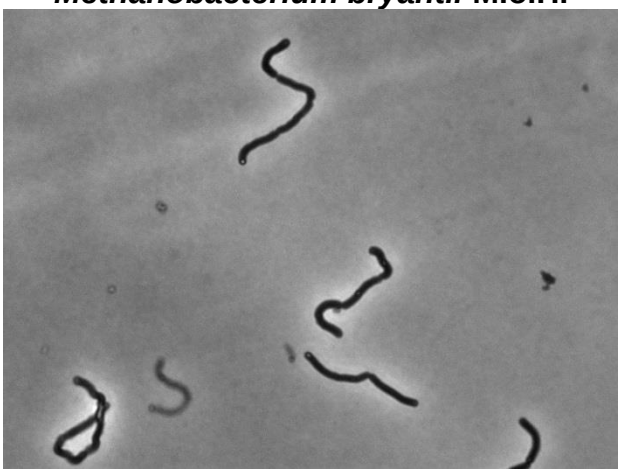
***Megasphaera elsdenii* B159**



***Megasphaera elsdenii* T81**



***Methanobacterium bryantii* M.o.H.**



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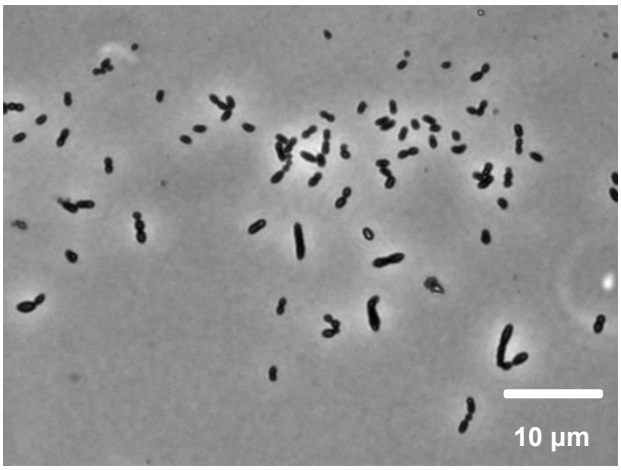

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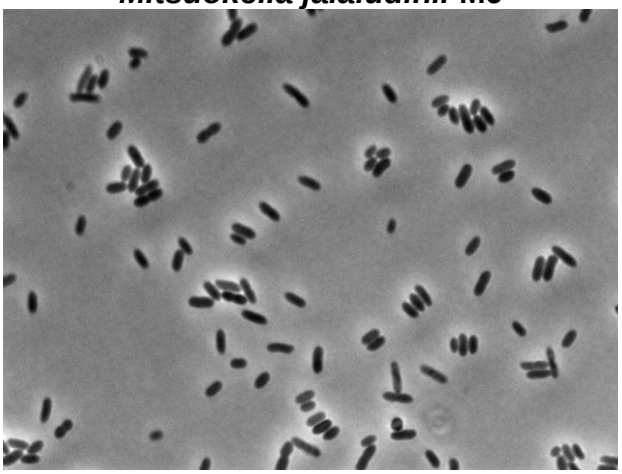

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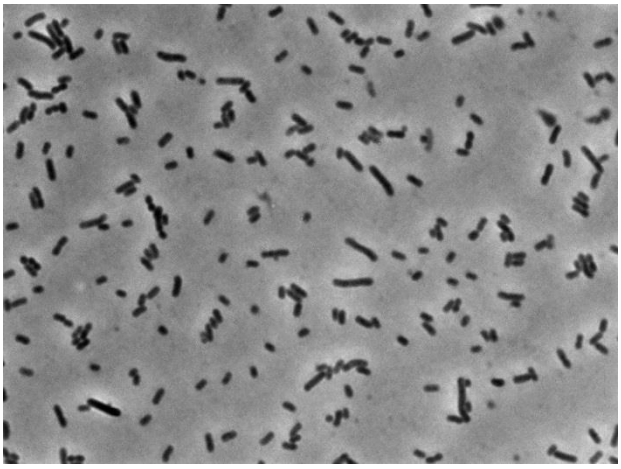
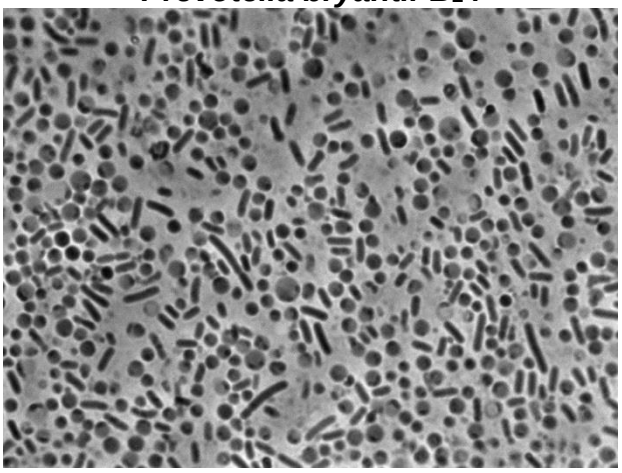
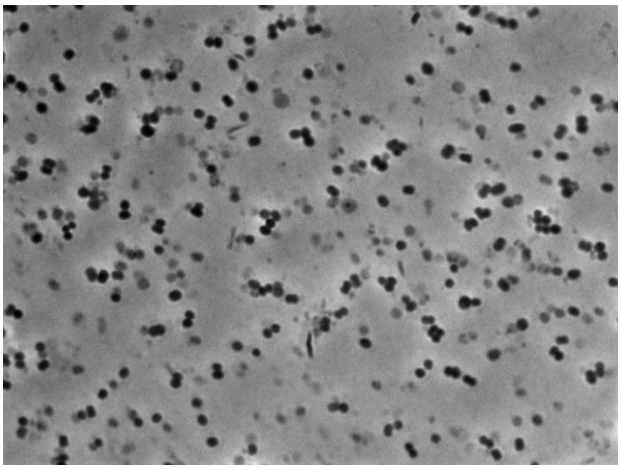


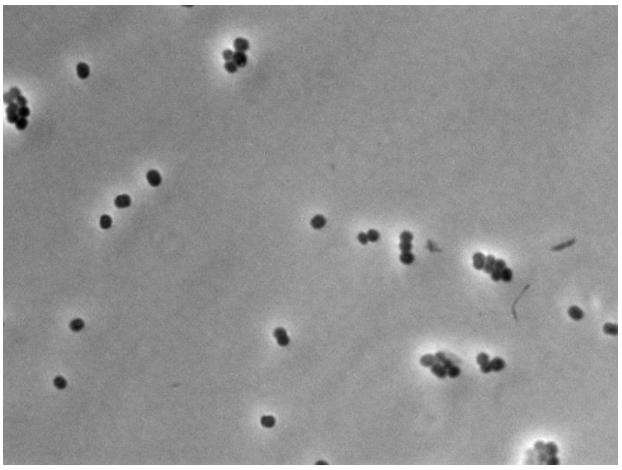
***Prevotella bryantii* $B_1$4**



***Ruminococcus albus* 7**



***Ruminococcus albus* 8**



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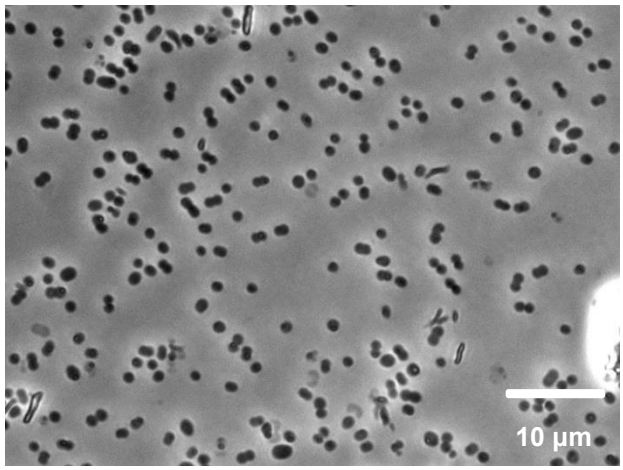
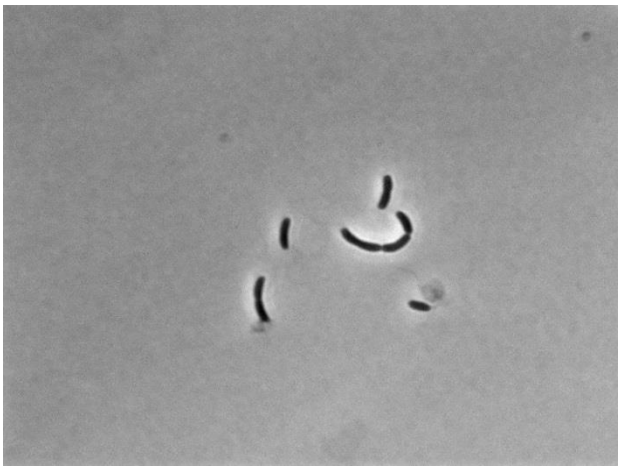
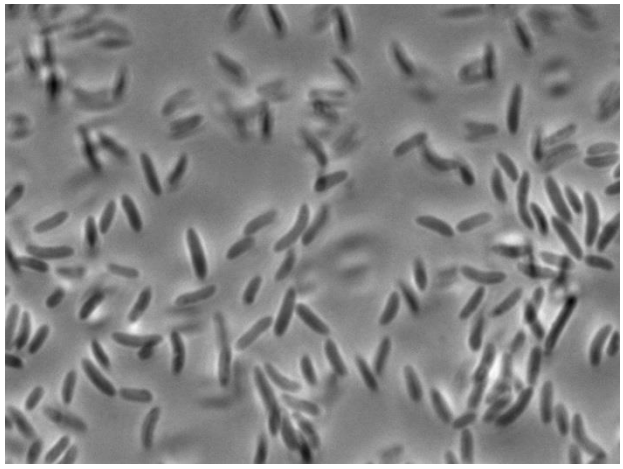


***Selenomonas bovis* WG**



***Selenomonas ruminantium* GA-192**



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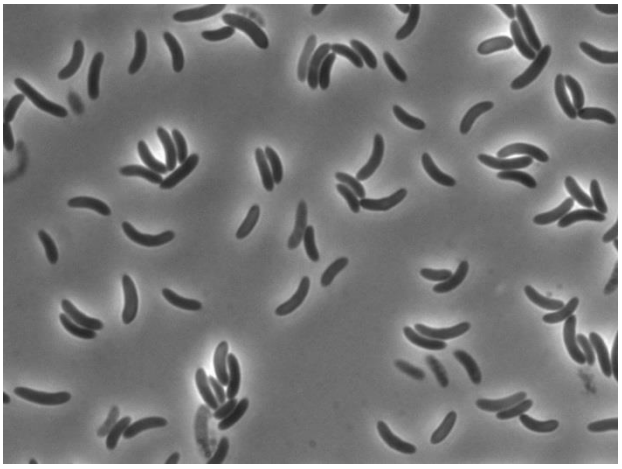
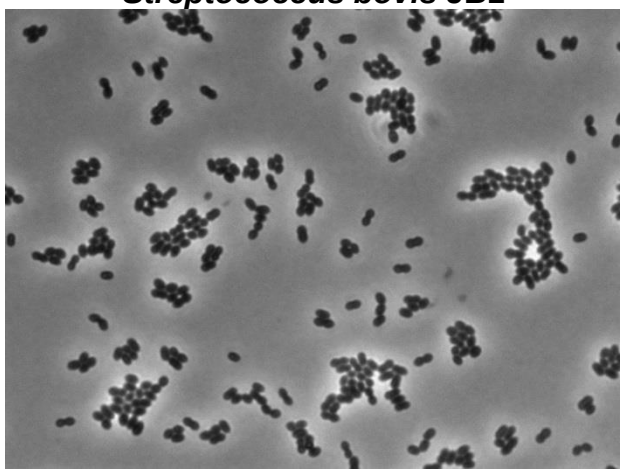
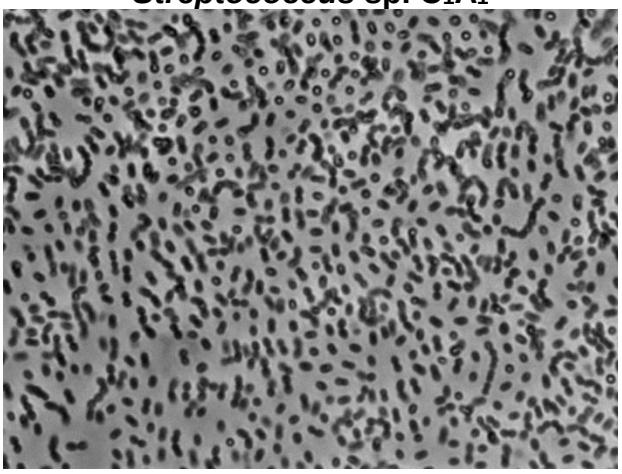


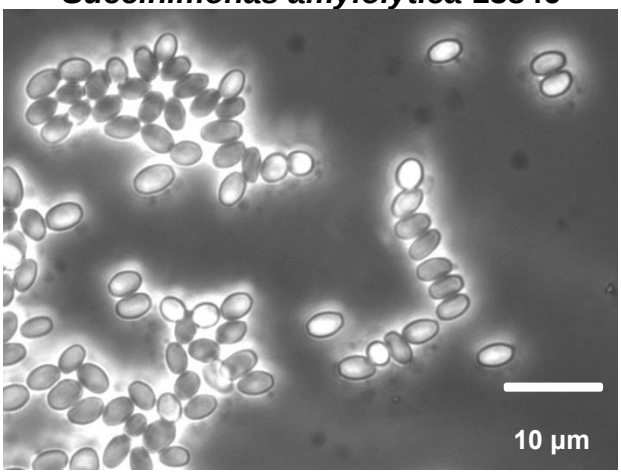
***Streptococcus bovis* JB1**



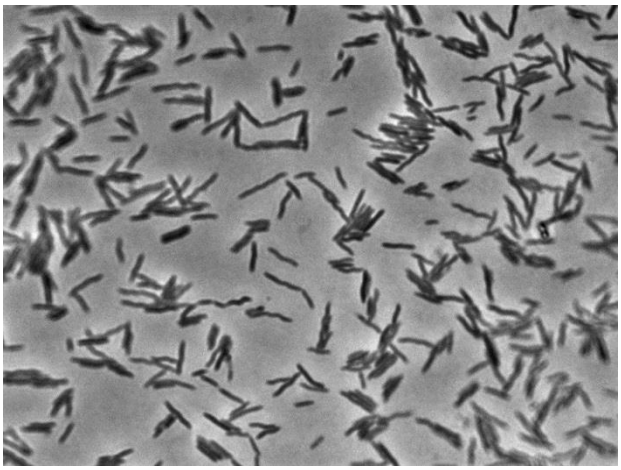
***Streptococcus* sp. C_1A_1**



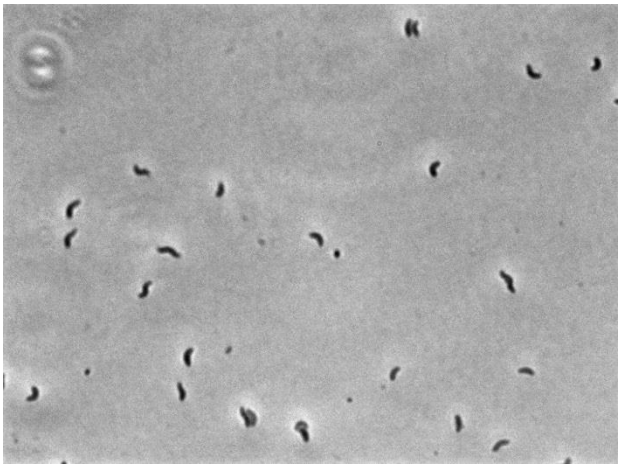
***Succinimonas amylolytica* 13846**



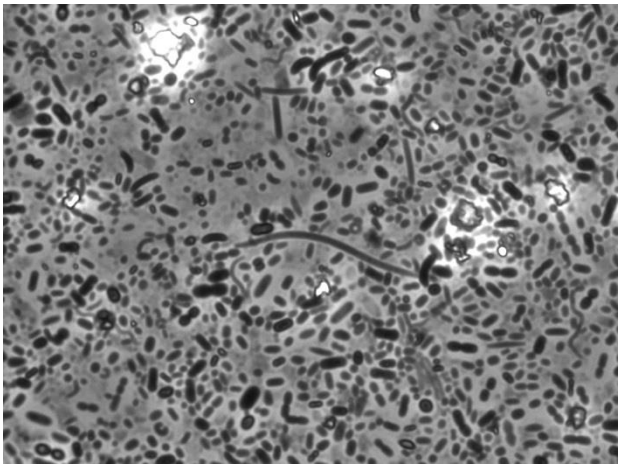
***Succinivibrio dextrinosolvens* 24**



***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.

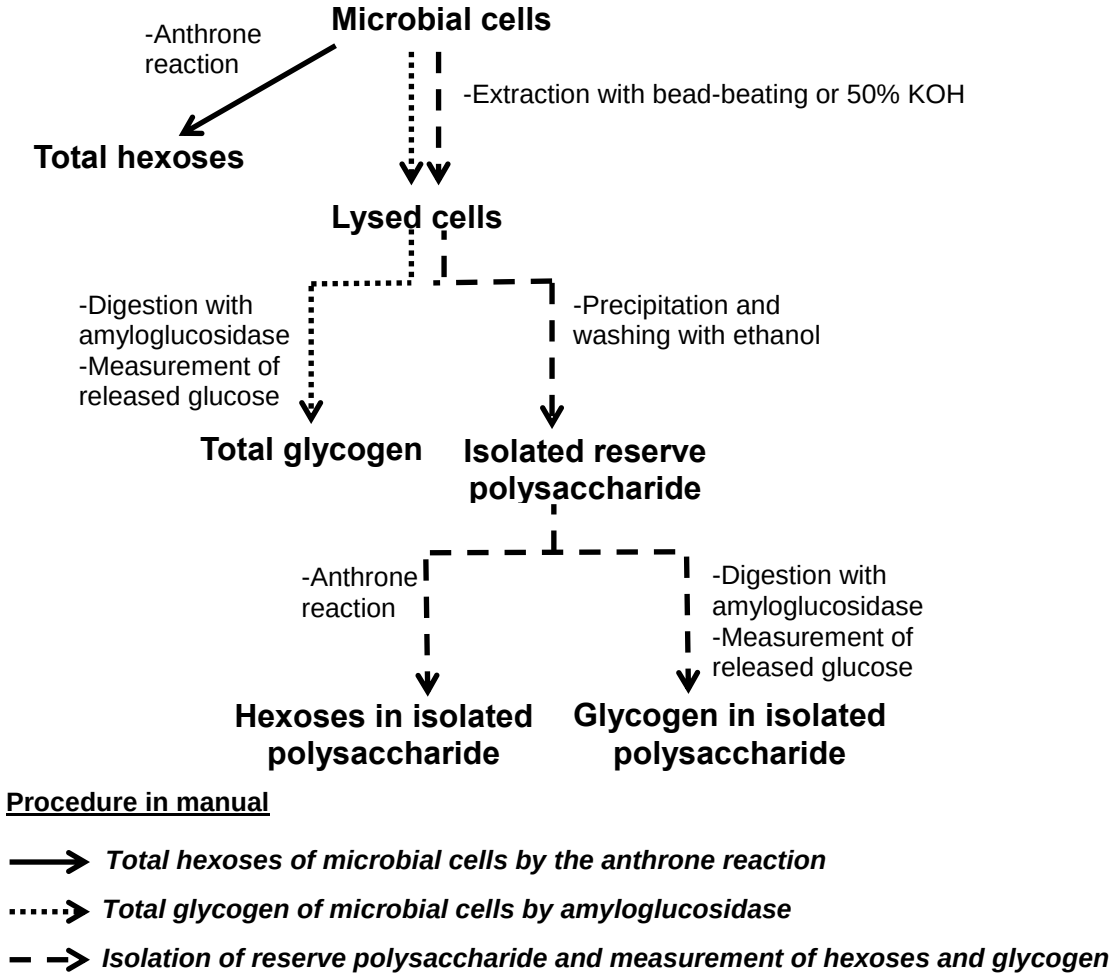


Table 1. Comparison of methods to measure glycogen.

Trait	Type of measurement			
	Total hexoses	Total glycogen	Hexoses in isolated polysaccharide	Glycogen in isolated polysaccharide
Quantitive 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.

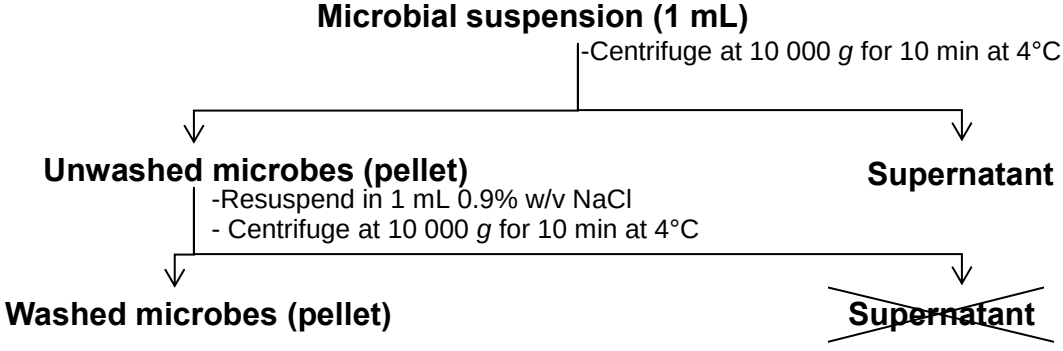
2.) After resuspension, immediately boil for 15 min to inactivate degradative enzymes.

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 105ºC 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 dH_2O 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 105ºC oven (usually overnight) and record mass (hot pan + sample mass).

4.) Incinerate dry matter at 550ºC in muffle furnace overnight. Cool sample enough to transfer to 105 ºC 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_2O (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 µg/mL BSA stock from Pierce BCA assay kit according to the following:

Tube	Volume and source of BSA (µL)	Volume of H_2O (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 dH_2O 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 μL sample or standard in triplicate. Then add 200 μL 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 (μg/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 dH_2O in step #4 of the main procedure), then multiply by either 3 (dilution factor for expected protein mass <2700 μg/tube in step #3 of the main procedure), 8.93 (2700 to 8100 μg/tube), or 25 (8100 to 22500 μg/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 dH_2O that gives (i) final concentration of NaOH of 0.2 N; (ii) cell protein between 300 to 900 μg/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 dH_2O 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 dH_2O. 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 dH_2O. Chill acetaldehyde to 4°C before opening because boiling point is 21°C. Likewise, chill dH_2O 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 H_2SO_4. 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 dH_2O.

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_2O (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 $FeCl_3$ solution. Dissolve 0.540 g $FeCl_3$ in 1 L concentrated HCl. Solution is stable indefinitely.

Prepare aqueous orcinol. Dissolve 0.5 g orcinol in 50 mL dH_2O. 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 $FeCl_3$ 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_2O (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 $MgCl_2 \cdot 6\ H_2O$ solution by dissolving 6 g $MgCl_2 \cdot 6\ H_2O$ 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 $MgCl_2 \cdot 6\ H_2O$ 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 N_2 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 N_2. 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 N_2 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 H_2SO_4 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 dH_2O to a 1 L Berzelius beaker. Stir and heat gently to dissolve. Transfer quantatively 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_2O (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 dH_2O 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 dH_2O 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 dH_2O, pellets should not be re-frozen/thawed because doing so lowers the concentration of hexoses. Incubating at 100ºC 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, 4ºC) 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 µL dH_2O. Add 120 µL, 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 μL 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·0.9.

Notes

(i) An advantage of this method is that it is specific to glycogen because amyloglucosidase digests glycogen only. A disadvatange is that measures less polysaccharide than the anthrone method and probably does not measure all polysaccharide that can indeed serve 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 dH_2O, pellets should not be re-frozen/thawed because doing so lowers the concentration of glycogen. Incubating at 100ºC 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 quantative; 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.

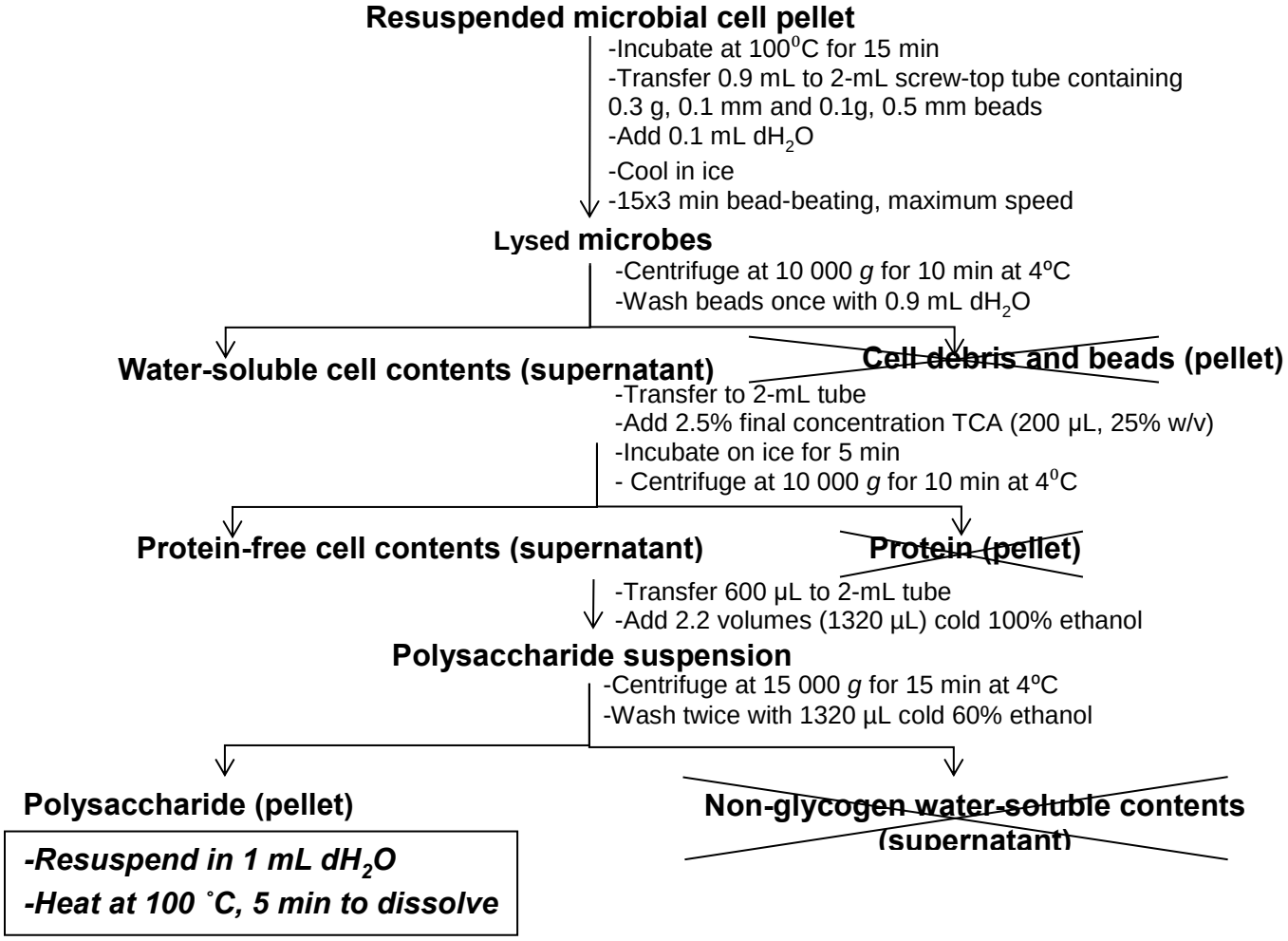
Notes

(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.

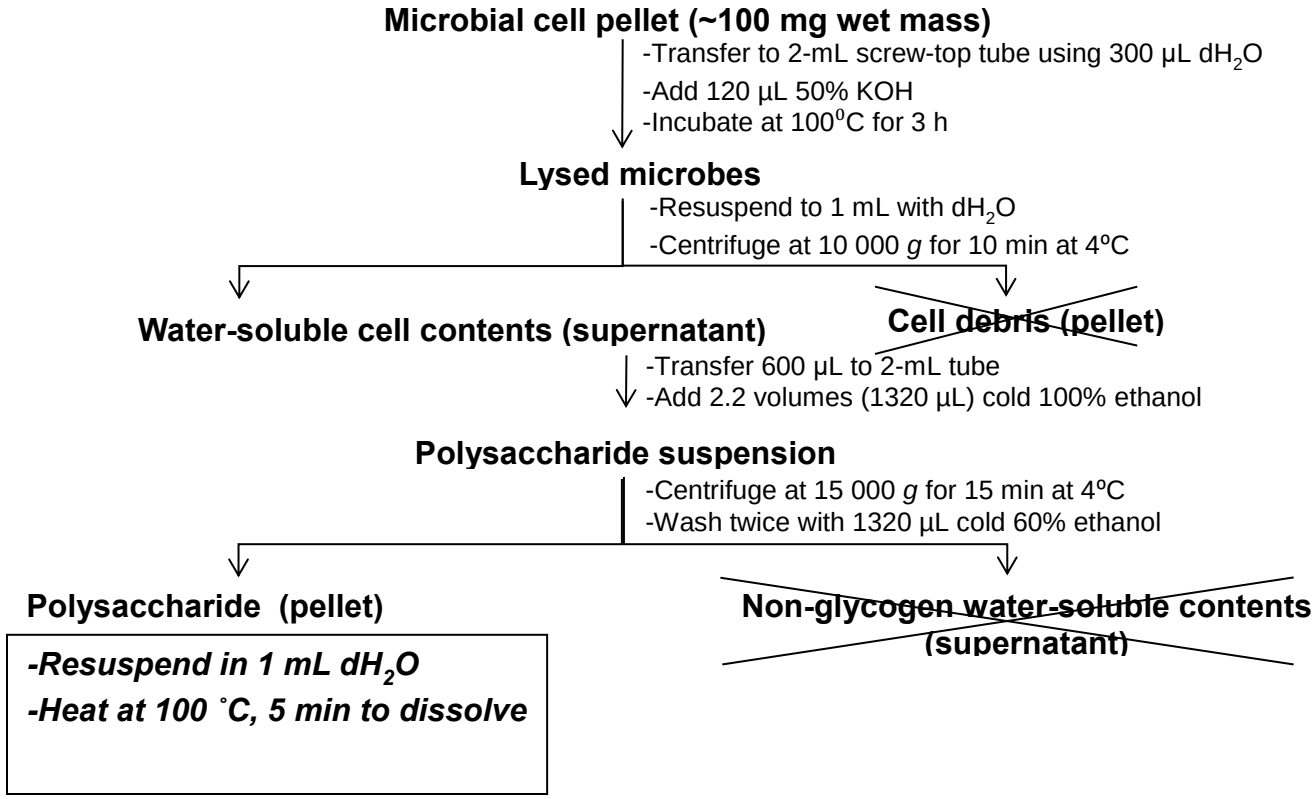
(ii) Measurement of reserve polysaccharide is higher for extraction by bead-beating vs. extraction with 50% KOH (Appedix II). Bead-beating requires special equipment (bead-beater) while 50% KOH does not.

(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)


Microbial cell pellet (~100 mg wet mass)
-Transfer to 2-mL screw-top tube using 300 µL dH2O
-Add 120 µL 50% KOH
-Incubate at 100°C for 3 h
Lysed microbes
-Resuspend to 1 mL with dH2O
-Centrifuge at 10 000 g for 10 min at 4ºC
Water-soluble cell contents (supernatant)
Cell debris (pellet)
-Transfer 600 µL to 2-mL tube
-Add 2.2 volumes (1320 µL) cold 100% ethanol
Polysaccharide suspension
-Centrifuge at 15 000 g for 15 min at 4ºC
-Wash twice with 1320 µL cold 60% ethanol
Polysaccharide (pellet)
-Resuspend in 1 mL dH2O
-Heat at 100 ˚C, 5 min to dissolve
Non-glycogen water-soluble contents (supernatant)

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 dH_2O. For maltose, repeat but with 0.3 g maltose monohydrate. For maltotriose hydrate, add 0.02915 g maltotriose hydrate to 10 mL dH_2O (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 dH_2O (assuming 94% maltoheptaose; adjust for actual purity). For beef liver glycogen, add 0.0314 g to 10 mL dH_2O (assuming 90.04% DM and 94% glucose; adjust for actual DM and purity).

Plate preparation

1.) Prepare solvent (1-propanol:acetone:dH_2O; 2:2:1 by volume).

2.) Cut plates (20 x 20 cm, Merck Si 60 F_{254}, 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 (H_2SO_4: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 Berzulius 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 "Aquire 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.

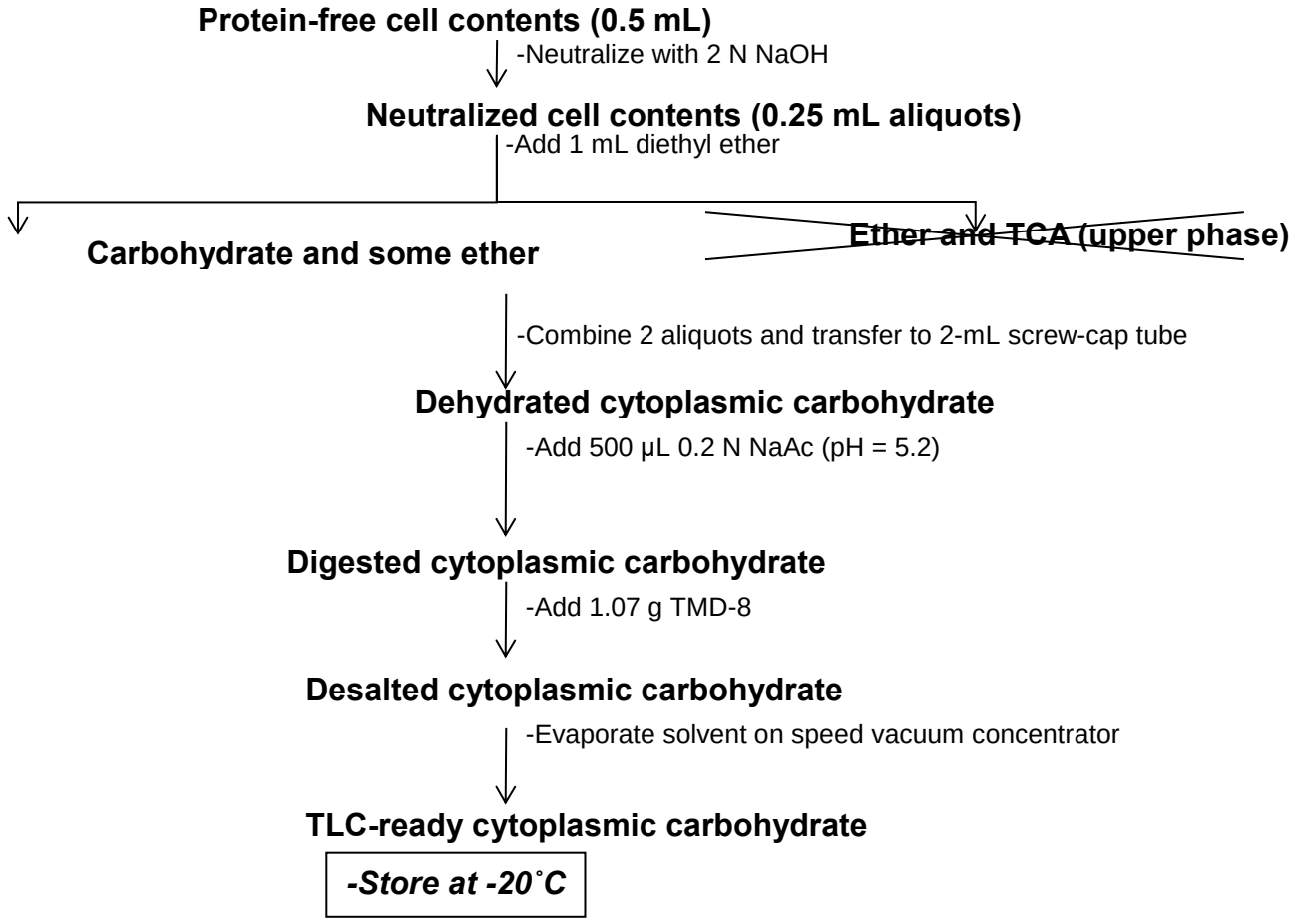


Fig. 2. Diagram of TLC plate marked for development.

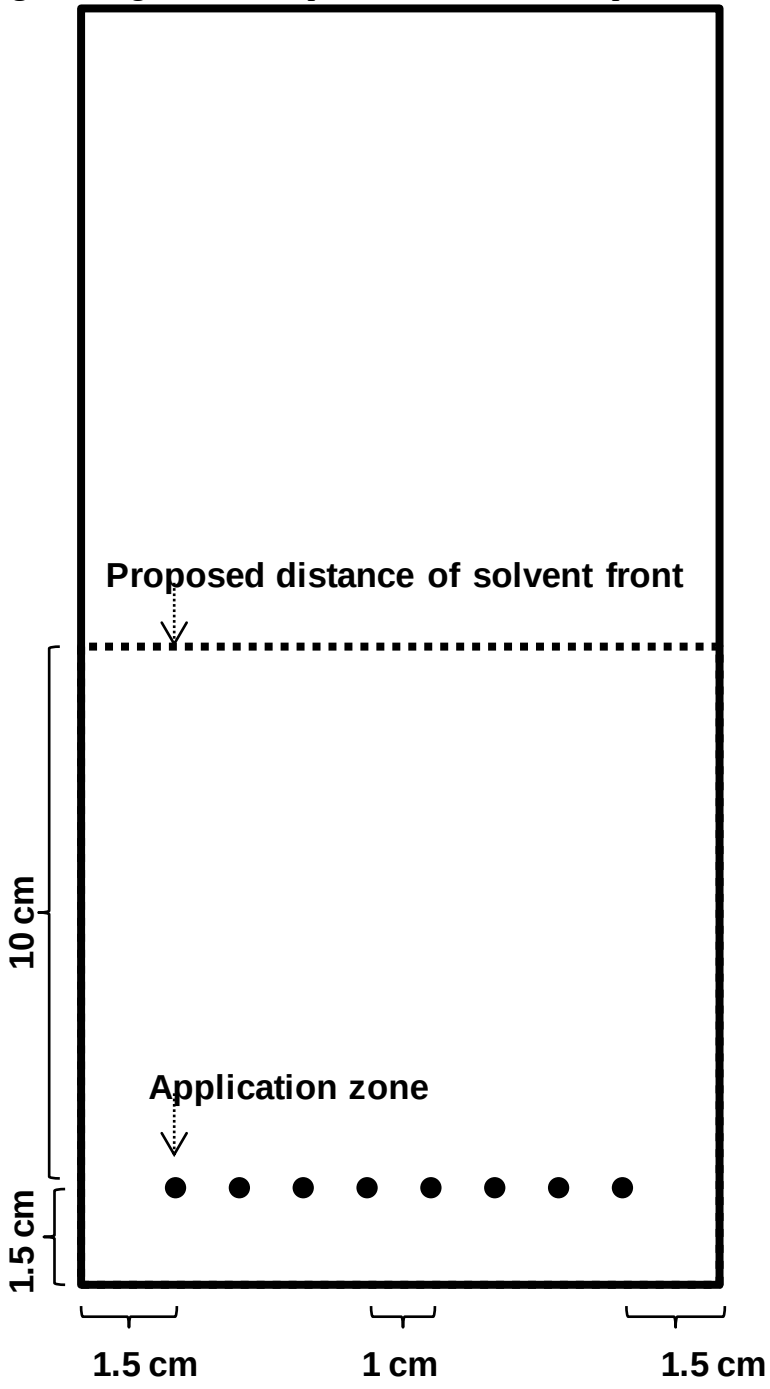
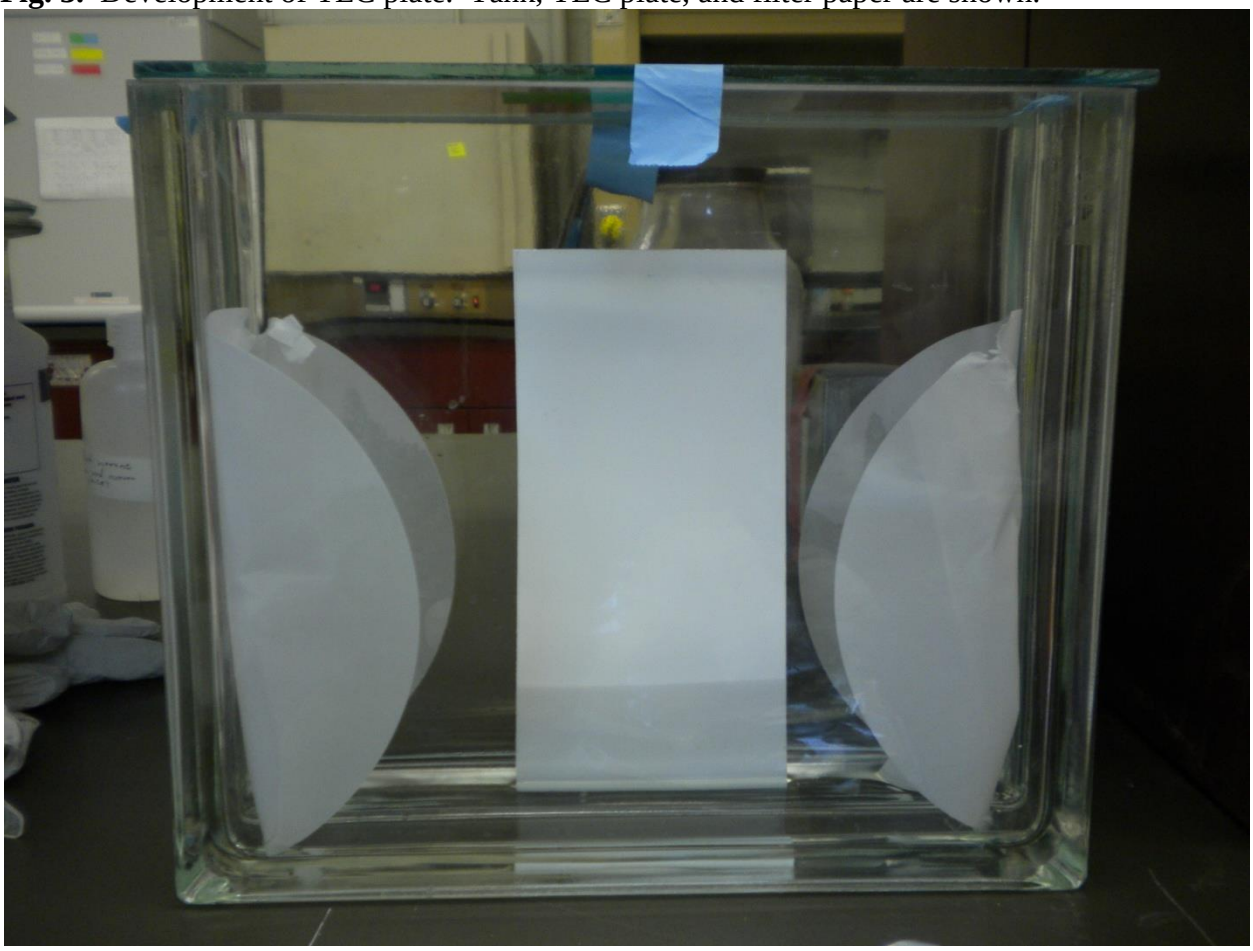


Fig. 3. Development of TLC plate. Tank, TLC plate, and filter paper are shown.



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 I_2 and 1.3 g KI to 5 mL dH2O. Store at RT.
Prepare saturated $CaCl_2$. Add 30 g $CaCl_2 \cdot 2H_2O$ to 25 mL dH_2O and stir to dissolve. Heating is not necessary because reaction is exothermic. Cool on ice to fully crystallize $CaCl_2$. 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 $CaCl_2$. Thaw saturated $CaCl_2$ 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 $CaCl_2$ to each well of a microplate. Include blank wells with 30 µL dH_2O and 97.5 µL saturated $CaCl_2$.

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

The set of absorbances is called A_1(reserve polysaccharide) for wells to which reserve polysaccharide was added, and $A_1(dH_2O)$ for wells to which dH_2O 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 A_2(reserve polysaccharide) for wells to which reserve polysaccharide was added, and $A_2(dH_2O)$ for wells to which dH_2O was added.

5.) Determine the absorbance spectrum of the reserve polysaccharide. Determine [A_2(reserve polysaccharide) – A_1(reserve polysaccharide)] – [$A_2(dH_2O) – A_1(dH_2O)$].

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 A_1(reserve polysaccharide) in #5 corrects for absorbance of reserve polysaccharide and saturated $CaCl_2$. Further subtracting [$A_2(dH_2O) – A_1(dH_2O)$] corrects for absorbance of 2x iodine reagent.

(iii) Saturated $CaCl_2$ is added in #3 to bring solution to a volume readable by the spectrophotometer. Saturated $CaCl_2$, rather than dH_2O, must be used because $CaCl_2$ 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 Na_2HPO_4, 5 g KH_2PO_4, 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 Na_2HPO_4 and KH_2PO_4 in dH_2O (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 dH_2O. 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 dH_2O 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_2O (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 dH_2O 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 dH_2O.

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 shchould 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 dessicant. Glucose oxidase and peroxidase should be stored at -20˚C in a sealed jar containing dessicant.

(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.) Inactive 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 µL supernatant and 200 µL dH_2O.

4.) Make lactic acid standards (Table 1). Make fresh (long-term stability is unknown).

5.) Make solution 2. Add 6 mL dH_2O 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 µL solution 1, 20 µL solution 2, and 2 µL solution 3. Include one extra reaction to avoid running out. Make fresh daily.

8.) Pipette 122 µL 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 µL 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 A_1.

11.) Add 2 µL 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 A_2.

13.) Add 2 µL 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_2O
		μL	
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.) Inactive 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 µL supernatant and 50 µL dH_2O.

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 dH_2O. Make standards by diluting stock (Table 1). Store at -20°C or use fresh.

5.) Make solution 1. Add 4 mL dH_2O 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 dH_2O 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 µL dH_2O, 20 µL solution 1, and 20 µL solution 2. Include one extra reaction to avoid running out. Prepare immediately before use.

9.) Pipette 240 µL working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.

10.) Pipette 10 µL 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 A_1.

This set of absorbances is called A_1.

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	dH_2O
	μL	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.) Inactive 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 µL supernatant and 50 µL dH_2O.

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 dH_2O. 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 dH_2O 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 dH_2O 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 µL dH_2O, 20 µL solution 1, 20 µL solution 2, 20 µL solution 3, and 2 µL solution 4. Include one extra reaction to avoid running out. Prepare immediately before use.

9.) Pipette 262 µL working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.

10.) Pipette 10 µL 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 A_1.

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	dH_2O
	μL	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.) Inactive 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 µL supernatant and 50 µL dH_2O.

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 dH_2O 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 µL dH_2O, 20 µL solution 1, 20 µL suspension 2, and 5 µL solution 3. Include one extra reaction to avoid running out. Make fresh daily.

8.) Pipette 245 µL working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.

9.) Pipette 10 µL 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 A_1.

11.) Add 2 µL 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 A_2.

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	dH_2O
	μL	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 dH_2O. Prepare Brilliant green dye by diluting 2 g and 2 mL of glacial acetic acid to 100 mL with dH_2O. Prepare 30% v/v glycerol by diluting 300 mL glycerol to 1000 mL with dH_2O.

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 10^5 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 mm^2), G is the number of fields (100), A_g is the area of each field (0.3128 mm^2), 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 4^0C.

(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

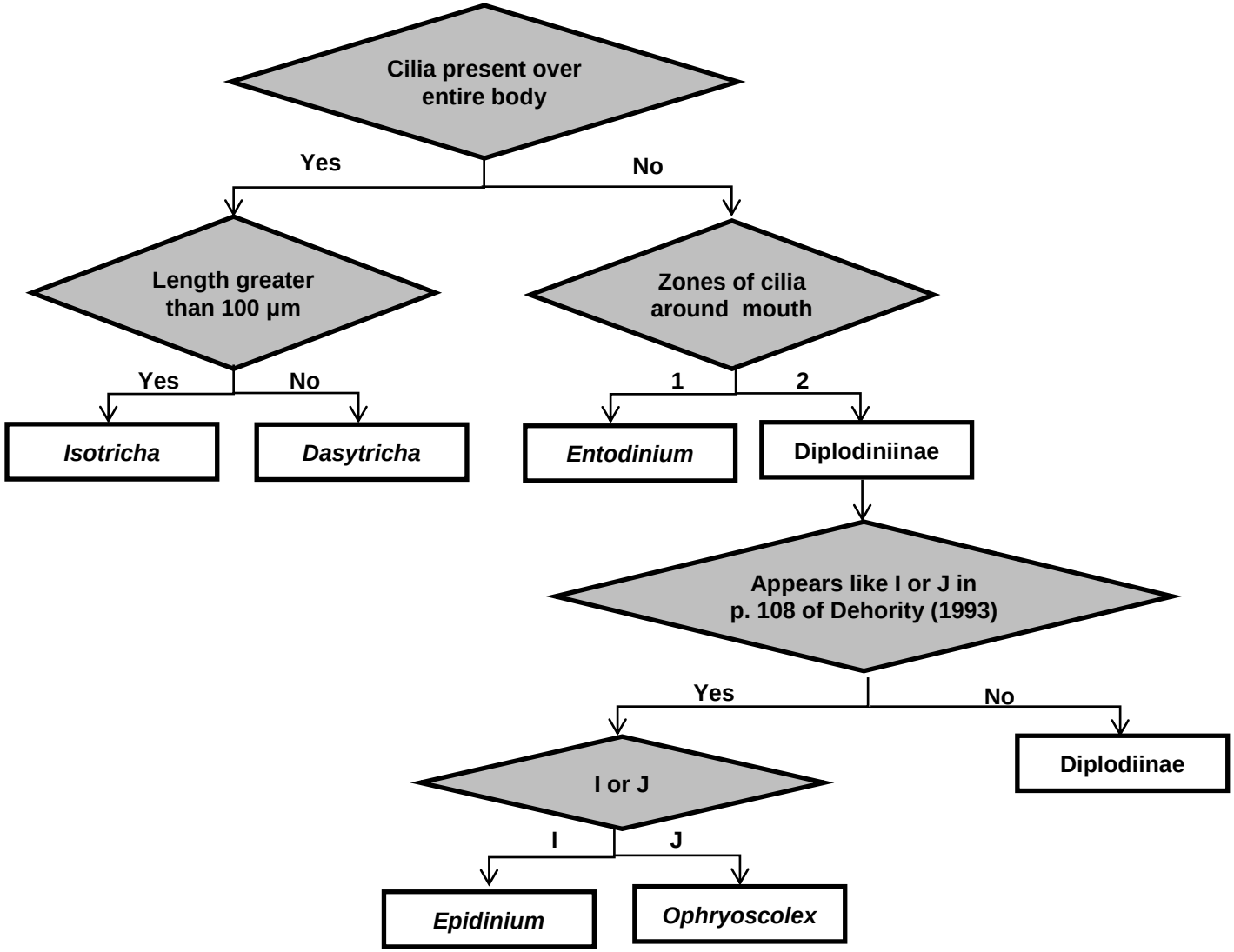


Fig. 1. Rules for counting protozoa inside counting grid. To avoid a bias that will lead to overcounting, protozoa touching the 2 sides marked with red X's should excluded from counting. Protozoa touching the 2 sides marked with green O's should be included, however. Actually, the arrangement of the 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.

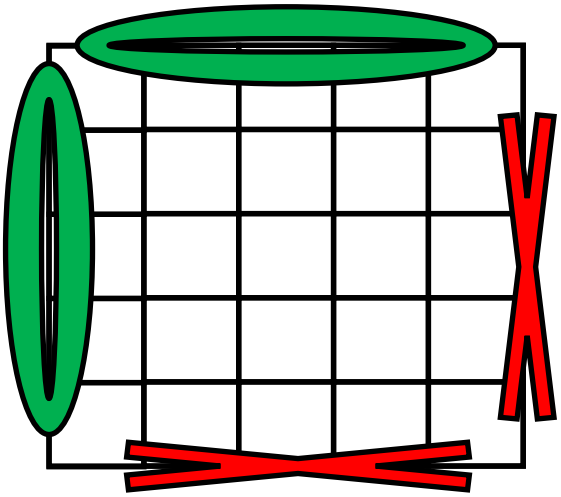
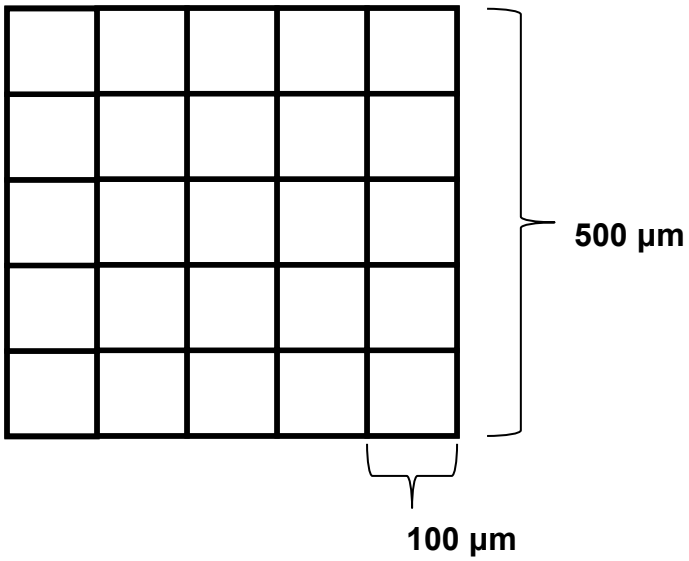


Fig. 2. Dimensions of counting grid at 150x (10x objective, 15x ocular) magnification. For 300x (not shown in figure), the corresponding dimensions are 250 µm and 50 µm; for 600x, they are 125 µm and 25 µm; and for 1500x, they are 50 µm and 10 µm.



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 dH_2O 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 dH_2O 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 dH_2O (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 uncentrifiged samples), proceed to #2.

2.) Dilute sample with diluent water (see composition below). Prepare 10^4 dilution when expecting 10^9 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 um 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 1000x magnification is 210 μm.

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 ddH_2O 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 Pastuer 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 CHROMOTOGRAPHY

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 chromotography for measurement of volatile fatty acids (VFA) and gases (H_2, CH_4).

Operation of gas chromatograph

Risks of operation

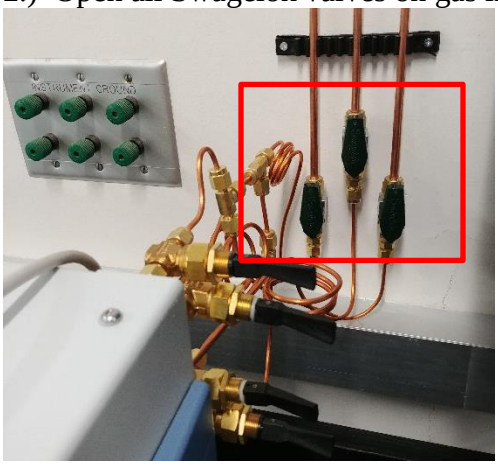
Do not operate the gas chromatograph (GC) until understanding the risks. The GC will be damaged if

- 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.

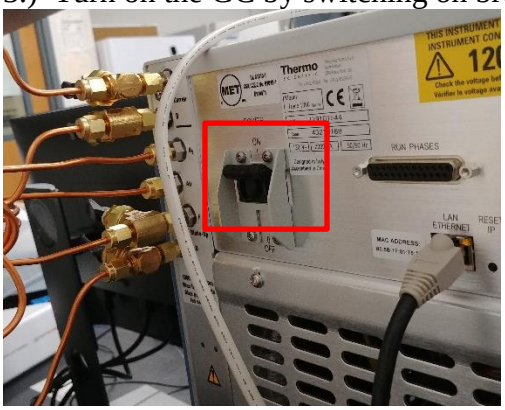
Turning on and placing into standby mode

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.

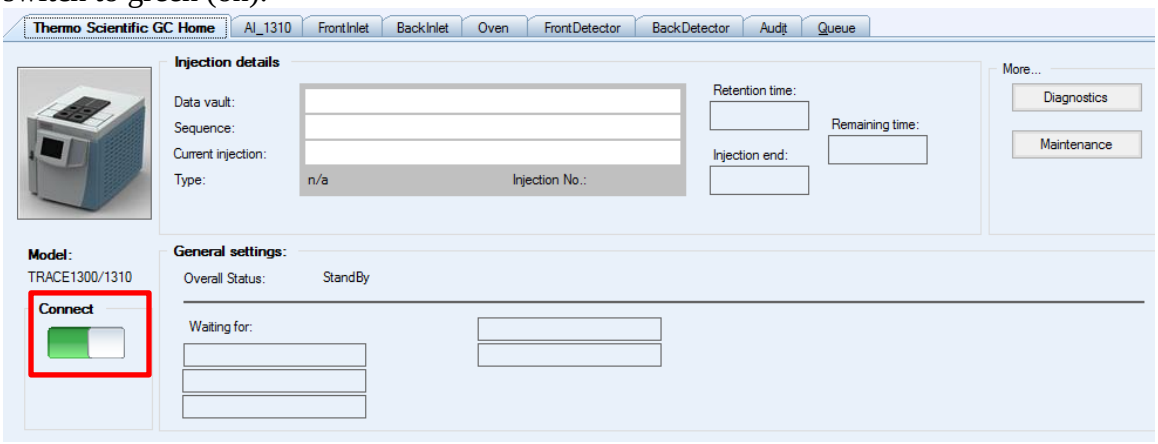


3.) Turn on the GC by switching on breaker on back of instrument.

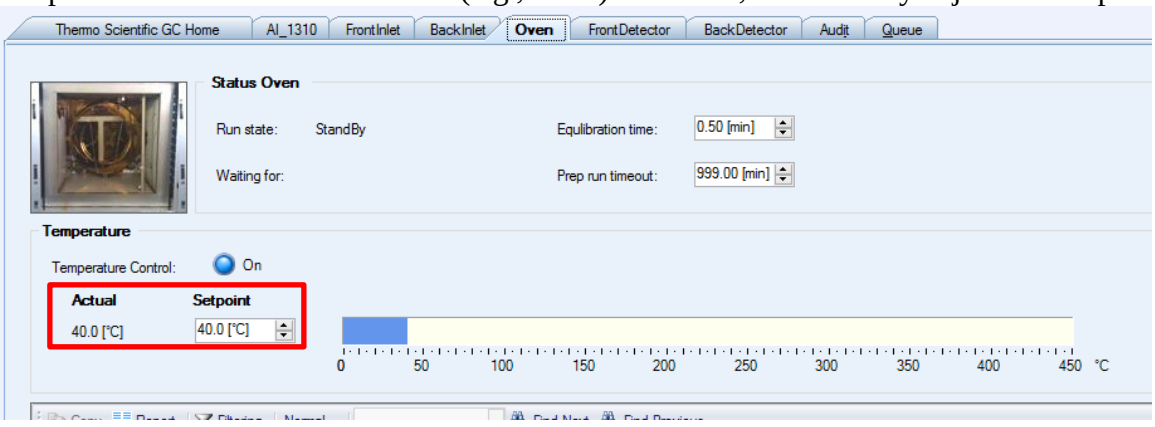


4.) Start Chromeleon 7 software on GC computer.

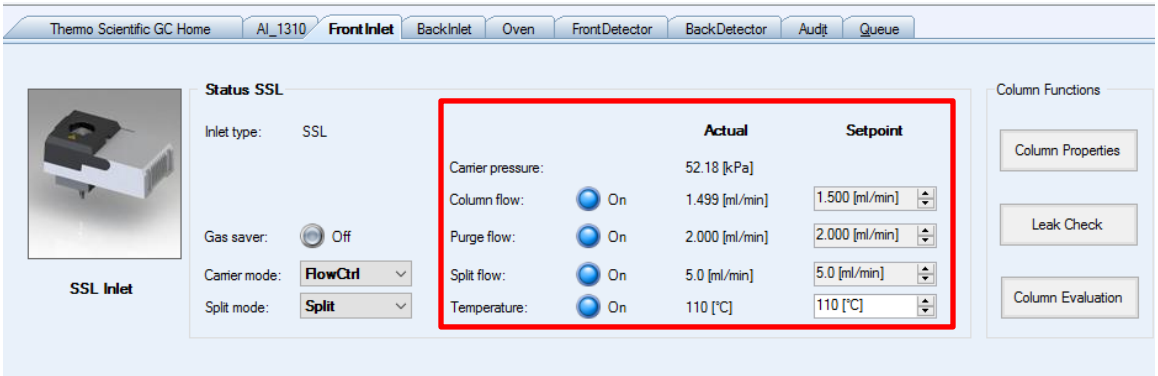
5.) Connect to Chromleon to instrument. In Thermo Scientific GC Home tab, toggle Connect switch to green (on).



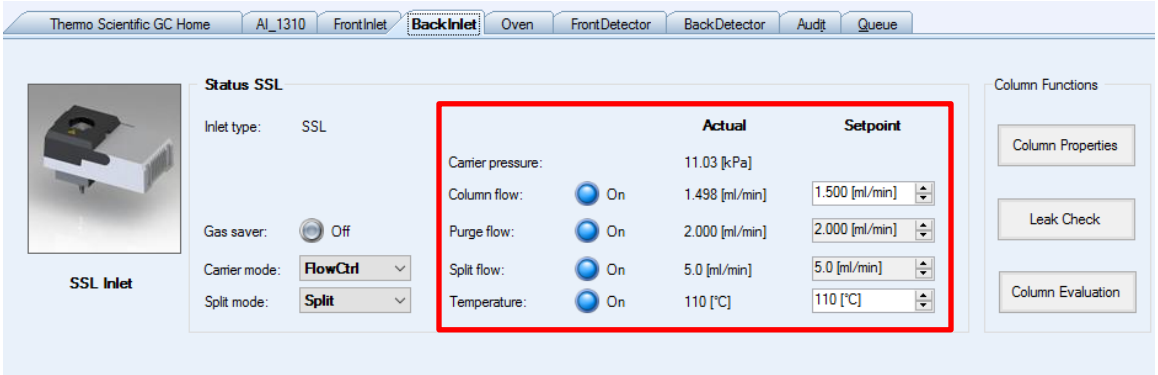
6.) Verify that oven is at safe temperature. In Oven tab, check that Actual and Setpoint temperatures are well below 100°C (e.g., 40°C). If above, immediately adjust the set point.



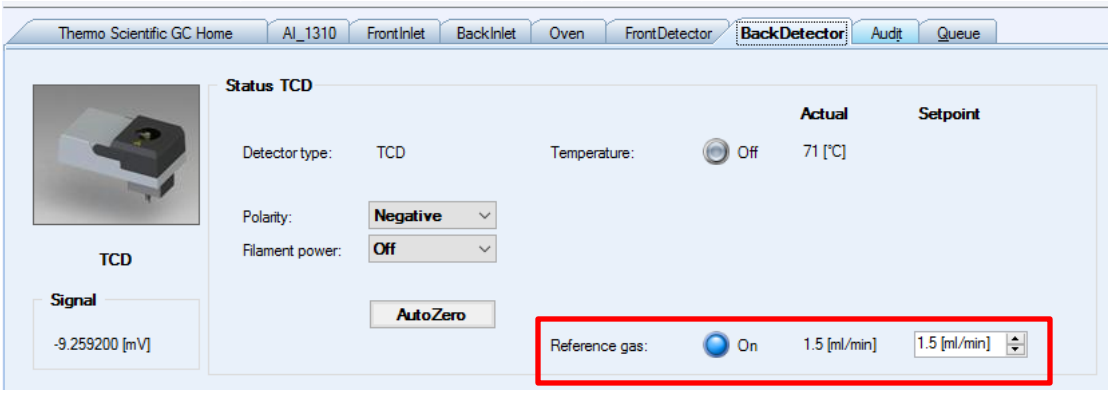
7.) Turn on carrier gas and temperature for front inlet. Go to FrontInlet tab, and turn Column flow to On. Repeat for Purge flow, Split flow, and Temperature. Choose setpoints shown (minimum needed to purge system and prevent condensation of water).



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



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



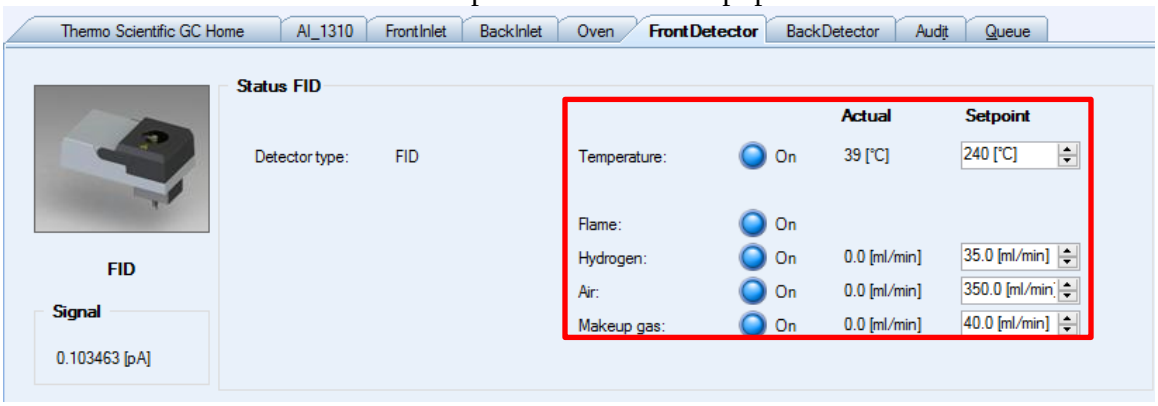
10.) Continue to monitor the compressed gases, and adjust back to 100 psi as needed.

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

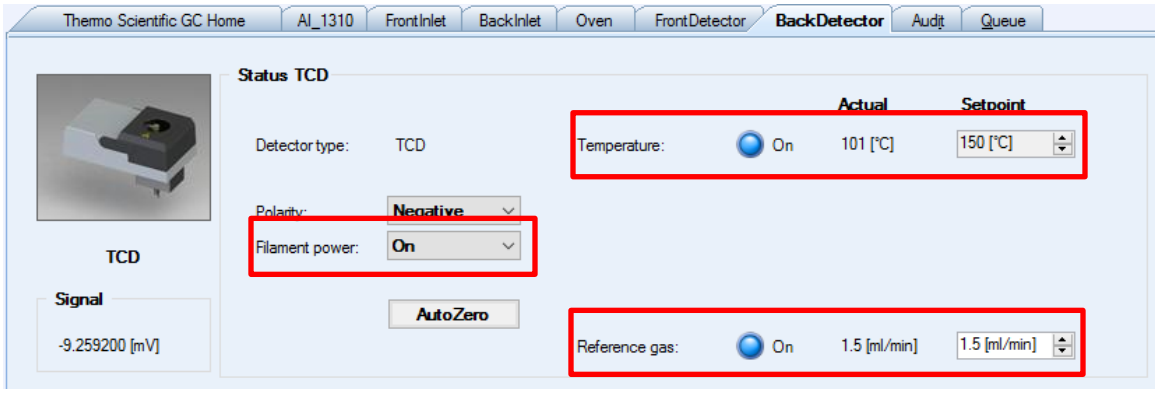
There are two detectors (front and back). Only the detector that is needed should be turned on. The front detector is needed for VFA analysis, and the back detector is needed for gas analysis.

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.



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.



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.

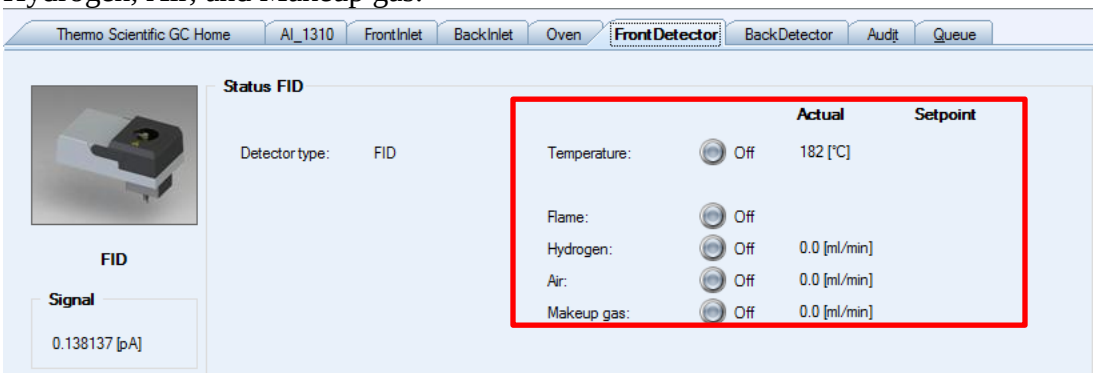
Running samples

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

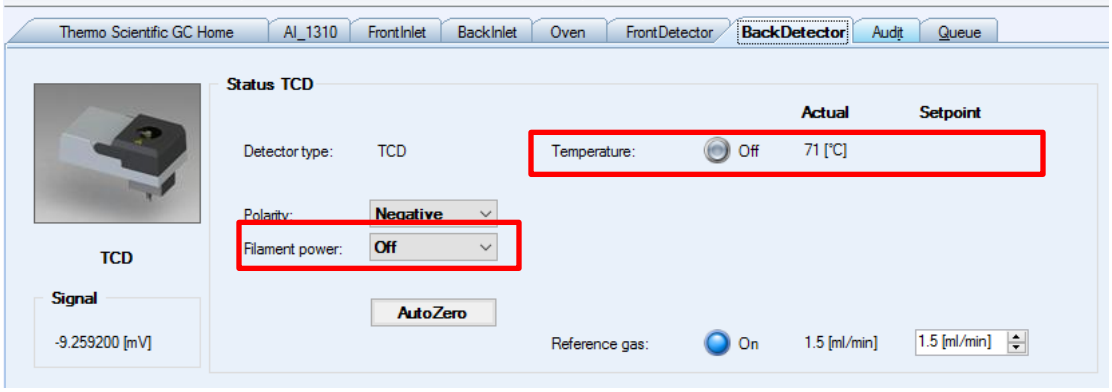
Placing back into standby mode

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

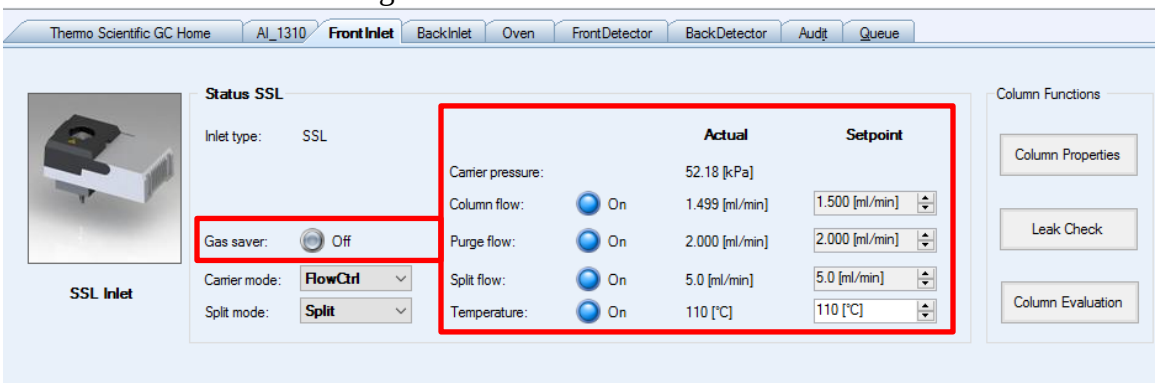
1.) Turn off the front detector (FID). In FrontDetector tab, turn off Temperature, Flame, Hydrogen, Air, and Makeup gas.



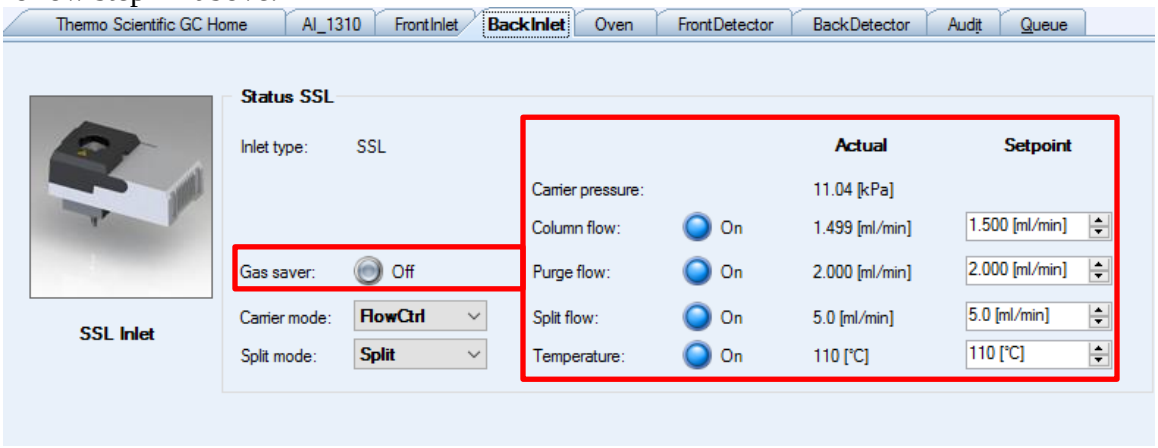
3.) Turn off back detector. In BackDetector tab, turn Temperature and Filament to Off. Do not turn off reference gas yet; filament will be damaged.



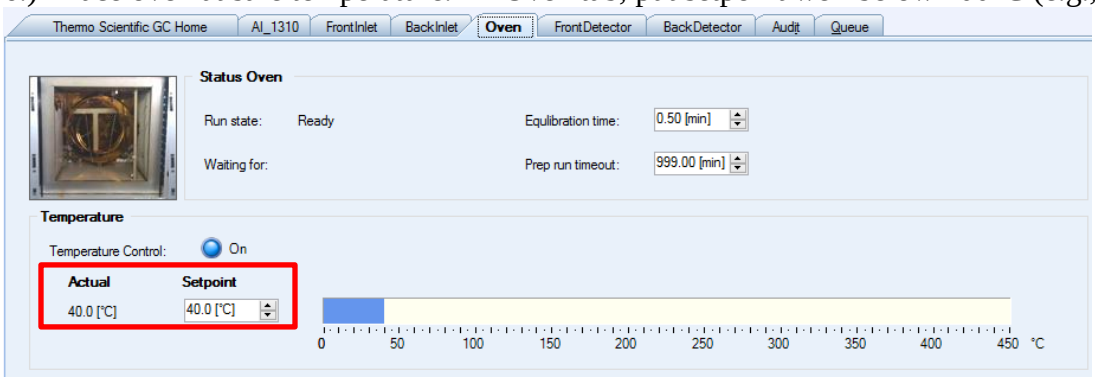
4.) Adjust carrier gas and carrier gas and temperature for front inlet. Go to FrontInlet tab, and change Column flow to value below (1.5 mL/min). Repeat for Purge flow (2 mL/min), Split flow (5 mL/min), and Temperature (110°C). Turn off Gas saver. Turning down carrier gas flow to these values will conserve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



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



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



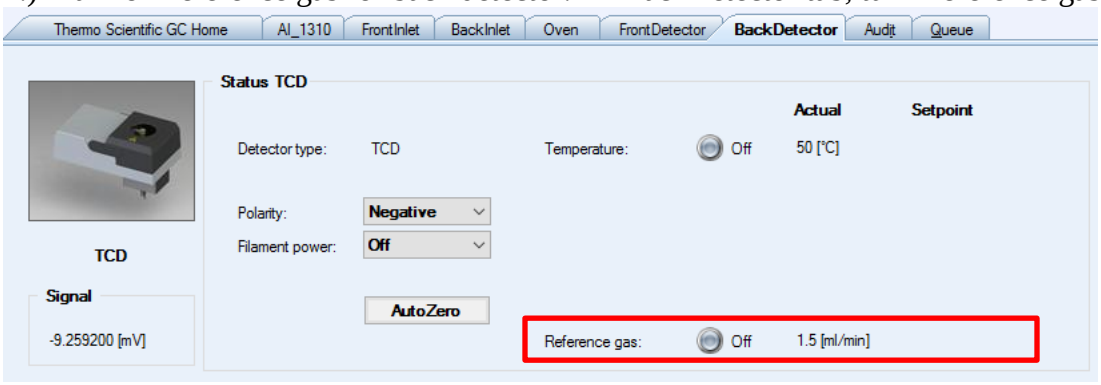
7.) Allow temperature of oven and detectors to cool down to well below 100°C (e.g., 50°C).

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

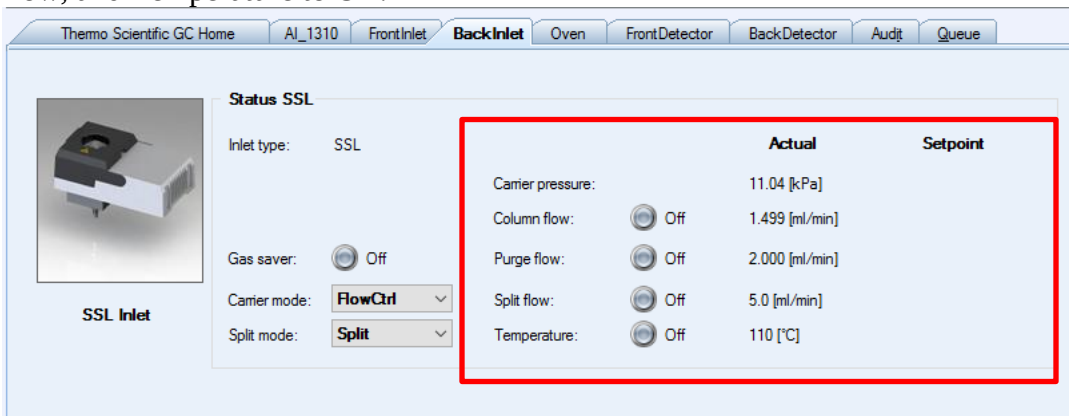
Turning off

1.) Follow all steps for ***Placing back into standby mode***. Verify detectors and oven are well below 100°C before proceeding.

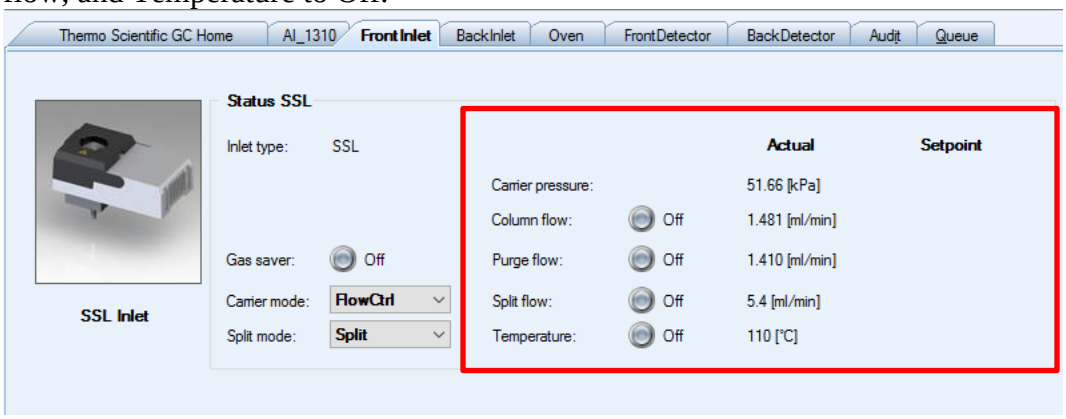
2.) Turn off reference gas for back detector. In BackDetector tab, turn Reference gas to Off.



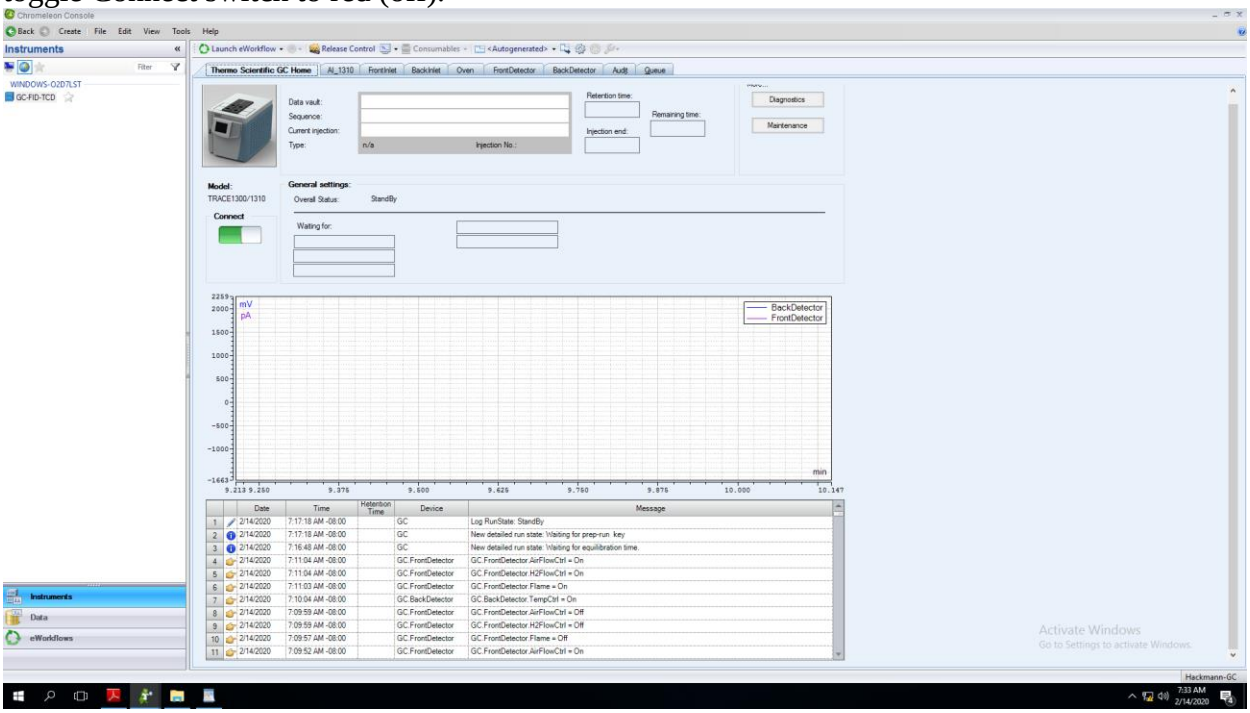
3.) Turn off carrier gas for back inlet. In BackInlet tab, turn Column flow, Purge flow, Split flow, and Temperature to Off.



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

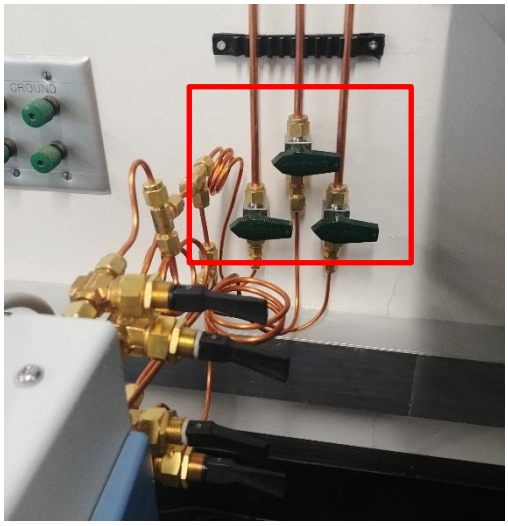


5.) Optional: disconnect Chromleon from instrument. In Thermo Scientific GC Home tab, toggle Connect switch to red (off).



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.

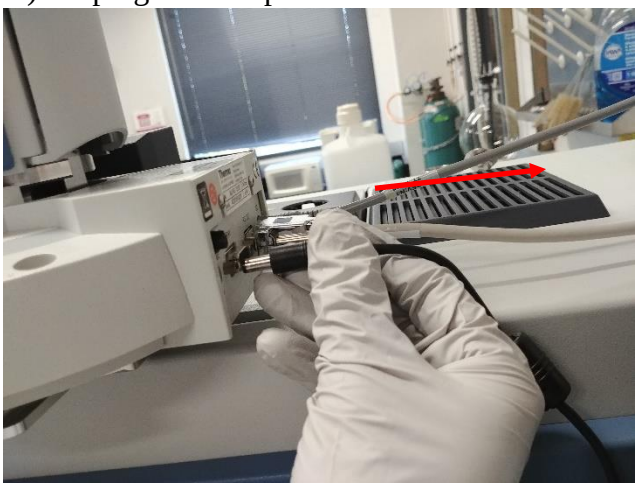
Maintenance of gas chromatograph

Replacing injector septum

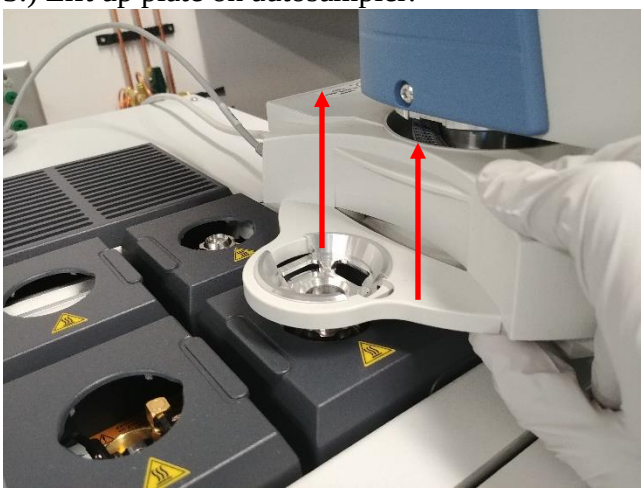
Injector septum will get dirty and should be replaced after a certain number of injections..

1.) Follow steps 1 to 4 for ***Turning off***. Make sure that all components cool down to ≤60°C before proceeding.

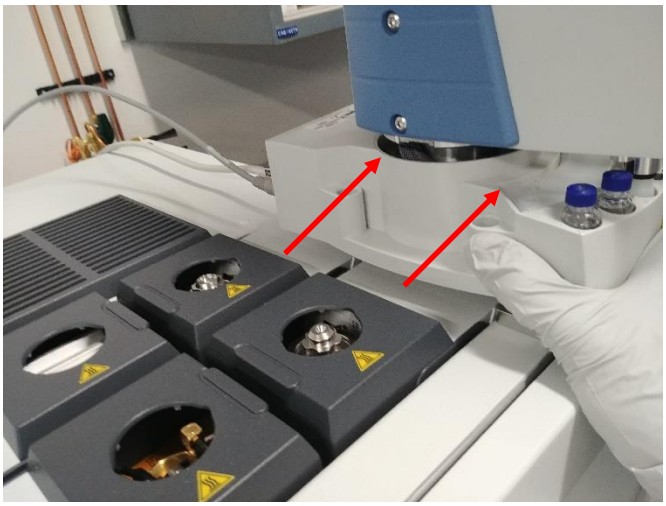
2.) Unplug autosampler.



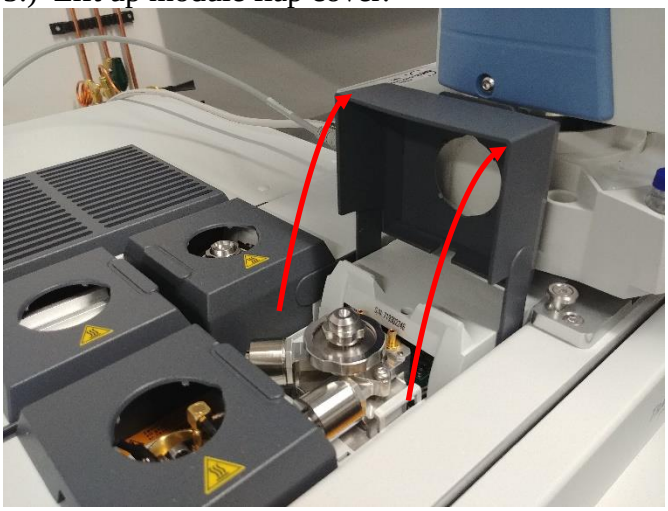
3.) Lift up plate on autosampler.



4.) Push autosampler away from injector.

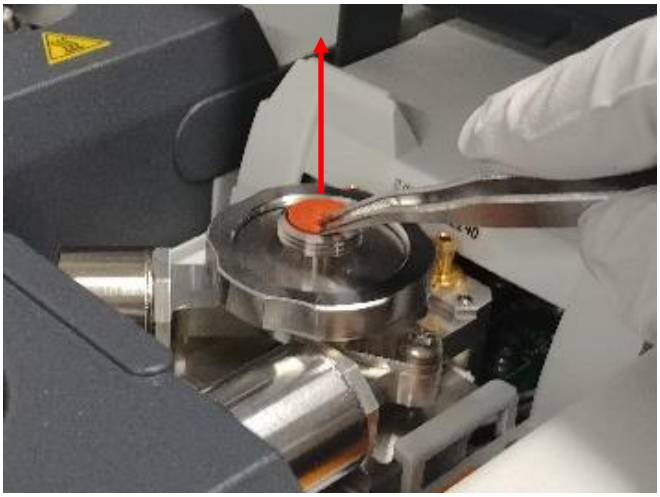


5.) Lift up module flap cover.



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).

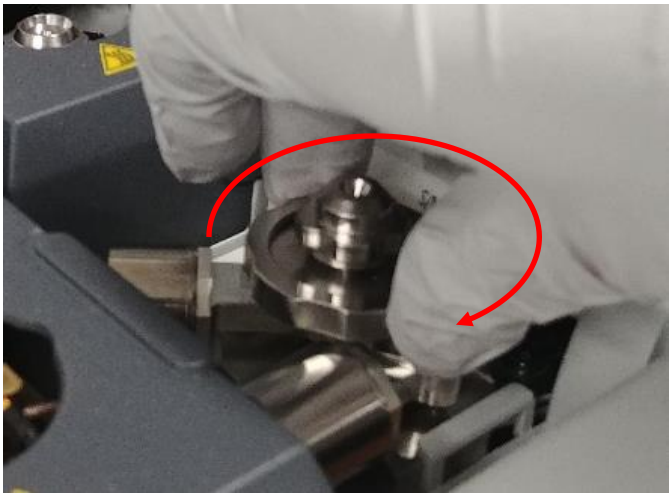
Replacing injector liner

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

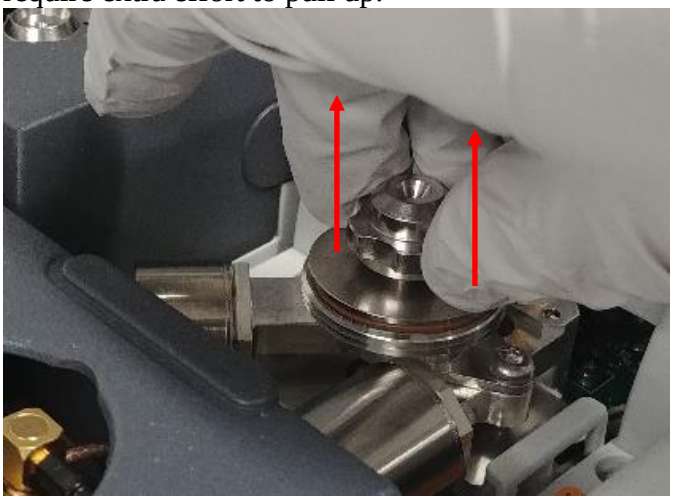
1.) Follow steps 1 to 3 for ***Turning off***. Make sure that all components cool down to ≤60°C before proceeding.

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

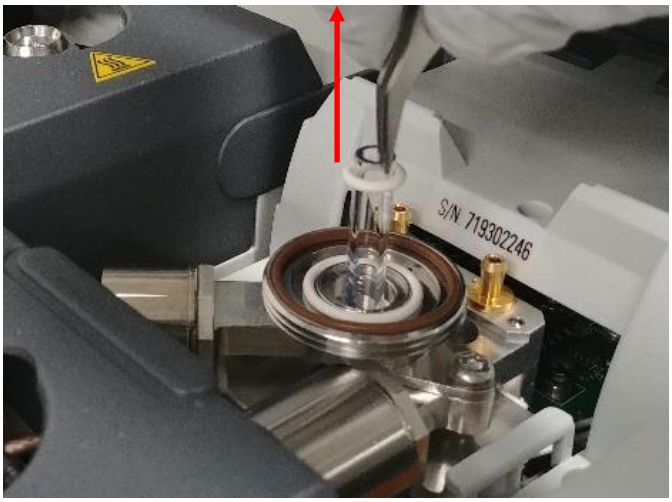
3.) Unscrew and lift up ring nut.



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.



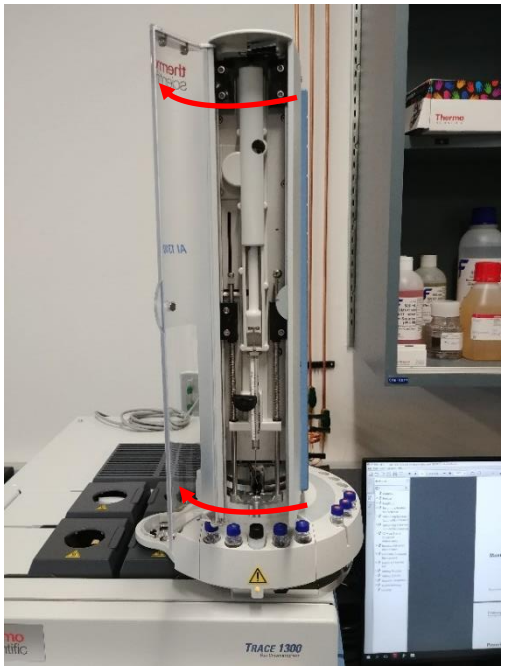
6.) Replace injector liner. Make sure to transfer white o-ring from old liner to new one.

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).

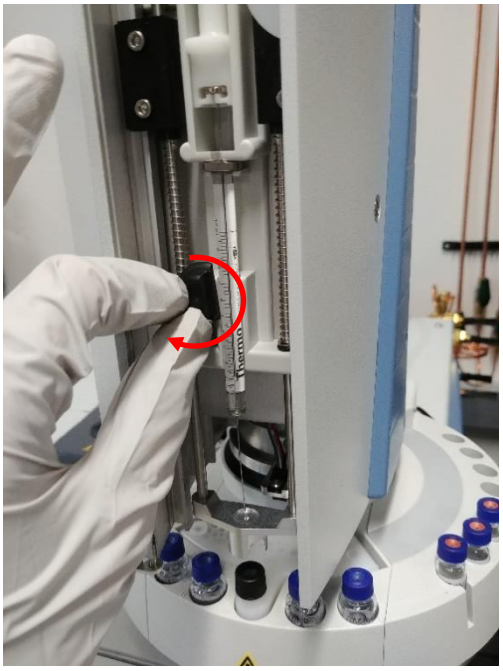
Replacing autosampler syringe

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

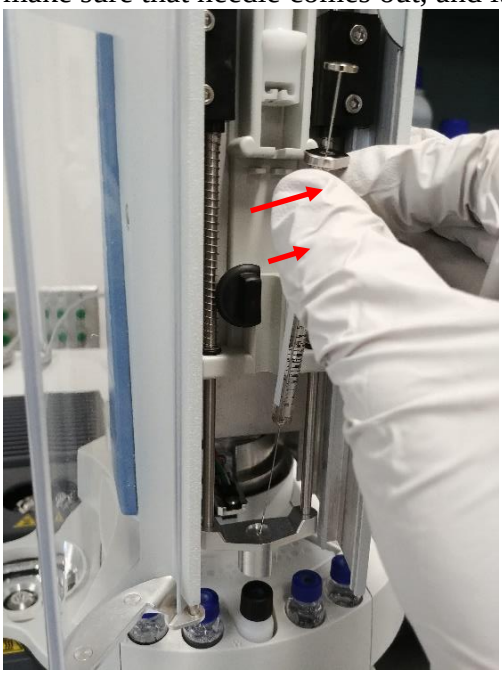
1.) Open door of autosampler.



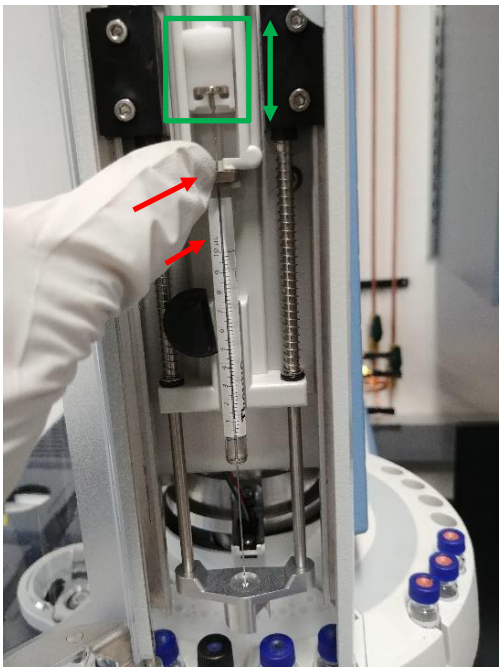
2.) Unlock syringe by turning black half circle clockwise.



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.

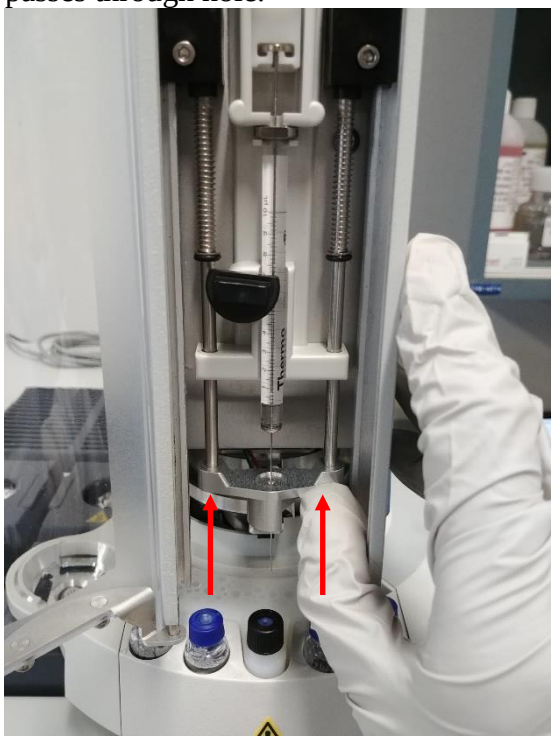


4.) Replace syringe. Slide needle through hole of vial capture device. Line up plunger in plunger head guide, sliding guide up or down by hand as needed. Push back into original position by pushing top of barrel.



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

Compressed gases must be of high purity. Gases routinely used are Airgas NI UHP300 (Ultra High Purity 5.0 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 N_2 and H_2 have purity 99.999% or higher. They should have maximum of 1 ppm each for H_2O, O_2, and THC

Air should have maximum of 1 ppm for THC. Maximum of 1 ppm for H_2O 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 ddH_2O. Store in autoclaved culture bottle with bung (to prevent volatilization). Solution is stable indefinitely at 4ºC.

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 ddH_2O.

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 4ºC.

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

Standard	External standard	ddH_20	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 ddH_2O, and 500 µL of methanol.

5.) Prepare blank samples autosampler vials. To a 2-mL vial, add 500 µL ddH_2O 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.

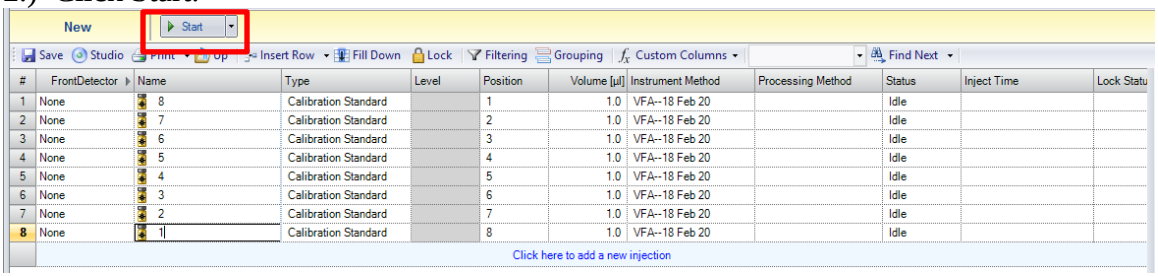
3.) Set up autosampler according to ***Setting up autosampler*** in ***Operation of gas chromatograph*** procedure. Fill wash vial A with 2 mL of ddH_2O and 2 mL methanol (1:1 ratio). Fill vial C with 4 mL ddH_2O and vial D with 4 mL methanol.

4.) In Chromeleon software, create a sequence (list of samples to be analyzed). Follow ***Setting up a sequence*** below.

Analyzing samples and standards

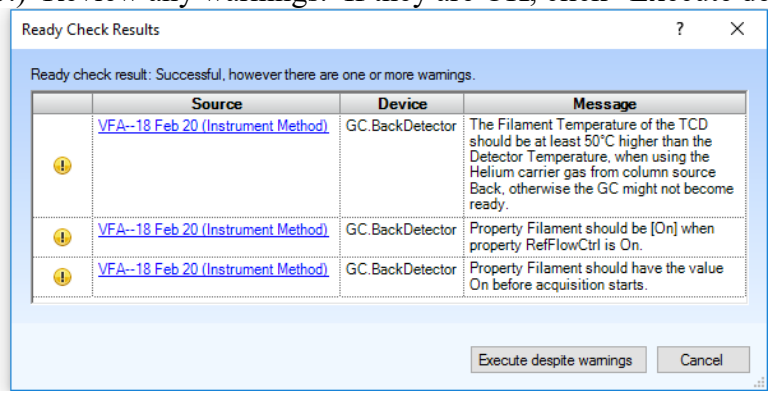
1.) Open a sequence.

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

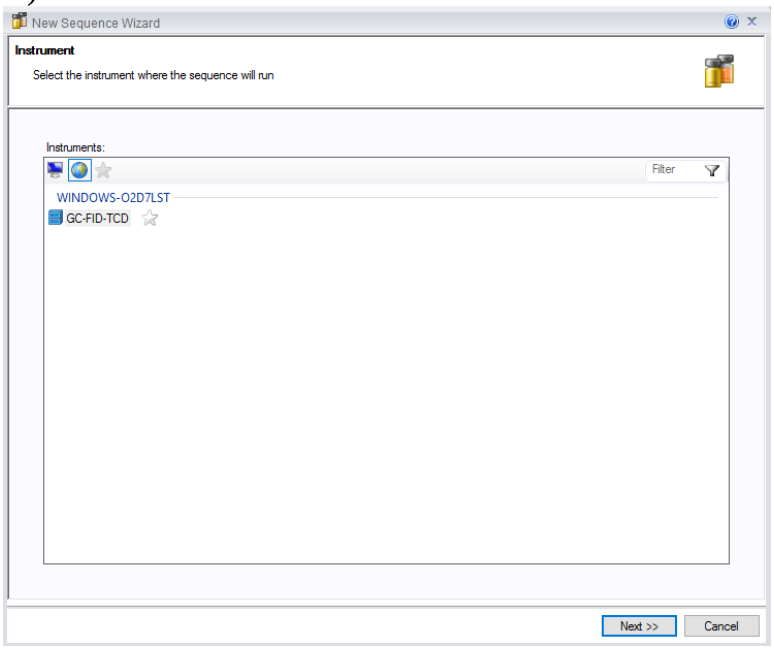
1.) Place the instrument back into standby mode according to ***Placing back into standby mode***.

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

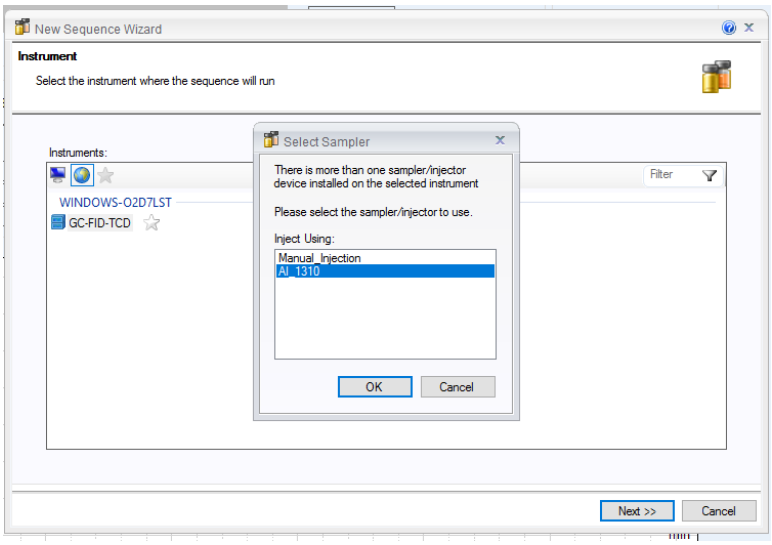
Setting up a sequence

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

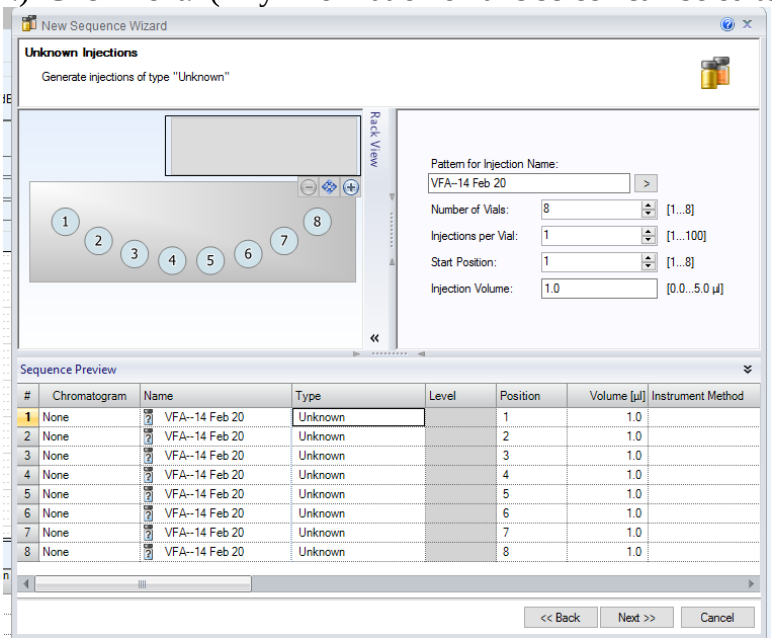
2.) Choose GC-FID-TCD.



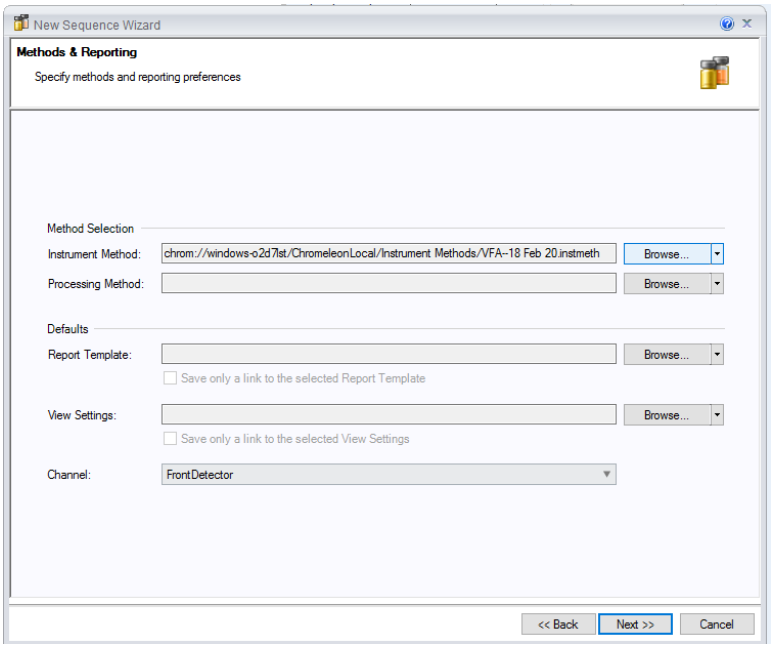
3.) Choose AI_1310 (the autosampler).



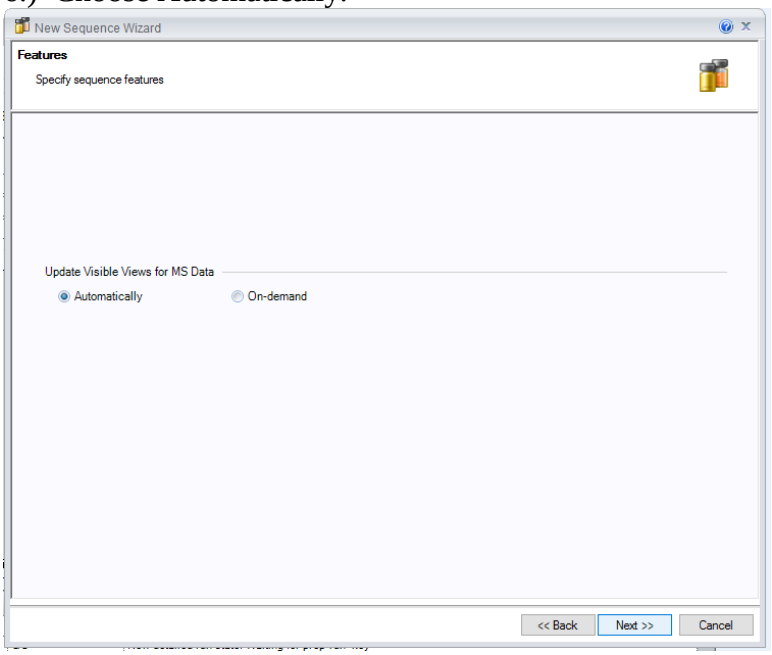
4.) Click Next. (Any information on this screen can be edited later.)



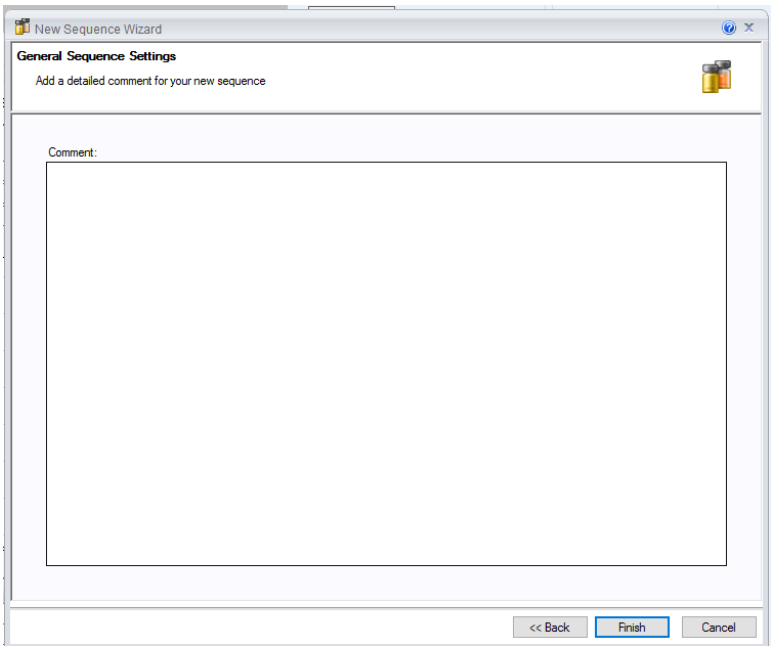
5.) Under Instrument Method, choose "VFA—18 Feb 20.instmethod". See details for this method under ***Setting up a method***.



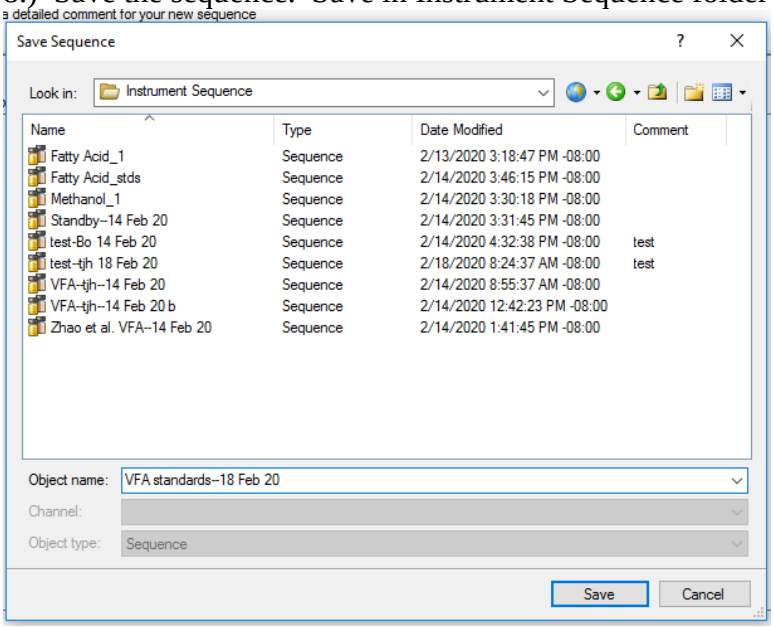
6.) Choose Automatically.



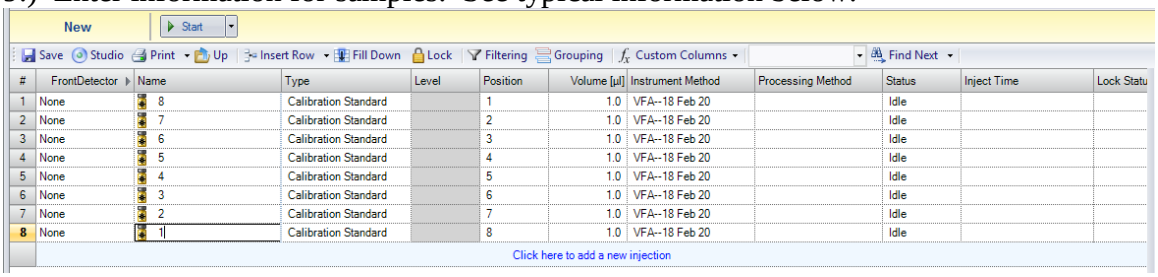
7.) Enter comments (optional).



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

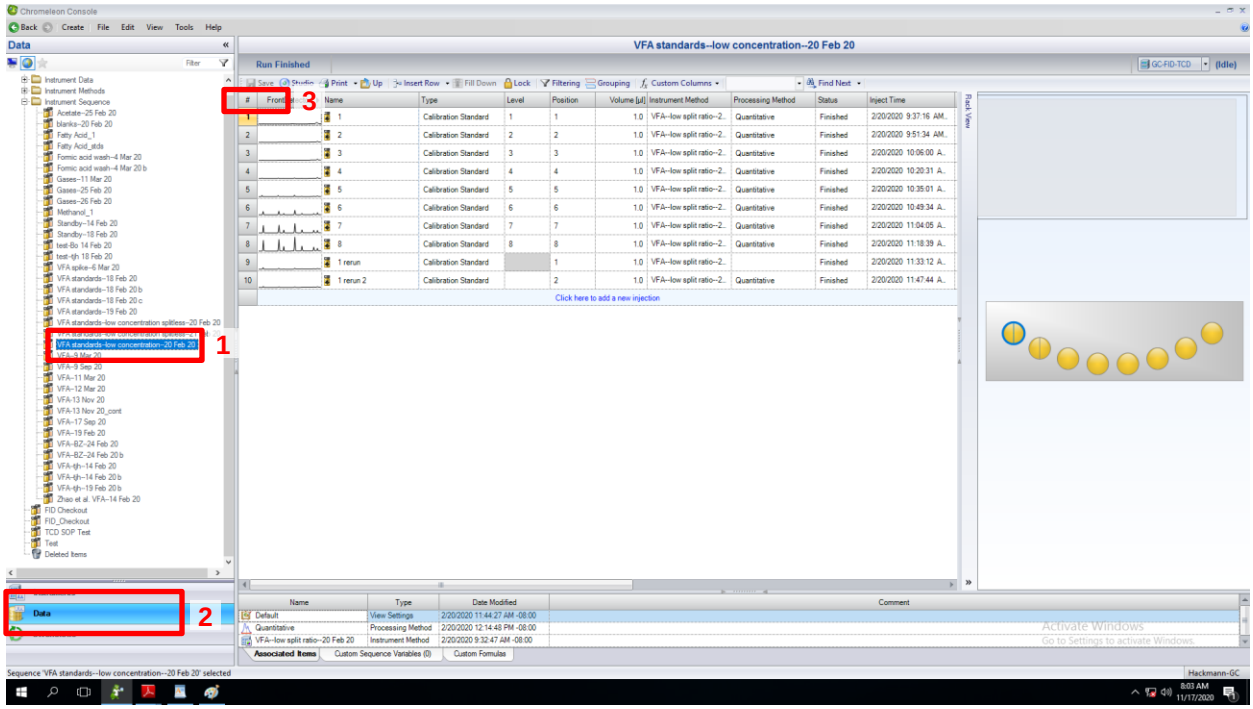


9.) Enter information for samples. See typical information below.

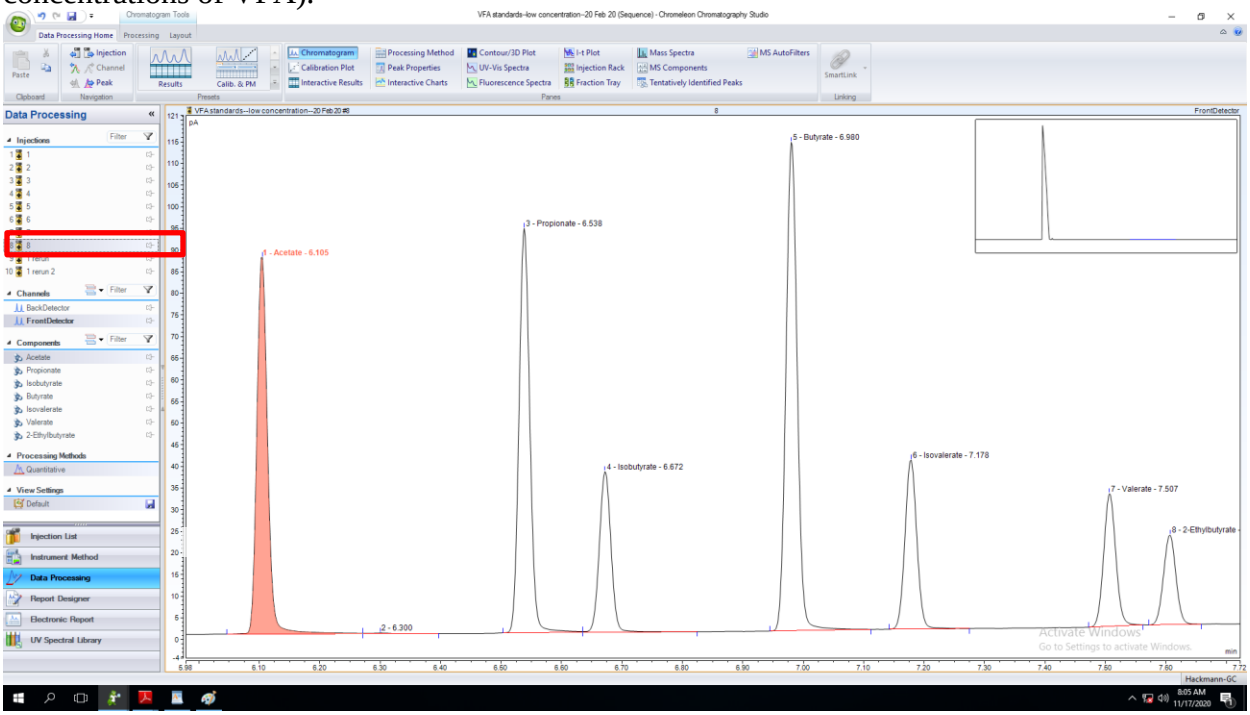


Constructing a standard curve and quantifying VFA concentrations

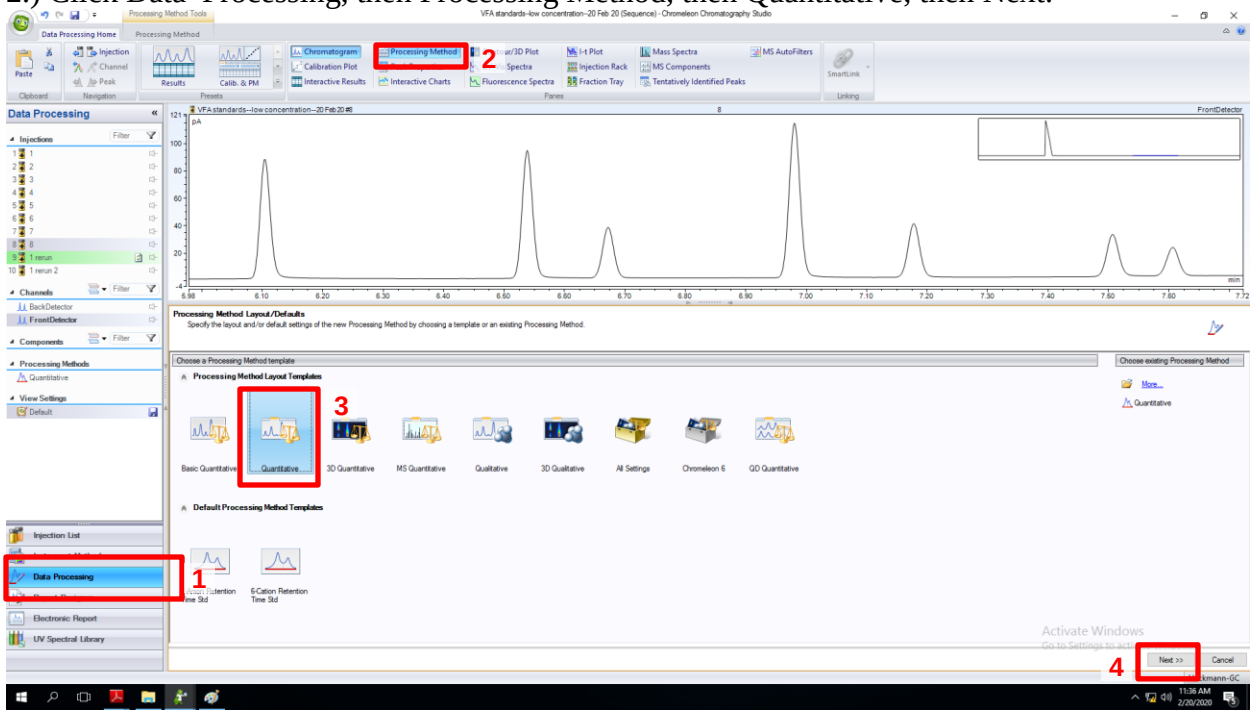
1.) In Chromeleon 7 software, click on the sequence that should be analyzed. Click on Data, then click on Studio.



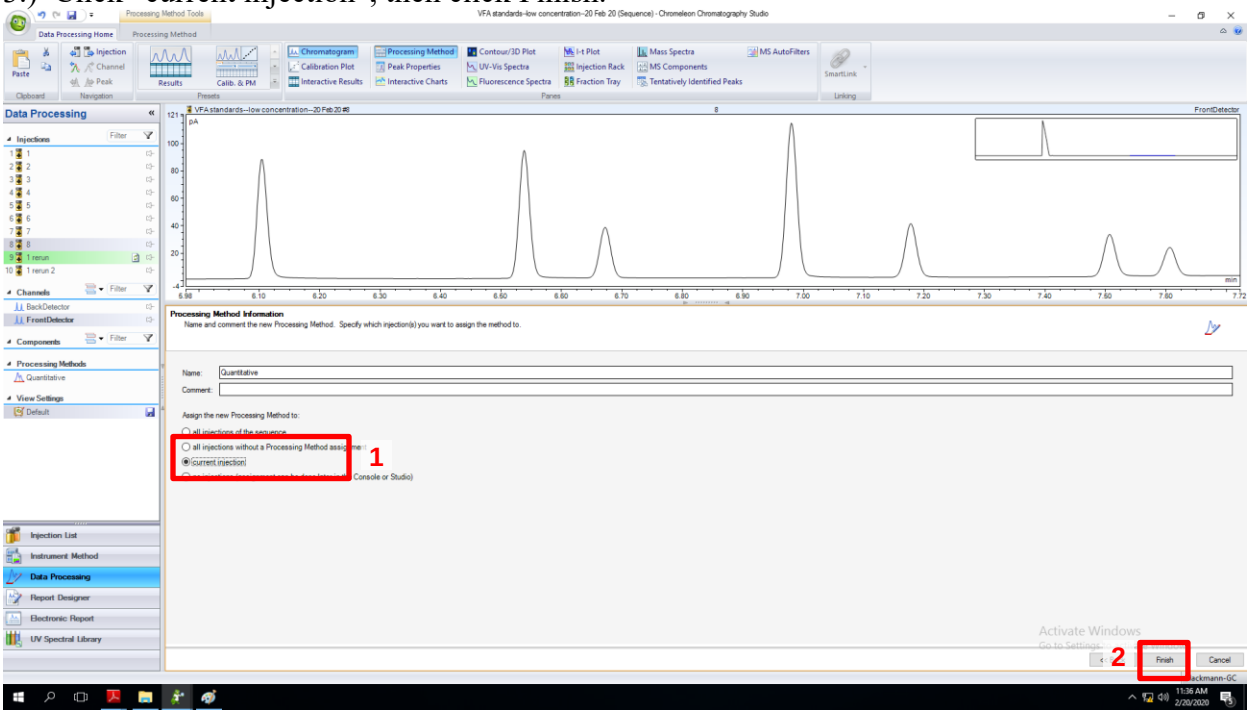
2.) In the window that appears, click on an injection of the last standard (the one with the highest concentrations of VFA).



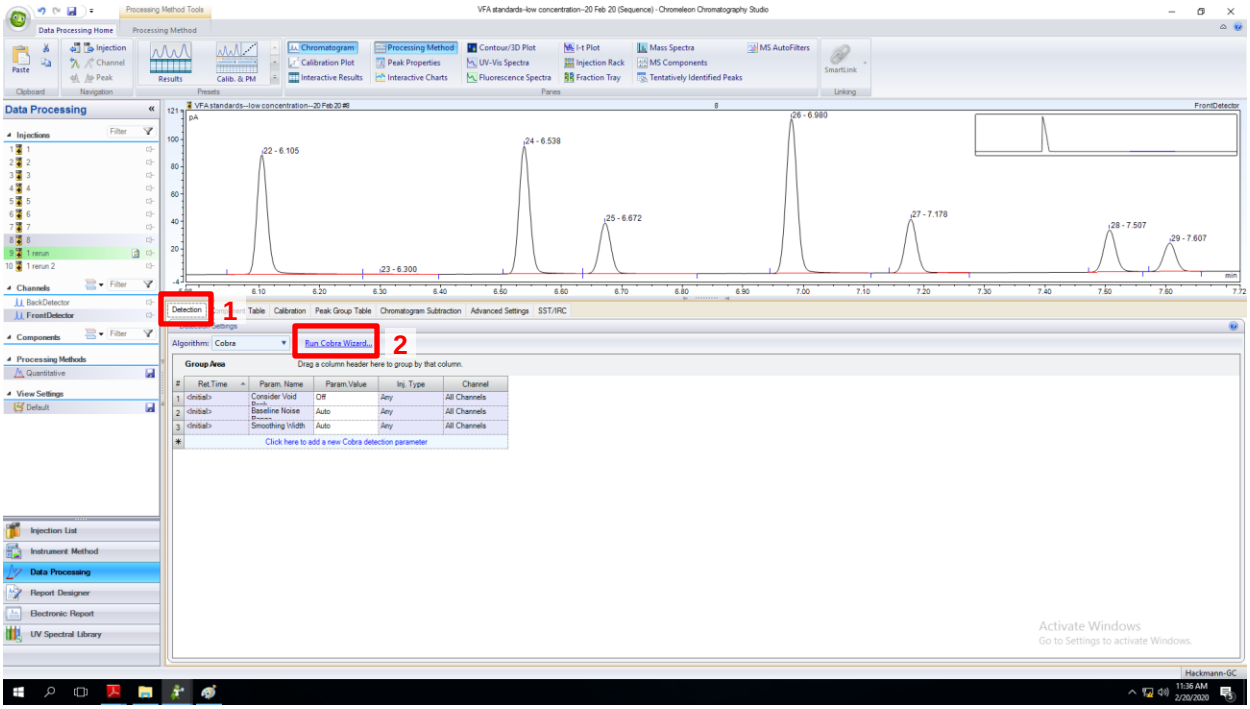
2.) Click Data Processing, then Processing Method, then Quantitative, then Next.



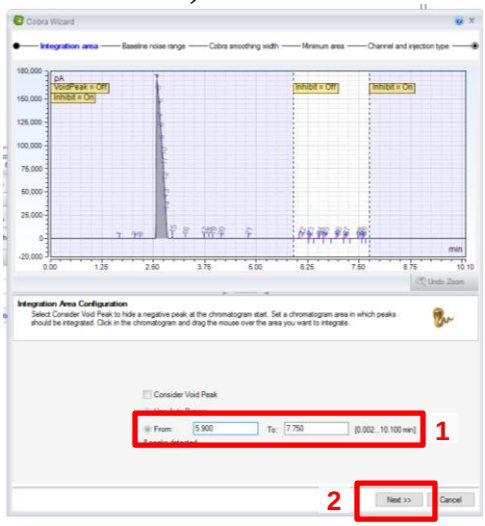
3.) Click “current injection”, then click Finish.



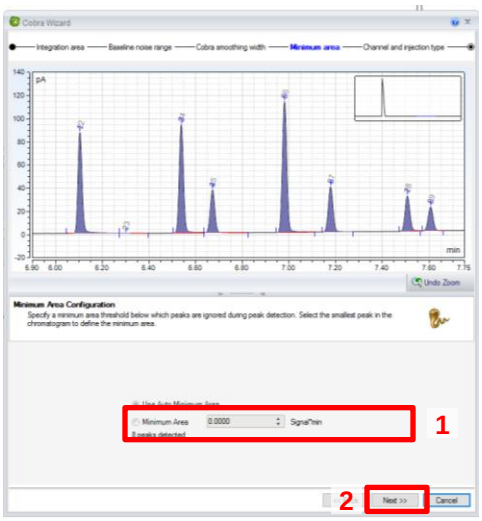
4.) Click Detection, then Click Run Cobra Wizard.



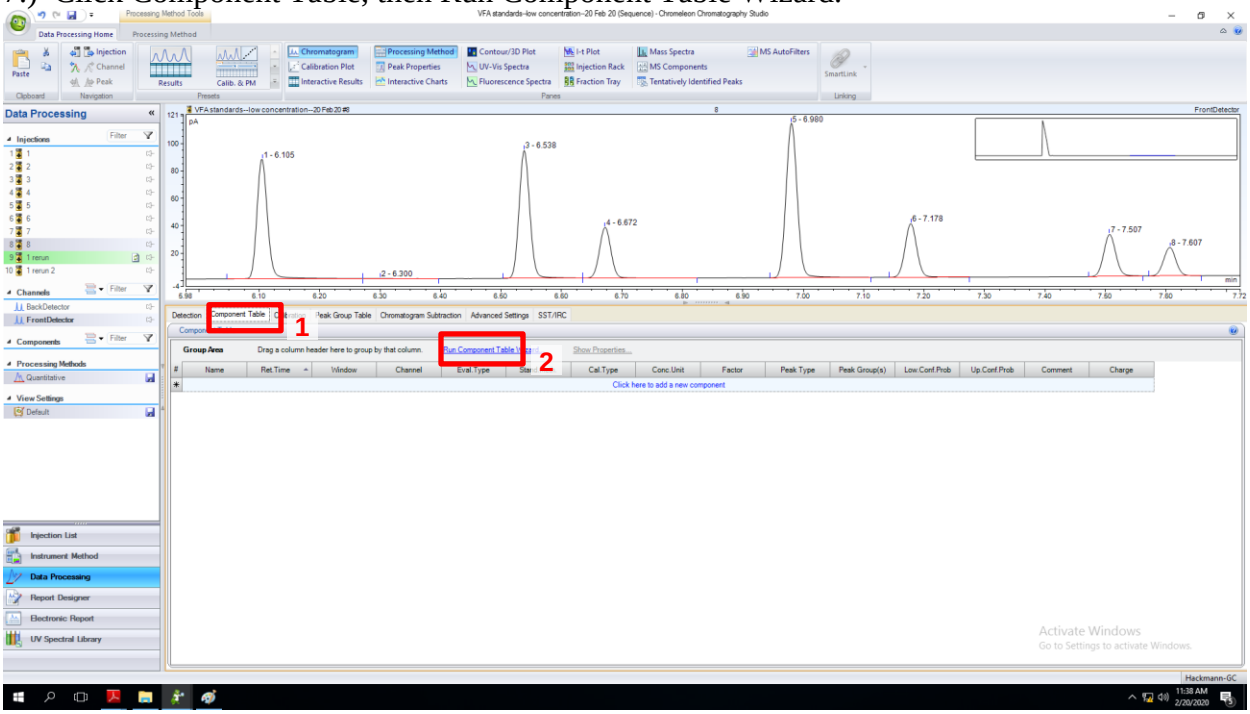
5.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next.



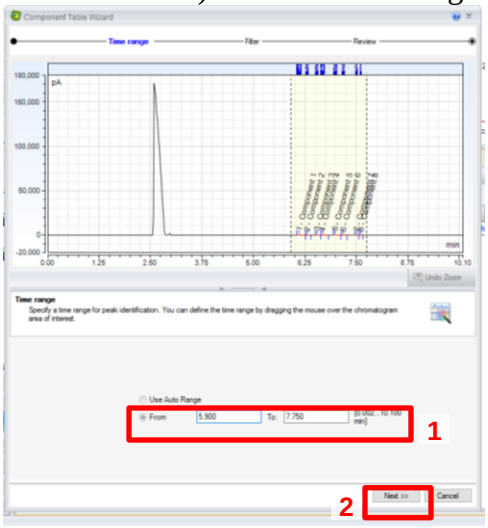
6.) Continue to click Next until reaching window below. If unexpected, small peaks are detected, enter a value of Minimum Area to suppress these small peaks. Otherwise, click Next, then click Finish.



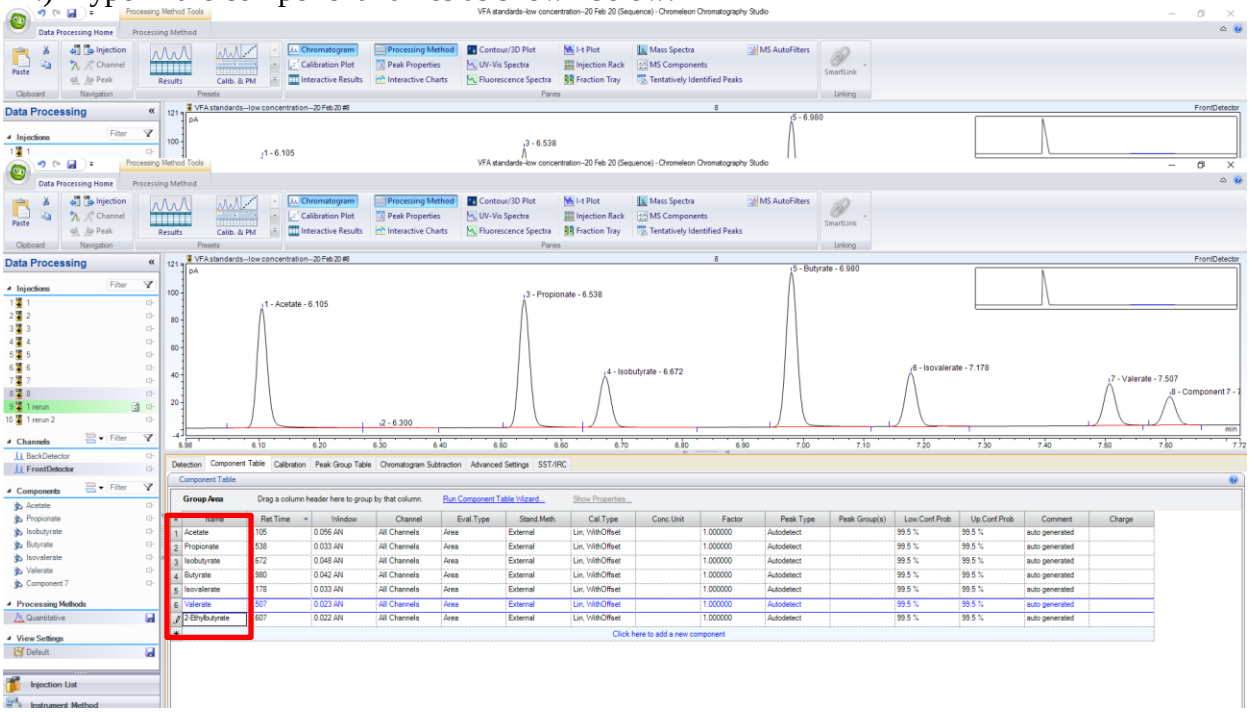
7.) Click Component Table, then Run Component Table Wizard.



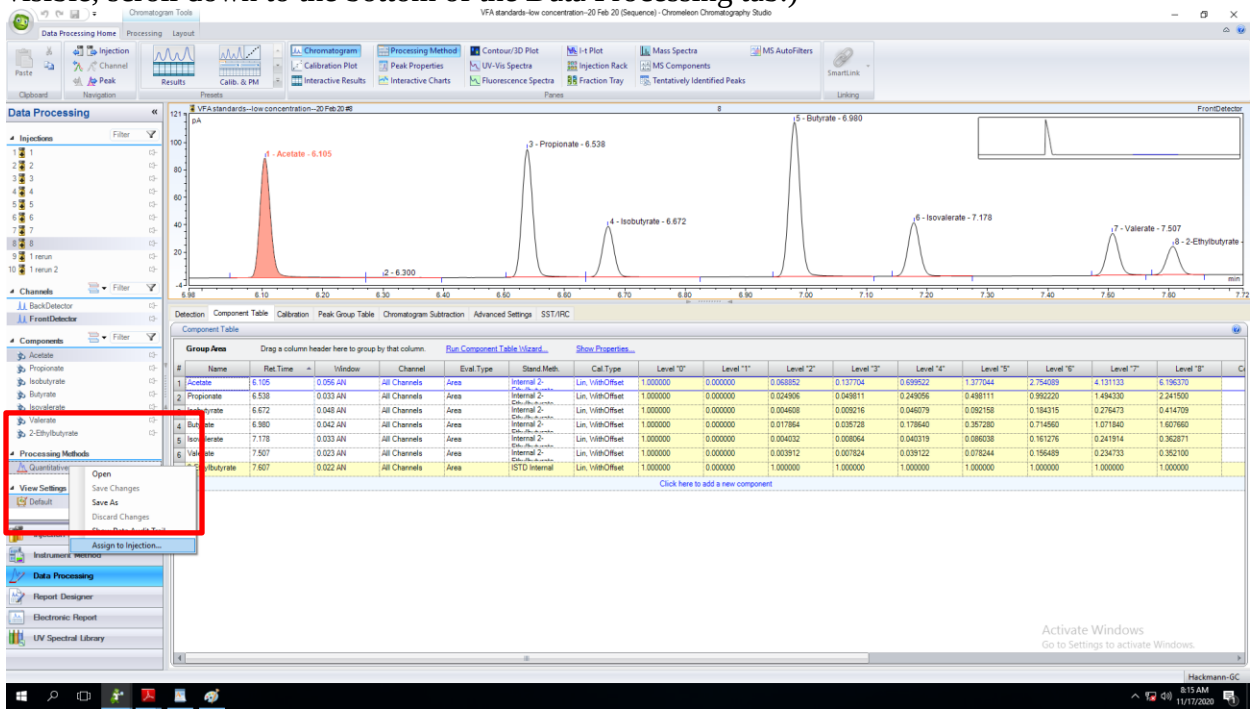
11.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next through all subsequent windows, then click Finish.



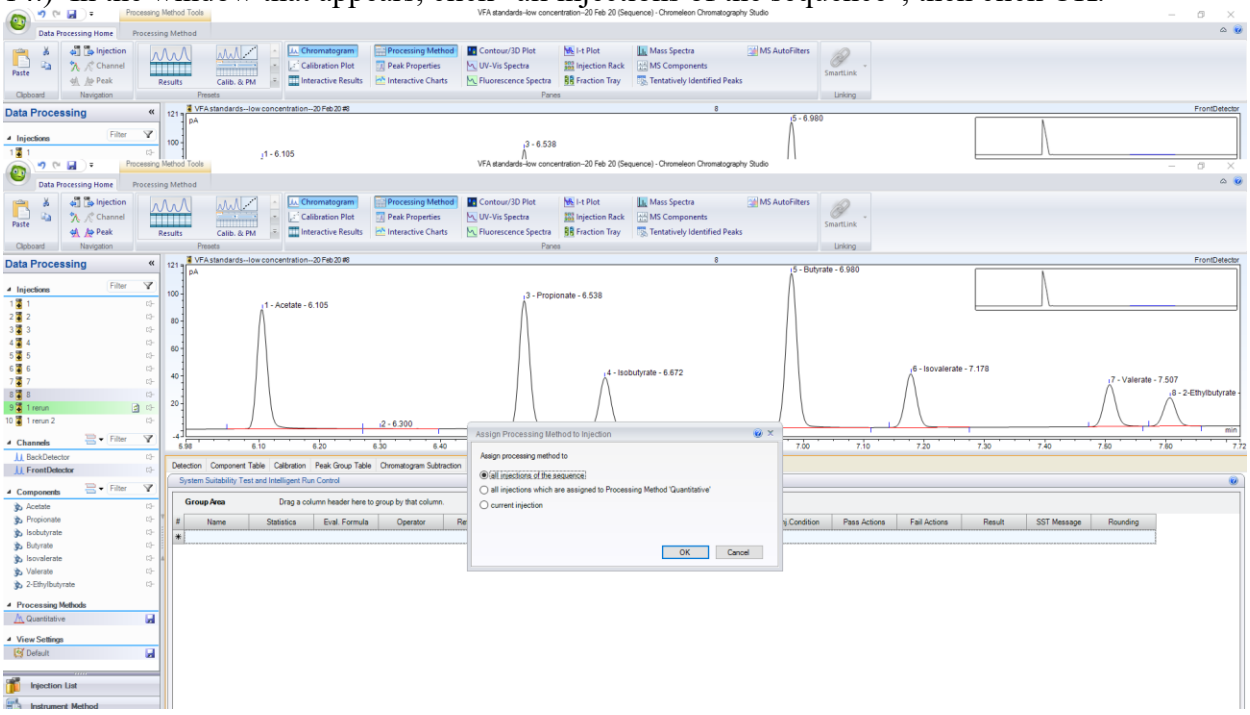
12.) Type in the component names as shown below.



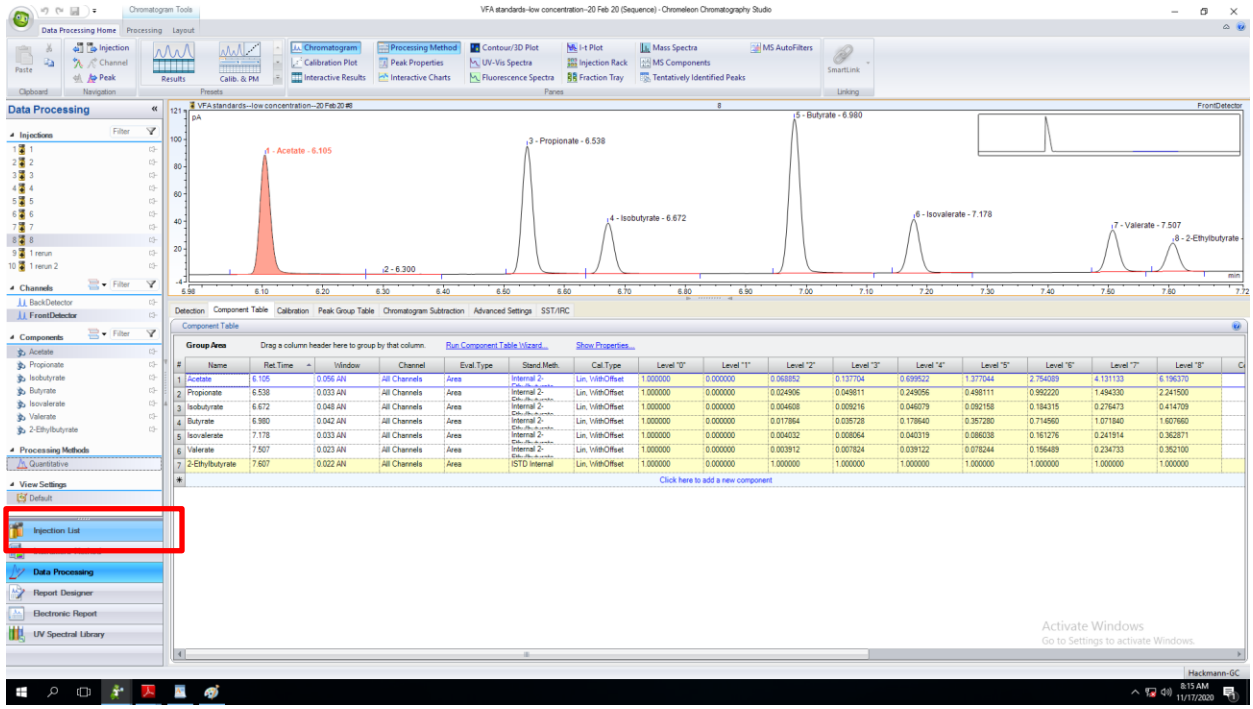
13.) Right click Processing Method, then click Assign to Injection. (If Processing Method is not visible, scroll down to the bottom of the Data Processing tab.)



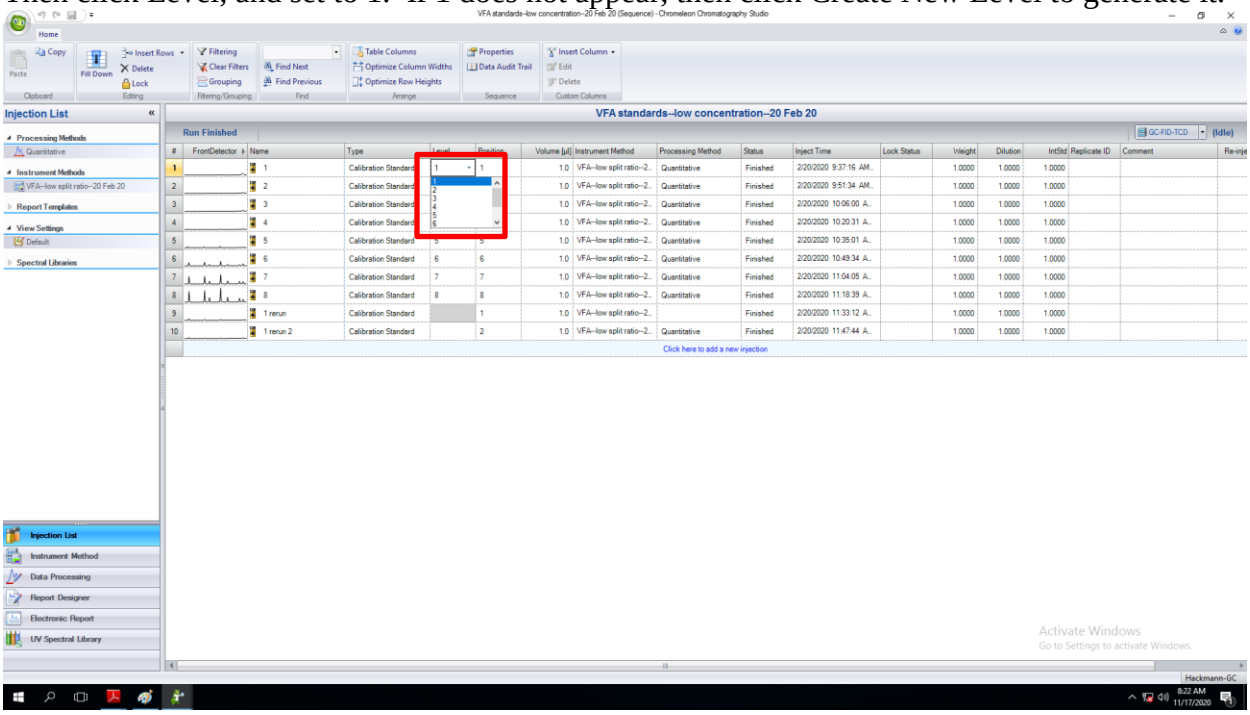
14.) In the window that appears, click “all injections of the sequence”, then click OK.



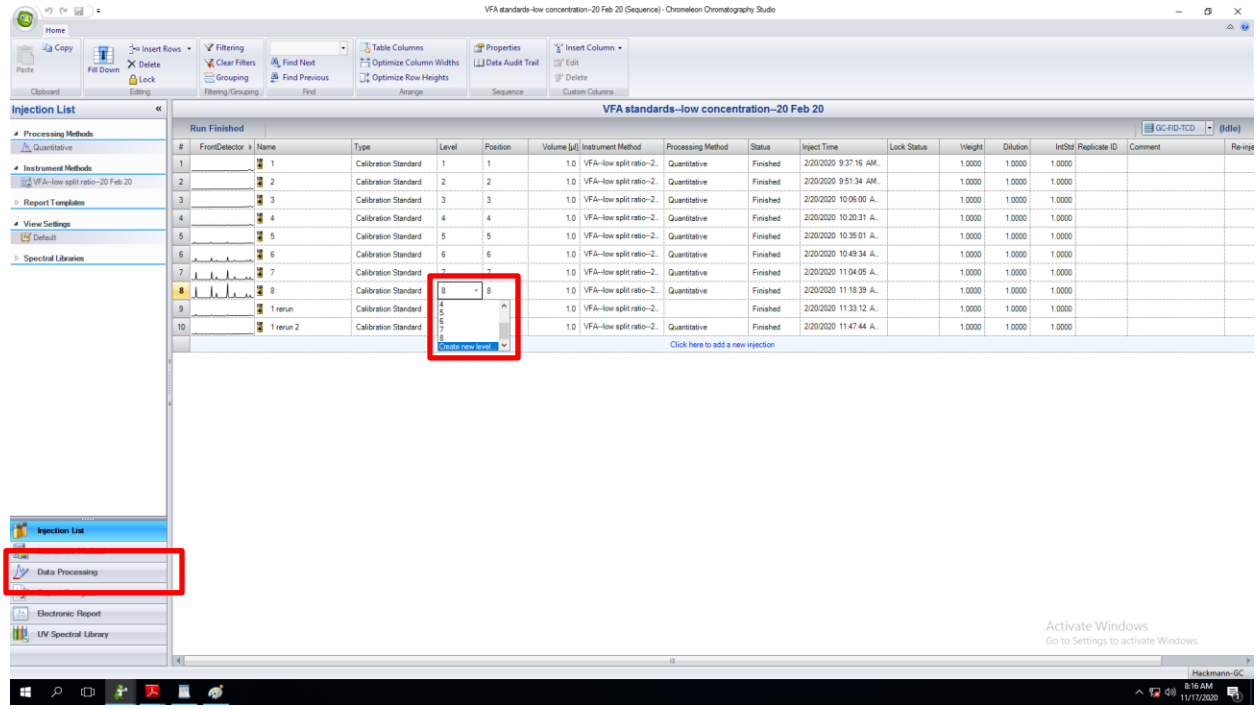
15.) Click Injection List.



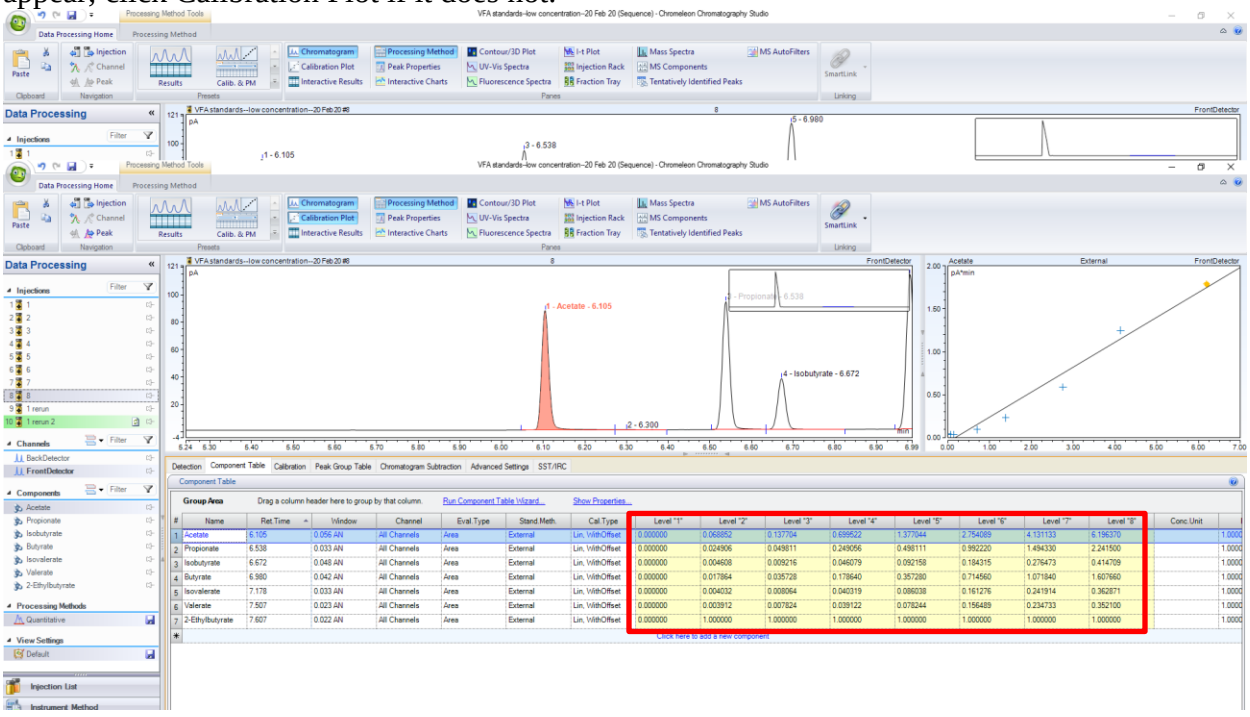
16.) Click on the injection for the first standard (the one with the lowest concentrations of VFA). Then click Level, and set to 1. If 1 does not appear, then click Create New Level to generate it.



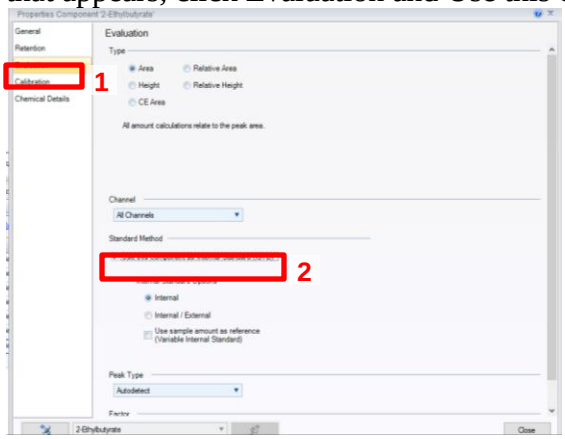
17.) Repeat for the second standard, but set Level to 2. Repeat similarly for all other standards. Go back by clicking Data Processing.



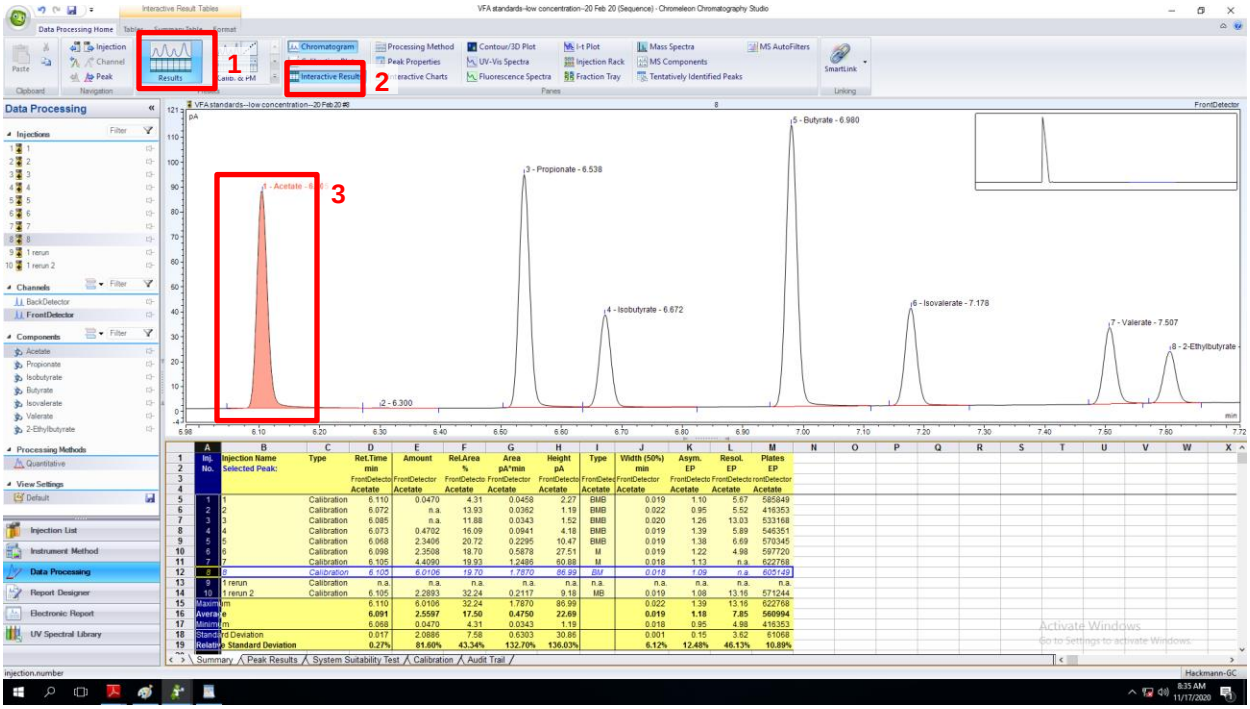
18.) Under Level, type in concentrations of standards (in mmol/L). Standard curve should appear; click Calibration Plot if it does not.



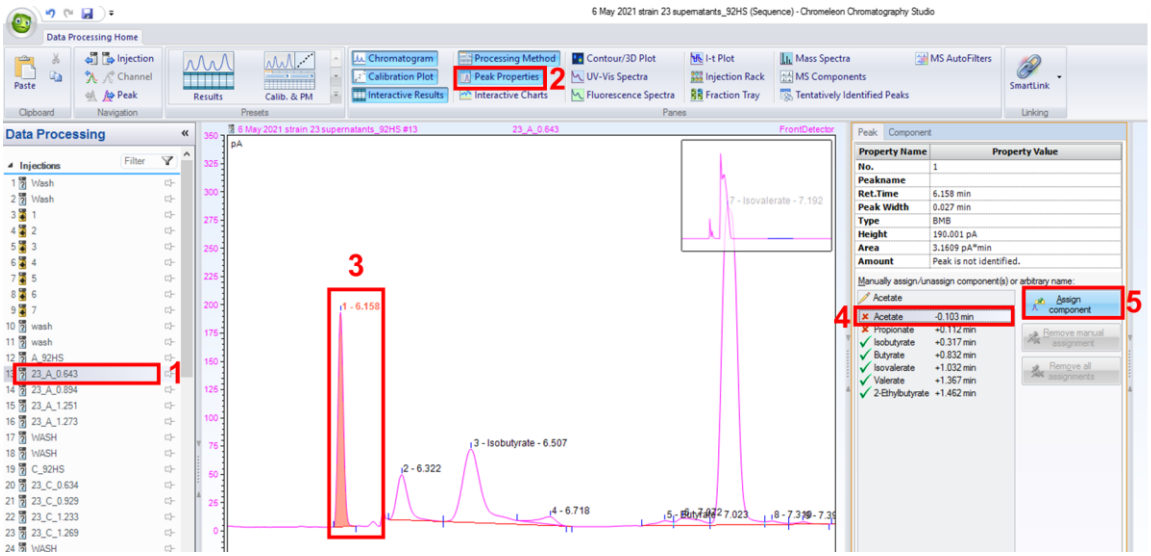
20.) Optional: set 2-ethylbutyrate as an internal standard by double clicking on it. In window that appears, click Evaluation and Use this component as Internal Standard (ISTD).



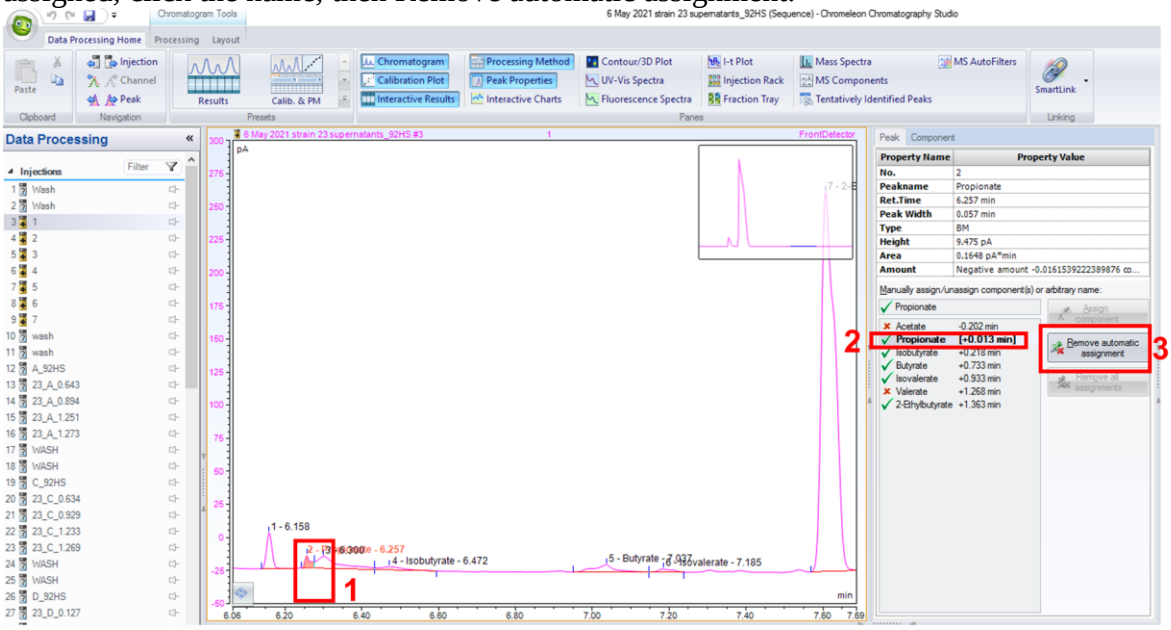
21.) Click Results, then Interactive Results, and then the acetate peak in the chromatogram. The concentration of VFA is reported under Amount. Click the remaining peaks to see their concentrations.



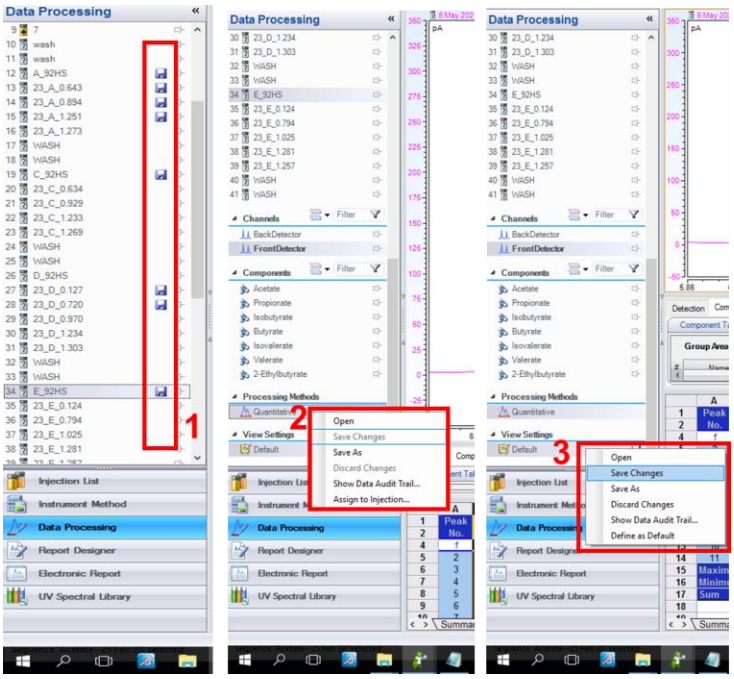
22.) Optional: Rename any peaks that are misidentified by the software. Click the sample, Peak Properties, the misidentified peak, the correct name, then Assignment component.



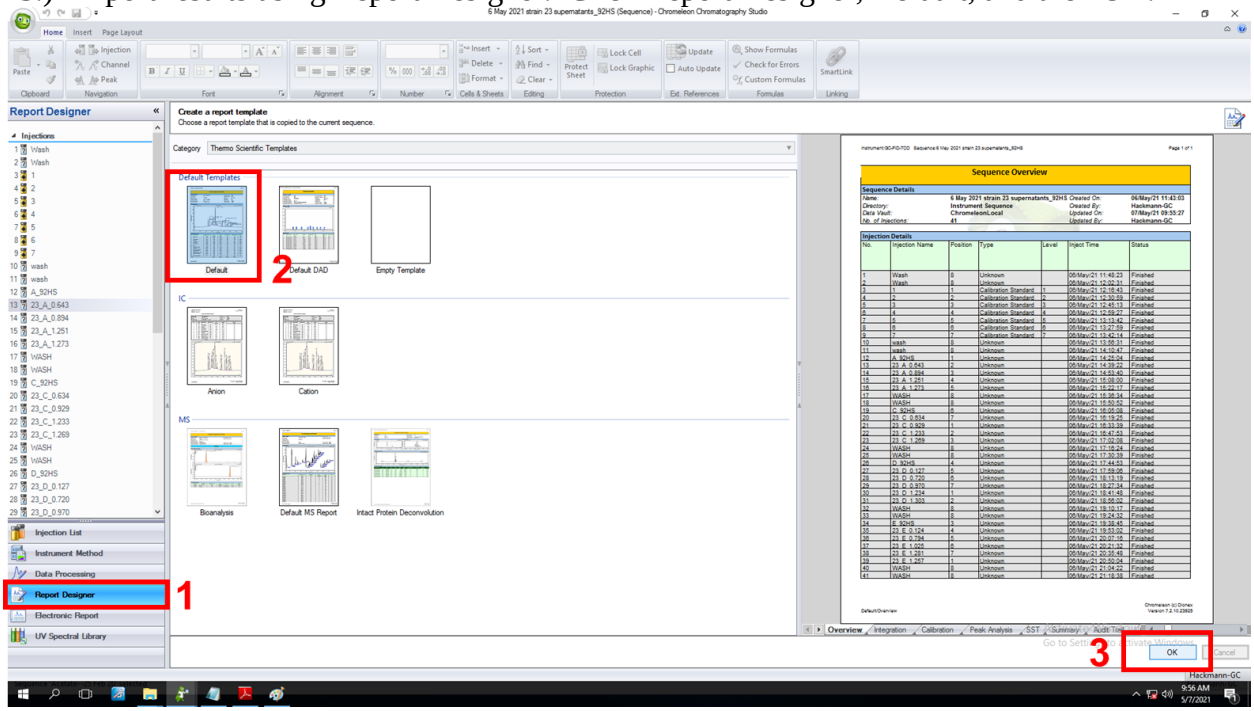
22.) Optional: Remove any peak assignments that are spurious. Click the peak that is wrongly assigned, click the name, then Remove automatic assignment.



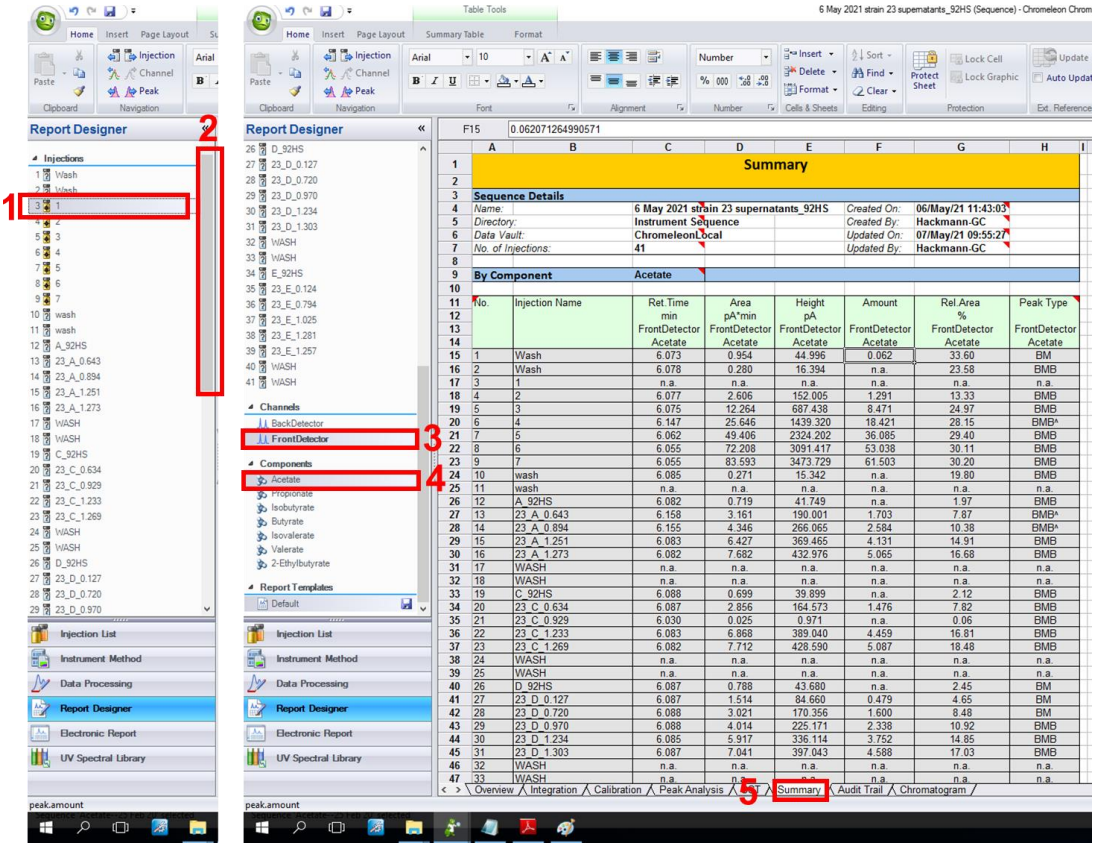
24.) Save all changes.



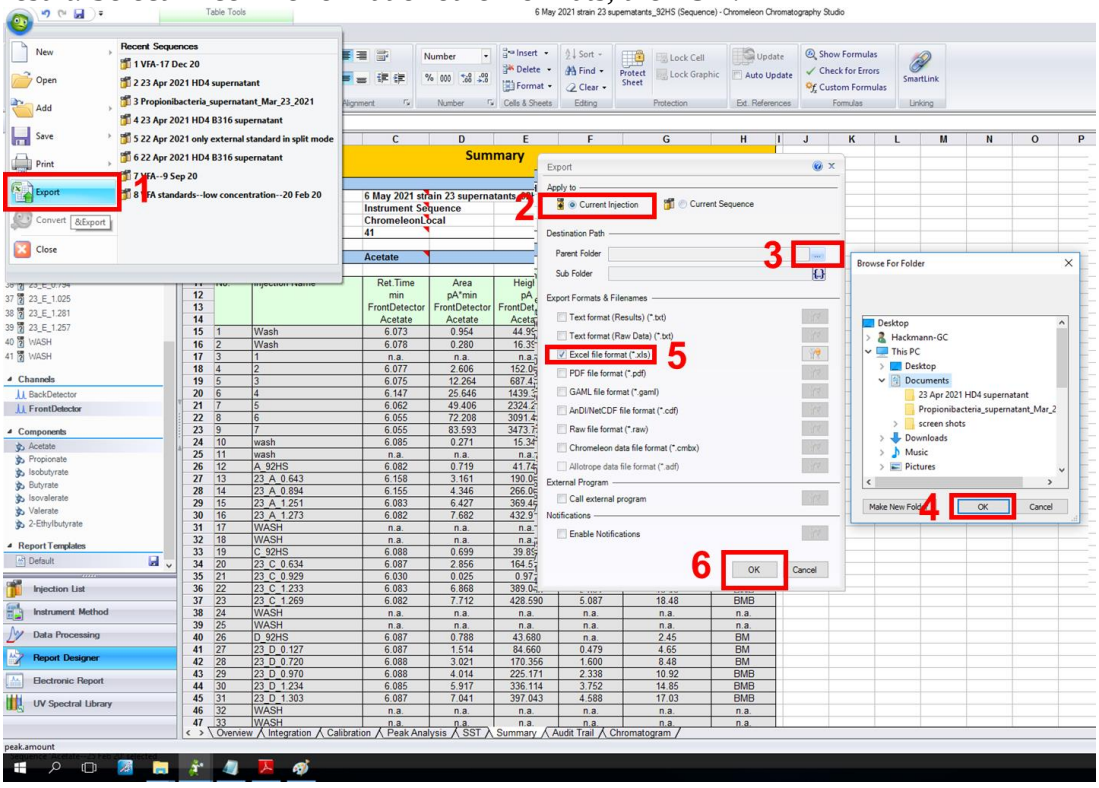
25.) Export results using Report Designer. Click Report Designer, Default, and then OK.



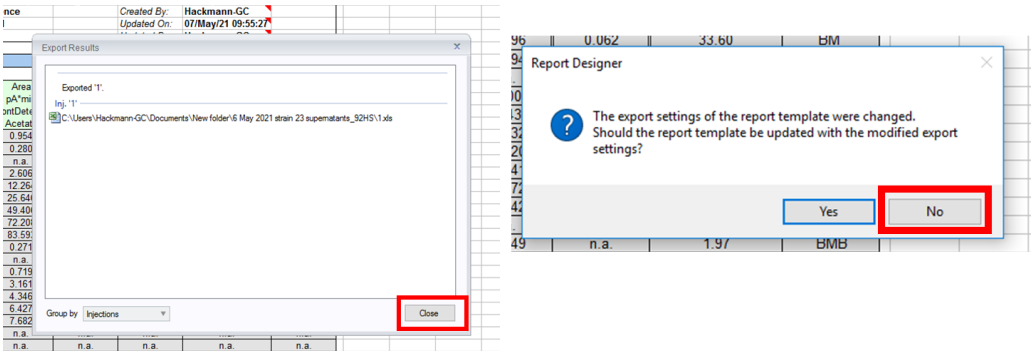
26.) In the window that appears, click any injection. Scroll down, Click FrontDector in Channels; click Acetate in Components; then click Summary.



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.

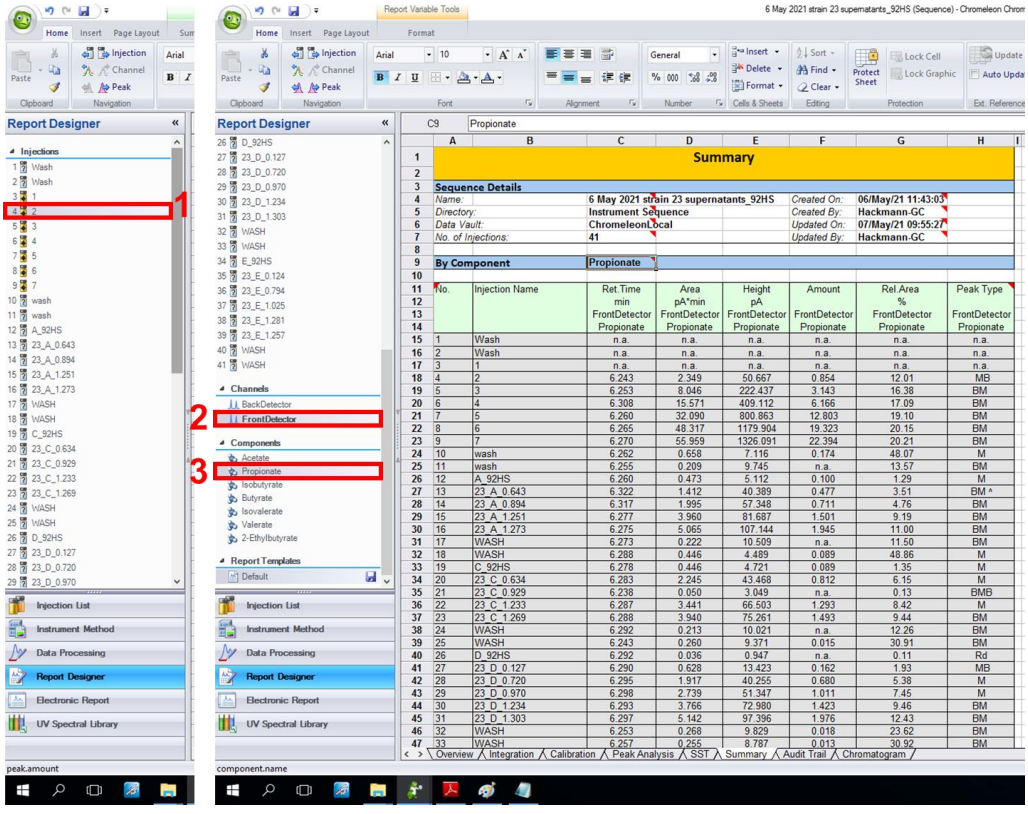


28.) In the window that appears, select Close. Then select No update.

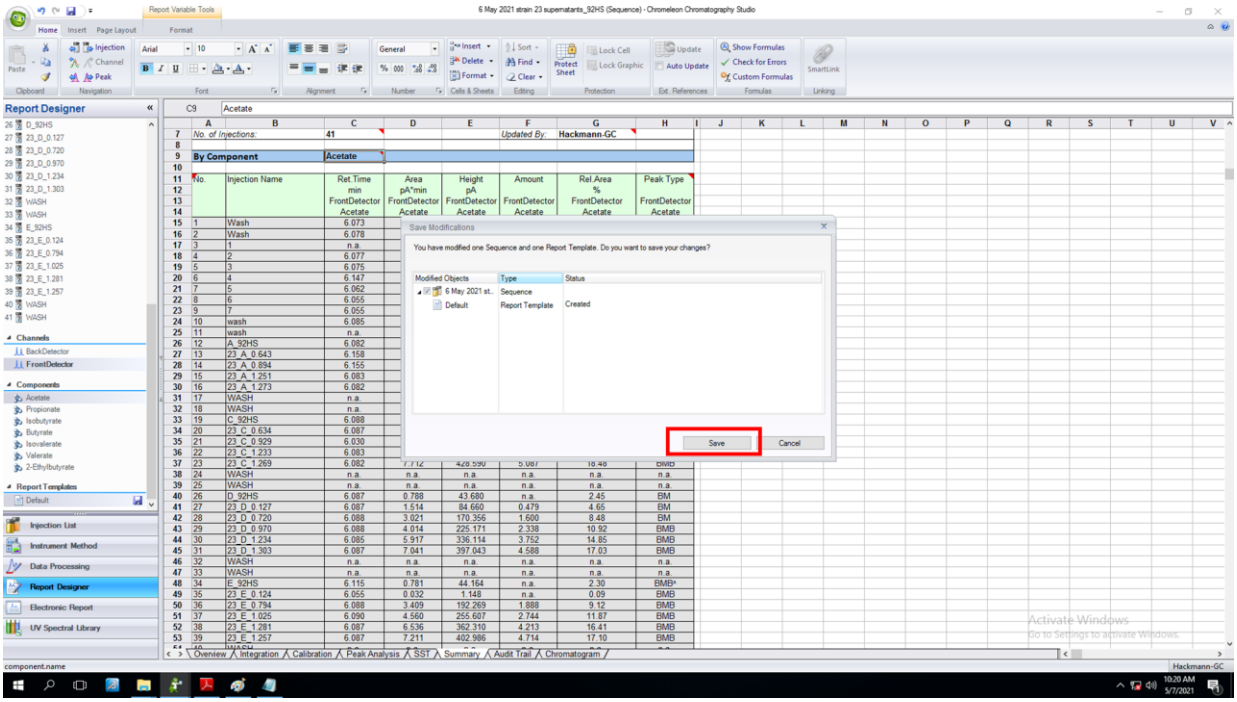


29.) Alternatively, instead of following step 7 and 8, select the value shown in the sheet summary of step 6, copy the value to Wordpad or Excel for analysis.

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.



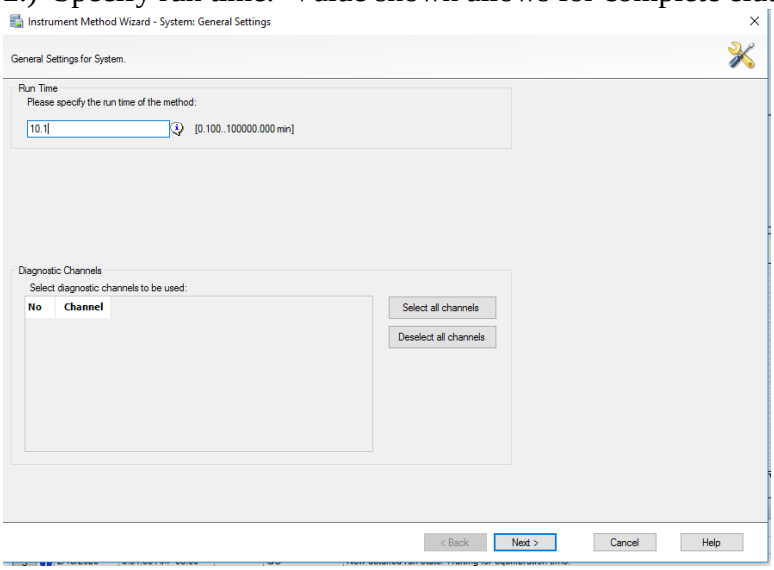
31.) Save the changes.


6 May 2021 strain 23 supernatants_92HS (Sequence) - Chromeleon Chromatography Studio
Report Designer
Save Modifications
You have modified one Sequence and one Report Template. Do you want to save your changes?
Modified Objects
Type
Status
6 May 2021 st...
Sequence
Default
Report Template
Created
Save
Cancel
Activate Windows
Go to Settings to activate Windows.

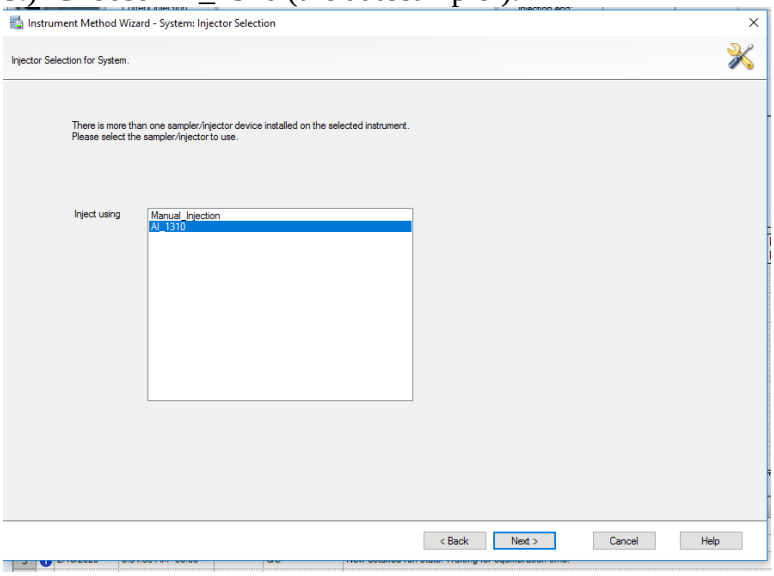
Setting up a method

1.) Go to Create -> Instrument Method.

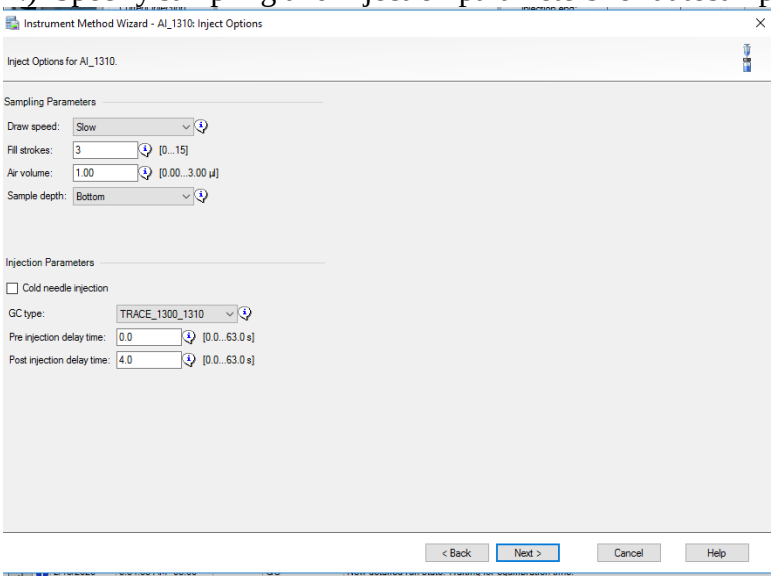
2.) Specify run time. Value shown allows for complete elution of VFAs.



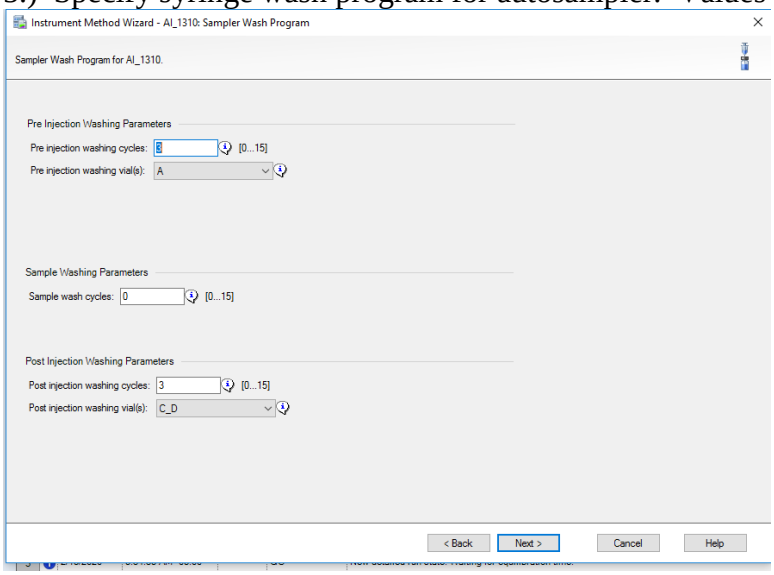
3.) Choose AI _1310 (the autosampler).



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

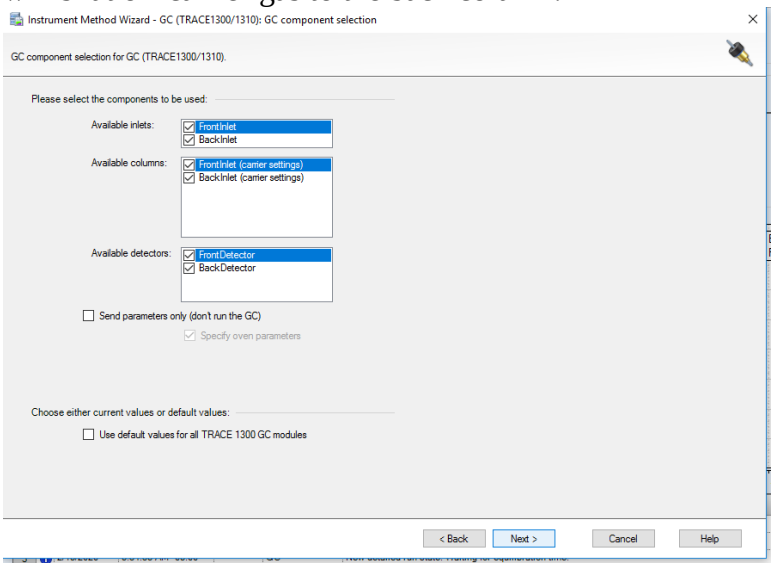


5.) Specify syringe wash program for autosampler. Values shown are typical.

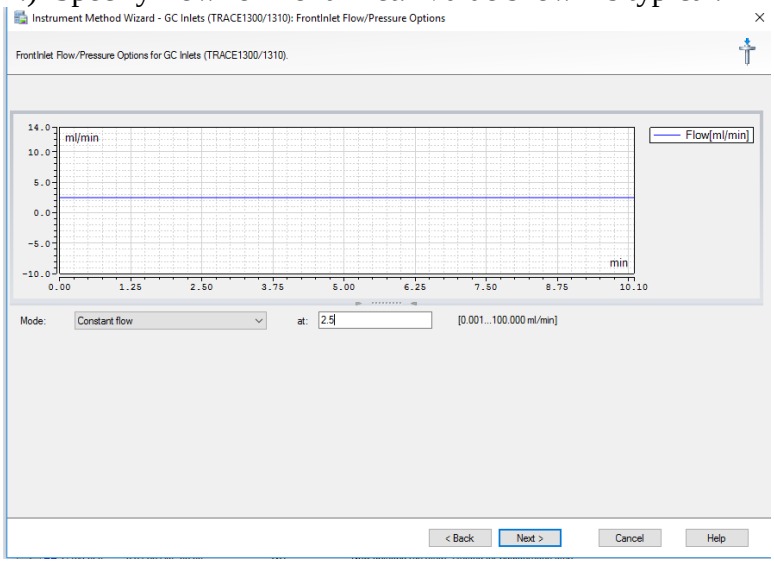


6.) Select all GC components. Even though not all components are used for VFA analysis, they still need to be selected to avoid damaging the instrument. Unselecting BackInlet, for example,

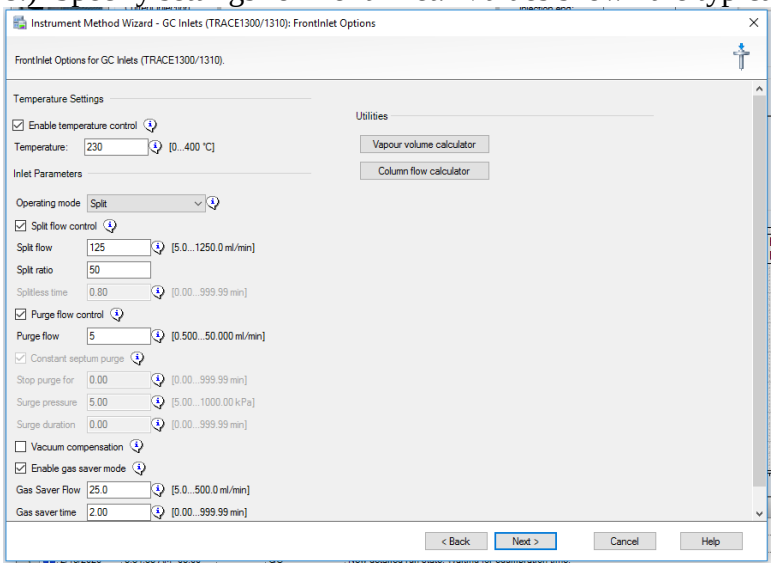
will shut off carrier gas to the back column.



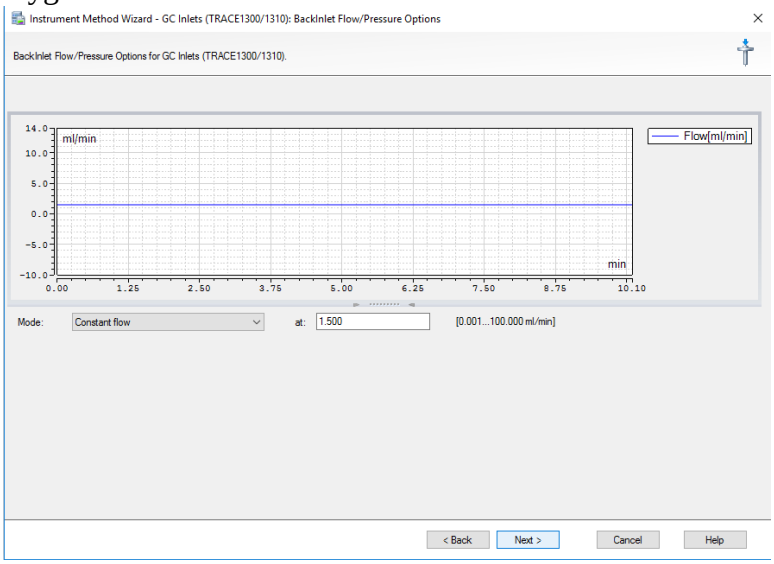
7.) Specify flow for front inlet. Value shown is typical.



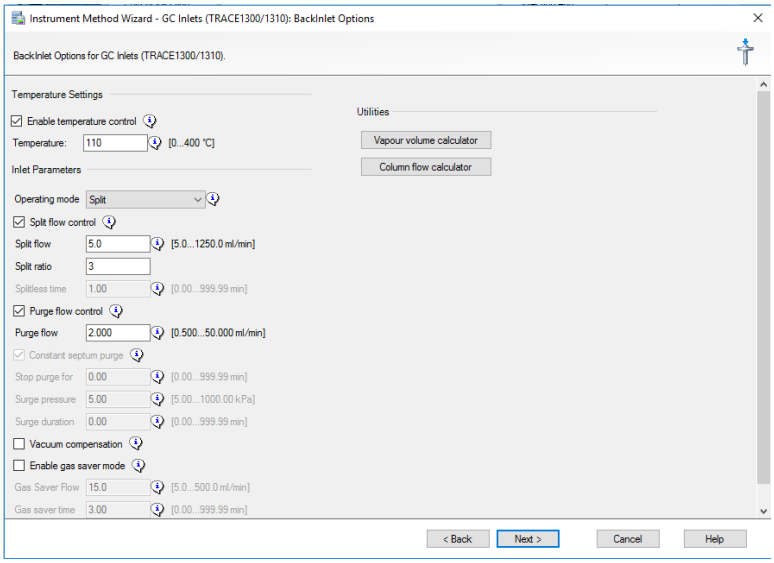
8.) Specify settings for front inlet. Values shown are typical.



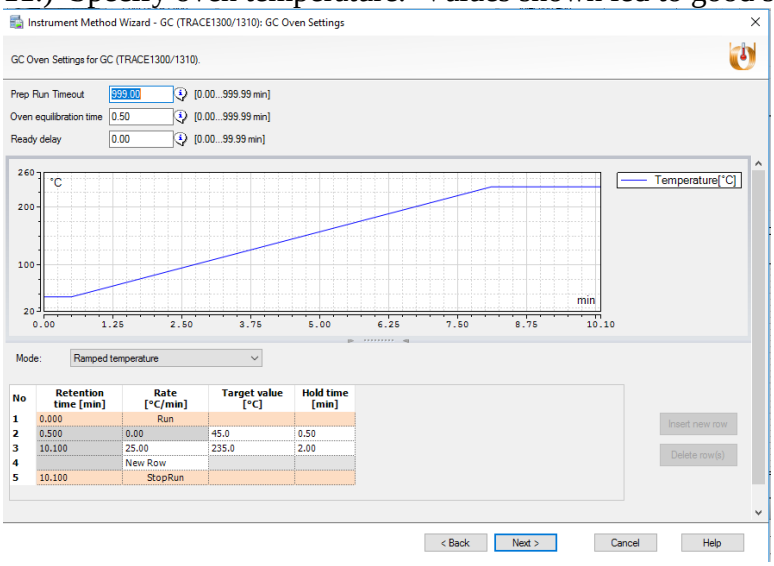
9.) Specify flow for back inlet. Value shown is minimum needed to purge the back column of oxygen.



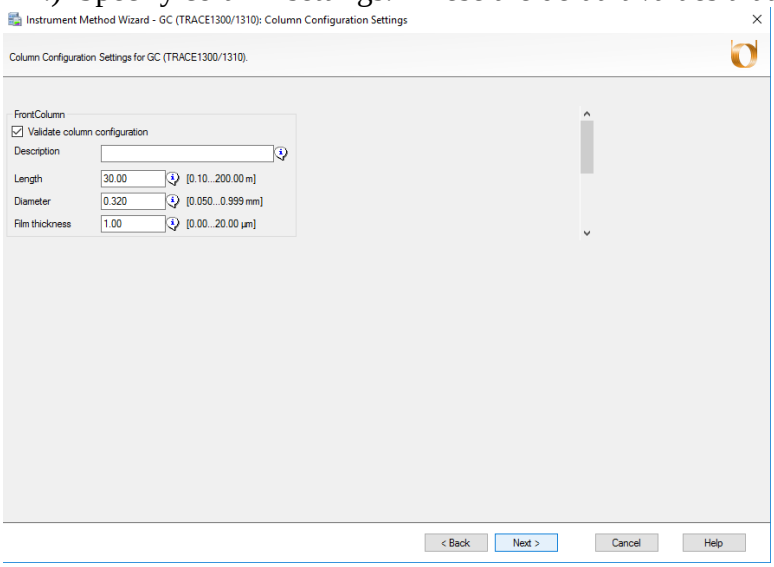
10.) Specify settings for back inlet. Valued shown are minimum needed to purge the system of oxygen and prevent condensation of water.



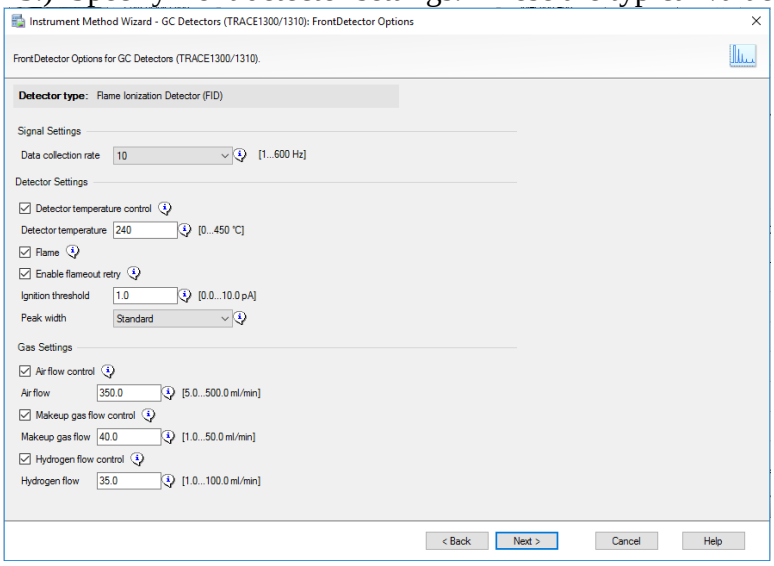
11.) Specify oven temperature. Values shown led to good separation of VFAs.



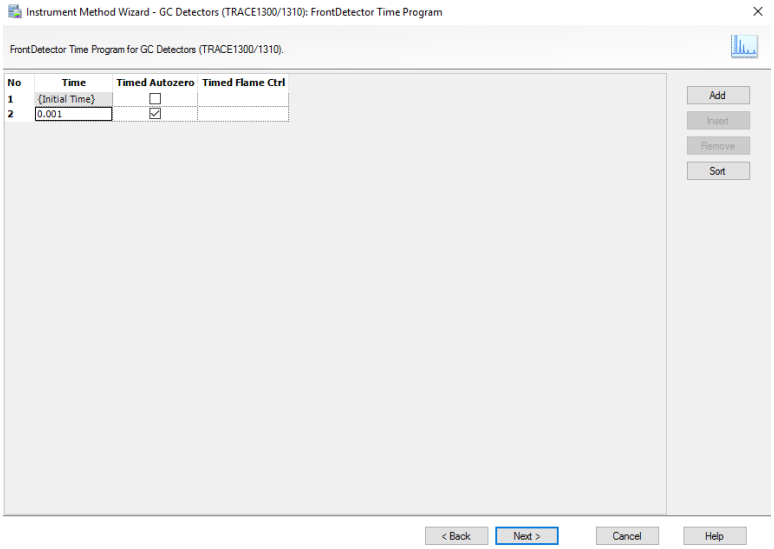
12.) Specify column settings. These are default values that should not be changed.



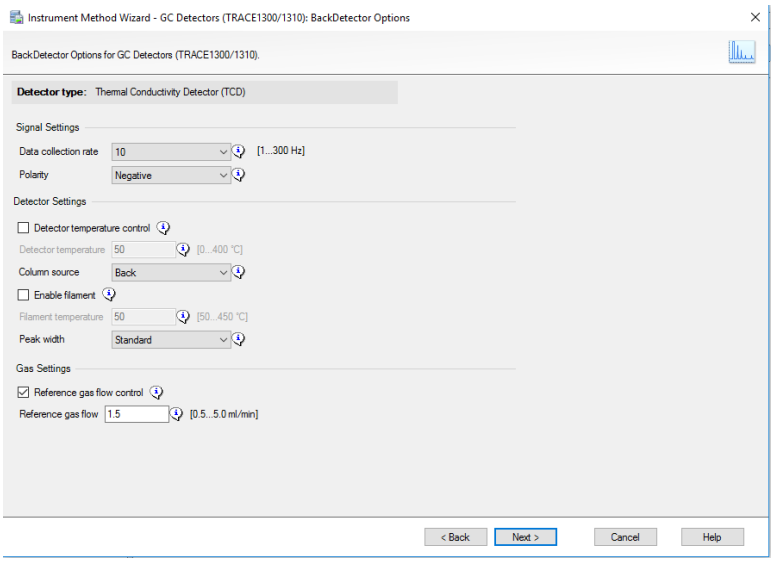
13.) Specify front detector settings. These are typical values.



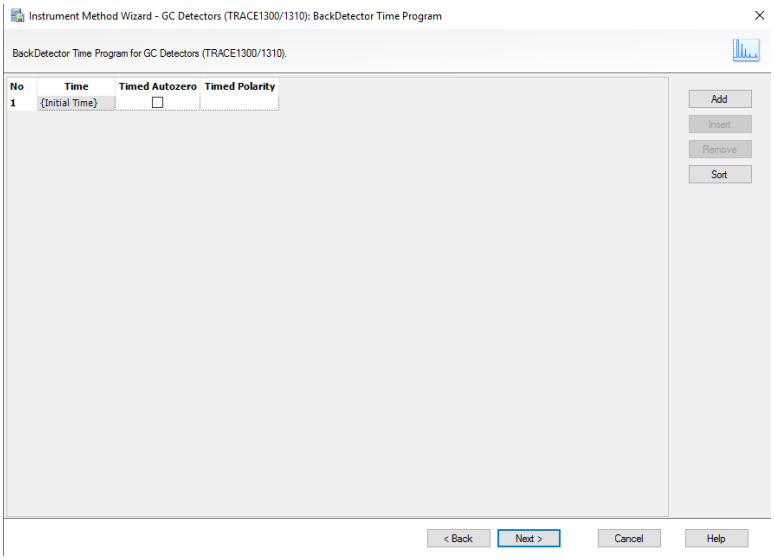
14.) Autozero front detector near beginning of program. This will set the signal to zero.



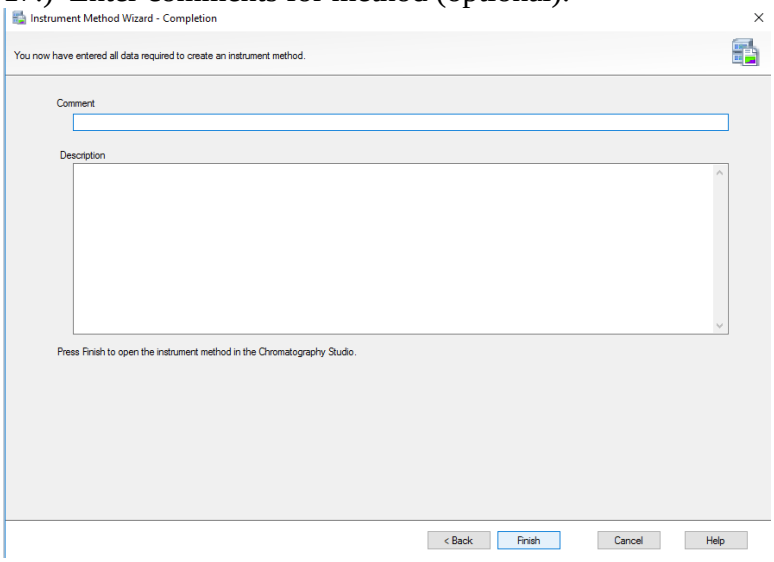
15.) Specify settings for back detector. Turn filament off to preserve life. Turn reference gas as a precaution: it will prevent filament from being damaged if filament is still hot from being turned on.



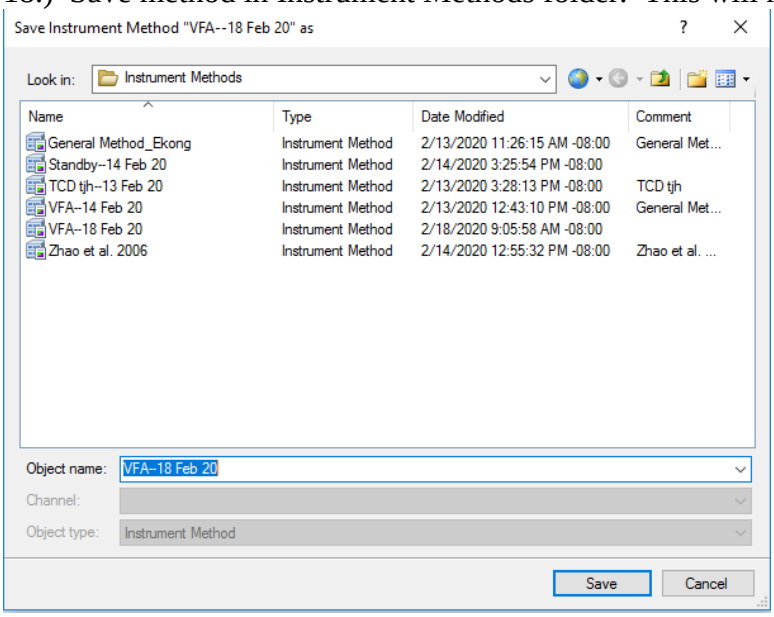
16.) Do not autozero back detector near beginning of program. Back detector will not be used, so autozeroing is unnecessary.



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 CO_2. Insert syringe into gassing probe dispensing CO_2, 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 H_2 gas can be used as standard for H_2.

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.***

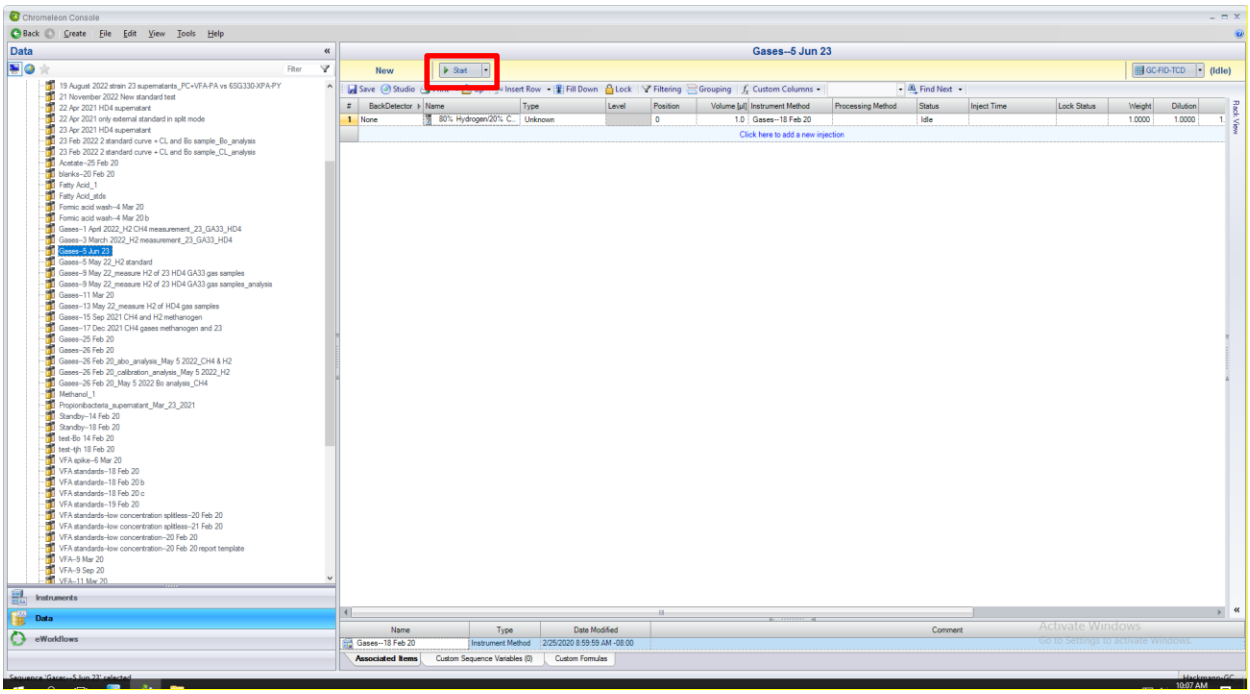
2.) Turn on TCD detector according to ***Turning on detectors***.

3.) In Chromeloen software, create a sequence (list of samples to be analyzed). Follow ***Setting up a sequence***.

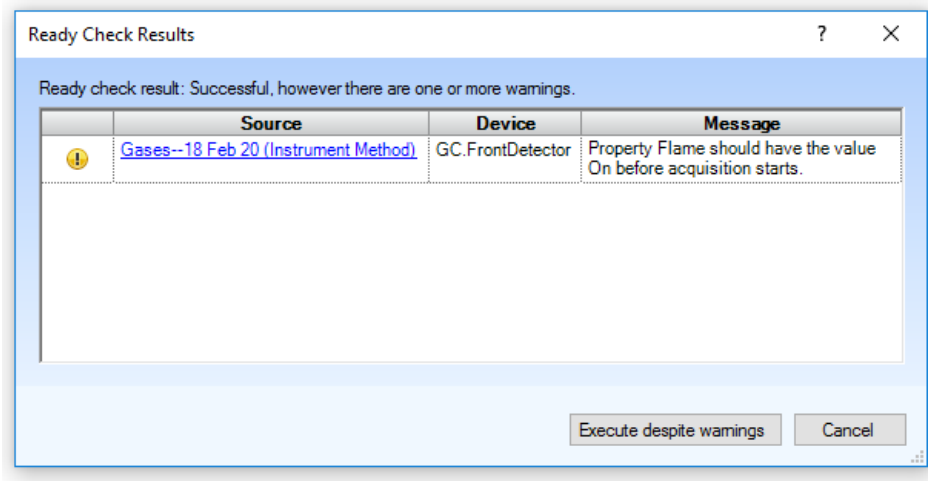
Analyzing samples and standards

1.) Open the sequence.

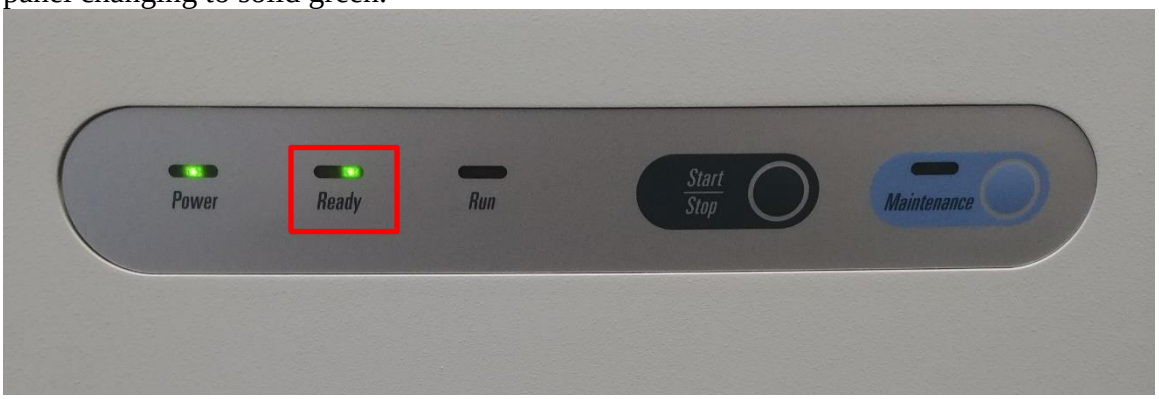
2.) Click Start.



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

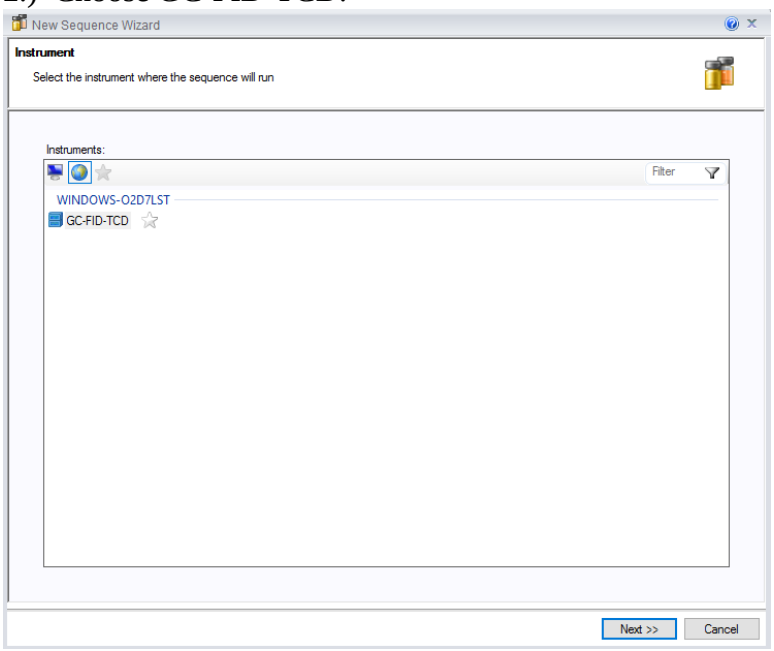
1.) Place the instrument back into standby mode according to ***Placing back into standby mode***.

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

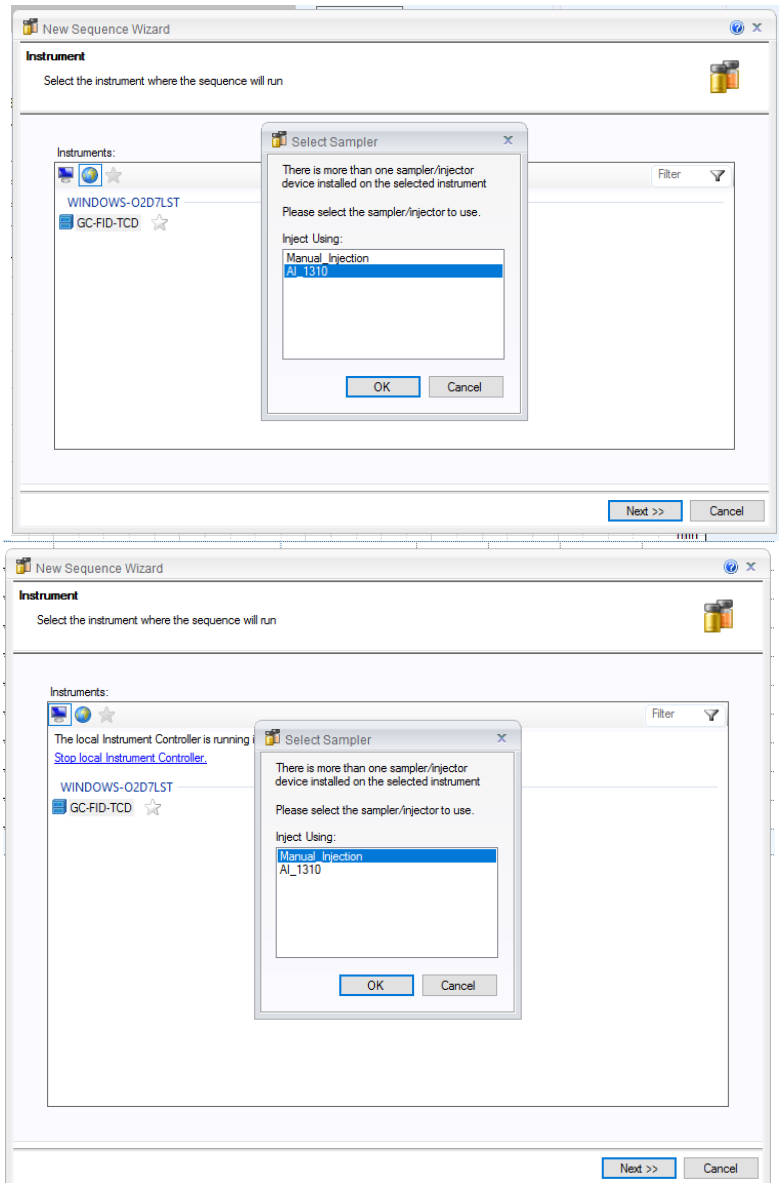
Setting up a sequence

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

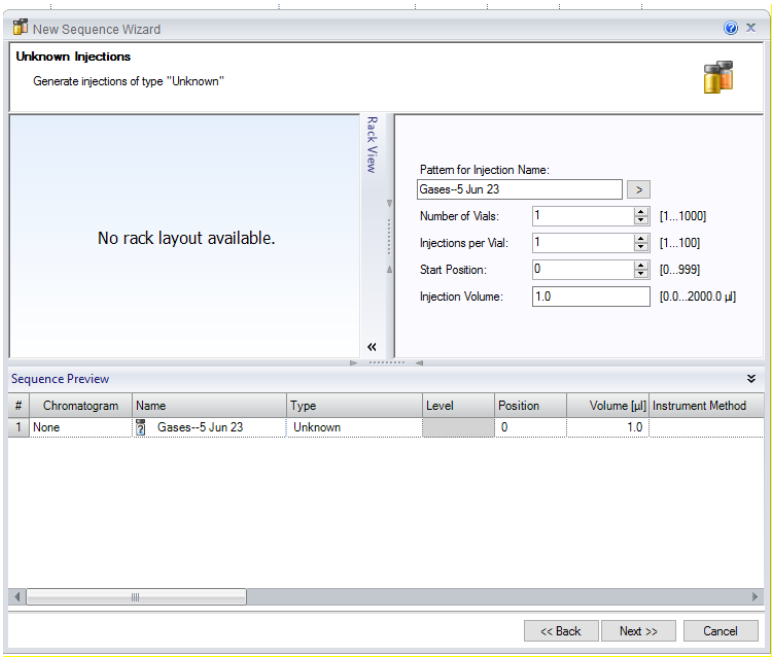
2.) Choose GC-FID-TCD.



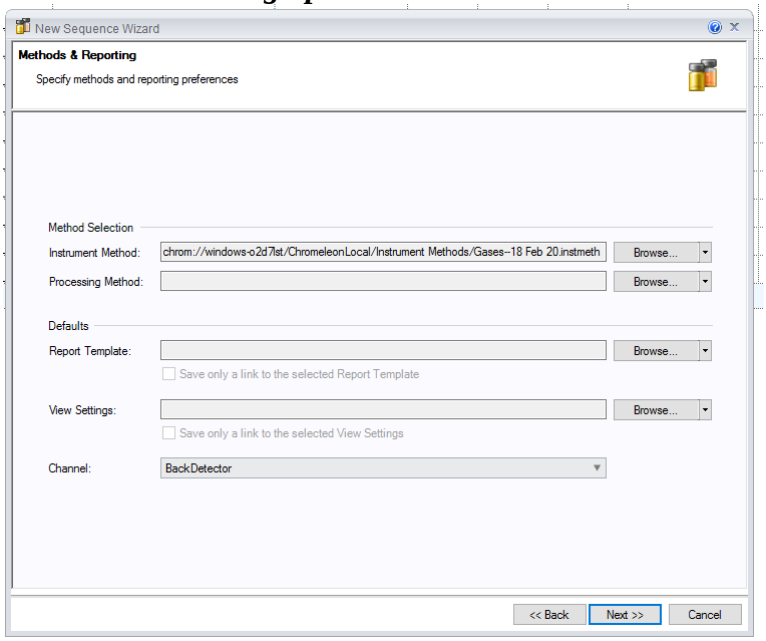
3.) Choose Manual_injection (the autosampler).



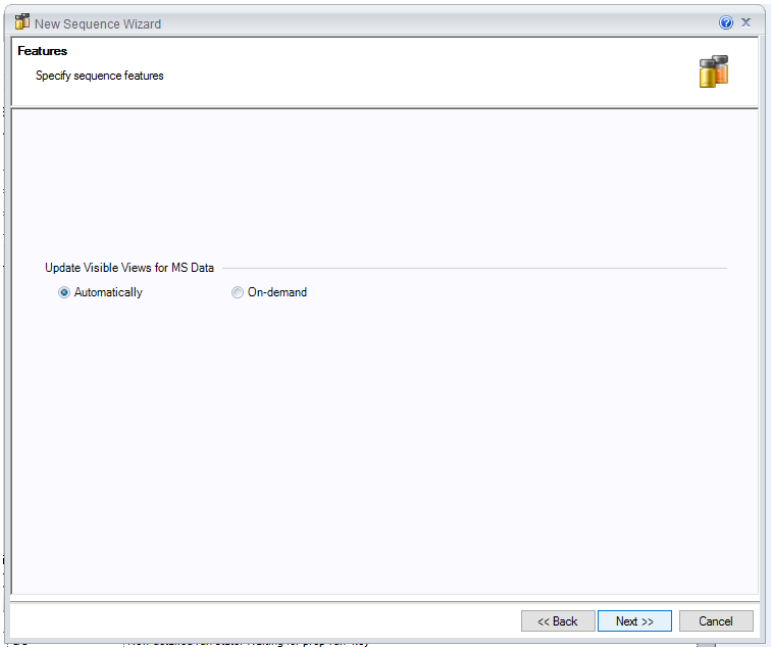
4.) Click Next. (Any information on this screen can be edited later.)



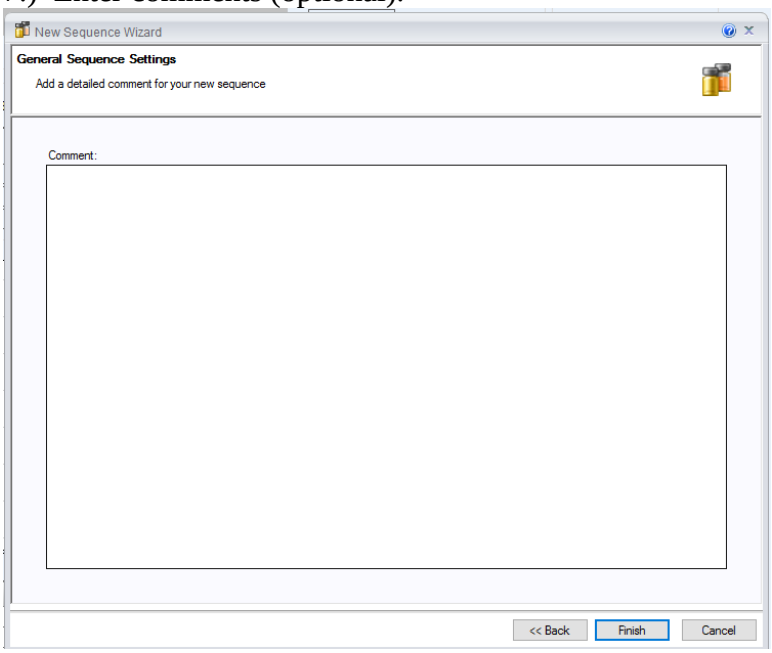
5.) Under Instrument Method, choose "Gases—25 Feb 20.instmethod". See details for this method under ***Setting up a method***.



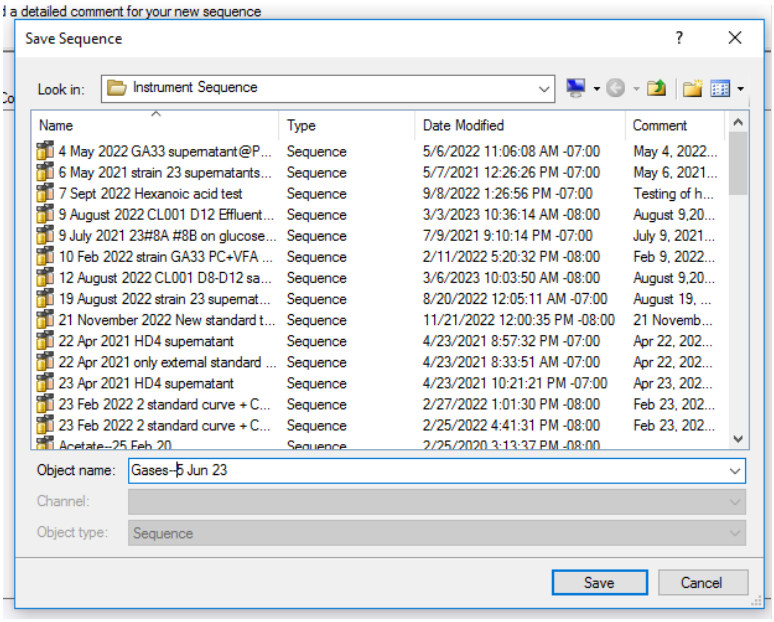
6.) Choose Automatically.



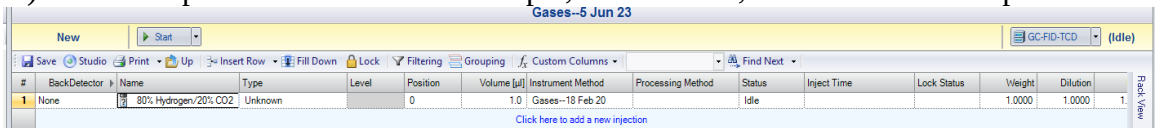
7.) Enter comments (optional).



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



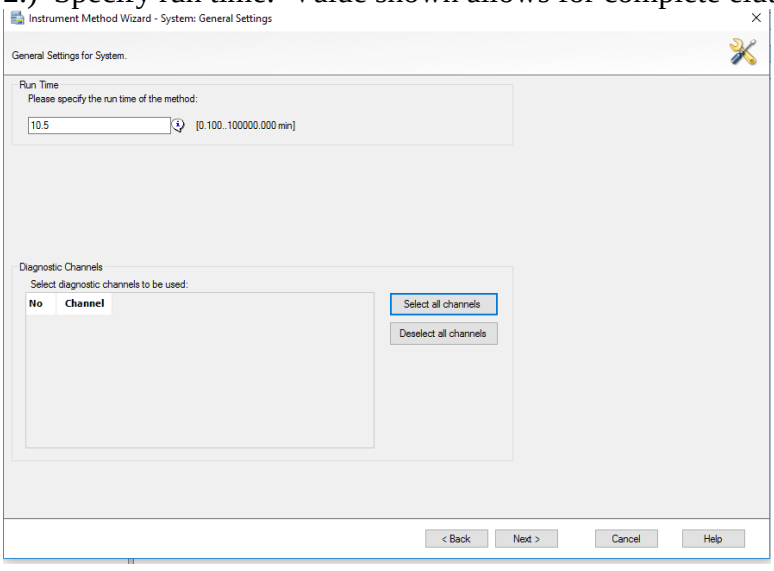
9.) Enter sample information. For example, under Name, enter names of samples.



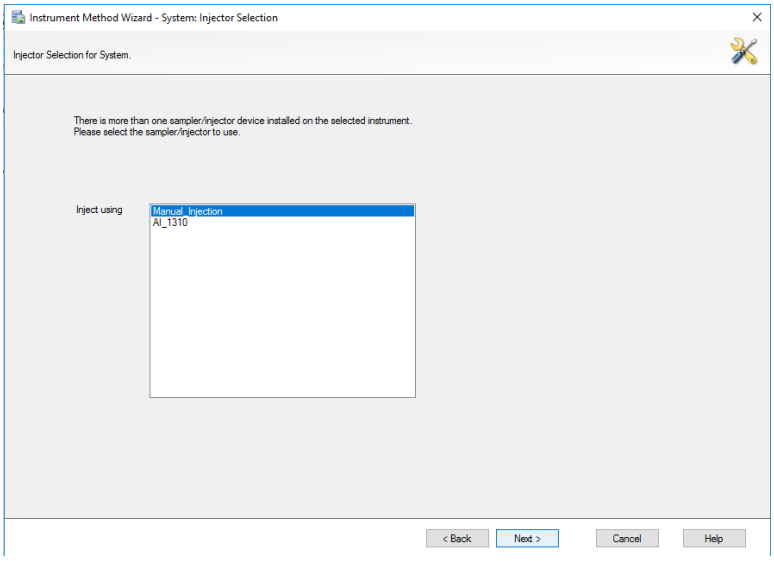
Setting up a method

1.) Go to Create -> Instrument Method.

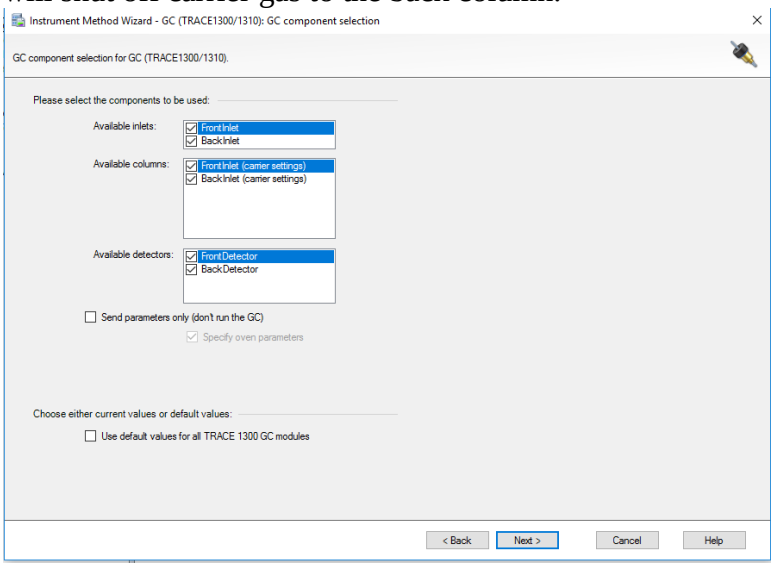
2.) Specify run time. Value shown allows for complete elution of gases.



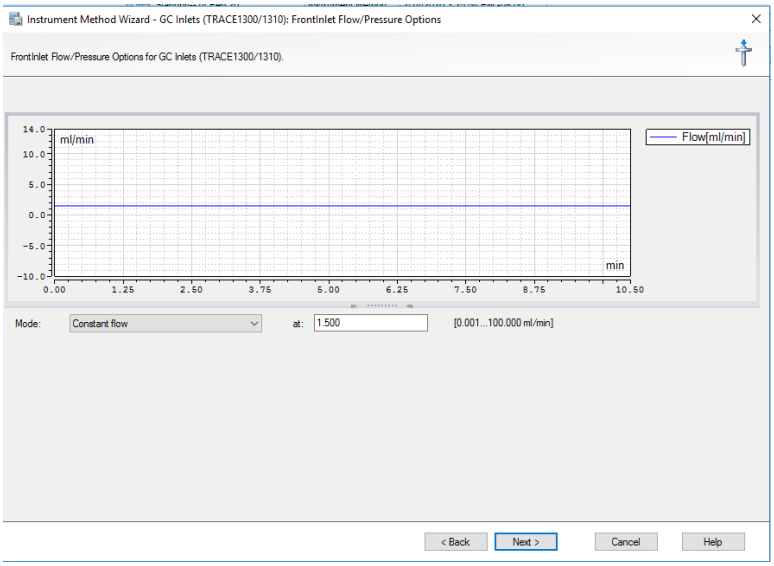
3.) Choose Manual_Injection



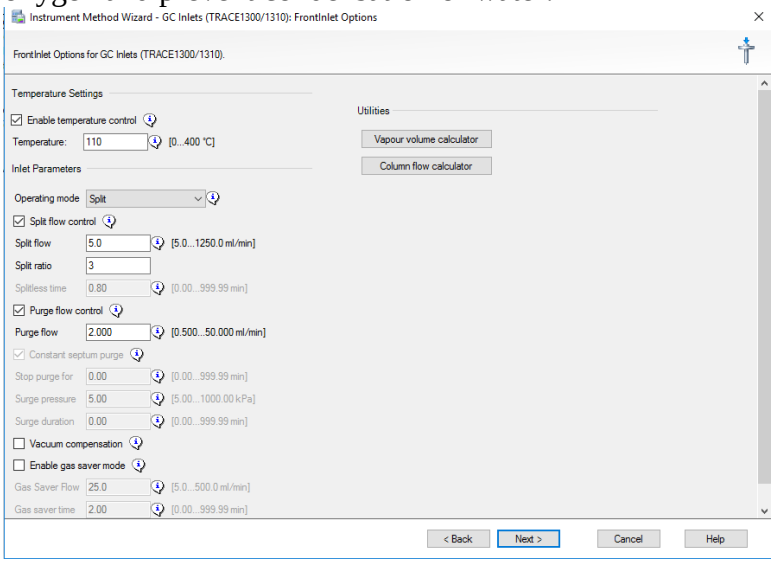
4.) Select all GC components. Even though not all components are used for VFA analysis, they still need to be selected to avoid damaging the instrument. Unselecting BackInlet, for example, will shut off carrier gas to the back column.



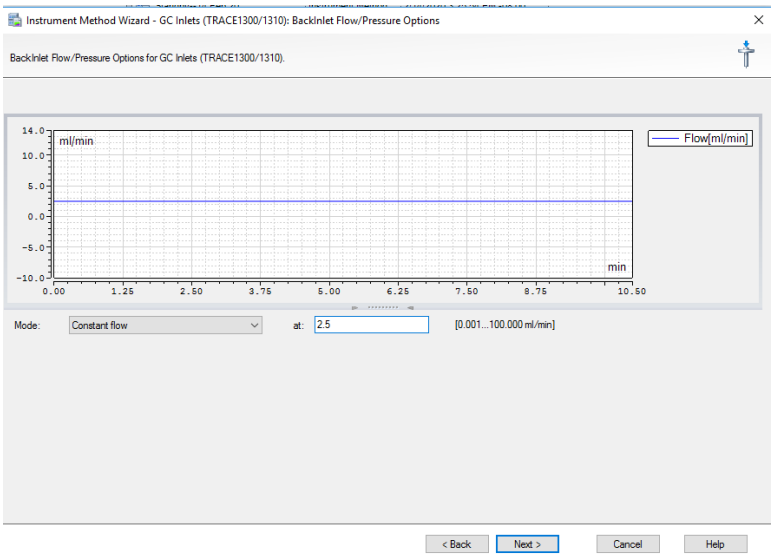
5.) Specify flow for front inlet. Value shown is minimum needed to purge the back column of oxygen.



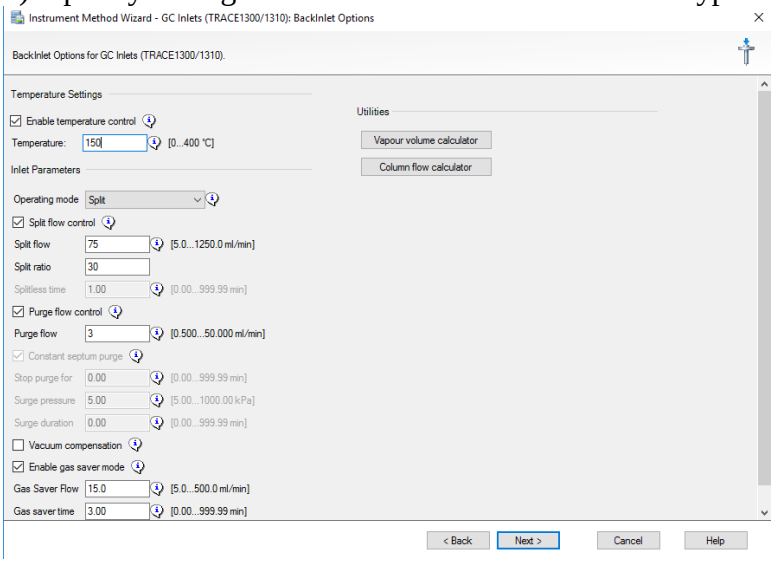
6.) Specify settings for front inlet. Values shown are minimum needed to purge the system of oxygen and prevent condensation of water.



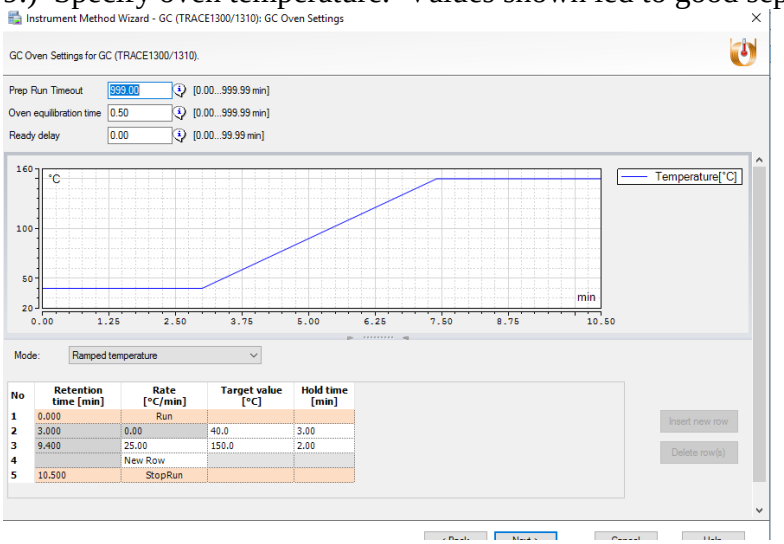
7.) Specify flow for back inlet. Value shown is typical.



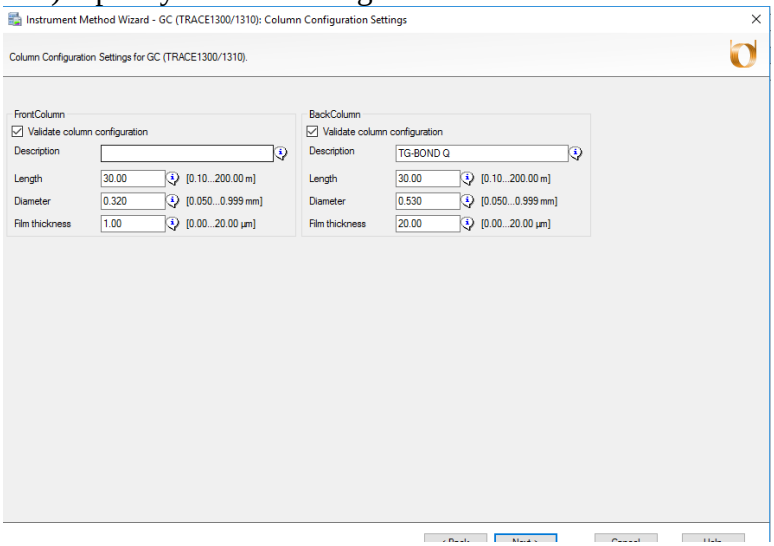
8.) Specify settings for back inlet. Values shown are typical.



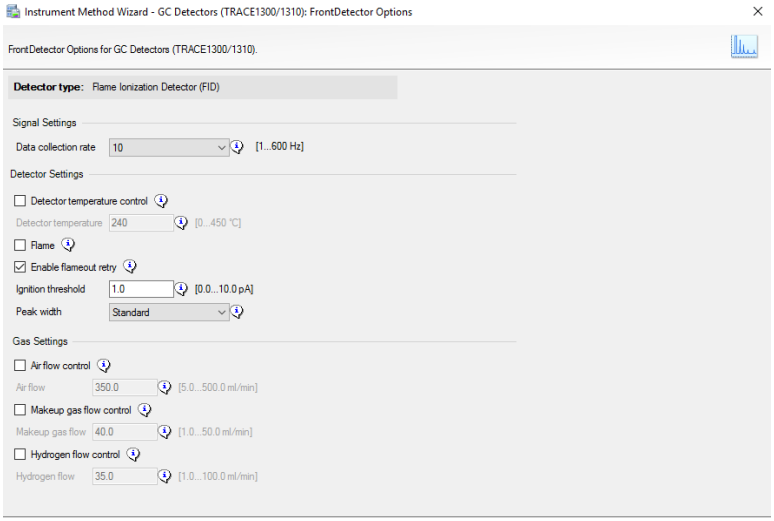
9.) Specify oven temperature. Values shown led to good separation of gases.



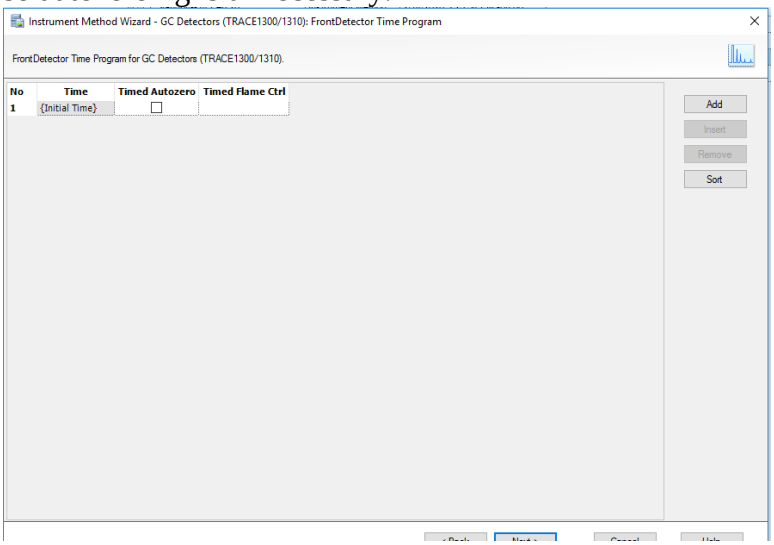
10.) Specify column settings. These are default values that should not be changed.



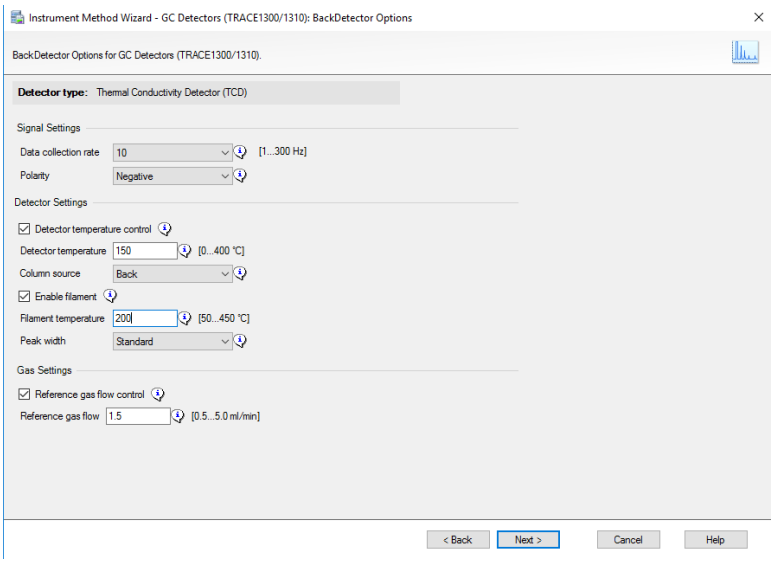
11.) Specify settings for front detector. These values will ensure detector is off.



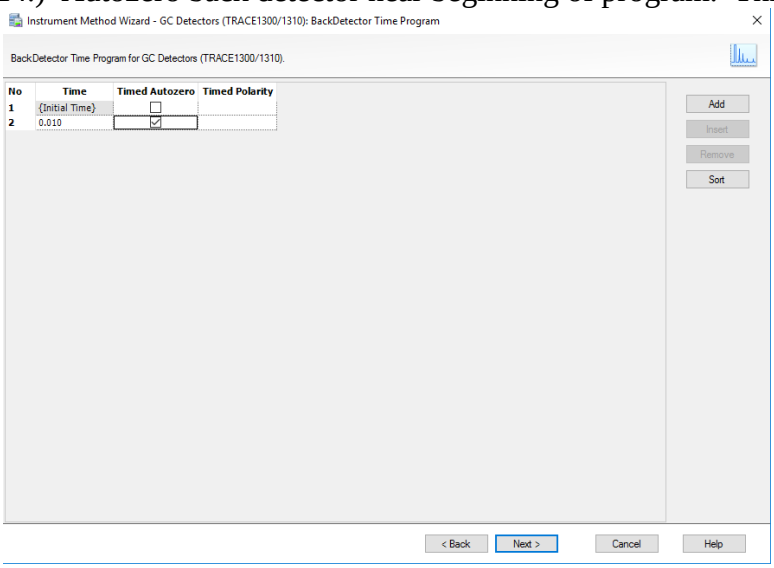
12.) Do not autozero front detector near beginning of program. Front detector will not be used, so autozeroing is unnecessary.



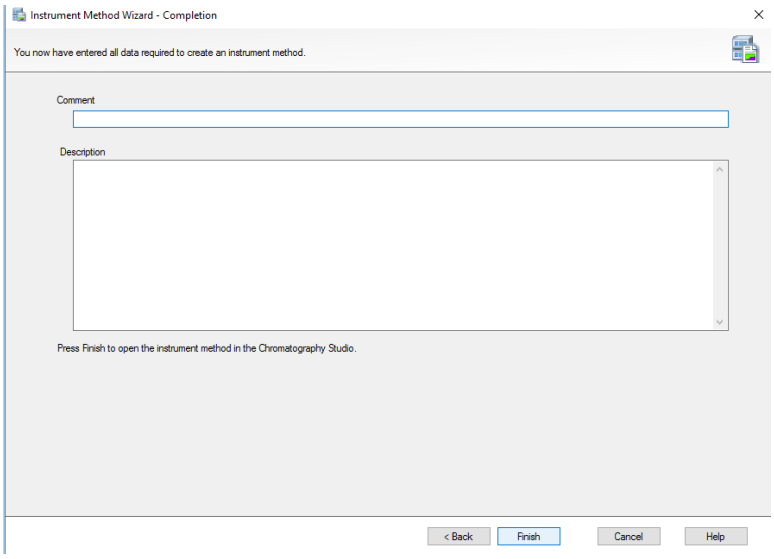
13.) Specify settings for back detector. Values shown are typical



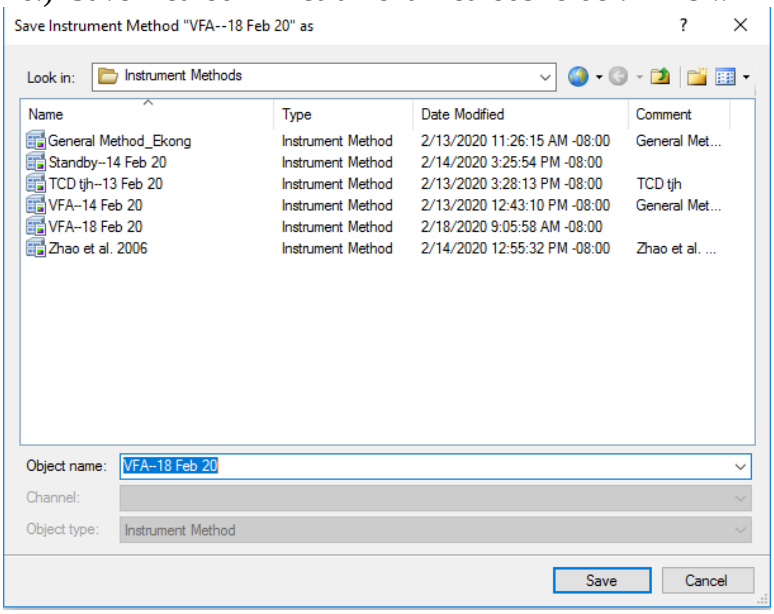
14.) Autozero back detector near beginning of program. This will set the signal to zero.



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_wI4iYQb1Azhpo8l0ZW2VBKosj9rVGJ

Preparation of cell suspension

1.) Inoculate cultures. Inoculate 4 tubes containing 9-mL media with 0.1 mL bacteria. Record OD_{600} 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.

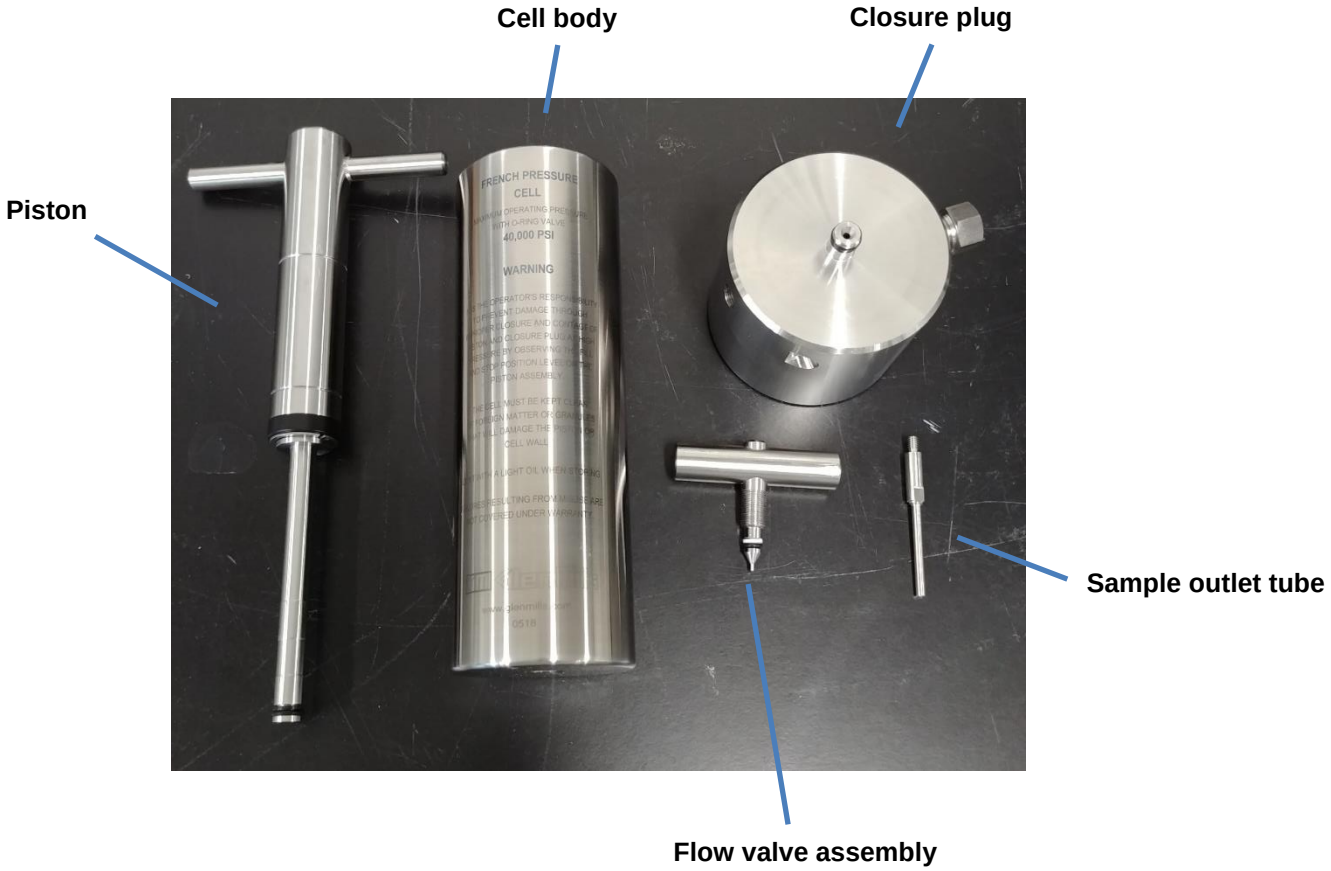


Fig. 2. Pressure cell assembled and loaded on stand

Fig. 3. Overview of cell press.

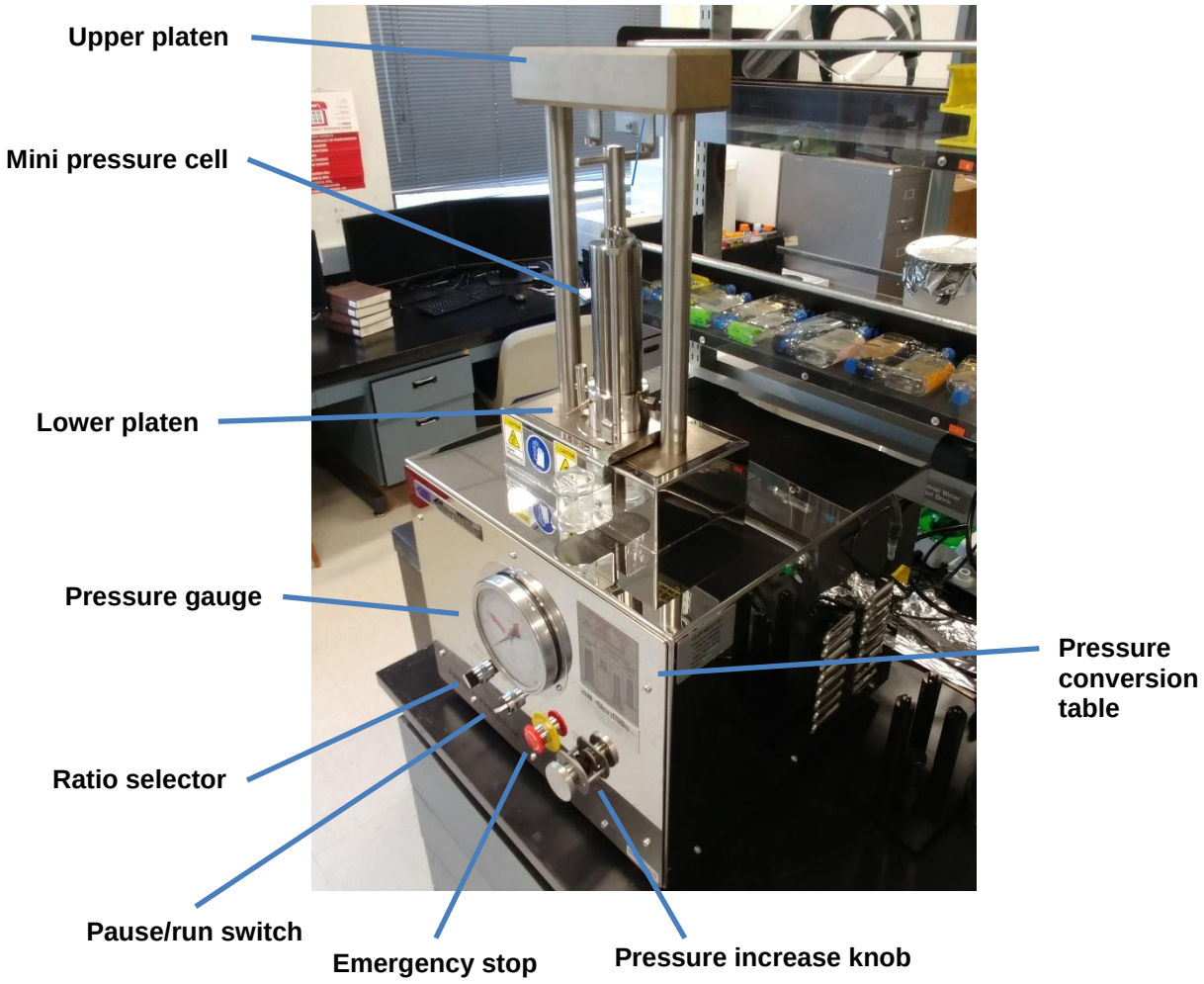


Fig. 4. Safety clamp and thumb screws used to secure cell body

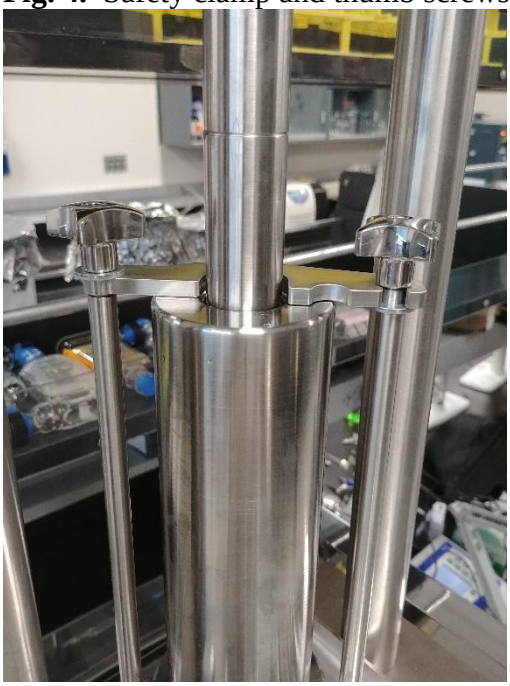


Fig. 5. Operation of cell press. (A) Before operation. Cell body and lower platen are in fully lowered position. (B) During operation. Cell body and lower platen are in intermediate position. Piston has made contact with upper platen. (C) At end of operation. Cell body and lower platen are in fully raised 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***.

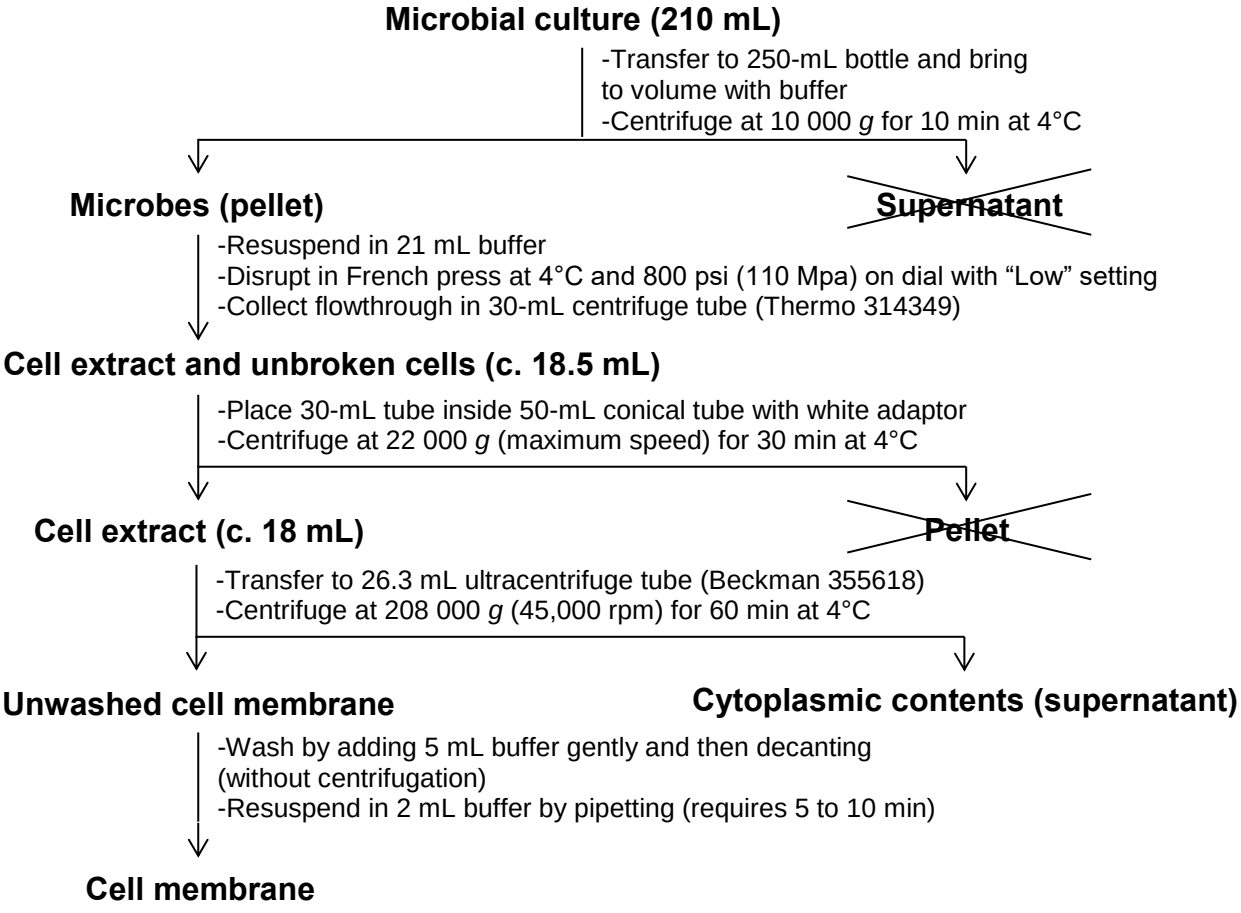
2.) Store cell membrane and cytoplasmic contents at -80°C.

Notes

(i) For samples that need to be prepared anaerobically, flush tubes with N_2 while open and before sealing. Replace the 250-mL bottle with 40-mL centrifuge tubes (Thermo 3139-0050), which are easier to flush with N_2. Use anaerobic buffers and store cytoplasmic contents and cell membrane under N_2.

(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 dH_2O. 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 dH_2O 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 dH_2O per 0.004807 g acetyl-CoA (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH_2O assumes acetyl-CoA has purity of 84.2%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 dH_2O.

The volume of dH_2O assumes acetyl phosphate A has purity of ~100%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 dH_2O. Adjust pH to a value between 7 to 9 with NaOH (e.g., 4 N). Bring to 5 mL with dH_2O (using the remaining ~1 mL dH_2O 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 $AlCl_3 \cdot 6H_2O$ in ~75 mL ddH_2O, then bring to 100 mL in a volumetric flask. Use $AlCl_3 \cdot 6H_2O$, not anhydrous $AlCl_3$, as the latter reacts violently with water.

Ammonium chloride (1 M)

Dissolve 5.3491 g ammonium chloride ~75 mL dH_2O, 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 dH_2O per 0.002 g 3-acetylpyridine adenine dinucleotide (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH_2O assumes APAD has purity of 93%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 dH_2O.

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 dH_2O 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 dH_2O. 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 dH_2O 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 dH_2O per 0.003904 g coenzyme A (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH_2O assumes coenzyme A has purity of 98.3%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 N_2 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 N_2. Add 75 mL of water (anaerobic) while flushing with N_2 and stopper. Filter sterilize into a sterile serum bottle previously gassed with N_2. 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 N_2. Add 75 mL of water (anaerobic) while flushing with N_2 and stopper. Filter sterilize into a sterile culture bottle previously gassed with N_2. 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 KPO_4 (pH = 7). Solution is stable for up to 6 months at 4°C and protected from light.

To prepare 0.1 M KPO_4 (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·$2H_2O$ to 400 mL dH_2O. 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 dH_2O.

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 dH_2O.

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 (OD_{600} = 1.0 to 1.2 for *P. brevis* GA33 and OD_{600} = 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-$MgSO_4$ (50 mM Tris-Cl [pH 7.6], 20 mM $MgSO_4$, 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 µg/µL.

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 ddH_2O in a beaker. Add 41.3 mL concentrated (12.1 M) HCl, then bring to volume with ddH_2O 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 ddH_2O. 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 dH_2O. 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 dH_2O 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 dH_2O. 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 dH_2O 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 ddH_2O 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 dH_2O. 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 dH_2O 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 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. 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 N_2 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 N_2. 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 dH_2O. 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 dH_2O. 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 dH_2O 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 dH_2O, and dissolve by pipetting up and down. Long-term stability is not established.

$MgCl_2$ (1 M)
Dissolve 10.1655 g of $MgCl_2.6H_2O$ in ~40 mL dH_2O, then bring to 50 mL in a volumetric flask.

$MgCl_2$ (1 M) (anaerobic)
Add 1.6785 g $MgCl_2 \cdot 6\ H_2O$ to a Balch tube. Flush tube with N_2. 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 N_2. 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 dH_2O. 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 dH_2O.

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 N_2 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 dH_2O 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 dH_2O.

The volume of dH_2O assumes NADP has purity of 89.9%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 ddH_2O. 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 dH_2O in a volumetric flask.

Phosphenolpyruvate (100 mM)

Reference: (74)
Add 0.0206 g phosphoenolpyruvic acid monopotassium salt (Fisher AAB2035806) to a small (10 mL) beaker. Add 1 mL of dH_2O. 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 dH_2O 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 dH_2O in a volumetric flask.

Potassium bicarbonate (1 M)

Add 10.0115 g of potassium bicarbonate to a beaker. Bring to ~75 mL with dH_2O, then stir to dissolve. To prevent decomposition and loss as CO_2, do not heat. Bring to 100 mL with dH_2O in a volumetric flask. Store in serum bottle and stopper to prevent volatilization as CO_2.

Potassium hydroxide (8 N)

Add 44.88 g of KOH pellets to a beaker. Bring to ~75 mL with dH_2O, then stir to dissolve. After cooling, bring to 100 mL with dH_2O in a volumetric flask.

Potassium phosphate buffers (1 M) (pH = 5.8 to 8.0)

Reference: (78, 81)

Mix 1 M K_2HPO_4 and 1 M KH_2PO_4 in volumes indicated

pH	1 M K_2HPO_4 (mL)	1 M KH_2PO_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 K_2HPO_4 and 1 M KH_2PO_4 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 K_2HPO_4 and 1 M KH_2PO_4 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 K_2HPO_4, bring 87.1 g K_2HPO_4 to 500 mL in a volumetric flask.

To prepare 1 M KH_2PO_4, bring 68.043 g KH_2PO_4 to 500 mL in a volumetric flask.

Potassium phosphate buffer (pH = 7.0) (1 M) (anaerobic)

Add 1.0713 g K_2HPO4 and 0.5239 g KH_2PO4 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°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°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°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°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 dH_2O. 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 dH_2O in a volumetric flask.

Sodium fluoride (100 mM)

Add 0.4199 g NaF to a screw-cap plastic bottle. Add 100 mL ddH_2O with a volumetric pipette. Dissolve. Solution will etch glass bottles and should not stored in them.

Sodium vanadate (200 mM) (pH = 10.0)

Reference: (82)

Dissolve 3.6781 g Na_3VO_4 in ~50 mL ddH_2O. 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 dH_2O. 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 dH_2O 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 H_2SO_4 directly to 0.005 g succinyl-CoA (Sigma S1129-5MG) in the manufacturer's vial. Dissolve by pipetting up and down.

The volume of H_2SO_4 assumes succinyl-CoA has purity of 78.7%; see certificate of analysis for exact purity and adjust volume of dH_2O 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 H_2SO_4, add 275.9 µL of concentrated H_2SO_4 to ~125 mL in a beaker. Bring up to 500 mL in a volumetric flask. The volume of H_2SO_4 assumes assay of 96.6 % w/w; see pack label for exact assay and adjust volume of dH_2O 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 N_2 for several minutes. Store at -20°C.

Trichoroacetic acid (30 % w/v)

Dissolve 30.0 g trichloroacetic acid in ~30 mL ddH_2O, 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 dH_2O 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 dH_2O 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 dH_2O. 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 N_2. Mix and stopper.

To prepare 50 mL anaerobic Tris HCl (1 M), add 7.88 g Tris HCl to a serum bottle flushed with N_2 for 10 min, add 44.32 mL anaerobic dH_2O to the bottle and cap it with butyryl stopper under N_2. 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 dH_2O to the bottle and cap it with butyryl stopper under N_2. 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 B_{12} (1 mg/mL)

Dissolve 0.01 g vitamin B_{12} in 10 mL ddH_2O. Filter through 0.22 μm membrane. Store at 4°C.

Water (anaerobic)

Boil ~350 mL water under N_2 for 5 to 7 min (timed after first bringing to a boil). Dispense ~75 mL aliquots in 160-mL culture bottles while flushing with N_2 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 dH_2O.

1 M Acetic acid
Add 5.72 mL glacial acetic acid to a 100 mL volumetric flask. Bring to volume with dH_2O.

Ammonium sulfate (10% saturated, pH = 6.8)
Add 5.3 g ammonium sulfate and 100 mL dH_2O 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 dH_2O 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 dH_2O 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 K_2HPO_4 and 3.85 g KH_2PO_4 to 1 L volumetric flask. Bring to volume with dH_2O. 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 dH_2O.

NaCl (2 M)
Add 11.688 g NaCl to ~75 mL dH_2O in beaker and stir to dissolve. Transfer to a 100 mL volumetric flask and bring to volume with dH_2O.

NaCl (100 mM)
Add 2.3376 g NaCl to 250 mL volumetric flask. Bring to volume with dH_2O.

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 dH_2O 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 dH_2O. Add 10% HCl drop-by-drop to adjust pH to 8. Transfer to a 100 mL volumetric flask and bring to volume with dH_2O.

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 dH_2O. Transfer to a 250 mL volumetric flask and bring to volume with dH_2O.

NaOH (0.5 N)
Add 4.9996 g of NaOH to ~200 mL dH_2O. Transfer to a 250 mL volumetric flask and bring to volume with dH_2O.

Water (anaerobic)
Boil ~350 mL water under N_2 for 5 to 7 min (timed after first bringing to a boil). Dispense ~75 mL aliquots in 160-mL culture bottles while flushing with N_2 and stopper. Autoclave.

Preparing medium for C. pasteurianum

C. pasteurianum minerals A

Add following to a 1-L volumetric flask
153.29 g K_2HPO_4 to 1 L
Bring to volume to 1 L with dH_2O and store at 4°C.

C. pasteurianum minerals B

Add the following to a 1-L volumetric flask

15 g KH_2PO_4
1 g NaCl
0.9766 g $MgSO_4$
0.1 g $Na_2MoO4 \cdot 2H_2O$
10 g NH_4Cl

Bring to volume with dH_2O and store at 4°C.

C. pasteurianum iron sulfate

Add 0.5 g $FeSO_4 \cdot 7\ H_2O$ to a 1-L volumetric flask. Bring to volume with dH_2O 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 dH_2O and store at 4°C.

Glucose (40% w/v, anaerobic)

Add 40 g glucose to a 150-ml serum bottle. Flush the bottle under N_2 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 N_2, 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 dH_2O
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 N_2 (requires about 5 min). Cool it down in ice water under N_2 and bubble for 5 min. Approximately 50 mL will be lost, giving 300 mL final volume. pH should be approximately 7.1.

Bubble with CO_2 to decrease pH to 6.8 (requires about 3 min).

Under CO_2, 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 dH_2O to a 1-L Pyrex bottle. Stir to dissolve, then bring to 540 mL with dH_2O. Wire a stopper in place, cover with aluminum foil, and autoclave at 121°C for 15 min. Pressurize the bottle with N_2, cool, then remove stopper and flush with N_2.

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 N_2 for 4 min, then continue bubbling with CO_2 for 4 min. Drain contents of separatory funnel into Pyrex bottle with glucose solution. Bubble with CO_2 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 dH_2O, transfer ion exchanger to Buchner funnel lined with 2 layers of #1 Whatman filter paper.
(e) Wash ion exchanger with dH_2O. Fill a 1 L graduated cylinder with dH_2O, 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 dH_2O).
(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 dH_2O, 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 dH_2O).

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 (OD_{600} 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 CO_2). In cuvette (1-cm pathlength), dilute 1→10 with 0.9% NaCl. Measure OD_{600}.

5.) Harvest culture when it reaches mid-exponential phase (OD_{600} = 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 (OD_{600} = 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 $MgCl_2$. 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 × OD_{600}^{-1} × mL $culture^{-1}$ (e.g, 5.4 g wet cells for 900 mL of culture grown to OD_{600} = 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 (23-25°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 (~ 4-5 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 μL 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 μL 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-uL 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 #2can be extended to over-night. 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 scale from λ = 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

$$A_{390} = c * \varepsilon_{390} * L$$

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 µM (~5300 mg/L) and purity of $A_{390}/A_{280} = 0.73$.

6.) Dispense ~150 µL aliquots in 500-µL 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_2O	425	NA
Cell extract	25	NA
Pyruvate (100 mM) or ddH_2O	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:

	Cuvette	
Addition	1	2
1	Mastermix A	
1	ddH_2O	
1	Cell extract	
2	Pyruvate	ddH2O

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 M^{-1} cm^{-1} is the extinction coefficient of NADH.

4.) Calculate the activity (mol L^{-1} 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

$$1\ \text{Succinyl-CoA} + 1\ \text{Acetate} \xrightarrow{SCACT} 1\ \text{Acetyl-CoA} + 1\ \text{Succinate}$$

$$1\ \text{Acetyl-CoA} + 1\ P_i \xrightarrow{PTA} 1\ \text{Acetyl-Phosphate} + 1\ \text{CoA}$$

$$1\ \text{CoA} + 1\ \text{DNTB} \rightarrow 1\ \text{TNB-CoA} + 1\ TNB^{-2}$$

where SCACT is succinyl-CoA:acetate CoA-transferase and PTA is phosphotransacetylase. TNB^{2-} 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/µL.

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 (µL)	Final concentration (mM or U/mL)
ddH_2O	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_2O	30	300
Enzyme sample or ddH_2O	6	NA
Succinyl-CoA (5 mM) or ddH_2O	30	1.5
Final volume	100	

Some components are added as master mixes, not individually, for convenience and uniformity.

Component	Master mix
ddH_2O	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:

	Well			
Addition	1	2	3	4
1	Mastermix A			
1	Potassium acetate	ddH_2O	Potassium acetate	Potassium acetate
1	Enzyme sample	Enzyme sample	ddH_2O	Enzyme sample
2	SucCoA	SucCoA	SucCoA	ddH_2O

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

Rate = (slope/14 150) / path length

where 14 150 M^{-1} 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 (mol L^{-1} 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_2O	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:

	Cuvette			
Addition	1	2	3	4
1		ddH_2O		
1		Mastermix A		
1		Mastermix B		
1	Cell extract	Cell extract	Cell extract	ddH_2O
2	Acetate	Acetate	ddH_2O	Acetate
3	SucCoA	ddH_2O	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

$$\text{Rate} = (\text{slope}/6\,220)$$

where 6 220 M^{-1} cm^{-1} is the extinction coefficient of NADH.

4.) Calculate the activity (mol L^{-1} 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_2O	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_2O	20.8	1
Mastermix A	25.2	1
Succinate or ddH_2O*	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 ddH_2O

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

$$1\ Fd_{ox} + 1\ Pyruvate \xrightarrow{PFOR} 1\ Fd_{red} + 1\ Acetyl\text{-}CoA$$

$$1\ Fd_{red} + 1\ NAD_{ox} + 1\ Na^+(in) \xrightarrow{Rnf} 1\ Fd_{ox} + 1\ NAD_{red} + 1\ Na^+(out)$$

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-$MgSO_4$ (50 mM Tris-Cl [pH 7.6], 20 mM $MgSO_4$, 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_2O (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_2O (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	

[a]µg protein/mL
[b]U/mL

Add components to the cuvette anaerobically. Transfer small aliquots of components to 0.5 mL centrifuge tubes flushed with N_2. While flushing the cuvette with N_2, transfer the component to the cuvette with a micopipette. Cap the cuvette once done. Tap the cuvette to remove any bubbles.

Measurement of activity

Add components to cuvettes in the following order:

	Cuvette	
Addition	1	2
1	dH_2O	
1	Tris	
1	$MgCl_2$	
1	Dithiothreitol	
1	NaCl	
1	Cell membrane	dH_2O
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 A_3.

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

Rate = (slope/6 200)

where 6 200 M^{-1} cm^{-1} is the extinction coefficient of NADH.

4.) Calculate the activity (mol L^{-1} min^{-1}) by correcting the reaction rate with control cuvettes.

$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}}$$

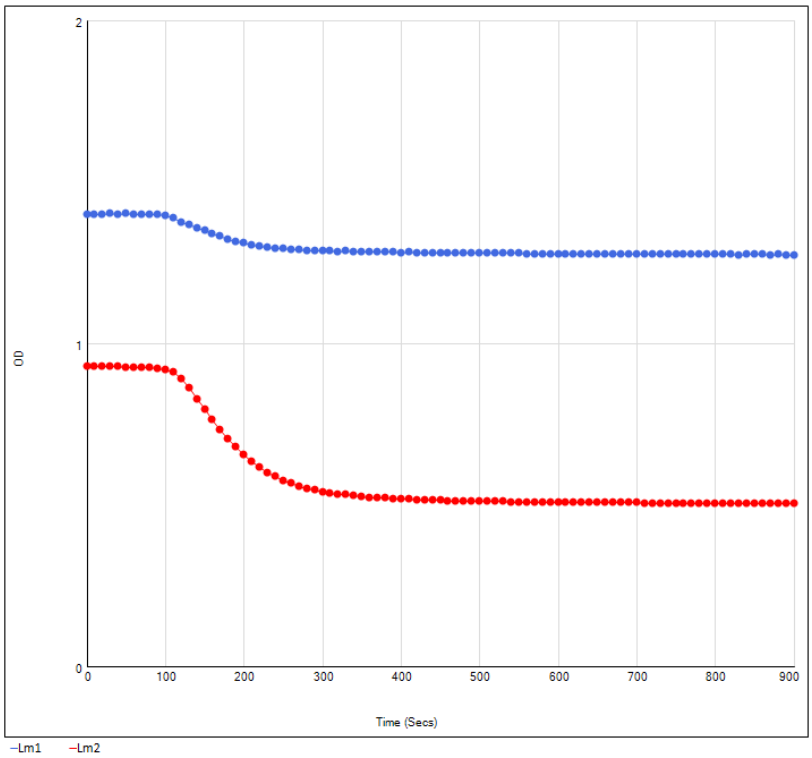
5.) Measure the protein of the membrane and express activity as mol mg $protein^{-1}$ min^{-1}.

Notes

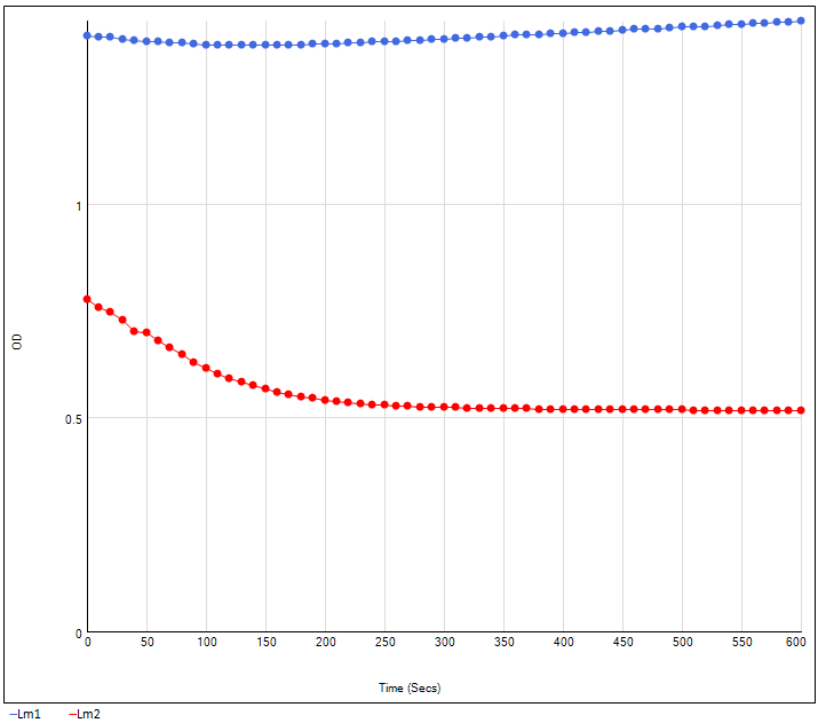
i) Low activity may be observed and be due to oxidation of NADH (by a NADH dehydrogenase). To correct for this oxidation, include a control cuvette containing NADH (in place of NAD).

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

$(A_2^*-A_1)/6\ 220 \times (500/435.91)$

where 6 220 M^{-1} 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 M^{-1} 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

$(A_3 - A_2)/6\ 220 \times \{1 + (A_2 - A_1)/[(A_2 - A_1) + (A_3 - A_2)]\} \times (500/401.25)$

where 6 220 M^{-1} 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

$(A_4^*-A_3)/6\ 220\times\{1+(A_3-A_1)/[(A_4*-A_3) + (A_3-A_1)]\} \times (333.57/401.25)$

where 6 220 M^{-1} 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_2O		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).

	Cuvette	
Addition	1	2
1	ddH_2O	ddH_2O
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).

	Well	
Addition	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 $A_{cuvette\ 1}$ and $A_{cuvette\ 2}$. $A_{well\ 1}$ and $A_{well\ 2}$ are the absorbances of well plates.

2.) Calculate path length (cm) as

$$\text{Path length} = (A_{well\ 1} - A_{well\ 2}) / (A_{cuvette\ 1} - A_{cuvette\ 2})$$

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 H_2O, 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

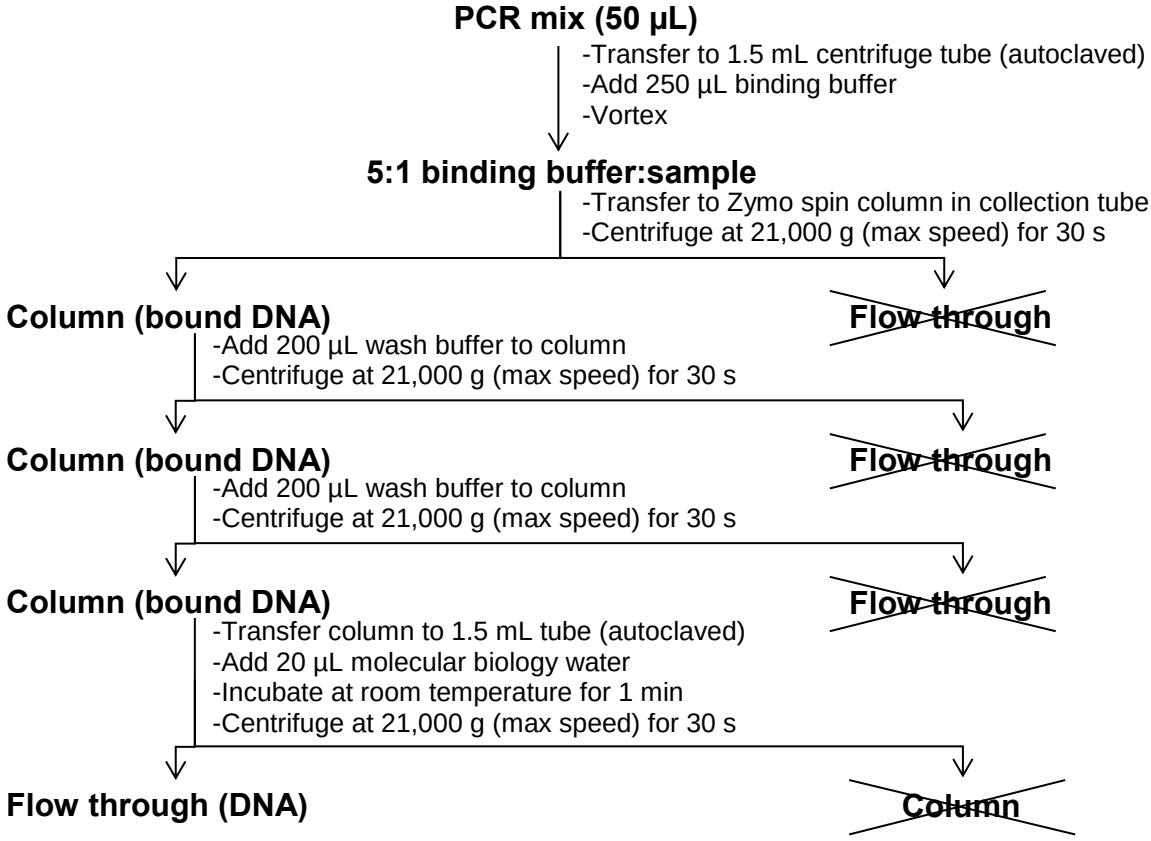
Component	µL per 50 µL reaction
5x Q5 reaction buffer (from NEB M04935)	10
10 mM dNTP (NEB N0447S)	1
Forward primer working concentration	2.5
Reverse primer working concentration	2.5
Molecular biology H_2O	32.5
Q5 DNA polymerase fidelity (from NEB M04935)	0.5

Table 2. PCR cycling conditions

	Temp (°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 Tm calculator (http://tmcalculator.neb.com/#!/main); typically 3°C above Tm of lower Tm 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 ng/µL 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 ng/µL 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 ng/µL, 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 ng/µL) 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 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, outfname
= forward_file_name_fastq)
    abifToFastq(seqname = reverse_file_name_no_extension, fname = reverse_file_path_abi, outfname
= 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(reve
rse_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·2H_2O to 400 mL dH_2O. 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 dH_2O. Bring to 500 mL final volume with dH_2O.

Make 5x TBE buffer. Add to 300 mL dH_2O
- 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

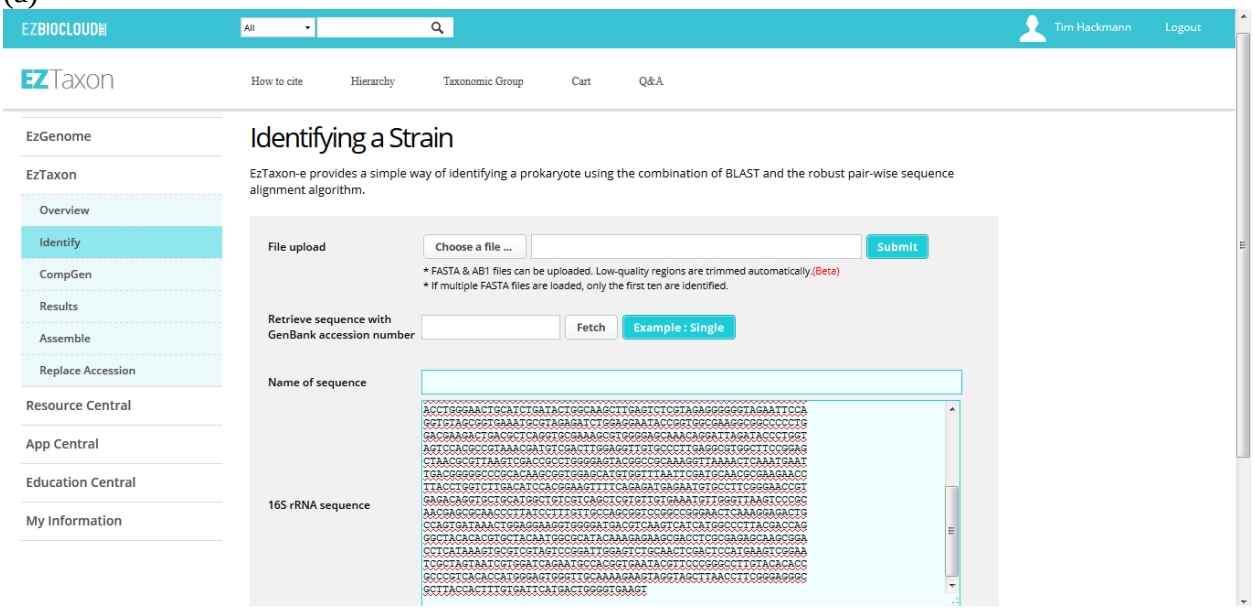
Temp (°C)		Length (min)
	98	3
35 cycles	98	0.167
	63	0.5
	72	1
	72	2
	4	∞

Fig. 1. Example consensus sequence of 16S rDNA gene.

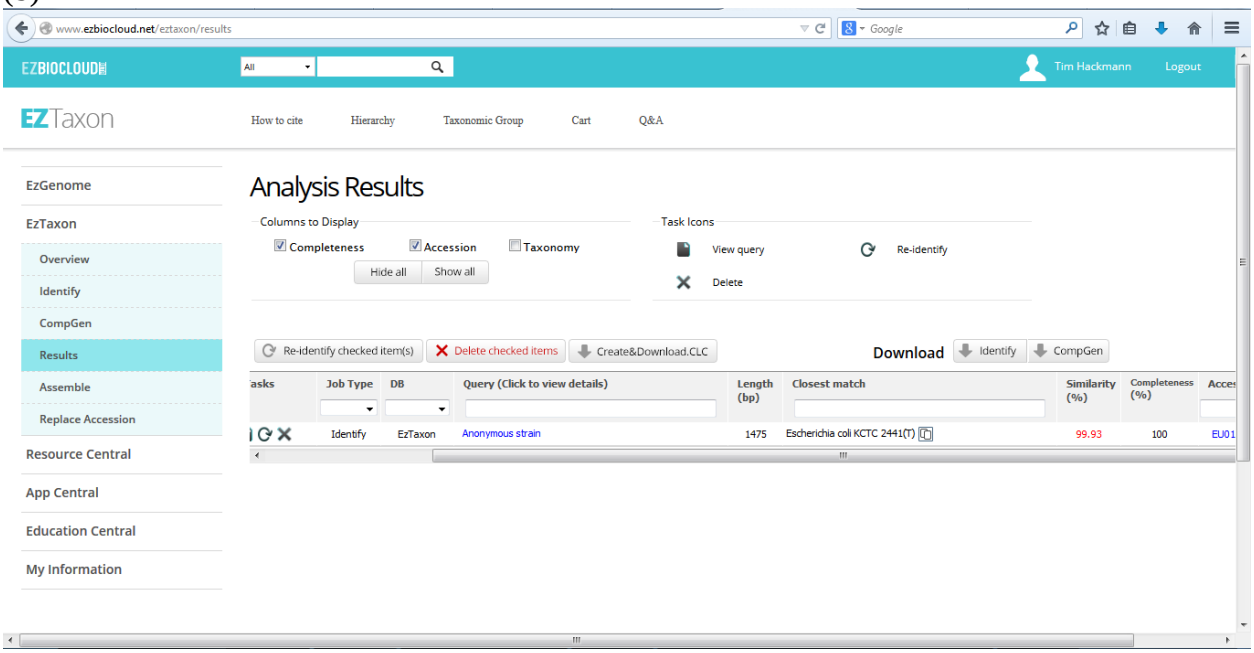
GCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTT
GCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGA
TAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG
CCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT
GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATAC
CTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAG
TCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCG
TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG
CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC
TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAAGGTTAAAACTCAAAT
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC
TTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCG
CAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAAC
TGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTA
CAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAAT
GCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCA
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%.

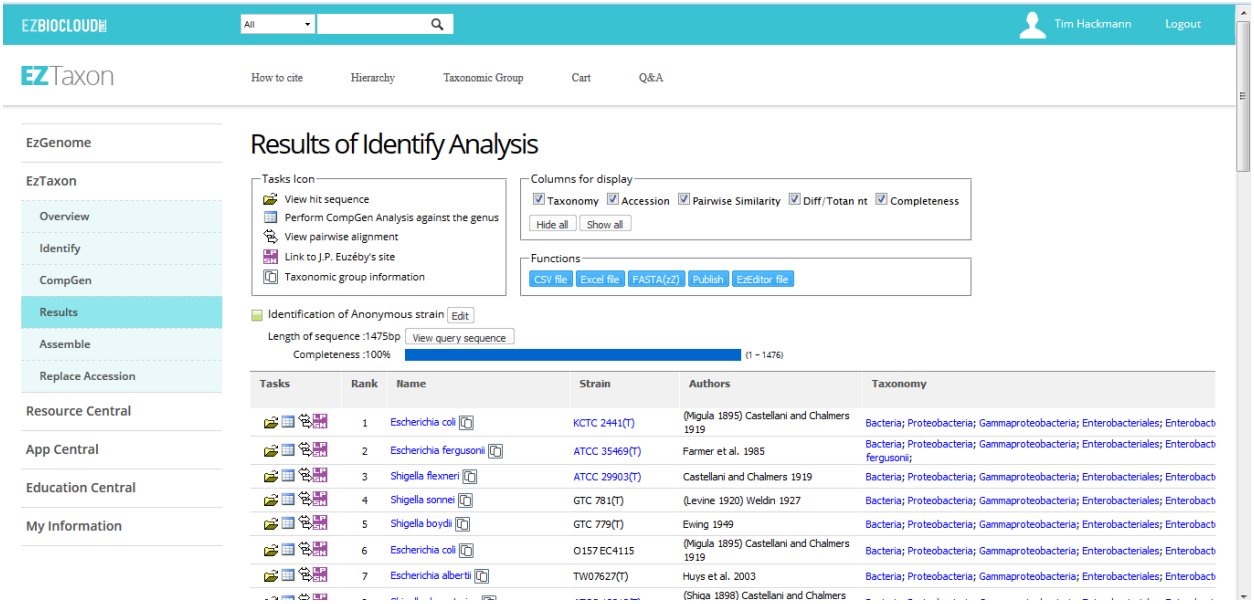
(a)



(b)



(c)


EZBIOCLOUD
All
Tim Hackmann
Logout
EZTaxon
How to cite
Hierarchy
Taxonomic Group
Cart
Q&A
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 for display
Taxonomy
Accession
Pairwise Similarity
Diff/Totan nt
Completeness
Hide all
Show all
Functions
CSV file
Excel file
FASTA(zZ)
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; Enterobacte
2
Escherichia fergusonii
ATCC 35469(T)
Farmer et al. 1985
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte fergusonii;
3
Shigella flexneri
ATCC 29903(T)
Castellani and Chalmers 1919
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte
4
Shigella sonnei
GTC 781(T)
(Levine 1920) Weldin 1927
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte
5
Shigella boydii
GTC 779(T)
Ewing 1949
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte
6
Escherichia coli
O157 EC4115
(Migula 1895) Castellani and Chalmers 1919
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte
7
Escherichia albertii
TW07627(T)
Huys et al. 2003
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacte
(Shiga 1898) Castellani and Chalmers

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	CATTGAAAACTTTTATAAACGAAAGGA
1936F	TTGCAGTACCAACTTCAGCAG
2439F	TGCTCTTGAACGTCACTTGG
2938F	TCCCTAAAGGAAACGTAACTGG
3447F	CGTAACAAAGTCAGCCGTCA
305R	CCGACACCCATTCTTGTTCT
787R	CACCGATGACAGTTCCTTCA
1313R	CCTGCTGAAAGCTTAATTCAAA
1785R	AGGAAAAGACCTGCAACAGC
2276R	GCCATGTTTGTACCCCTTTC
2808R	TGGATGATACCACCTGAAAGG
3299R	AGCCGTTAATTCCACGGTAG
3787R	GGTTCCATCCAGCTTCACAT
3927R	CAGCAAGTTTTTCAGCAGCA

Table 2. PCR cycling conditions

Temp (°C)		Length (min)
	94	2
35 cycles	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

TGCTGCTGAAAAACTTGCTG = last reverse primer (3957R)

>2601025965 PTS system, mannose-specific IIB component [Streptococcus bovis JB1 : Ga0062118_101] (+)strand

ACCTTTTCATGATTCTTTCAAAAAACATAGCGTTTTTCCCGAACGTTATT
TTGCGAATTATACAATTTAATCGCTTTATTTTGTGGGTTTCTGAAAATTT
TTCATGATTTCTGTTATTTTTTCTCAAAAAGCTATTGCATTTTTTGTGAA
AACGTTTTACAATAATATCGTTCTTAAAATTTCCTTGGAACGGAAAAGAA
TTGCCGGACTTGTCCGGAAGGAGATTATTCTTTTTTGTTTCAACGAAAAA
TAATCATAAGGAGGAAGAACAAGAATGGGTGTCGGTATTATTATTGCCAG
CCATGGTAAATTTGCTGAAGGTATTCATCAATCAGGTTCTATGATCTTTG
GTGACCAAGAGAAAGTTCAAGTTGTAACTTTCATGCCAAGCGAAGGACCT
GATGATTTATATGCACACTTCAACGATGCTATCGCACAATTTGATGCTGA
TGATGAAATCCTCGTACTAGCTGACCTTTGGAGTGGTTCTCCATTTAACC
AAGCTAGTCGCGTAATGGGTGAAAATCCAGACCGCAAGATGGCTATCATT
ACAGGTCTTAATTTGCCAATGCTTATCCAAGCCTACACTGAGCGTATGAT
GGACGCAAATGCAGGTGTTGAACAAGTTGTCGCAAATATCATTAAAGAGT
CTAAAGACGGTGTTAAAGCACTTCCTGAAGAACTCAACCCAGCTGAAACA
GCTGCTGCAGCGCCTGCCGCACAAGCTGCTCCTCAAGGTGCTATCCCTGA
AGGAACTGTCATCGGTGATGGTAAACTTAAAATCAACCTCGCTCGTATTG
ACACACGTCTTCTTCACGGACAAGTTGCAACAAACTGGACACCAGCTTCT
AAAGCTGATCGTATCATCGTTGCATCTGACACTGTATCTAAAGATGAATT
GCGTAAAGGCTTGATCAAACAAGCAGCACCAAACGGTGTTAAAGCAAACG
TTGTTCCAATCAAGAAATTGATTGAAGCTTCTAAAGACCCTCGTTTTGGT
AACACACACGCACTTATCTTGTTTGAAACACCTCAAGAAGCTCTTGAAGC
TATCGAAGGCGGTGTGCCAATTAAAGAACTTAACGTTGGTTCAATGGCTC
ACTCAACAGGTAAAACAATGGTTAACAACGTATTGTCAATGGACAAAGAT
GACGTTGCTACATTTGAAAAATTACGTGACCTTGGCGTAACATTCGATGT
TCGTAAAGTCCCTAATGATTCTAAAAAAGACTTGTTTGATCTAATCAAAA
AAGCAAACGTTCAATAATTTTTTGAATTAAGCTTTCAGCAGGGTGAAAAAG
AAAGCGTTTCTATGAAGGGCTTTCACCTGTCGTGAGAATCAAACTTTCCT
GTTTTTATCCAAAACACGTAAGCCTTGGTTTCTGGCTTACAATTGCACAT
TGAAAACTTTTATAAACGAAAGGAACTAGAATAATGTCAGTTATTTCTAT
GATTTTAGTCGTTGTAGTTGCCTTCTTCGCTGGTCTTGAAGGTATCCTTG
ACGAATTCCAATTCCACCAACCACTAGTTGCCTGCACACTTATCGGTCTT
GTTACTGGTAACCTTGAAGCAGGTATCATCCTTGGCGGTTCTCTACAAAT
GATCGCTCTTGGTTGGGCTAACATTGGTGCCGCTGTTGCGCCAGATGCTG
CCCTTGCTTCTGTAGCCGCTGCTATCATCATGGTTAAAGGTGGAGACTTC
ACTTCTAAAGGTATCGCCGTTGCAACAGCAACAGCTATCCCTCTTGCTGT
TGCAGGTCTTTTCCTTACTATGCTTGTTCGTACTGCTTCTGTTGCCCTTG
TTCACGGTGCTGACGCTGCTGCTAAAGAAGGTAACATCGCTGCAGTTGAA
CGTACTCACTTGGTAGCTCTATTCCTTCAAGGTCTTCGTATTGCTGTTCC
TGCTGCTCTTCTTCTTGCAGTACCAACTTCAGCAGTACAATCTATCCTTA
ACGCTATGCCAGACTGGTTGTCAGGTGGTATGGCTGTCGGTGGTGGTATG
GTTGTTGCCGTAGGTTACGCTATGGTTATCAACATGATGGCTACAAGCGA
AGTATGGCCTTTCTTCGCAATCGGTTTTGCCGTTGCTGCTATCTCTGACC
TTACTCTTATCGCCCTTGGTACAATTGGTGTTGCTCTTGCCTTCATCTAC
CTTAACCTTTCAGAAAAAGGTGGAAATGGTGGCGGAACTATCTCAGGTTC

TGGTGACCCAATCGGCGACATCTTGGAAGACTACTAGAAAGGGGTACAAA
CATGGCTGAAAAACTTCAATTATCAAAATCTGATCGTCAAAAAGTTTGGT
GGCGTTCTACTTTCCTACAAGGTTCTTGGAACTACGAACGTATGCAAAAC
TTAGGTTGGGCATACGCTTTGATCCCTGCTATCAAAAAACTTTATACATC
TAAAGAAGACCGAGCTGCTGCTCTTGAACGTCACTTGGAATTCTTCAATA
CTCACCCATACGTTGCTGCTCCAATCATCGGTGTAACTCTTGCCCTTGAA
GAAGAACGCGCAAATGGTGCTGAAATTGATGACACAGCTATCCAAGGGGT
TAAAATCGGTATGATGGGACCTCTTGCTGGTGTTGGTGACCCAGTCTTCT
GGTTTACAGTTCGTCCTATCCTTGGTGCCCTTGGTGCTTCACTTGCTATG
GCAGGTAACATCGTTGGTCCACTTCTATTCTTCTTTGGATGGAACATCAT
CCGTATGGCATTCTTGTGGTACACTCAAGAACTTGGTTACAAAGCTGGTT
CTGAAATCACTAAAGACCTTTCAGGTGGTATCATCCAAAAAATCACTAAA
GGTGCTTCAATCCTTGGTATGTTCATCTTGGCTGTCTTGGTTGAACGTTG
GGTATCAATTAACTTCACTGTTGATCTTCCTTCAACTAAACTTTCAGAAG
GTGCCTACATCGAATTCCCTAAAGGAAACGTAACTGGTACTGAACTTCAA
GGTATTCTTGGTAAAGTGGCTGATGGACTTAGCCTTTCACCAGAAAAAGC
TAACACACTTCAAGGTCAATTGAACTCATTGATTCCTGGTTTGATGGGAC
TTGCCCTTACATTCCTTTGCATGTGGTTGCTTAAGAAAAAAGTTTCTCCA
ATCACAATCATCATCGGATTGTTCATCGTTGGTATCATCGCTCGTTTCTT
CGGAATCATGTAATAAAAGAAAGGAAGGATTTCGGTCCTTCTTTTTTTAT
TATGTTATAATAGACTAAAAGTTTAATCAGAGGAGATTACATGGCACAAT
CACTTAACTCTACCGTGGAATTAACGGCTACTGGTGTTTCTTACCTTGGT
ATGGGAGGAAAAGTAGGGAAATTCCTTCTCGGTAACAAAGGTCTTGAATT
TTATAGCGACGCTAACGTCGAAGATTATATCCAAATTCCTTGGGAAAACA
TCGAAAAAATCGGCGCTAACGTTTCTCGTAACAAAGTCAGCCGTCACTTT
GAAATCTTTACAGACAAAGGGAAATTTCTTTTTGCCTCAAAAGACTCAGG
AAAAATCTTAAAAGTGGCTCGCCAGCATATCGGAAACGATAAAGTTGTTC
GTATGCTTACGCTTGTTCAAGTCCTTATGAAAAAACTAACAGGATTTGTC
AAAAAGAAATAAATCTAGTATAATAAATAGTACGGATAAGTAAGAGTGGA
GCTCTTTCAGAAAGTCTGCGGTTGCTGTGAGCAGATAGAGCGACAGGTCT
GAAATTCTACCGTTATATCAATTAAAAATAGAAATTAAGTGCACAGGATG
TGAAGCTGGATGGAACCGCGCGATTGCGCTCCAGCAGTTAACATGCTGTG
CTTTTTGTTCATCTGGGCACAGCTAACCCTATGAGCGCTAAAAAAACTGG
AGGAAACTTATGTTAGACATCAAACGTATTCGTAACGATTTCGACGAAGT
CGCTAAAAAATTAGCTACACGTGGCGTTGCTGCTGAAAAACTTGCTGAAC
TTAAAGAACTTGACGACAAACGTCGTGAATTGCTT

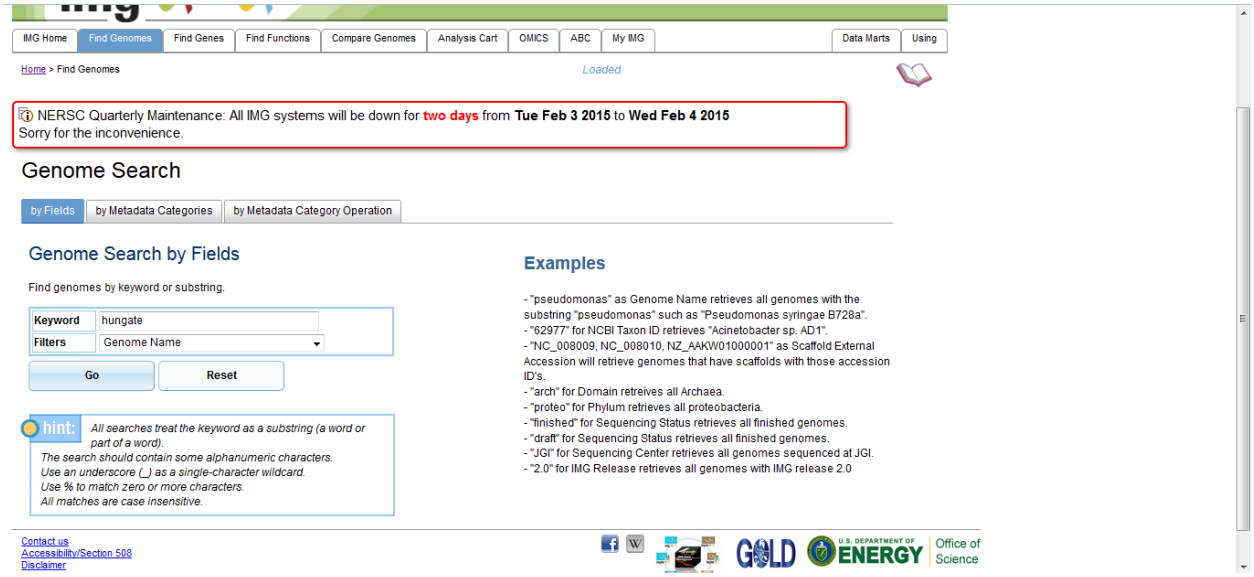
Example experiment: Finding the sequence of mannose phosphotransferase (PTS) genes with IMG

Modified from (100)

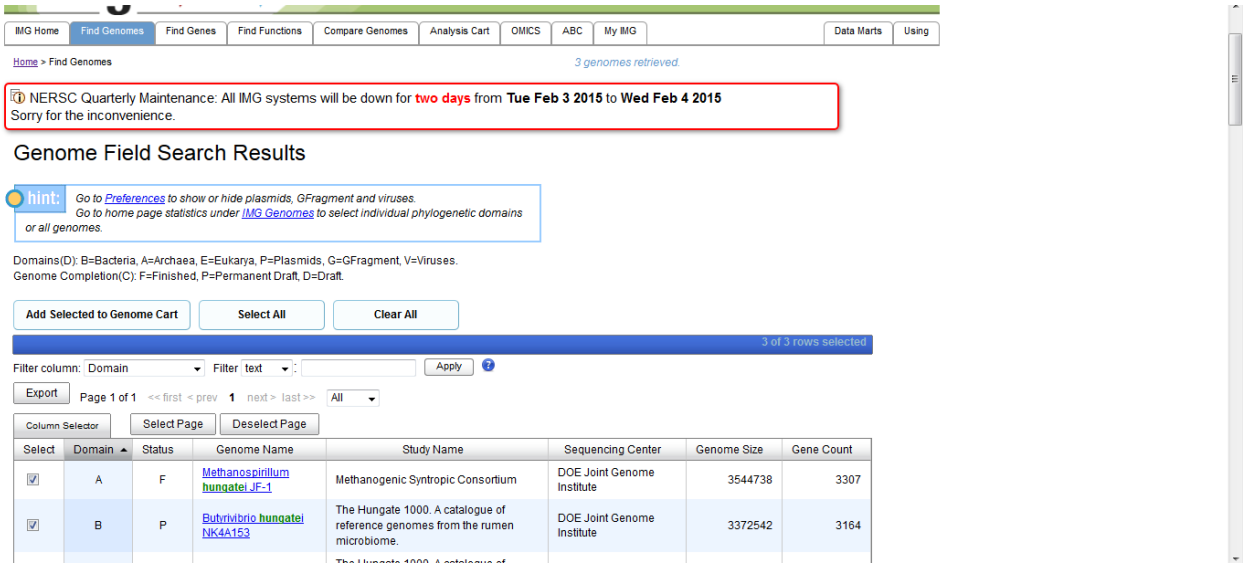
1.) Go to https://img.jgi.doe.gov/cgi-bin/m/main.cgi. You may be prompted to register and log in first.

2.) Go to “Genomes” -> “Genome search”.

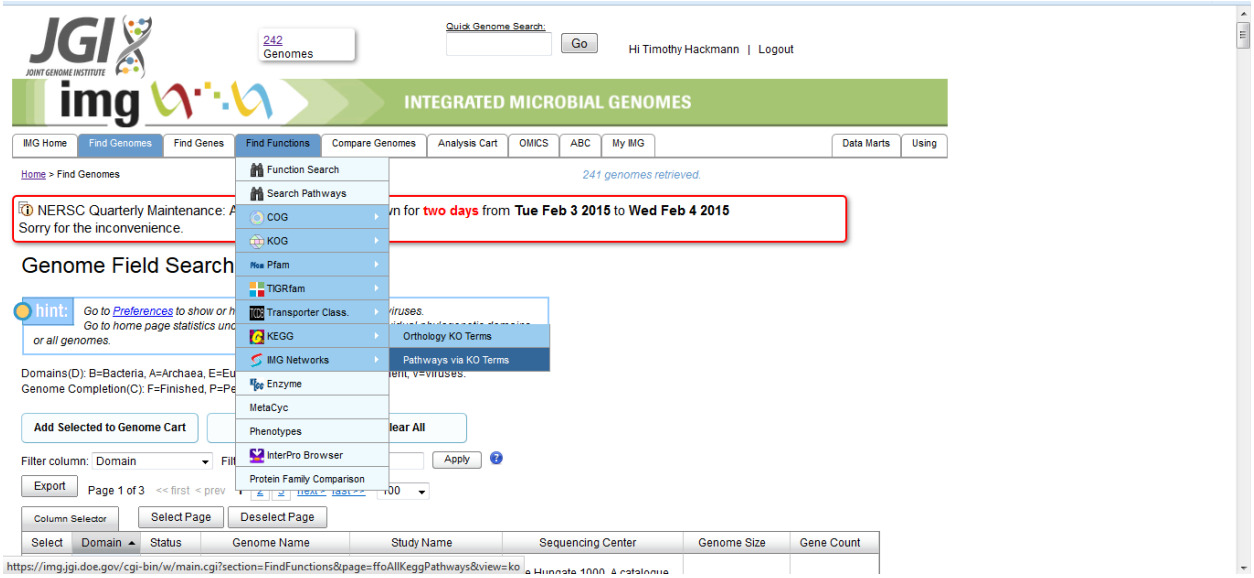
3.) In “Keyword”, type the genome of interest. Click “Go”.
For a large set of rumen bacterial genomes, enter “Hungate 1000”. Under “Filters”, find “Study Type”.



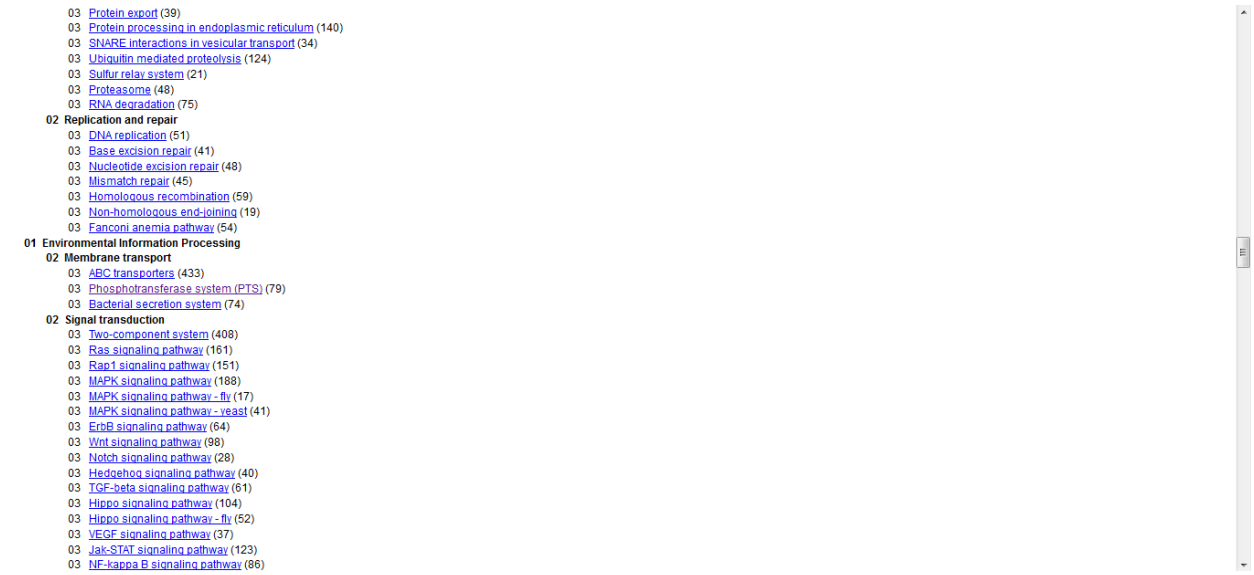
4.) Click “Select All” and “Add Selected to Genome Cart”. Make sure that you are showing all entries on one page; select “All” from the drop down menu.



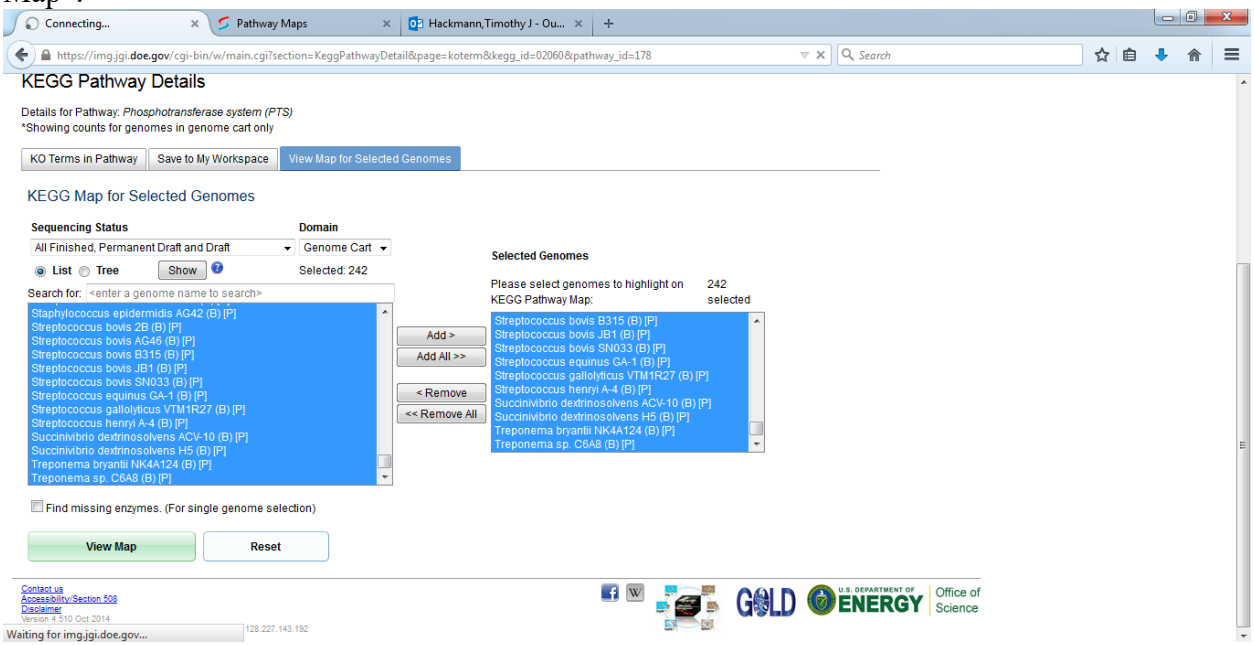
5.) In the menu tabs, point to “Find Functions”, then “KEGG”, then “Pathways via KO terms”. Click.



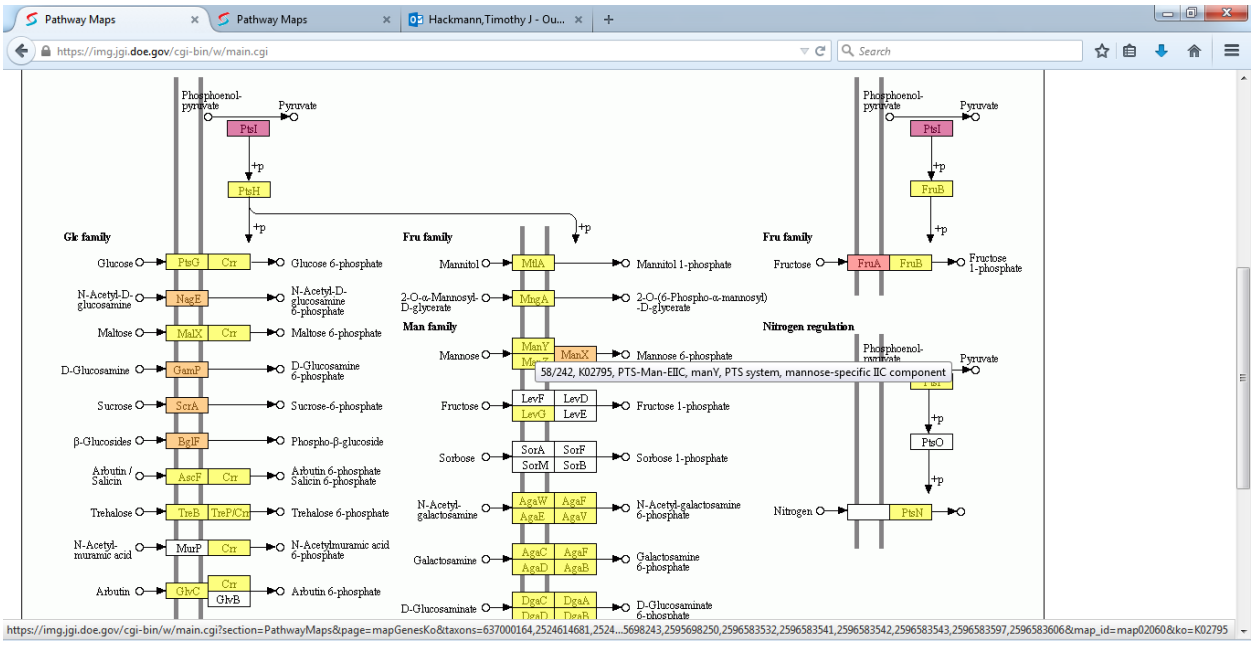
6.) Search for and click on “Phosphotransferase system (PTS)”



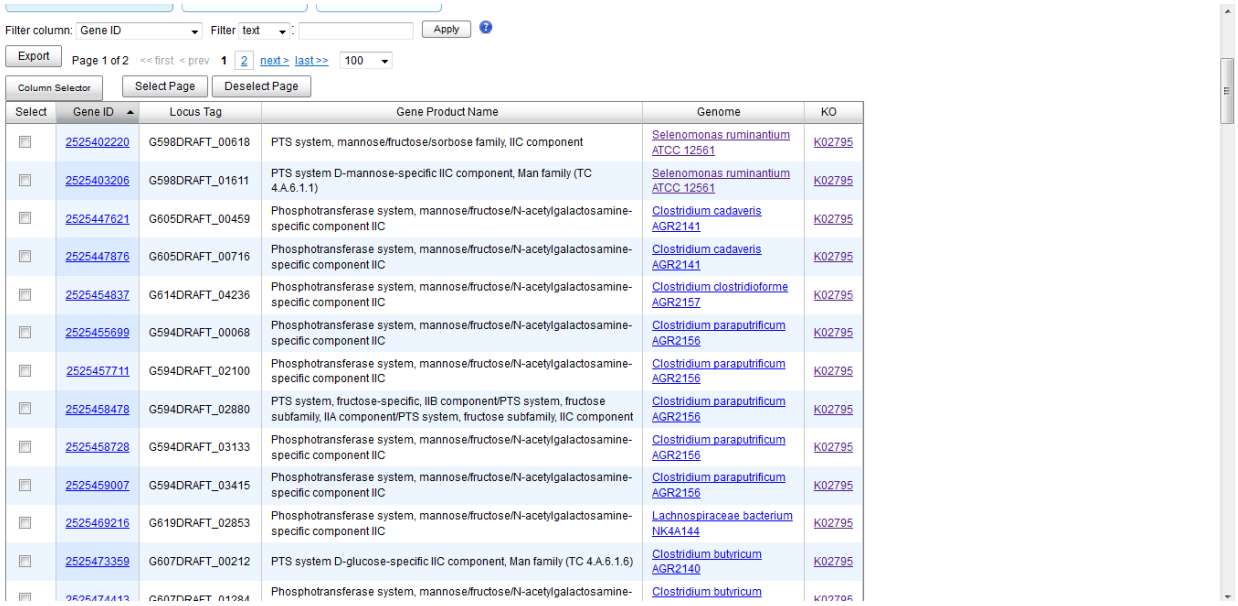
7.) Click tab for "View Map for Selected Genomes". Click "Add All>>", then click "View Map".



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.



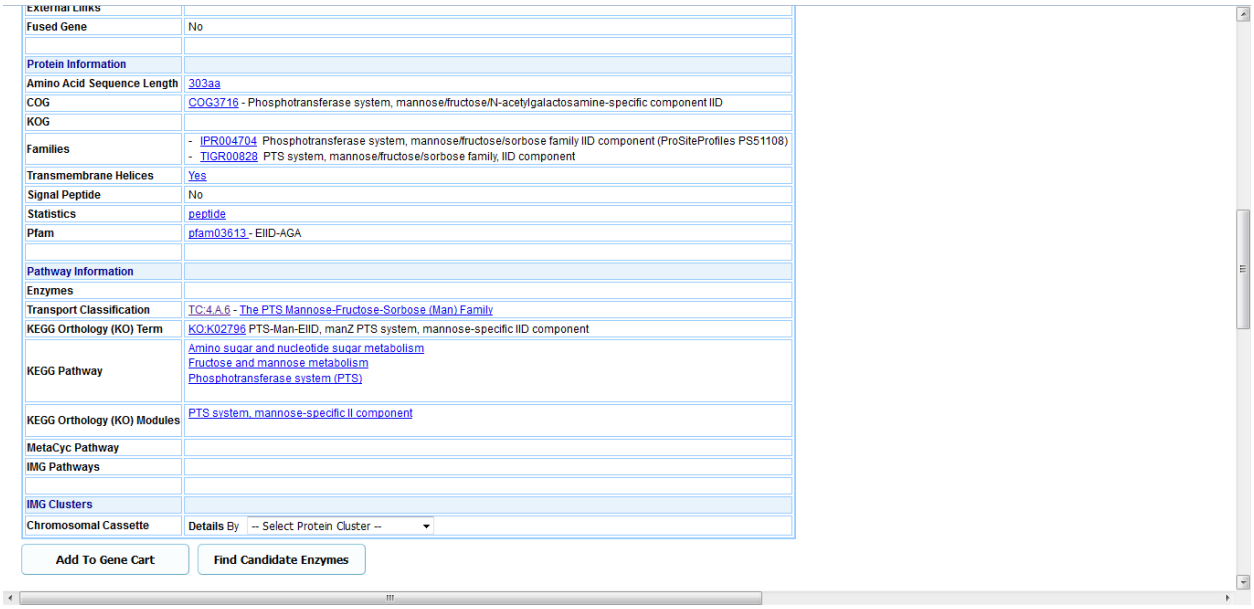
Filter column: Gene ID Filter text: Apply

Export Page 1 of 2 << first < prev 1 2 next > last >> 100

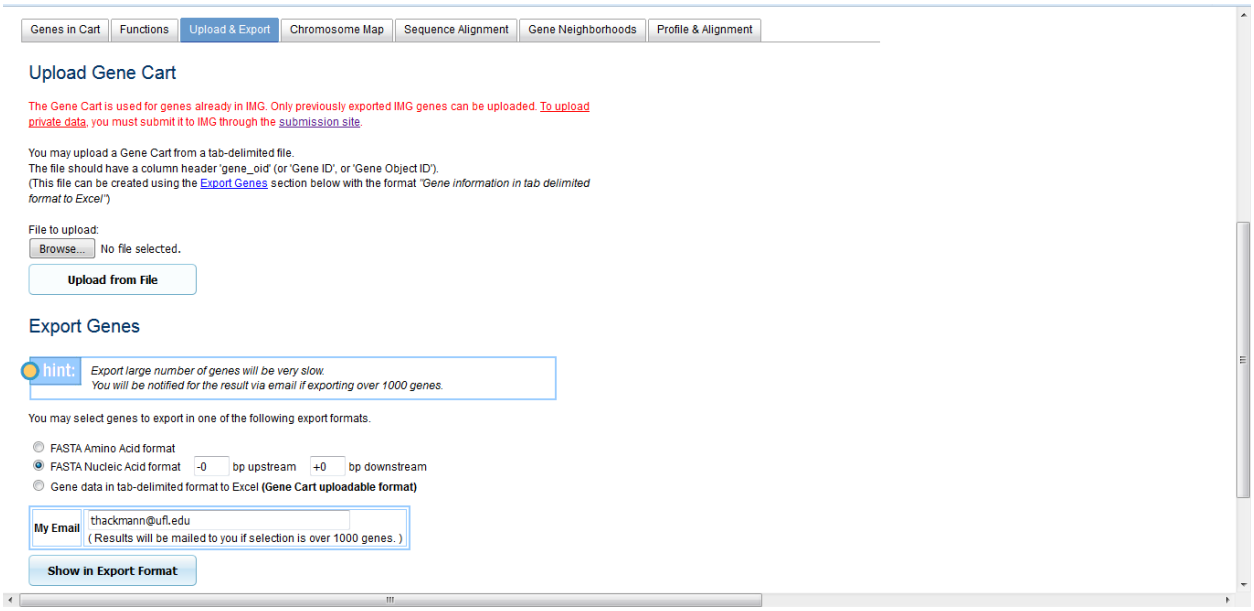
Column Selector Select Page Deselect Page

Select	Gene ID	Locus Tag	Gene Product Name	Genome	KO
	2525402220	G598DRAFT_00618	PTS system, mannose/fructose/sorbose family, IIC component	Selenomonas ruminantium ATCC 12561	K02795
	2525403206	G598DRAFT_01611	PTS system D-mannose-specific IIC component, Man family (TC 4.A.6.1.1)	Selenomonas ruminantium ATCC 12561	K02795
	2525447621	G605DRAFT_00459	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium cadaveris AGR2141	K02795
	2525447876	G605DRAFT_00716	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium cadaveris AGR2141	K02795
	2525454837	G614DRAFT_04236	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium clostridioforme AGR2157	K02795
	2525455699	G594DRAFT_00068	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
	2525457711	G594DRAFT_02100	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
	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
	2525458728	G594DRAFT_03133	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
	2525459007	G594DRAFT_03415	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Clostridium paraputrificum AGR2156	K02795
	2525469216	G619DRAFT_02853	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	Lachnospiraceae bacterium NK4A144	K02795
	2525473359	G607DRAFT_00212	PTS system D-glucose-specific IIC component, Man family (TC 4.A.6.1.6)	Clostridium butyricum AGR2140	K02795
	2525474413	G607DRAFT_01284	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-	Clostridium butyricum	K02795

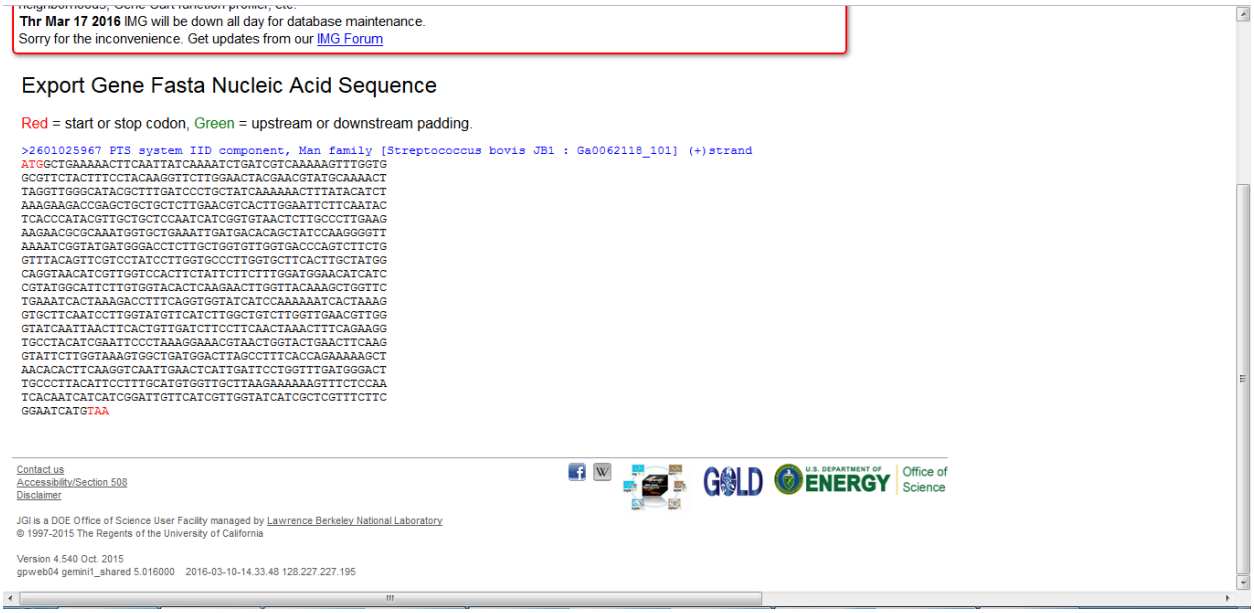
10.) Scroll down and click “Add to Gene Cart.”



11.) Click “Upload & Export” tab, select the “FASTA Nucleic Acid format” radio button, and click “Show in Export Format”.

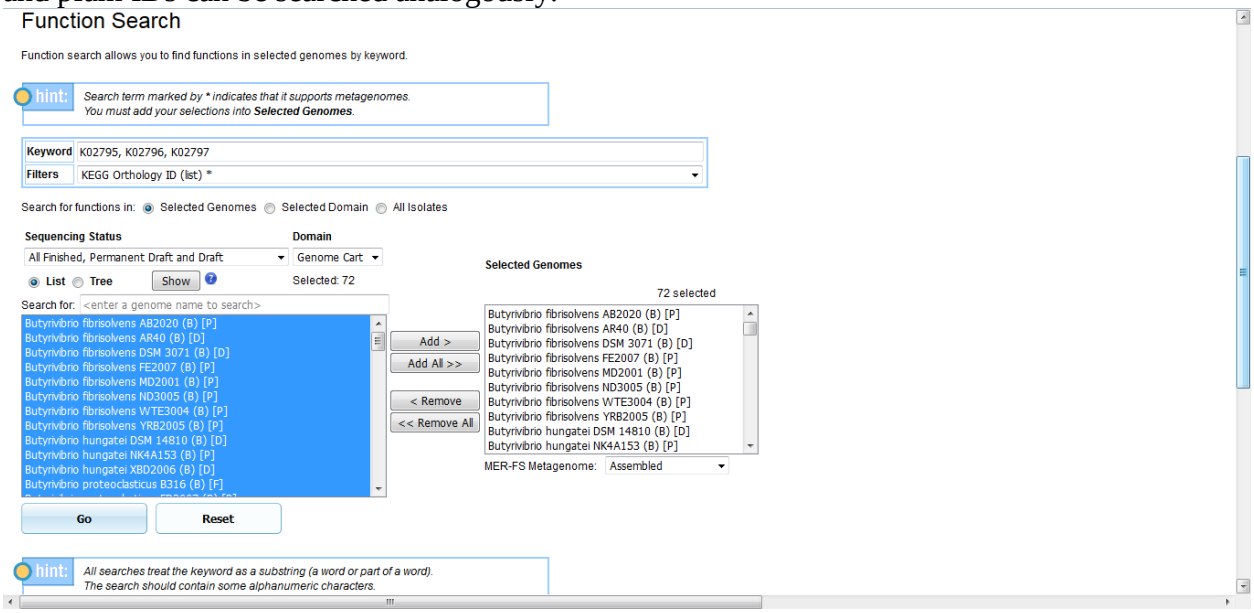


12.) Examine the sequence that is displayed. Copy and paste into Notebook and save as a *.fasta file.



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.



Amplification of DNA from small numbers of rumen bacteria cells

Adapted from (94-97)

Preparation of equipment and other materials

1.) Gather 10 µl pipette tips (Phenix TS-060BR); 30 µl pipette tips (Diamond F171303); 300 µl pipette tips (Eppendorf 0030 077.636); 1000 µl pipette tips (Fisher 02707404); 1.5 mLcentrifuge 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 µl H_2O 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 µL 10,000X SYBR Green (Fisher S7563) into 199 µL molecular biology water. Dispense 50 µL aliquots into 1.5-mL PCR microcentrifuge tubes. Store at -20°C.

3.) Order primers (Table 1). Prepare 10 µM 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 µl of each sample (serial dilution of mixed bacteria, positive controls, negative controls) to strip tubes in duplicate.

2.) Add 1.5 µl 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 µl 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 µl 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 radiant exposure in that reference was 4 mW/cm^2, whereas the exposure of our multilinker only 3.75mW/cm^2. Thus, 75×4/3.75 = 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_2O 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℃	6 min
	Plate read	30℃	NA
	Heat inactivation	65℃	3 min
	Hold	4℃	∞

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
	Initial denaturation	98°C	3 min
40 cycles	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	∞

[a]Use 0.5°C increments

Fig. 1. Startup Wizard of Bio-Rad CFX Manager.

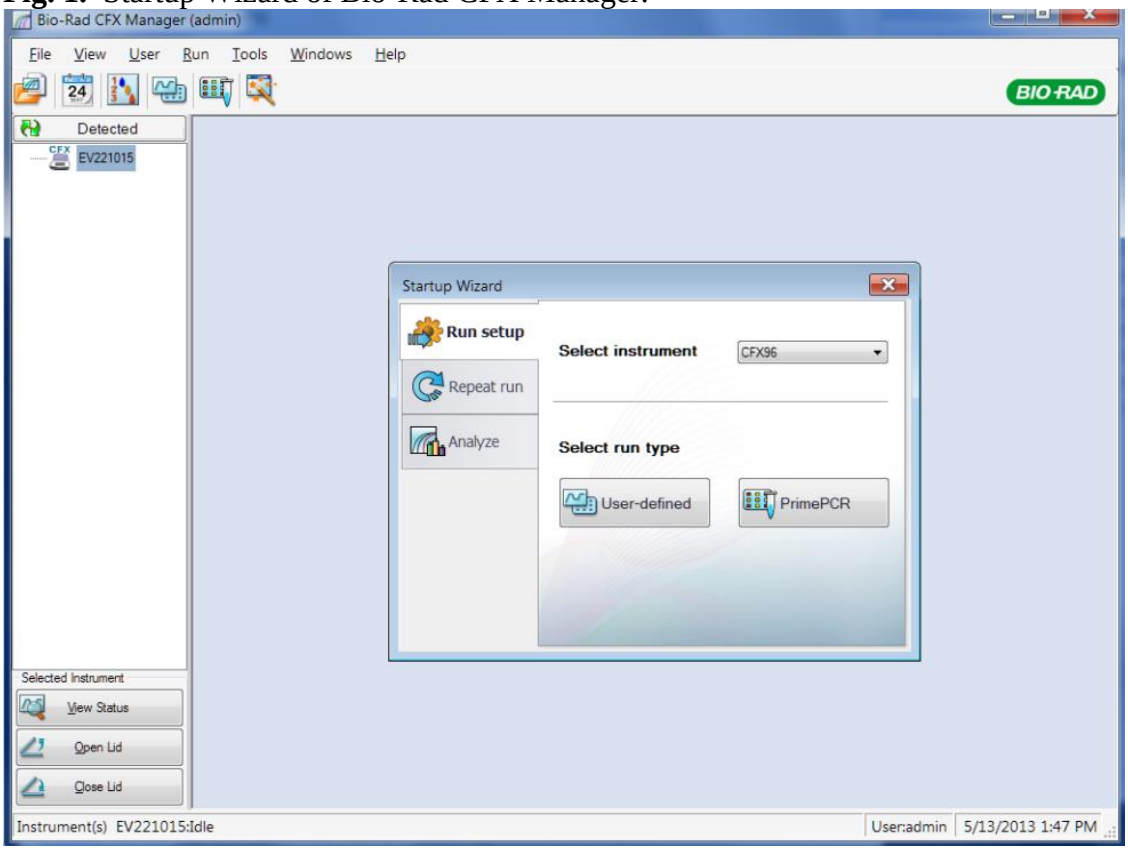


Fig. 2. Run Setup.

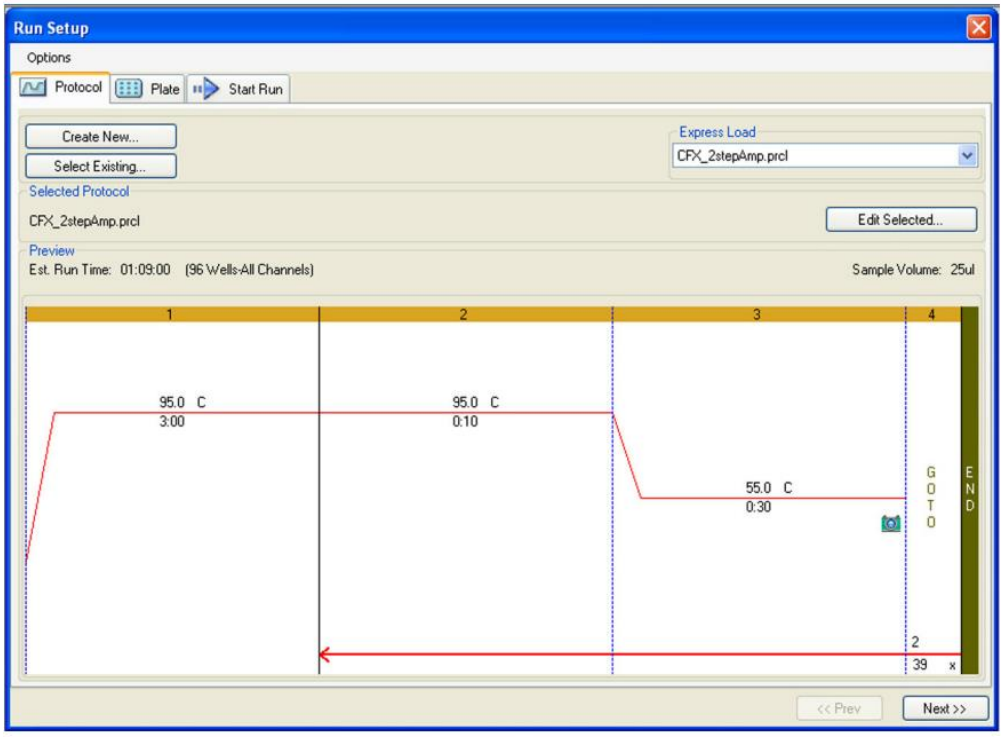


Fig. 3. Protocol Editor window

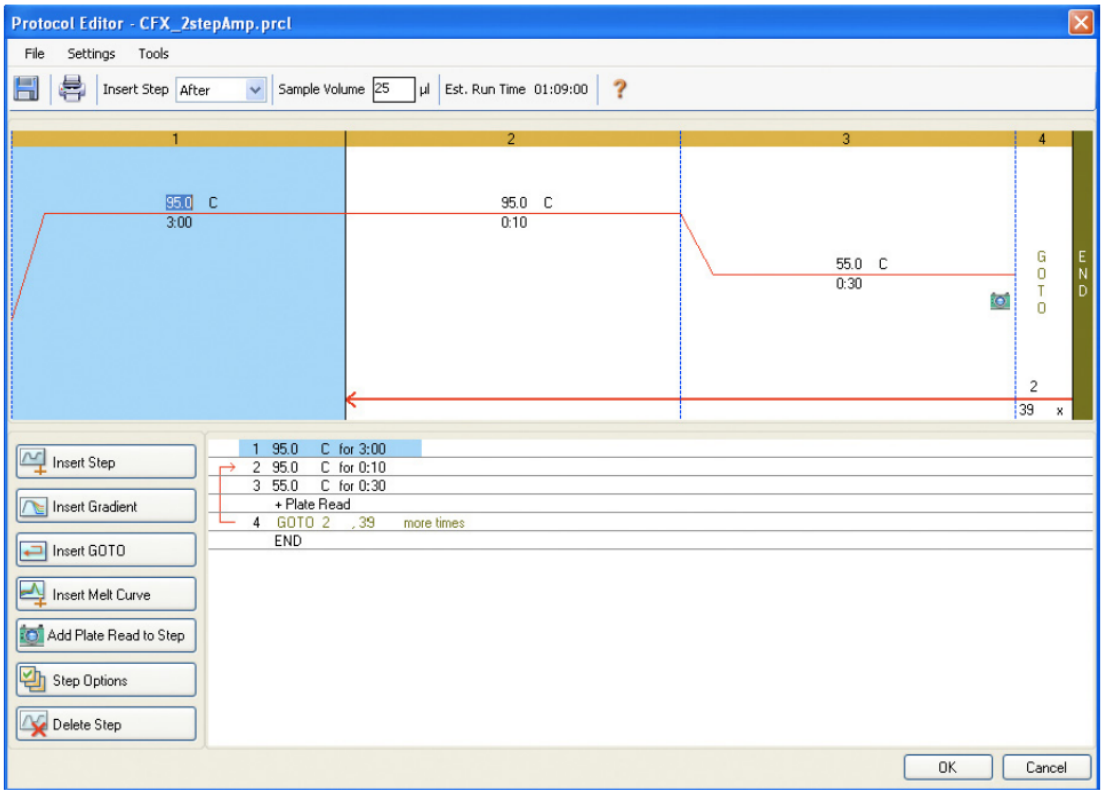


Fig. 4. Plate Editor window

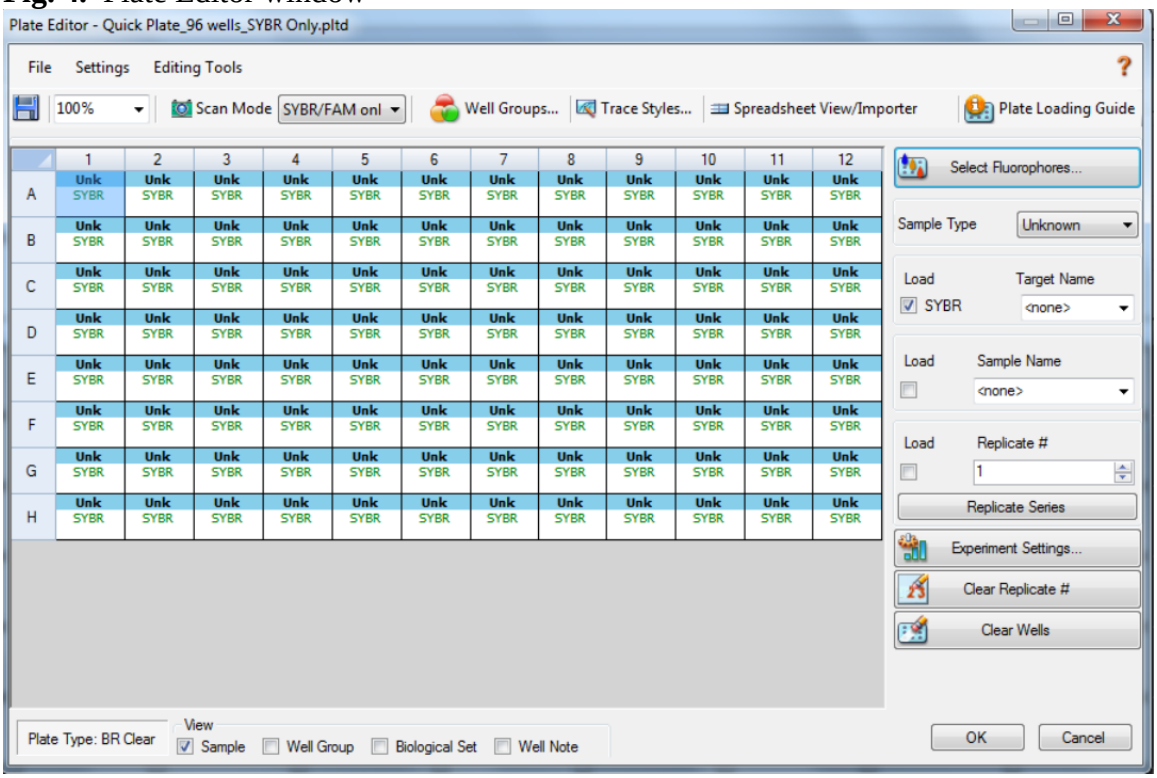


Fig. 5. Start Run tab in Run Setup.

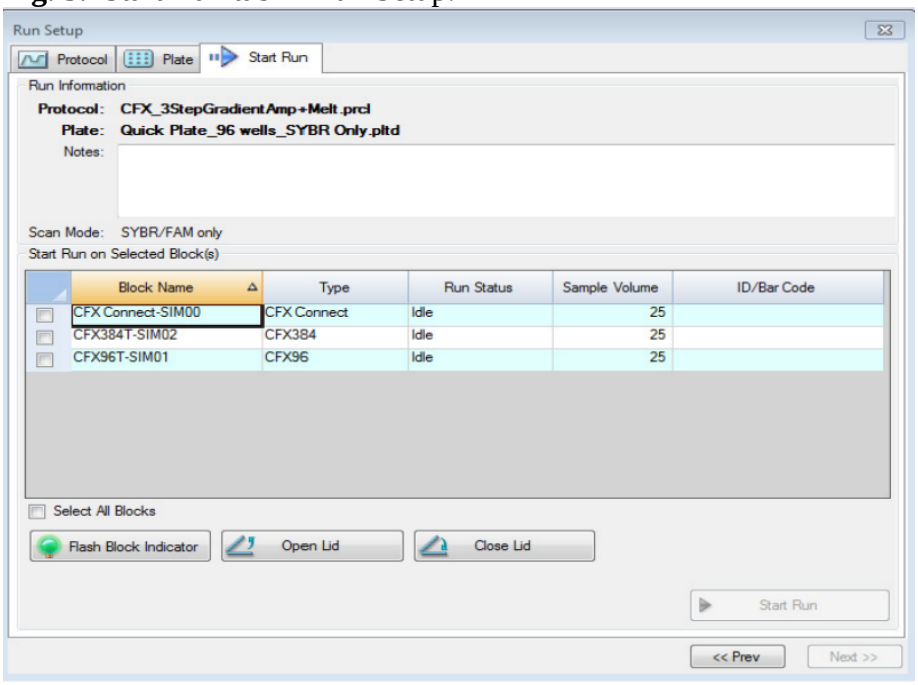


Fig. 6. Balancing of strip tubes.

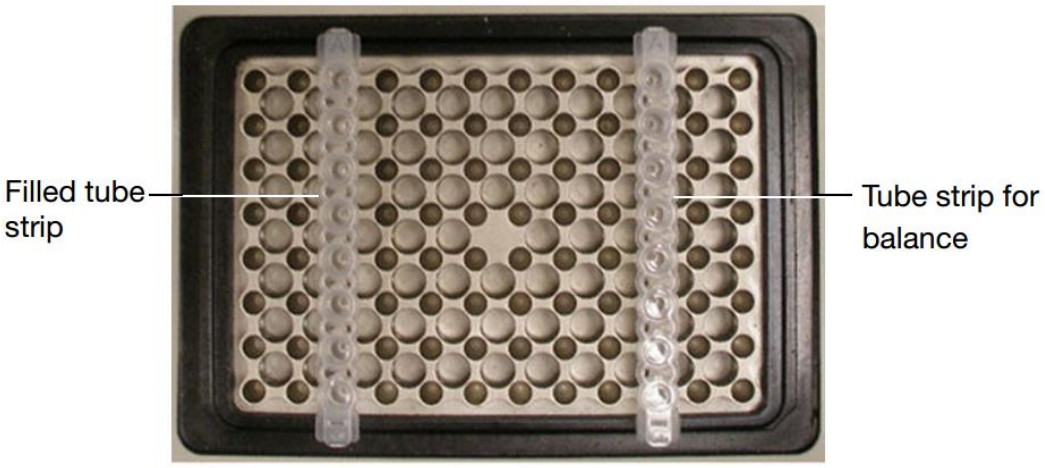


Fig. 7. Run Details window with Real-time Status tab.

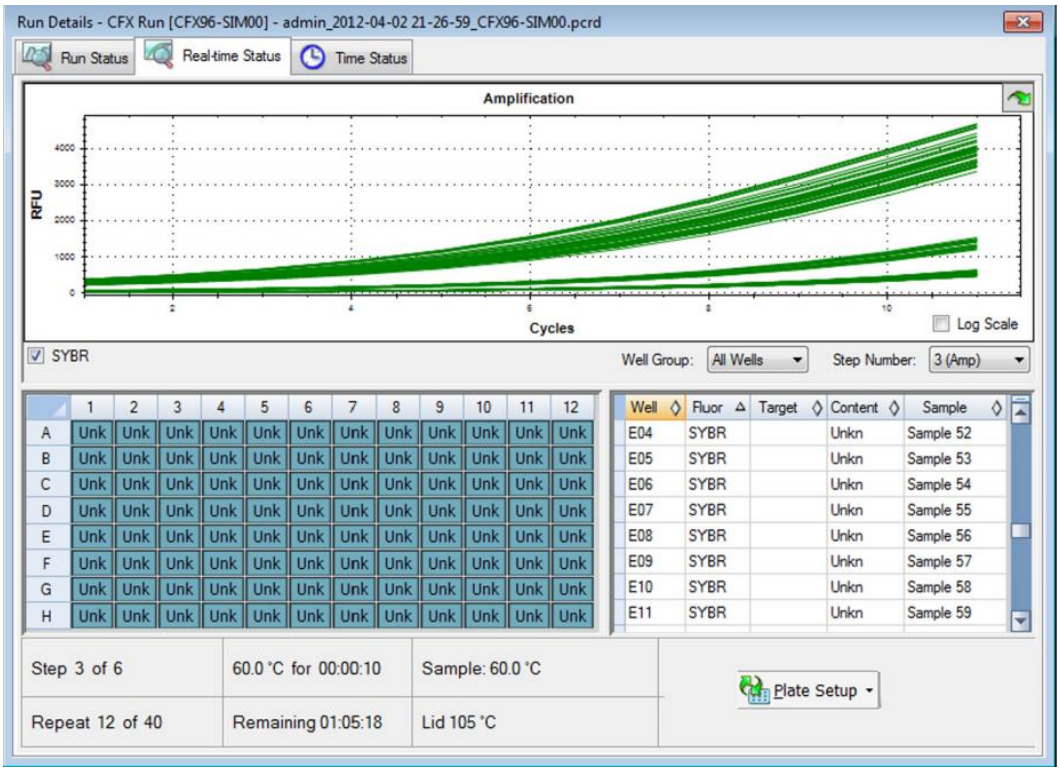


Fig. 8. Melt Curve tab, showing melt curve with sharp peak.

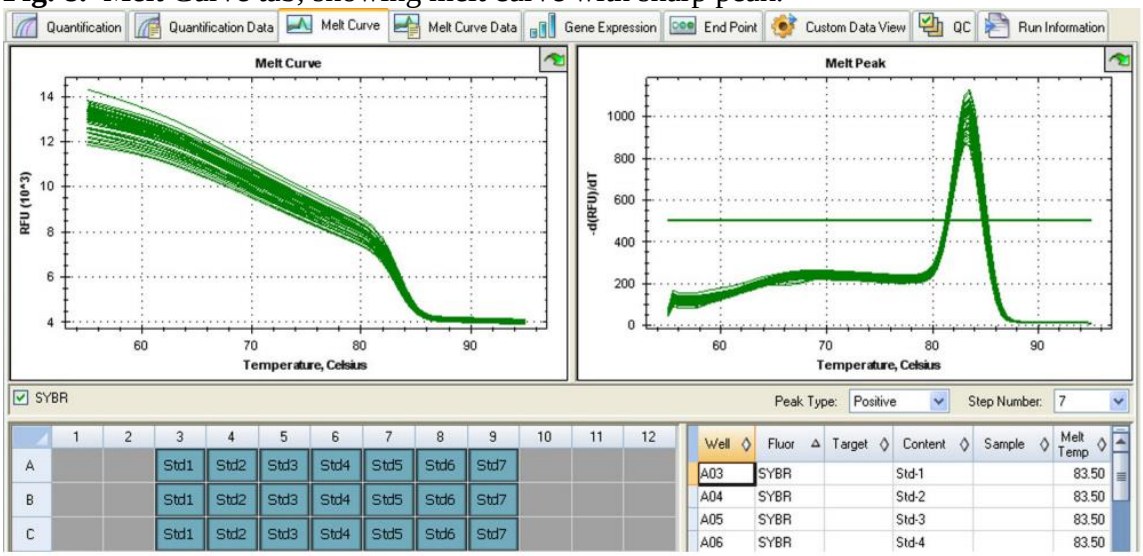
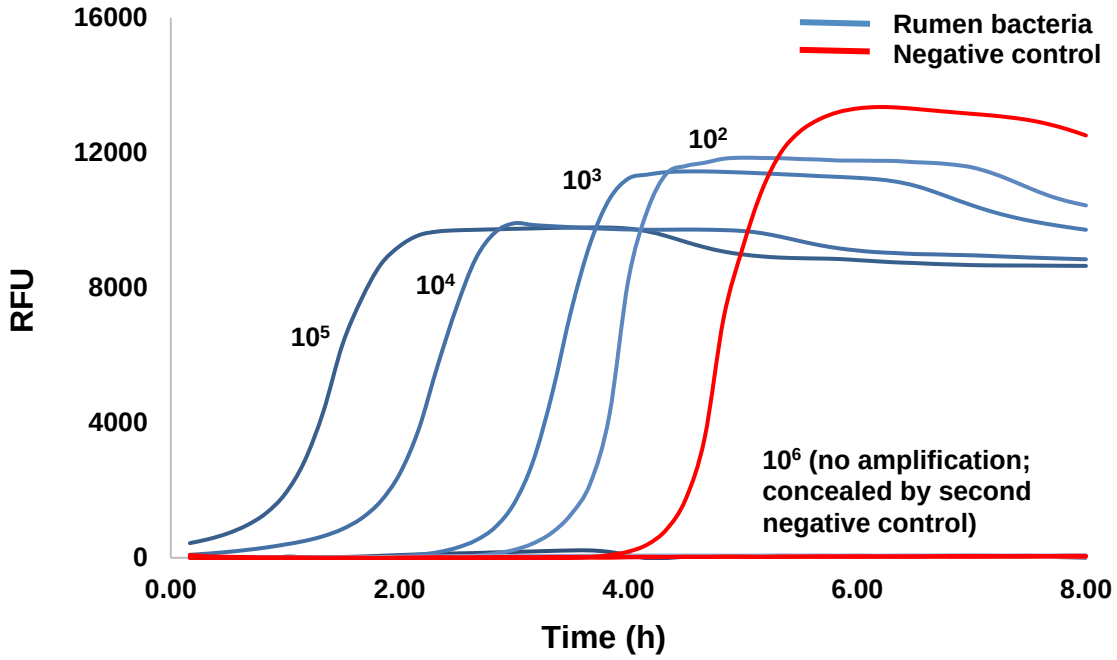


Fig. 9. Amplification of genomic DNA from rumen bacteria. Number of bacteria in each sample is shown alongside curves. All amplifications were run in duplicate, but only one replicate is shown for brevity. Both replicates are shown for the negative control because one amplified while the other did not.



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 4ºC) and 0.002 g dithioerythritol (DTT) in a 2-ml tube. Dissolve in 1 mL acetone and chill at -20ºC. 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 4ºC.

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 4ºC 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 -80ºC.

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 µg/µL).

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 -80ºC.

3) Thaw at room temperature (requires approximately 3 min).

4) Continue incubation overnight at -20ºC.

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 ºC 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 µL. If the volume is not 100 µL, adjust the volumes of the DTT, IAM, and AMBIC in the following steps accordingly.

5) Add 2.5 µl of 200 mM DTT in AMBIC to the 100 µL of dissolved sample in the 1.5 mL tube. Incubate at 37°C for 30 min.

6) Add 7.7 µl of 194.6 mM IAM in AMBIC. Incubate at room temperature in dark for 30 min.

7) Quench IAM by adding 20 µl 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 µg).

9) Activate trypsin by adding 550 µl 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 µg.

Desalting with ZipTips

1) Attach a Millipore C18 ZipTips (ZTC18S096) to a 10 µL-pipette. Make sure that tip is well-seated. Set pipette to 10 µL.

2) Wet tip by aspirating 10 µL 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 µL 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).

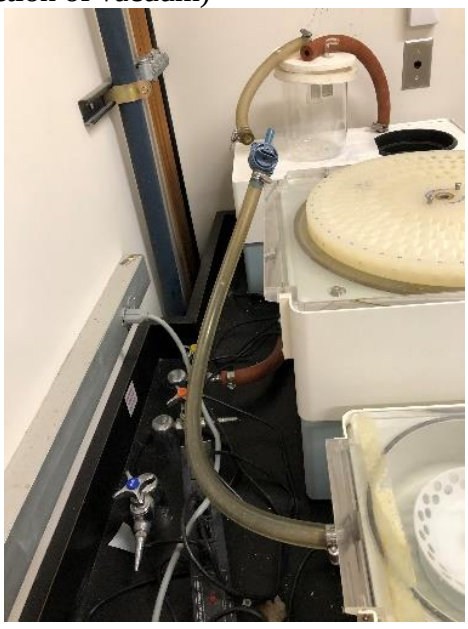
7) Use the 3 µl aliquot to measure peptides concentration. Use the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo 23290) according to instructions. Store sample at -80°C.

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)



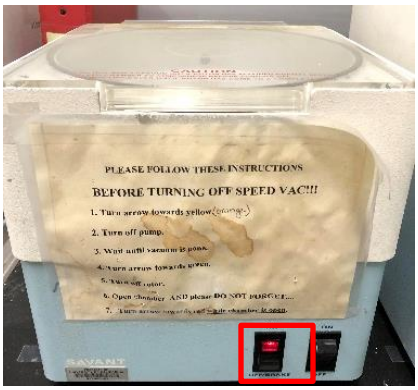
(C) Turn on trap and wait for 1 hour to allow the temperature to decrease to about -50ºC.

2) Load samples in SpeedVac concentrator.

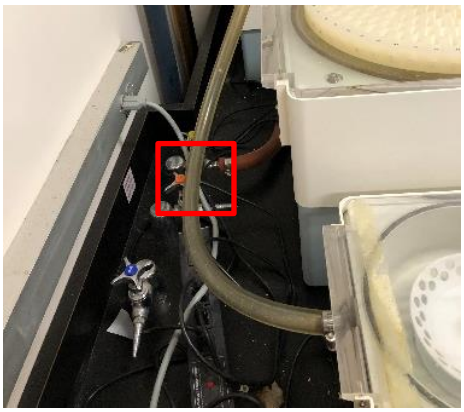
(A) Take out samples from -80ºC and thaw at room temperature. Samples should be in 1.5-ml tubes.

(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 warms 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 ng/µL, 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

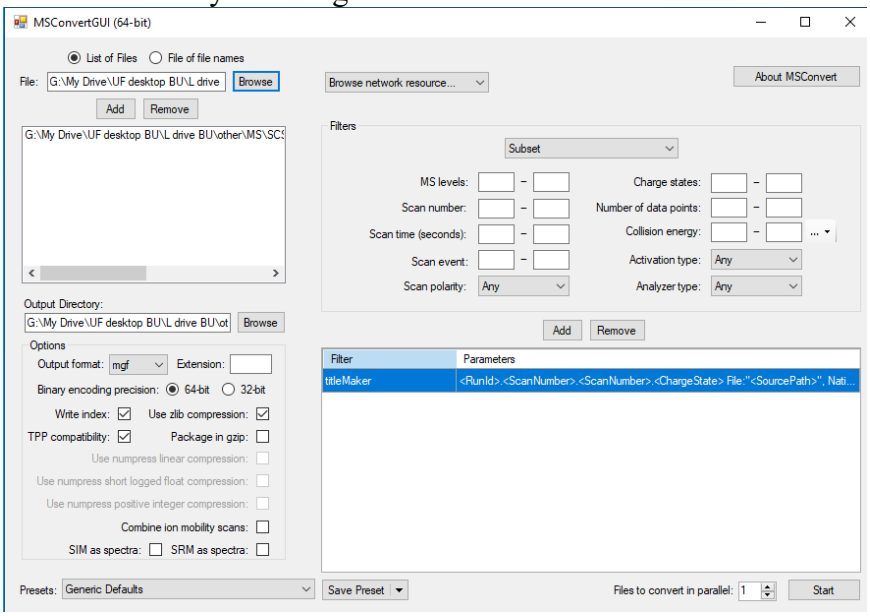
- MSConvert
- X!Tandem pipeline

- 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).

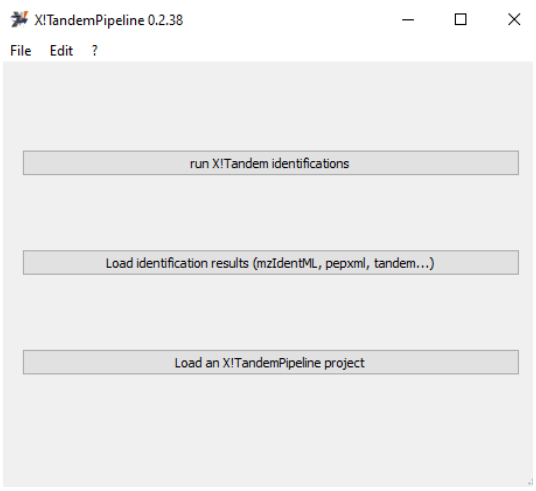
(A) Open MSConvert.
(B) Select *RAW file by choosing "Browse"



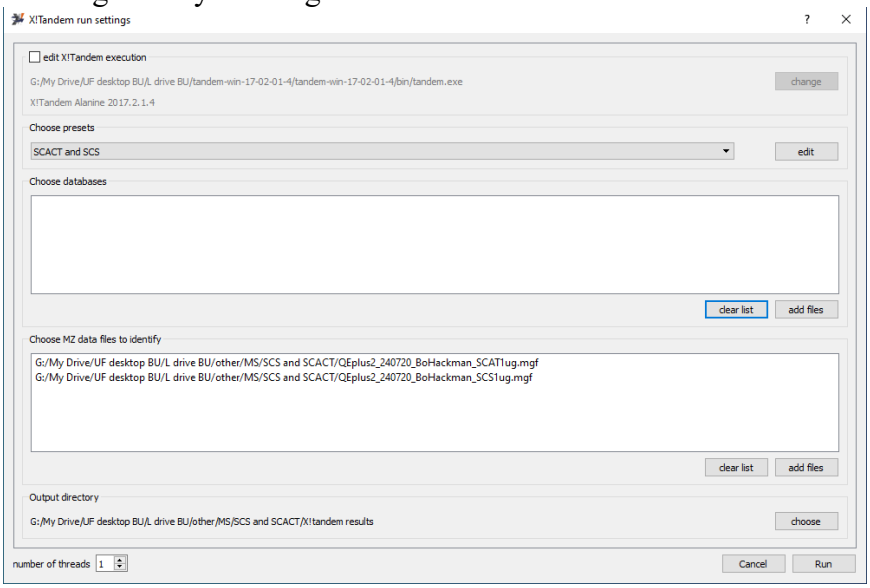
Click "Add." Click "Start."

4.) Obtain a *faa or *fasta file containing the sequence of the protein. The file should 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

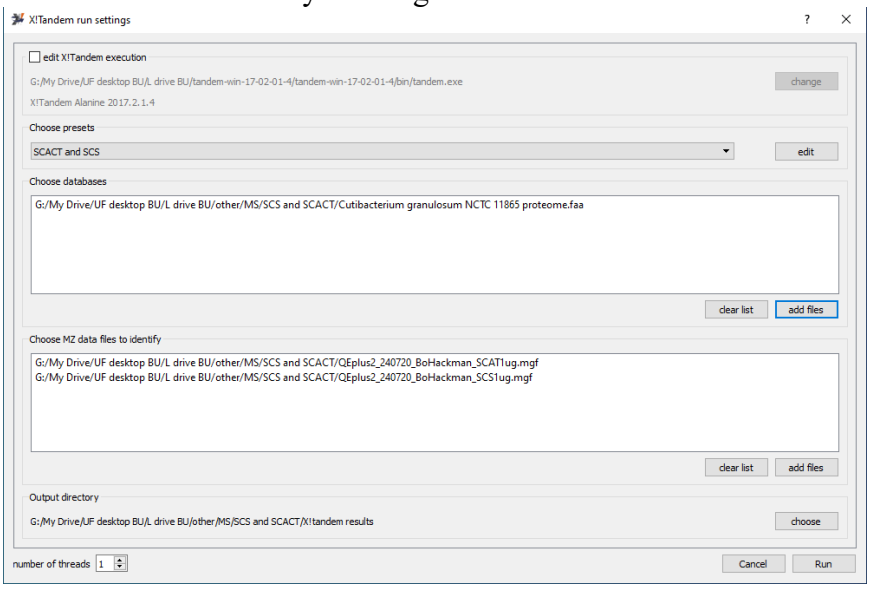
5.) Identify proteins in *mgf file.
(A) Open X!Tandem pipeline (xtcpp.exe).
(B) Click "run X!Tandem identifications"



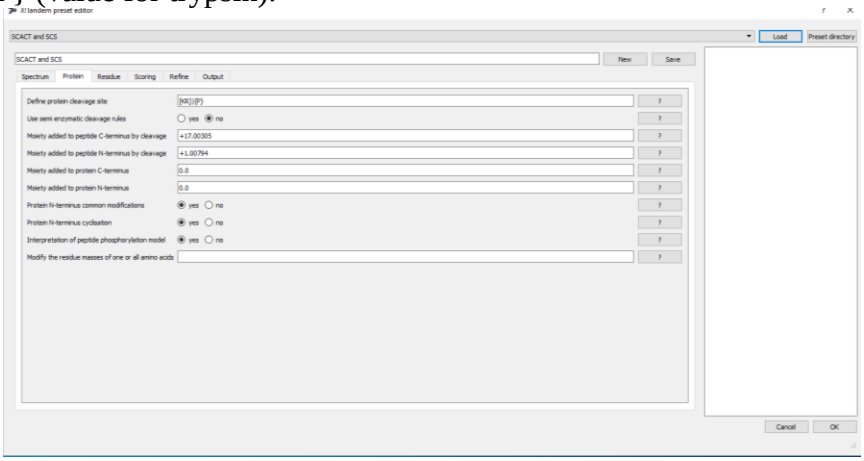
(C) Select *mgf file by clicking “add files” under “Choose MZ data files to identify”.



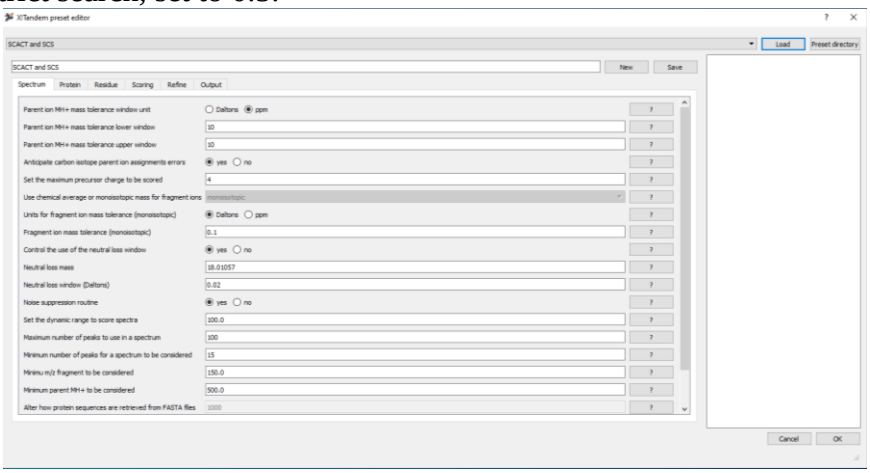
(D) Select *faa or *fasta file by clicking “add files” under “Choose database”



(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).



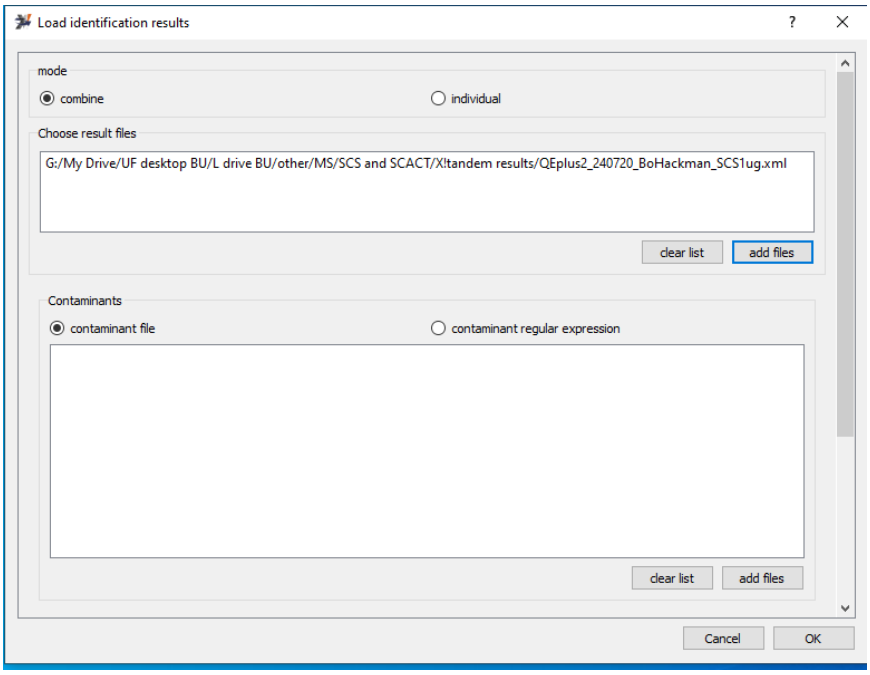
b. Click the “Spectrum” tab. Set the “Fragment ion mass tolerance (monoisotropic) to 0.1. For a less strict search, set to 0.5.



c. Inspect other parameters. They can be left at default values if appropriate.

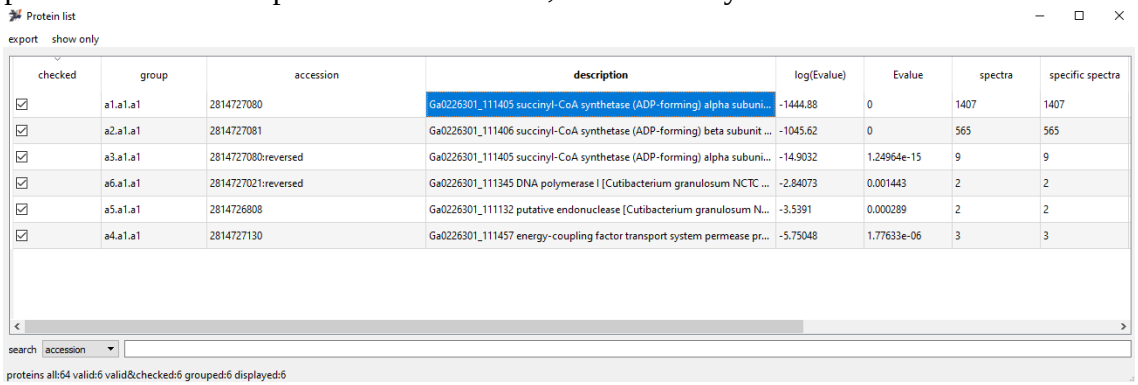
(G) Click “Run”

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.

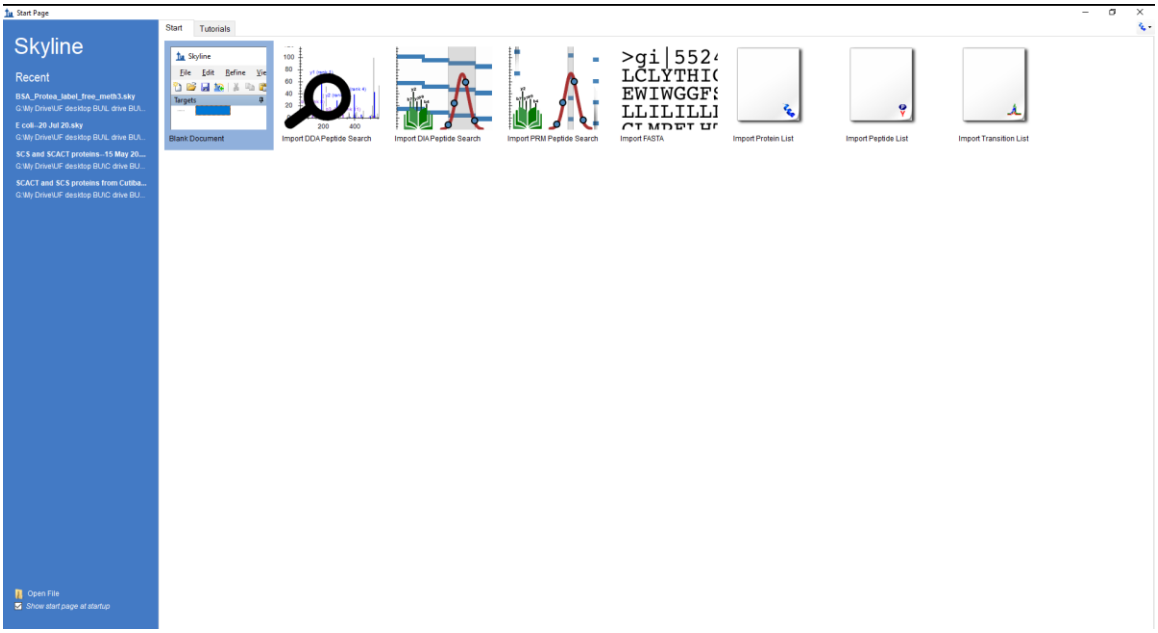


checked	group	accession	description	log(Evalue)	Evalue	spectra	specific spectra
☑	a1.a1.a1	2814727080	Ga0226301_111405 succinyl-CoA synthetase (ADP-forming) alpha subuni...	-1444.88	0	1407	1407
☑	a2.a1.a1	2814727081	Ga0226301_111406 succinyl-CoA synthetase (ADP-forming) beta subunit ...	-1045.62	0	565	565
☑	a3.a1.a1	2814727080:reversed	Ga0226301_111405 succinyl-CoA synthetase (ADP-forming) alpha subuni...	-14.9032	1.24964e-15	9	9
☑	a6.a1.a1	2814727021:reversed	Ga0226301_111345 DNA polymerase I [Cutibacterium granulosum NCTC ...	-2.84073	0.001443	2	2
☑	a5.a1.a1	2814726808	Ga0226301_111132 putative endonuclease [Cutibacterium granulosum N...	-3.5391	0.000289	2	2
☑	a4.a1.a1	2814727130	Ga0226301_111457 energy-coupling factor transport system permease pr...	-5.75048	1.77633e-06	3	3

7.) Optional: view chromatograms and proteins in Skyline.

(A) Open Skyline.

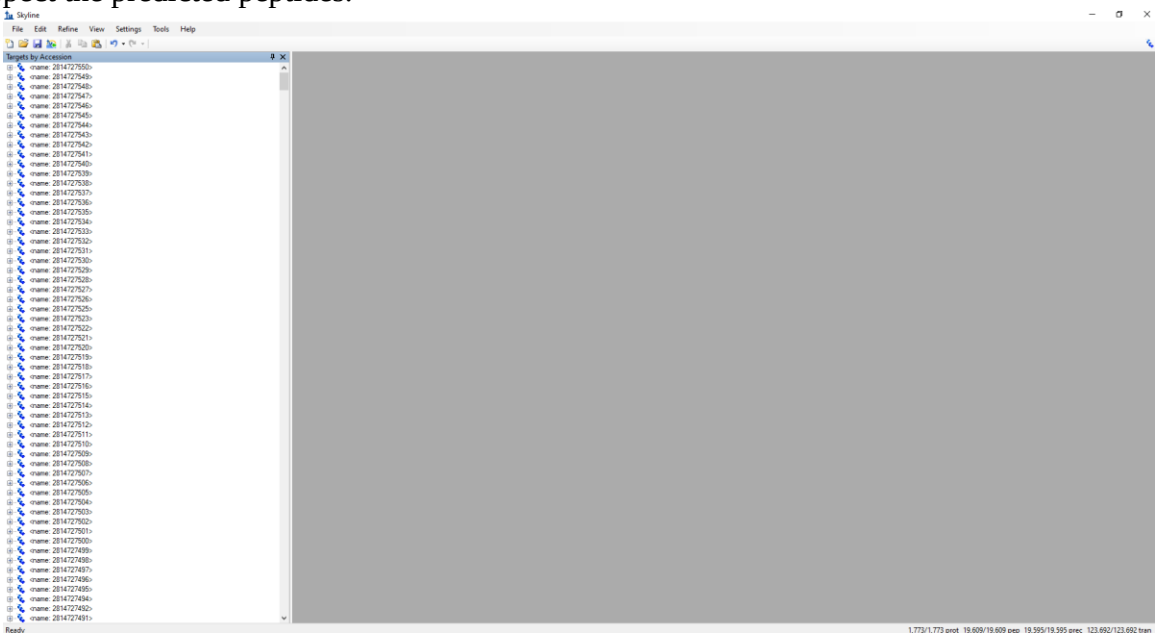
(B) Click “Blank Document”



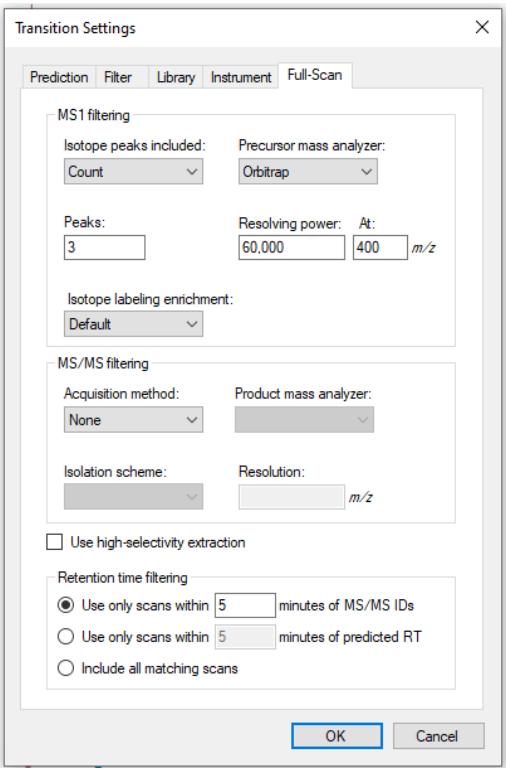
(C) Save file via File -> Save As… (must do this to proceed).

(D) Load the *faa or *fasta file via File -> Import -> Fasta…

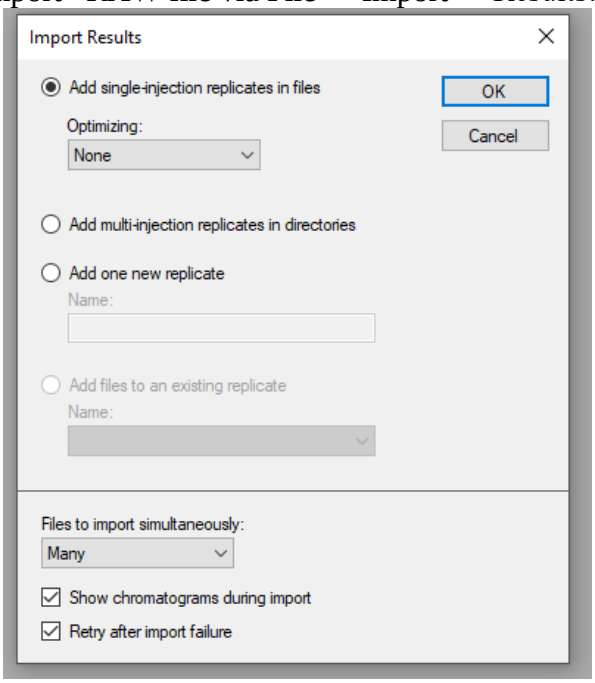
(E) Inspect the predicted peptides.



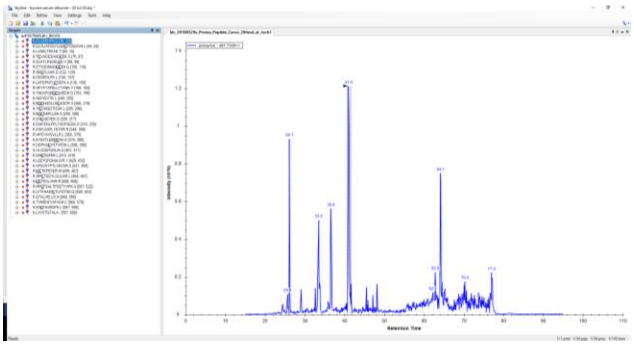
(F) Specify settings for chromatogram via Settings -> Transition Settings…. See example below.



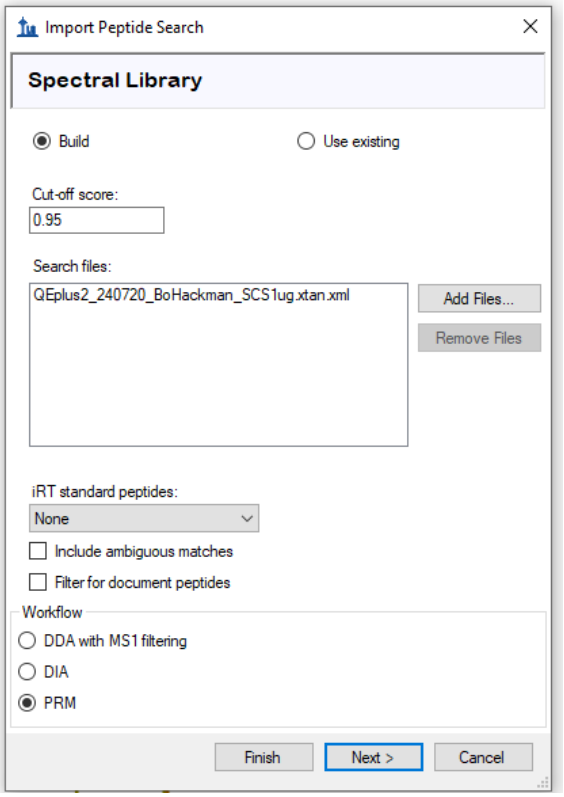
(G) Import *RAW file via File -> Import -> Results… When prompted, click OK.



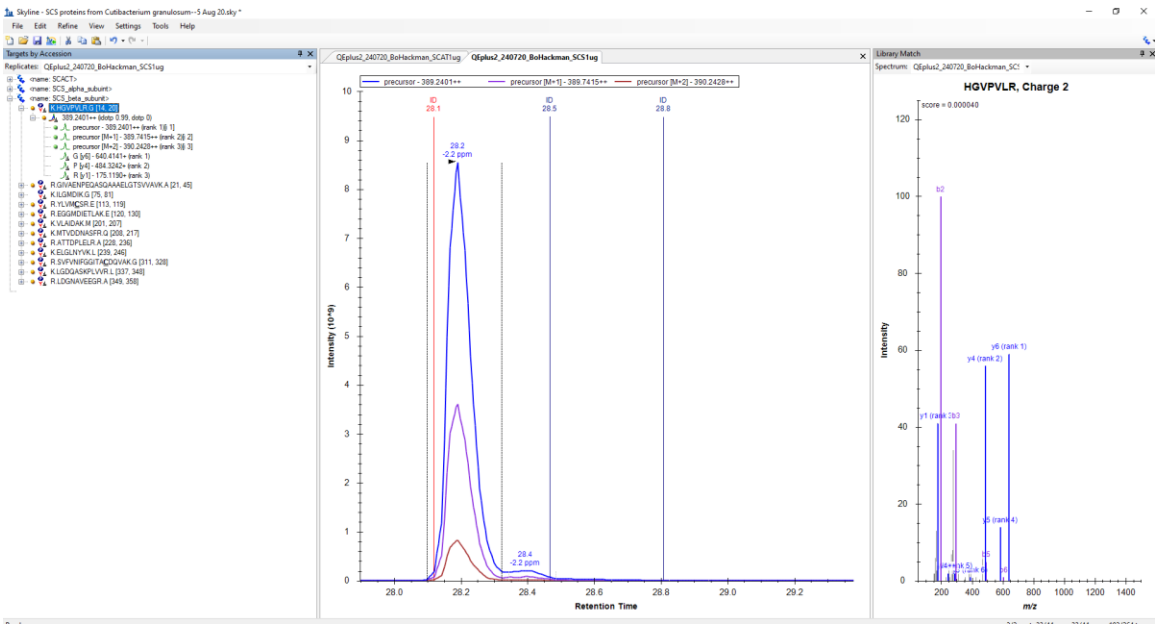
(H) Inspect the chromatograms.



(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 m*M*). It combines many of the procedures listed earlier in the manual.

Heat production of microbial cells by calorimetry

Adapted from Thermal Hazard Technology µRC manual and (104, 105)

Use

1.) Turn on calorimeter (Thermal Hazard Technology µRC) 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 CO_2 while screwing on cap with rubber septum. Pipette 1 mL of dH_2O 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 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 39ºC 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 25ºC (106). To correct to 39ºC, the calculation also assumes $\Delta_r C_p^\circ = 0.22\ kJ \cdot K^{-1} \cdot mol^{-1}$ (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.

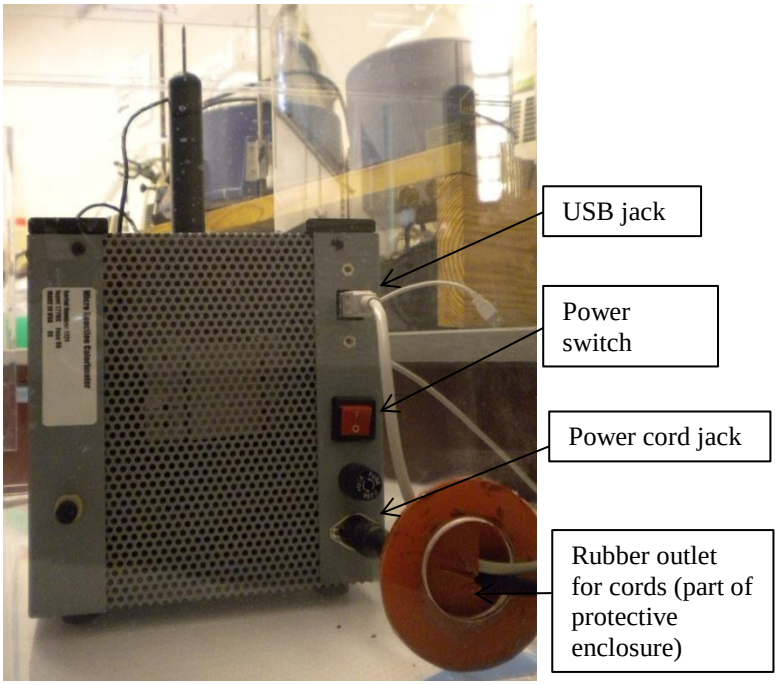
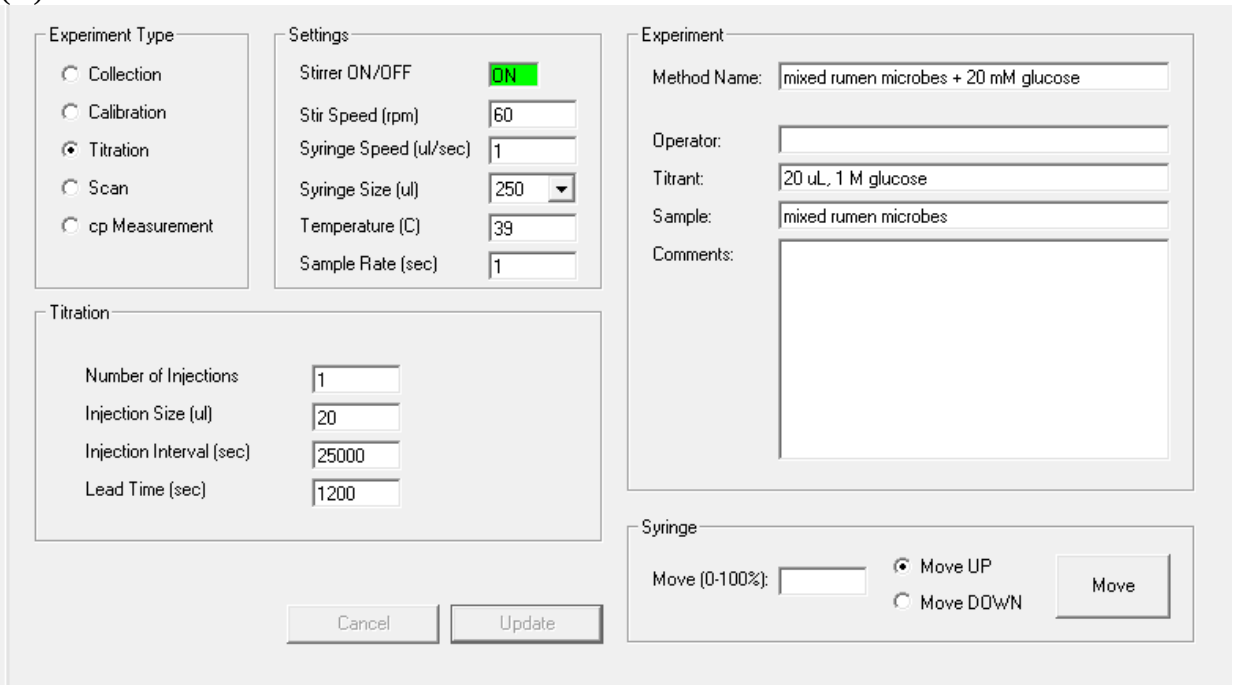


Fig. 2. Settings for typical experiments. (A) Titration. Titration setting is selected to make an injection, with injection number (1) and size (20 µL) indicated. Lead time (1200 s) is time before injection. Injection interval (25 000 s) is total length of experiment or, were multiple injections made, 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)



(B)

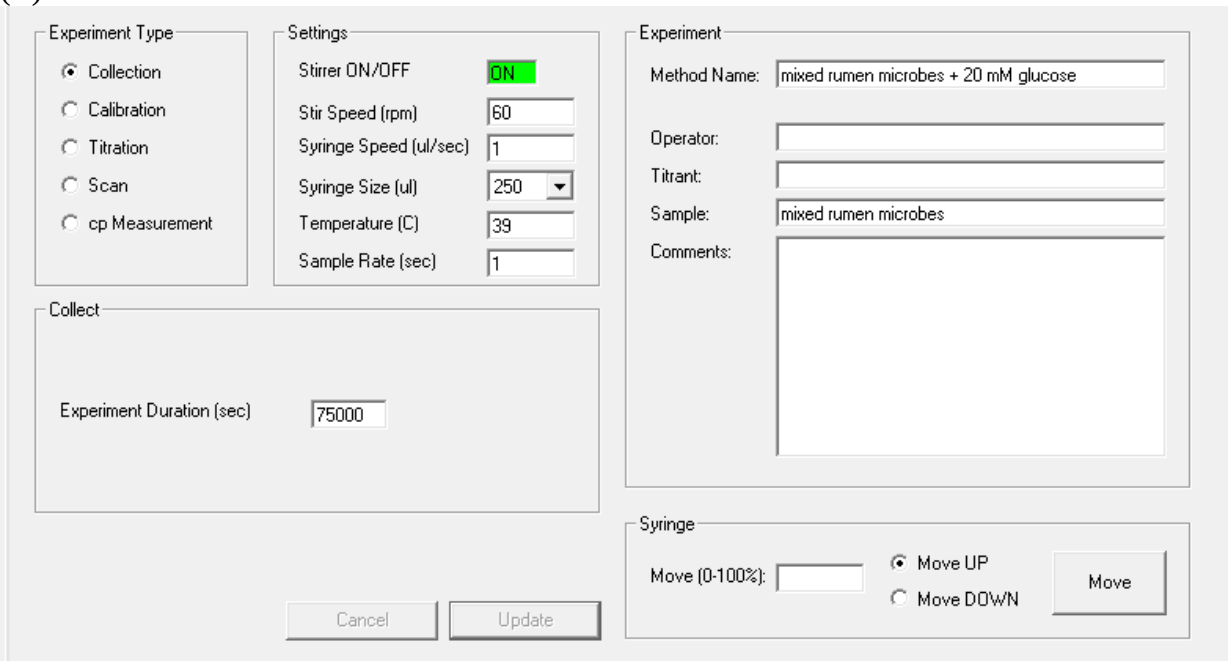


Fig. 3. Reference vial fully prepared. Grasped by long forceps, it is ready to be lowered into reference cell.

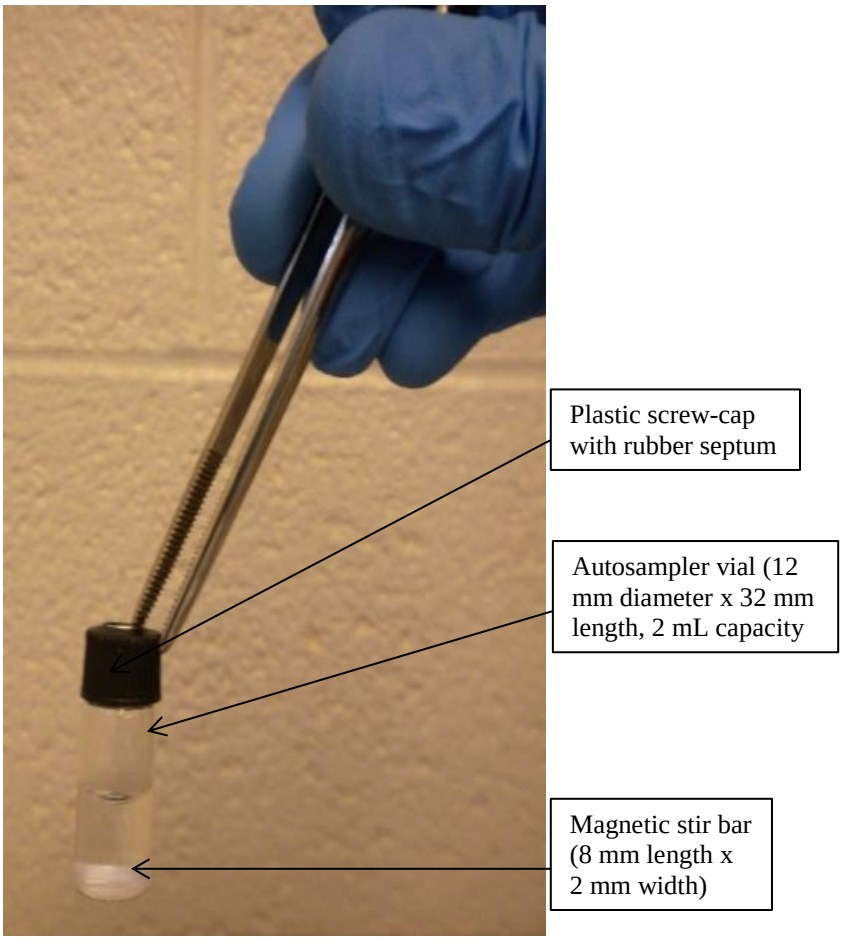


Fig. 4. Front view of calorimeter.

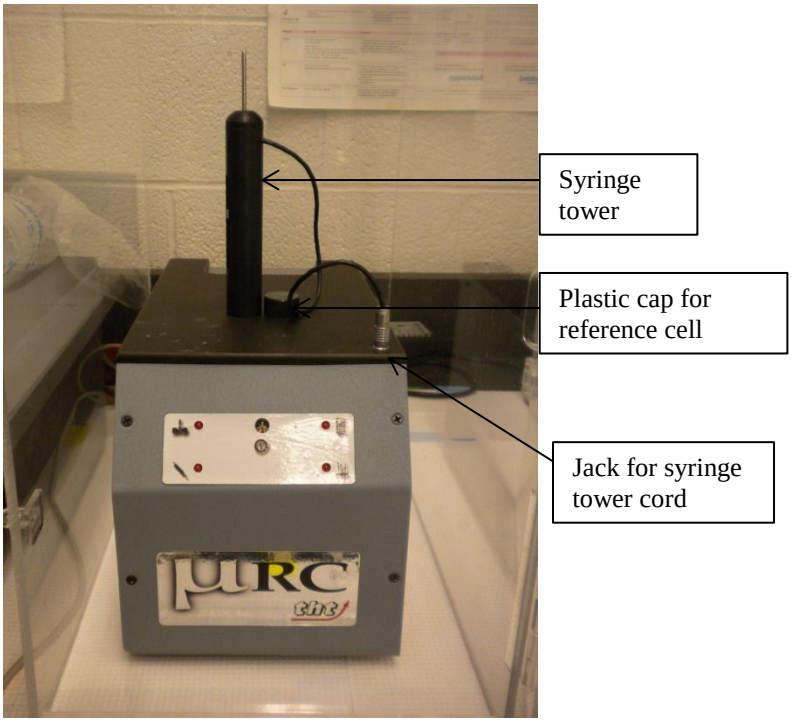


Fig. 5. Top view of calorimeter, with sample and reference cells exposed by removing syringe tower and plastic cap for reference cell.

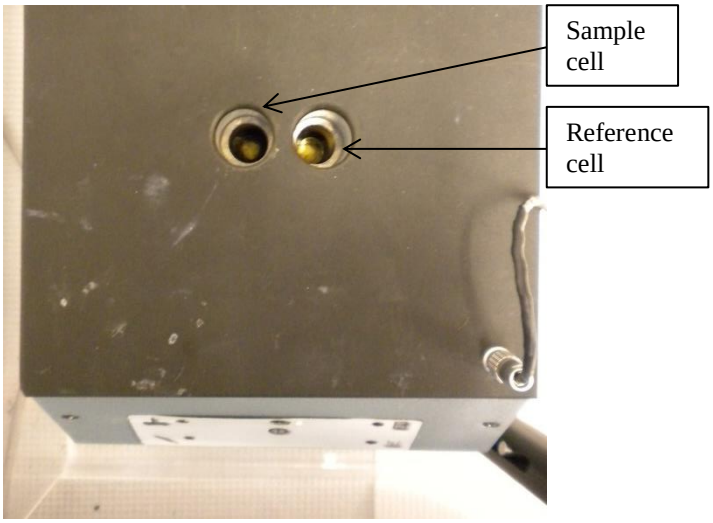


Fig. 6. Lowering metal stopper into reference vessel.

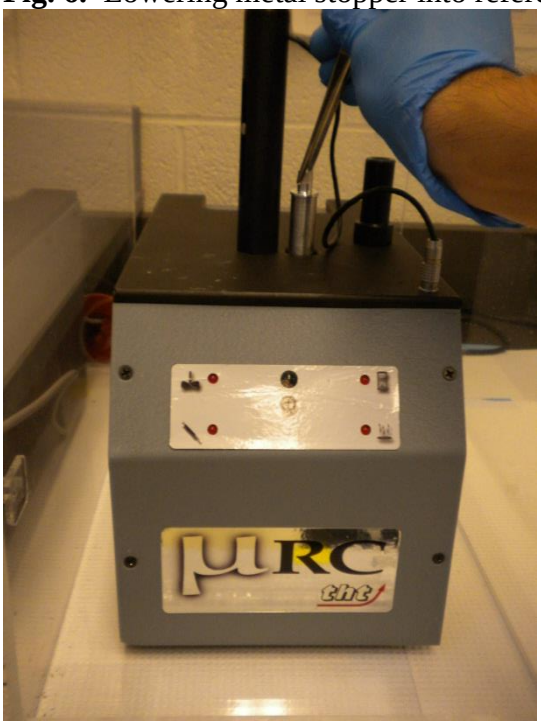
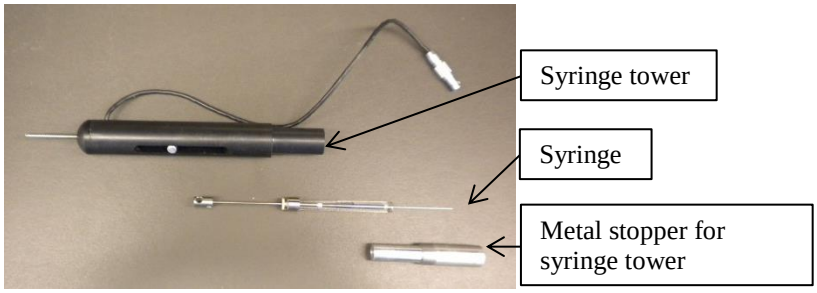
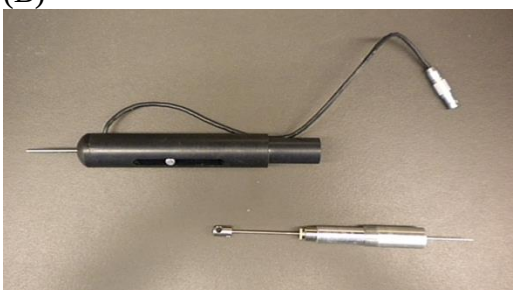


Fig. 7. Syringe tower. (A) Disassembled. (B) Insertion of syringe into metal stopper for sample vial holder. (C) Assembled.

(A)



(B)



(C)

Fig. 8. Typical settings for calibration

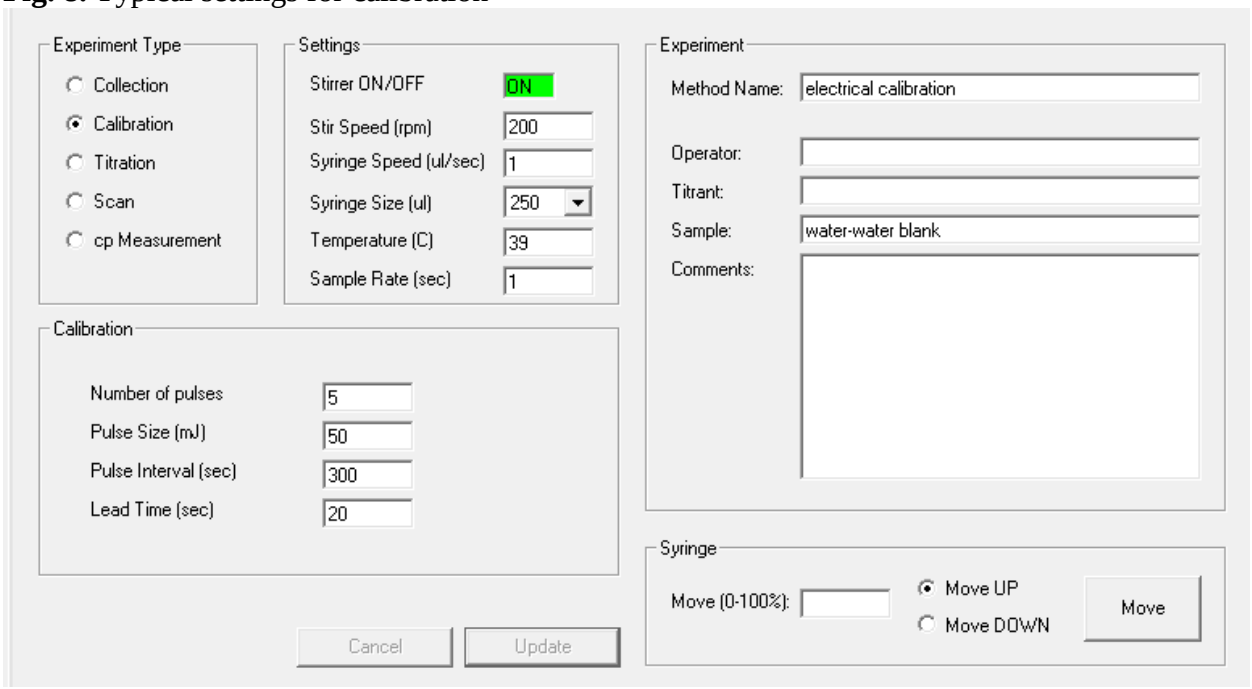
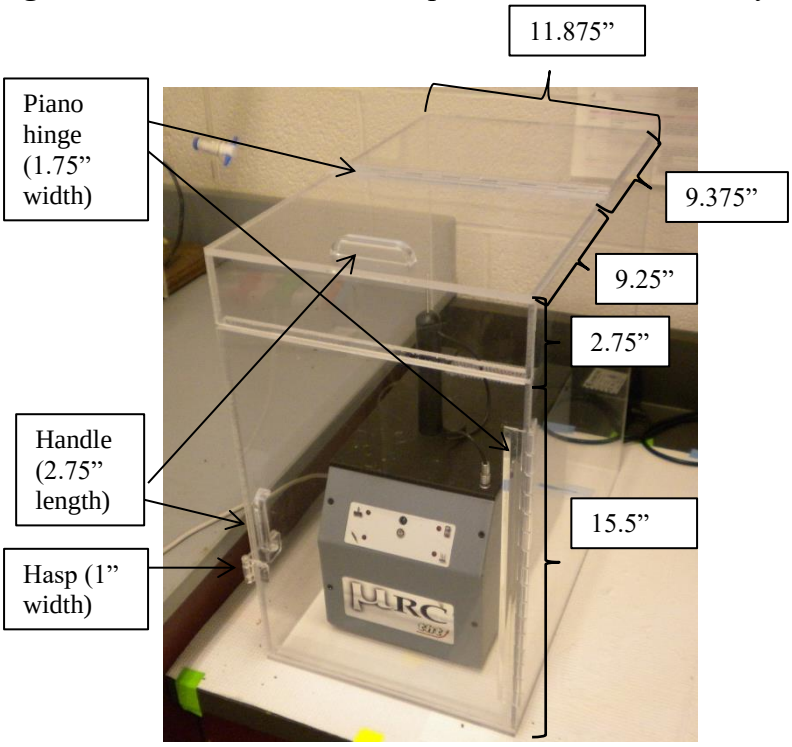


Fig. 9. Protective enclosure. All panels are 0.25” thick acrylic, welded together using solvent.



Example experiment: Measuring response of rumen microbes to 20 m*M* 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 µL GC syringe (with dH_2O). Run at 39ºC 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 µL 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 equilibriate. Start titration experiment. Inject 20 µL, 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 µL 1 *M* glucose into culture bottle (now containing 16 mL culture). Collect sample and place in P4.

7.) Centrifuge P1 to P4 at 4ºC 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 4ºC 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 4ºC at 10 000 *g* for 10 min.

12.) Return to main lab and place P1 to P4 and S1 to S4 in -20ºC 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 -20ºC.

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 dH_2O 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 analysese 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 ddH_2O. 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 dH_2O. 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 $CaCl_2$) into 30 mL dH_2O (instead of 300 mL) and omitting $CaCl_2$. 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 (OD_{600}) over time.

2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to $OD_{600} = 1$ and requires 3 to 4.5 h from inoculation. Place tubes on ice, transfer to 40-mL screw-cap centrifuge tube under CO_2. Centrifuge at 4°C, and wash twice with ice-cold

Simplex buffer under CO_2. 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 CO_2.

Incubation in 2-NBDG and radiolabeled sugars

1.) Prepare aliquots of culture and pre-incubate. Under CO_2, 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 μL 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 μm, 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 filer 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

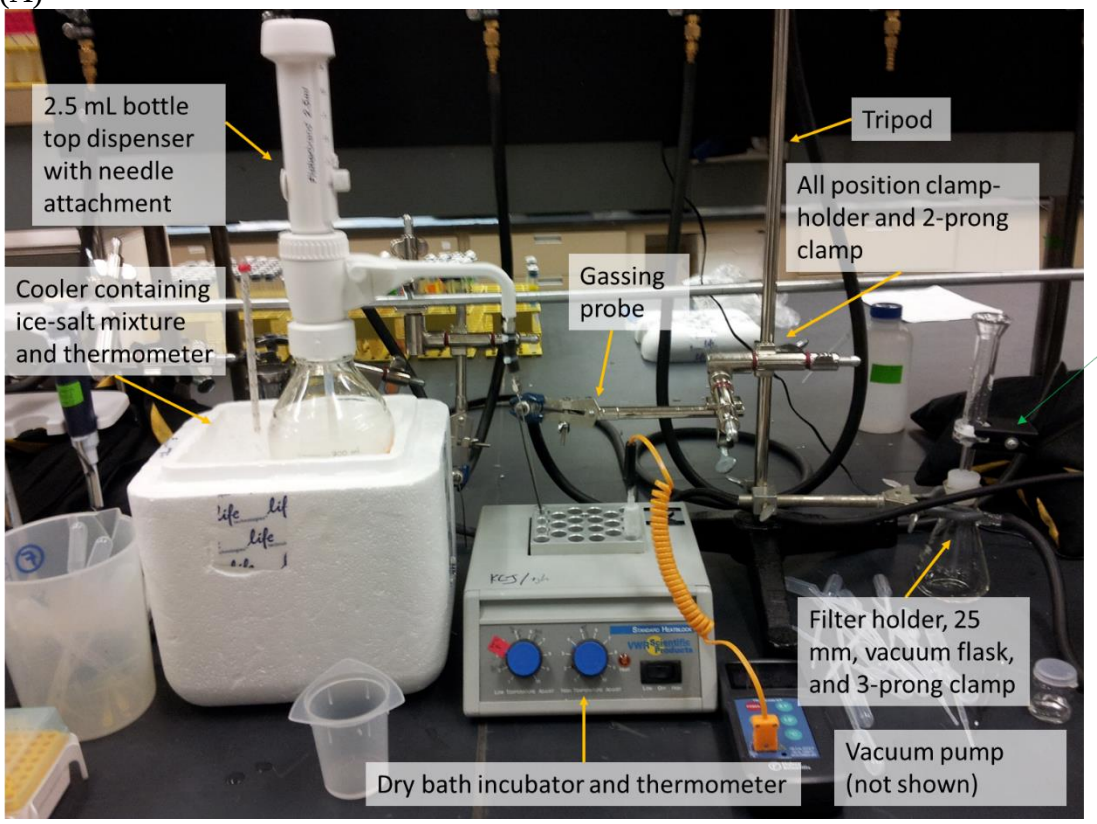
(i) For #3 of ***Inoculation of cultures***, cell protein can be roughly estimated by measuring optical OD_{600} and referring to past experiments that have measured both OD_{600} and protein. This rough estimate facilitates adjustment of protein to 0.4 mg/L.

(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.

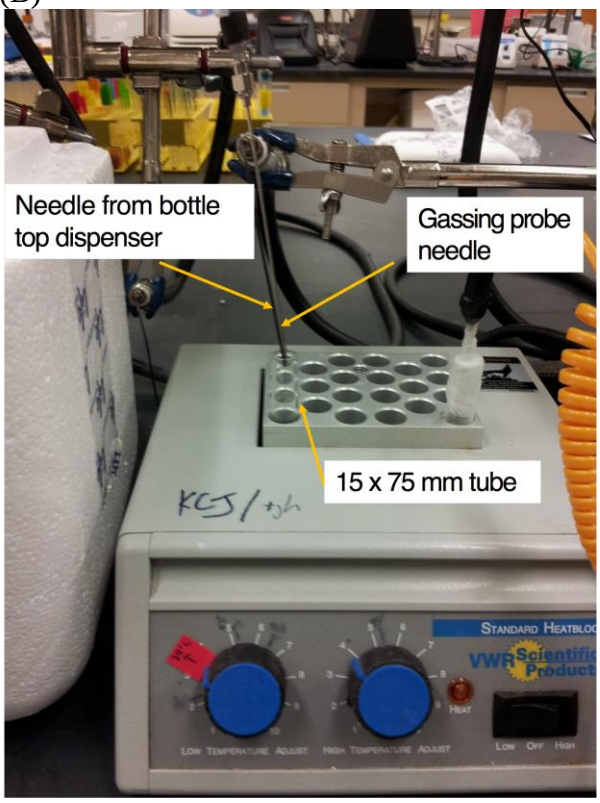
(iii) To avoid spread of radioactive contamination, radiolabeled sugar samples should be done only after all 2-NBDG samples.

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.

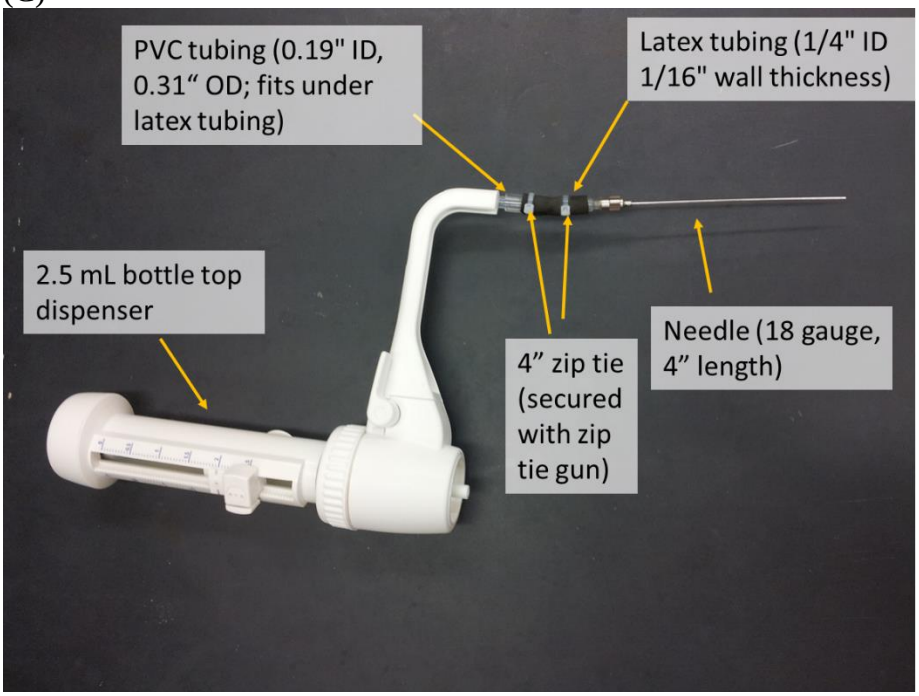
(A)



(B)


Needle from bottle top dispenser
Gassing probe needle
15 x 75 mm tube
KCS/tjh
STANDARD HEATBLOC
VWR Scientific Product
LOW TEMPERATURE ADJUST
HIGH TEMPERATURE ADJUST
HEAT
LOW OFF HIGH

(C)


PVC tubing (0.19" ID, 0.31“ OD; fits under latex tubing)
Latex tubing (1/4" ID 1/16" wall thickness)
2.5 mL bottle top dispenser
4” zip tie (secured with zip tie gun)
Needle (18 gauge, 4” length)

Stocks for radiolabeled sugar transport experiments

1.) Prepare 10 m*M* glucose. Add 0.18016 g glucose to 100 mL volumetric flask and bring to volume with ddH_2O. Sterilize by filtering through 0.2 μm membrane into autoclaved Balch tube and store at -20°C.

Prepare 10 m*M* mannose. Repeat procedure for 10 m*M* glucose but use 0.18016 g mannose.

Prepare 10 m*M* 2-deoxyglucose. Repeat procedure for 10 m*M* 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 ddH_2O and 10 m*M* 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. ^{14}C-glucose stocks (presuming 0.1 mCi/mL and 289 mCi/mmol ^{14}C-glucose)

Total glucose	Activity	Sterile ddH_2O	10 m*M* 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_2O	10 m*M* deoxyglucose	^{14}C-deoxyglucose
µM	µCi/5 µL	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. ^{3}H-mannose stocks (presuming 1 mCi/mL and 19.3 Ci/mol ^{3}H-mannose)

Total mannose	Activity	Sterile ddH_2O	10 mM mannose	^{3}H-mannose
µM	µCi/5 µL	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 m*M* 2-NBDG by adding 584 µL ddH_2O 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_2O (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	ddH2O	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, ddH_2O, and paper towels. Collect contaminated liquids in liquid waste container. Discard disposable, incinerable materials in polypropylene barrel for Type A waste; discard disposable, non-incernerable 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 ^{3}H 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 cm^2 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 ^{14}C 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	☒	☐	☐
Waste Facilities Posted & Neat	☒	☐	☐
Inventory up to Date	☐	☐	☒
Work Areas Clean	☒	☐	☐
Survey Meter Calibrated	☐	☐	☒
Survey Meter Operation Verified with Dedicated Check Source	☐	☐	☒

Survey Meter Model & Serial #
NA

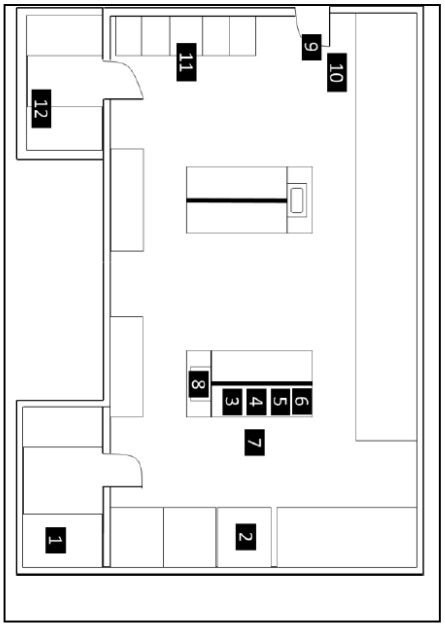
Work Area	NA	mR/hr
Waste Facilities	NA	mR/hr
Storage Areas	NA	mR/hr

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 m*M* NADH. Weigh out 0.025 g NADH (Sigma 43420-100MG) in a sterile 2-mL centrifuge tube and add 1762 µL molecular-biology ddH_2O. 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* phosphenolpyruvate (PEP) by adding 970.2 µL molecular-biology ddH_2O to 0.1 g PEP monopotassium salt. Do this by adding 500 µL molecular-biology ddH_2O 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 ddH_2O 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 m*M* glucose.

7.) Prepare 5 mM 2-NBDG. Add 584 µL ddH_2O 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 OD_{600} over time.

2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD_{600} = 1 and requires 3 to 4.5 h from inoculation. Place tubes on ice, transfer to 10-mL screw-cap centrifuge tube under N_2. Centrifuge at 4°C, and wash twice with ice-cold phosphate buffer + 2 m*M* DTT under N_2. For *S. bovis*, 10 000 *g* for 5 min is sufficient for each centrifugation.

3.) Resuspend pellet in 3-mL phosphate buffer + 2 m*M* DTT (or that volume which achieves 0.1 to 1 mg protein/mL). Transfer to 12x75 mm culture tube. Keep on ice and under N_2.

Preparation of toluene-treated cells

1.) Transfer 0.5 mL aliquot of re-suspended culture to a 12x75 mm culture tube.

2.) While under N_2, 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 N_2

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 N_2, stopper, and pre-incubate in 39°C air incubator for 10 min.

2.) Initiate the reaction. Add 20 µL 5 m*M* glucose to one cuvette, 20 µL 5 m*M* 2-NBDG a second cuvette, and 20 µL phosphate buffer (without DTT) to a third cuvette. Immediately mix, flush with N_2, 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}).

REFERENCES

1. Hungate R. 1969. A roll tube method for cultivation of strict anaerobes. Methods in Microbiology 3:117-132.
2. Wolfe RS. 2011. Techniques for cultivating methanogens. Methods Enzymol 494:1-22.
3. Dehority BA. 2003. Rumen microbiology. Nottingham University Press, Nottingham, UK.
4. Bryant MP, Burkey LA. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J Dairy Sci 36:205-217.
5. Olson KD. 1992. Modified bottle plate for cultivation of strict anaerobes. J Microbiol Methods 14:267-9.
6. Teather RM. 1982. Maintenance of Laboratory strains of obligately anaerobic rumen bacteria. Appl Environ Microbiol 44:499-501.
7. Bryant MP. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am J Clin Nutr 25:1324-8.
8. Dehority BA. 1998. Generation times of Epidinium caudatum and Entodinium caudatum, determined in vitro by transferring at various time intervals. J Anim Sci 76:1189-96.
9. Dehority BA. 2008. Improved in vitro procedure for maintaining stock cultures of three genera of rumen protozoa. J Anim Sci 86:1395-401.
10. Sylvester JT, Karnati SK, Dehority BA, Morrison M, Smith GL, St-Pierre NR, Firkins JL. 2009. Rumen ciliated protozoa decrease generation time and adjust 18S ribosomal DNA copies to adapt to decreased transfer interval, starvation, and monensin. J Dairy Sci 92:256-69.
11. Leedle JAZ, Hespell RB. 1983. Brief incubations of mixed ruminal bacteria: effects of anaerobiosis and sources of nitrogen and carbon. J Dairy Sci 66:1003-1014.
12. Russell JB, Strobel HJ. 1987. Concentration of ammonia across cell membranes of mixed rumen bacteria. J Dairy Sci 70:970-6.
13. Coleman GS. 1992. The rate of uptake and metabolism of starch grains and cellulose particles by *Entodinium* species, *Eudiplodinium maggii*, some other entodiniomorphid protozoa and natural protozoal populations taken from the ovine rumen. J Appl Bacteriol 73:507-13.
14. Macy JM, Snellen JE, Hungate RE. 1972. Use of syringe methods for anaerobiosis. Am J Clin Nutr 25:1318-1323.
15. Villas-Boas SG, Noel S, Lane GA, Attwood G, Cookson A. 2006. Extracellular metabolomics: a metabolic footprinting approach to assess fiber degradation in complex media. Anal Biochem 349:297-305.
16. Stams AJ, Van Dijk JB, Dijkema C, Plugge CM. 1993. Growth of syntrophic propionate-oxidizing bacteria with fumarate in the absence of methanogenic bacteria. Appl Environ Microbiol 59:1114-9.
17. Stewart CS, Flint HJ, Bryant MP. 1997. The rumen bacteria, p 10-72. *In* PN H, CS S (ed), The rumen microbial ecosystem, 2nd ed. Blackie Academic & Professional, New York, USA.
18. Chow JM, Van Kessel JA, Russell JB. 1994. Binding of radiolabeled monensin and lasalocid to ruminal microorganisms and feed. J Anim Sci 72:1630-5.
19. Chen G, Russell JB. 1989. More monensin-sensitive, ammonia-producing bacteria from the rumen. Appl Environ Microbiol 55:1052-7.
20. Leedle JA, Hespell RB. 1980. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. Appl Environ Microbiol 39:709-19.
21. Jones G, Pickard M. 1980. Effect of titanium (III) citrate as reducing agent on growth of rumen bacteria. Appl Environ Microbiol 39:1144-1147.
22. Holdeman L, Moore W (ed). 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University, Blacksburg, VA.

23. MA C, JB R. 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J Dairy Sci 65:226-234.
24. Balch WE, Wolfe RS. 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of Methanobacterium ruminantium in a pressureized atmosphere. Appl Environ Microbiol 32:781-91.
25. Derrien M, Vaughan EE, Plugge CM, de Vos WM. 2004. Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 54:1469-76.
26. Bond DR, Russell JB. 1998. Relationship between intracellular phosphate, proton motive force, and rate of nongrowth energy dissipation (energy spilling) in *Streptococcus bovis* JB1. Appl Environ Microbiol 64:976-981.
27. Bond DR, Russell JB. 1996. A role for fructose 1,6-diphosphate in the ATPase-mediated energy-spilling reaction of *Streptococcus bovis*. Appl Environ Microbiol 62:2095-9.
28. Cotta MA, Russell JB. 1982. Effect of peptides and amino acids on efficiency of rumen bacterial protein synthesis in continuous culture. J Dairy Sci 65:226-234.
29. Bond DR, Russell JB. 2000. Protonmotive force regulates the membrane conductance of *Streptococcus bovis* in a non-ohmic fashion. Microbiology 146:687-94.
30. Miller RS, Hoskins LC. 1981. Mucin degradation in human colon ecosystems. Fecal population densities of mucin-degrading bacteria estimated by a "most probable number" method. Gastroenterology 81:759-65.
31. Bryant MP, Robinson IM. 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J Dairy Sci 44:1446-56.
32. Sowers KR, Noll KM. 1995. Techniques for anaerobic growth, p 15-48. *In* Sowers KR, Schreier HJ, DasSarma S, Fleischmann EM (ed), Archaea: A laboratory manual, vol 2. Cold Spring Harbor Laboratory Press, New York, NY.
33. Schaefer DM, Davis CL, Bryant MP. 1980. Ammonia saturation constants for predominant species of rumen bacteria. J Dairy Sci 63:1248-63.
34. Martin SA, Russell JB. 1987. Transport and phosphorylation of disaccharides by the ruminal bacterium Streptococcus bovis. Appl Environ Microbiol 53:2388-93.
35. Kornberg HL, Reeves RE. 1972. Inducible phosphoenolpyruvate-dependent hexose phosphotransferase activities in Escherichia coli. Biochem J 128:1339-44.
36. Bond DR, Tsai BM, Russell JB. 1998. The diversion of lactose carbon through the tagatose pathway reduces the intracellular fructose 1,6-bisphosphate and growth rate of Streptococcus bovis. Appl Microbiol Biotechnol 49:600-5.
37. Prins RA, Lankhorst A, van der Meer P, Van Nevel CJ. 1975. Some characteristics of *Anaerovibrio lipolytica*, a rumen lipolytic organism. Antonie Van Leeuwenhoek 41:1-11.
38. Williams A, Coleman G. 1992. The rumen protozoa. Springer-Verlag, NY.
39. Moench TT, Zeikus JG. 1983. An improved preparation method for a titanium (III) media reductant. J Microbiol Method 1:199-202.
40. Caldwell DR, Bryant MP. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl Microbiol 14:794-801.
41. Herbert D, Phipps P, Strange R. 1971. Chemical analysis of microbial cells. Methods Microbiol 5B:209-344.
42. Schneider WC. 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol 3:684-690.
43. Russell JB. 1986. Heat production by ruminal bacteria in continuous culture and its relationship to maintenance energy. J Bacteriol 168:694-701.
44. Burton K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315-23.

45. Daniels L, Hanson RS, Phillips JA. 2007. Chemical analysis. *In* Reddy CA, Beveridge TJ, Breznak JA, Marzluf GA, Schmidt TM, Snyder LR (ed), Methods for general and molecular microbiology, 3rd ed. ASM Press, Washington, D.C.
46. Folch J, Lees M, Sloane Stanley GH. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509.
47. Hackmann TJ, Keyser BL, Firkins JL. 2013. Evaluation of methods to detect changes in reserve carbohydrate for mixed rumen microbes. J Microbiol Methods:In press.
48. Parrou JL, Francois J. 1997. A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. Anal Biochem 248:186-8.
49. Schulze U, Larsen ME, Villadsen J. 1995. Determination of intracellular trehalose and glycogen in *Saccharomyces cerevisiae*. Anal Biochem 228:143-9.
50. Gunja-Smith Z, Patil NB, Smith EE. 1977. Two pools of glycogen in *Saccharomyces*. J Bacteriol 130:818-25.
51. Hackmann T. Unpublished observation.
52. Lou J, Dawson KA, Strobel HJ. 1997. Glycogen biosynthesis via UDP-glucose in the ruminal bacterium *Prevotella bryantii* B1(4). Appl Environ Microbiol 63:4355-9.
53. Strange R, Dark F, Ness A. 1961. The survival of stationary phase *Aerobacter aerogenes* stored in aqueous suspension. J Gen Microbiol 25:61-76.
54. Ezeji TC. 2001. Production, purification and characterization of thermostable amylolytic enzymes from the newly isolated *Bacillus thermodenitrificans* HRO10. Ph.D. University of Rostock, Rostock, DE.
55. Hansen SA. 1975. Thin-layer chromatographic method for identification of oligosaccharides in starch hydrolyzates. J Chromatogr A 105:388-390.
56. Bauer K, Gros L, Sauer W. 1991. Thin layer chromatography: an introduction. Hüthig Buch Verlag GmbH, Heidelburg, DE.
57. Ohta M, Pan Y, Laine RA, D Elbein A. 2009. Trehalose ‐ based oligosaccharides isolated from the cytoplasm of Mycobacterium smegmatis. Eur J Biochem 269:3142-3149.
58. Krisman CR. 1962. A method for the colorimetric estimation of glycogen with iodine. Anal Biochem 4:17-23.
59. Karkalas J. 1985. An improved enzymic method for the determination of native and modified starch. J Sci Food Agric 36:1019-1027.
60. Haugaard N, Cutler J, Ruggieri MR. 1981. Use of *N*-ethylmaleimide to prevent interference by sulfhydryl reagents with the glucose oxidase assay for glucose. Anal Bioch 116:341-343.
61. Niederholtmeyer H, Wolfstadter BT, Savage DF, Silver PA, Way JC. 2010. Engineering cyanobacteria to synthesize and export hydrophilic products. Appl Environ Microbiol 76:3462-6.
62. Dehority BA. 1993. Laboratory manual for classification and morphology of rumen ciliate protozoa. CRC Press, Boca Raton, FL.
63. Hungate R. 1966. The rumen and its microbes. Academic Press, New York, NY.
64. Kepner RL, Pratt JR. 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev 58:603-15.
65. Sylvester JT, Karnati SK, Yu Z, Newbold CJ, Firkins JL. 2005. Evaluation of a real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal nitrogen. J Dairy Sci 88:2083-95.
66. Pfennig N, Wagener S. 1986. An improved method of preparing wet mounts for photomicrographs of microorganisms. J Microbiol Method 4:303-6.
67. Koval SF, Sprott GD. 2007. Cell Fractionation. *In* Reddy CA, Beveridge TJ, Breznak JA, Marzluf GA, Schmidt TM, Snyder LR (ed), Methods for general and molecular microbiology, 3rd ed. ASM Press, Washington, D.C.
68. Melville S, Michel T, Macy J. 1988. Pathway and sites for energy conservation in the metabolism of glucose by *Selenomonas ruminantium*. J Bacteriol 170:5298-304.

69. Bergmeyer HU, Graßl M, Walter H-E. 1983. Enzymes, p 126-328. *In* Bergmeyer HU, Bergmeyer J, Graßl M (ed), Methods of enzymatic analysis, 3 ed, vol 2. Weinheim, DE, Verlag Chemie.
70. Stadtman E. 1957. Preparation and assay of acetyl phosphate. Method Enzymol 3:228-31.
71. Beutler HO, Supp M. 1983. Coenzymes, metabolites, and other biochemical reagents, p 328-393. *In* Bergmeyer HA, Bergmeyer J, Gra ßl M (ed), Methods of enzymatic analysis, 3rd ed, vol 2. Verlag Chemie, Weinheim, DE.
72. Maurer C, Poppendiek B. 1974. Determination with lactate dehydrogenase and APAD, p 1472-1475. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, 2nd ed, vol 3. Verlag Chemie, Weinheim, DE.
73. Decker K. 1974. Acetyl-coenzyme A: UV-spectrophotometric assay, p 1988-1993. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, 2nd ed, vol 4. Verlag Chemie, Weinheim, DE.
74. Williamson JR, Corkey BE. 1969. Assays of intermediates of the citric acid cycle and related compounds by fluorometric enzyme methods. Method Enzymol 13:434-513.
75. Fowler ML, Ingram-Smith CJ, Smith KS. 2011. Direct detection of the acetate-forming activity of the enzyme acetate kinase. J Vis Exp doi:10.3791/3474.
76. Schut GJ, Menon AL, Adams MW. 2001. 2-keto acid oxidoreductases from Pyrococcus furiosus and Thermococcus litoralis. Methods Enzymol 331:144-58.
77. Ellman GL. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70-7.
78. Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed, vol 3.
79. Garland PB. Coenzyme A: fluorimetric assay, p 1981-7. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, 2nd ed, vol 4. Verlag Chemie, Weinheim, DE.
80. Lamprecht W, Trauschold I. 1974. Determination with hexokinase and glucose-6-phosphate dehydrogenase, p 2101-2111. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, 2 ed, vol 4. Verlag Chemie, Weinheim, DE.
81. Green AA. 1933. The preparation of acetate and phosphate buffer solutions. J Am Chem Soc 55:2331-6.
82. Protocols CSH. 2017. Na_3VO_4 Stock Solution. Cold Spring Harb Protoc:doi:10.1101/pdb.rec092940.
83. White H, Jencks WP. 1976. Mechanism and specificity of succinyl-CoA:3-ketoacid coenzyme A transferase. J Biol Chem 251:1688-99.
84. Schönheit P, Brandis A, Thauer RK. 1979. Ferredoxin degradation in growing Clostridium pasteurianum during periods of iron deprivation. Arch Microbiol 120:73-6.
85. Schönheit P, Wäscher C, Thauer RK. 1978. A rapid procedure for the purification of ferredoxin from Clostridia using polyethyleneimine. FEBS Lett 89:219-22.
86. Scherer PA, Thauer RK. 1978. Purification and properties of reduced ferredoxin: CO2 oxidoreductase from Clostridium pasteurianum, a molybdenum iron-sulfur-protein. Eur J Biochem 85:125-35.
87. Green M, Sambrook J. 2012. Molecular Cloning: A Laboratory Manual, 4th ed.
88. Hilpert W, Schink B, Dimroth P. 1984. Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion. EMBO J 3:1665-70.
89. Eyer P, Worek F, Kiderlen D, Sinko G, Stuglin A, Simeon-Rudolf V, Reiner E. 2003. Molar absorption coefficients for the reduced Ellman reagent: reassessment. Anal Biochem 312:224-7.
90. Schulman M, Wood HG. 1975. Succinyl-CoA: propionate CoA-transferase from Propionibacterium shermanii. EC 2.8.3.6 succinyl-CoA:propionate CoA-transferase. Methods Enzymol 35:235-42.
91. Dijkhuizen L, Van der Werf B, Harder W. 1980. Metabolic regulation in *Pseudomonas oxalaticus* OX1. Diauxic growth on mixtures of oxalate and formate or acetate. Archives of Microbiology 124:261-268.
92. Williamson JR. 1974. Succinate, p 1616-21. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, vol 3. Verlag Chemie, Weinheim, DE.

93. Bergmeyer HU, Möllering H. 1974. Acetate: determination with preceding indicator reaction, p 1520-8. *In* Bergmeyer HU (ed), Methods of enzymatic analysis, 2nd ed, vol 3. Verlag Chemie, Weinheim, DE.
94. Rinke C, Lee J, Nath N, Goudeau D, Thompson B, Poulton N, Dmitrieff E, Malmstrom R, Stepanauskas R, Woyke T. 2014. Obtaining genomes from uncultivated environmental microorganisms using FACS–based single-cell genomics. Nature protocols 9:1038-1048.
95. Qiagen. 2012. Repli-g Single Cell Handbook. https://wwwqiagencom/us/resources/downloadaspx?id=38faca1c-64b0-4281-aab3-aa8324bbd181&lang=en
96. BIO-RAD. SsoAdvanced™ Universal SYBR® Green Supermix manual. http://wwwbio-radcom/webroot/web/pdf/lsr/literature/10024379pdf.
97. BIO-RAD. 2013. Instruction manual for CFX 96. http://wwwbio-radcom/webroot/web/pdf/lsr/literature/10021337pdf.
98. Tamariz J, Voynarovska K, Prinz M, Caragine T. 2006. The application of ultraviolet irradiation to exogenous sources of DNA in plasticware and water for the amplification of low copy number DNA. Journal of forensic sciences 51:790-794.
99. Asanuma N, Yoshii T, Hino T. 2004. Molecular characteristics of phosphoenolpyruvate: mannose phosphotransferase system in Streptococcus bovis. Curr Microbiol 49:4-9.
100. Markowitz VM, Chen IM, Palaniappan K, Chu K, Szeto E, Pillay M, Ratner A, Huang J, Woyke T, Huntemann M, Anderson I, Billis K, Varghese N, Mavromatis K, Pati A, Ivanova NN, Kyrpides NC. 2014. IMG 4 version of the integrated microbial genomes comparative analysis system. Nucleic Acids Res 42:D560-7.
101. Lane DJ. 1991. 16S/23S rRNA sequencing, p 115-175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.
102. Tindall BJ, Rosselló-Móra R, Busse HJ, Ludwig W, Kämpfer P. 2010. Notes on the characterization of prokaryote strains for taxonomic purposes. Int J Syst Evol Microbiol 60:249-66.
103. Hackmann TJ, Diese LE, Firkins JL. 2013. Quantifying the responses of mixed rumen microbes to excess carbohydrate. Appl Environ Microbiol:In press.
104. Berger R. 1969. Calibration and test reactions for microcalorimetry, p 221-234. *In* Brown HD (ed), Biochemical microcalorimetry. Academic Press, New York, NY, USA.
105. Cooper A, Johnson CM. 1994. Differential scanning calorimetry. Method Mol Biol 22:125-136.
106. Wagman DD, Evans WH, Parker VB, Schumm RH, Halow I, Bailey SM, Churney KL, Nuttall RL. 1982. The NBS tables of chemical thermodynamic properties. selected values for inorganic and C_1 and C_2 organic substances in SI units. J Phys Chem Ref Data 11 Suppl 2:2-1-2-392.
107. Thomson J. 2012. Sample preparation techniques for liquid scintillation counting, p 575-623. *In* L'Annunziata M (ed), Handbook of radioactivity analysis. Elsevier, San Diego.
108. Kornberg HL, Reeves RE. 1972. Correlation between hexose transport and phosphotransferase activity in Escherichia coli. Biochem J 126:1241-3.