

HACKMANN LAB

# MANUAL *for* RUMEN MICROBIOLOGY

v. 5.2



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v. 5.2

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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

<b>SECTION I: ANAEROBIC TECHNIQUE AND CULTURING .....</b>	<b>1</b>
Constructing and using gassing station .....	2
Construction .....	2
Use .....	3
Culturing bacteria .....	16
Growth in broth .....	16
Growth on bottle plates .....	16
Growth on roll tubes .....	19
Growth on slants .....	20
Preparing colony picker .....	21
Measuring optical density for monitoring growth of bacterial cultures .....	26
Notes .....	26
Isolation of pure cultures of bacteria from rumen fluid .....	27
Dilution and inoculation .....	27
Notes .....	27
Ordering and growing bacteria from culture collections .....	28
Notes .....	28
Cryopreservation of bacteria .....	31
Freezing .....	31
Revival .....	31
Notes .....	31
Culturing methanogens .....	32
Growth in broth .....	32
Growth on bottle plates .....	32
Cryopreservation .....	33
Feeding and transferring protozoa in culture tubes .....	34
Schedule .....	34
Feeding .....	34
Transferring .....	35
Preparing blunt-tip pipette .....	35
Other recommendations .....	35
Making protozoal substrate .....	35
Growing large volumes of protozoa .....	36

Cryopreservation of rumen protozoa .....	41
Freezing.....	41
Revival .....	41
Notes .....	41
Collecting rumen fluid .....	42
Notes .....	42
Preparing and culturing mixed rumen microbes from rumen fluid.....	43
Notes .....	43
Preparing and culturing mixed bacteria from rumen fluid.....	47
Notes .....	47
Preparing and culturing mixed protozoa from rumen fluid.....	50
Notes .....	51
Preparing and culturing isolated protozoa from single-species cultures.....	54
Notes .....	54
Making buffer and media.....	58
Preparing small volumes of buffer/media .....	58
Preparing large volumes of buffer or media .....	59
Preparing buffer or media by distillation .....	60
Buffer and media recipes .....	77
6SG330 media.....	77
Anaerobic dilution solution (ADS) .....	78
Anaerobic dilution solution (50% glycerol).....	78
ATCC media 1015 for <i>Anaerovibrio lipolytica</i> .....	79
Basal Media .....	79
Branch-chained VFA solution .....	80
Cellulolytic media.....	80
Chen media .....	81
Chen media with yeast extract .....	81
Clarified rumen fluid.....	81
3% (w/v) cysteine HCl.....	81
0.1 M Dithiothreitol (DTT).....	82
Glucose (10% w/v) (anaerobic) .....	82
Hemin solution.....	82
Hemin solution (0.05%).....	82



Isaacson buffer .....	82
LH media .....	83
Media M.....	84
Media SP.....	84
Modified Methanobacterium medium (DSMZ 1523).....	85
Modified Methanobacterium medium for Methnobreivibacter ruminantium (DSMZ 1523) .....	86
Modified Peptone Yeast Extract Glucose (PYG; ATCC 1237).....	86
Modified Peptone Yeast Extract Glucose (PYG; DSMZ 104) .....	87
Mucin medium for <i>Akkermansia muciniphila</i> .....	87
Meat fluid and particles.....	88
5% (w/v) 2-mercaptoethanesulfonic acid.....	88
Minimal media for <i>Streptococcus bovis</i> .....	89
Minimal media plus VFA .....	89
Adapted from Minimal media for <i>Streptococcus bovis</i> .....	89
10% (w/v) NaOH .....	90
2.5% (w/v) Na <sub>2</sub> S·9 H <sub>2</sub> O .....	90
Orpin's Medium C with cellobiose.....	91
Orpin's Medium C with reed canary grass .....	91
Orpin's Medium C with 15% glycerol.....	92
PC minus VFA media .....	92
PC plus VFA media .....	92
Pfennigs heavy metal solution .....	93
Phosphate buffer for phosphotransferase assays .....	93
Prins semi-defined medium .....	93
Reinforced Clostridial Medium (RCM).....	94
Rumen bacteria medium (DSMZ 330).....	94
Russell vitamin solution.....	96
Schaefer's vitamin solution + THF .....	97
Selenomonas ruminantium medium (DSMZ 181).....	97
Selenite-tungstate solution .....	97
Seven vitamins solution .....	98
Simplex-type buffer .....	98
Weller and Pilgrim buffer .....	99
Titanium (III) nitrilotriacetate.....	100

Trace element solution SL-10.....	100
Treponema bryantii medium (DSMZ 159).....	101
<i>Treponema ruminis</i> medium.....	101
Vitamin K1 solution.....	102
Vitamin K <sub>3</sub> - Hemin solution .....	102
VFA solution.....	103
Images of pure cultures of bacteria and methanogens .....	105
<b>SECTION II: ANALYSIS OF MICROBIAL SAMPLES .....</b>	<b>110</b>
Preparing cells and culture supernatant for chemical analysis.....	113
Collecting cells and supernatant .....	113
Resuspending cell pellet with dH <sub>2</sub> O .....	113
Notes .....	113
Wet, dry, and organic matter content of microbial cells .....	114
Protein content of microbial cells .....	115
Notes .....	116
RNA and DNA content of microbial cells .....	117
Extraction of nucleotides .....	117
Measurement of DNA using diphenylamine.....	117
Measurement of RNA using orcinol .....	118
Notes .....	119
Lipid content of microbial cells .....	120
Notes .....	121
Total hexoses of microbial cells using the anthrone reaction .....	122
Notes .....	123
Total glycogen content of microbial cells using amyloglucosidase.....	125
Extraction.....	125
Amyloglucosidase digestion and measurement of glucose.....	126
Notes .....	126
Isolation of reserve polysaccharide and measurement of glycogen and total hexoses .....	127
Extraction.....	127
Measurement of hexoses and glycogen .....	127
Notes .....	128
Thin-layer chromatography (TLC) of cytoplasmic carbohydrate and reserve polysaccharide .....	130
Sample and standard preparation .....	130

Plate preparation .....	130
Development, charring, and visualization.....	131
Notes .....	131
Iodine absorbance spectrum of reserve polysaccharide .....	136
Notes .....	136
Free glucose content of culture supernatant.....	138
Notes .....	139
Lactic acid content of culture supernatant by enzymatic analysis .....	140
Notes .....	141
Formic acid content of culture supernatant by enzymatic analysis.....	143
Succinic acid content of culture supernatant by enzymatic analysis .....	146
Ethanol content of culture supernatant by enzymatic analysis .....	149
Concentration and taxonomic distribution of protozoa.....	152
Notes .....	152
Concentration of bacteria.....	156
Preparing DAPI stock and other reagents .....	156
Filtering and counting cells.....	156
Notes .....	157
Preparing wet mounts of bacteria for imaging.....	158
Notes .....	158
Measuring sample absorbance for microplate assays .....	159
Notes .....	159
<b>SECTION III: GAS CHROMATOGRAPHY .....</b>	<b>160</b>
Operation of gas chromatograph.....	161
Risks of operation .....	161
Turning on and placing into standby mode.....	161
Turning on detectors .....	163
Setting up autosampler.....	164
Running samples.....	165
Placing back into standby mode.....	165
Turning off.....	167
Maintenance of gas chromatograph .....	170
Replacing injector septum.....	170
Replacing injector liner.....	172
Replacing autosampler syringe .....	173

Selection of compressed gas and regulators for gas chromatography .....	177
Measuring VFA content of culture supernatant .....	178
Preparing standards and blanks.....	178
Preparing samples .....	178
Preparing GC for analysis of samples and standards .....	179
Analyzing samples and standards .....	179
Shutting down GC after analysis of samples and standards .....	180
Setting up a sequence.....	180
Constructing a standard curve and quantifying VFA concentrations .....	184
Setting up a method .....	198
Notes .....	206
Measuring hydrogen and methane of culture headspace samples.....	207
Preparing samples and standards. ....	207
Preparing GC for analysis of samples and standards .....	207
Analyzing samples and standards .....	207
Shutting down GC after analysis of samples and standards .....	209
Setting up a sequence.....	211
Setting up a method .....	215
<b>SECTION IV: ENZYMATIC ASSAYS .....</b>	<b>223</b>
Preparation of cell extracts for enzymatic assays using a French press .....	224
Preparation of cell suspension.....	224
Preparation of mini pressure cell .....	224
Preparation of the press.....	224
Operation of the press .....	225
Shutdown of cell press .....	225
Processing of cell extract .....	225
Clean up of pressure cell.....	226
Notes .....	226
Preparation of cell membrane and cytoplasmic contents by ultracentrifugation .....	233
Notes .....	233
Use of Beckman L8-70M ultracentrifuge .....	234
Reagents for enzymatic assays.....	235
Acetate kinase (1,000 U/mL) .....	235
Acetyl-CoA (5 mM).....	235

Acetyl-CoA synthetase (125 U/mL) .....	235
Acetyl phosphate (50 mM) .....	235
ADP (100 mM) .....	235
Aluminum chloride (100 mM).....	236
Ammonium chloride (1 M).....	236
APAD (50 mM) .....	236
ATP (100 mM).....	236
Blue dextran (1 mg/mL).....	236
Citrate synthase (90 U/mL).....	237
Coenzyme A (5 mM) Reference: (73) and Calbiochem website for product 234101-100MG.....	237
Coenzyme A (5 mM) (anaerobic) .....	237
Development solution .....	237
Dithionite (0.1 M) .....	237
Dithiothreitol (0.1 M).....	238
DTNB (10 mM) .....	238
EDTA (0.5 M) (pH = 8.0).....	238
$\alpha$ -ketoglutarate (100 mM) .....	238
$\alpha$ -ketoglutarate dehydrogenase .....	238
Pyruvate:ferredoxin oxidoreductase (crude).....	238
Ferric chloride-HCl.....	239
Glucose (1 M).....	239
Glucose-6-phosphate dehydrogenase (100 U/mL).....	239
Glutamate dehydrogenase .....	239
Hexokinase (270 U/mL).....	239
Hydroxylamine-HCl (pH = 7.0) (2 M).....	240
Lactate dehydrogenase (275 U/mL).....	240
DL-Lactate (1 M) (pH = 7.0) .....	240
Linoleic acid (5 mg/mL in 1% w/v Tween) .....	240
L-Malate (100 mM) .....	240
Malate dehydrogenase (180 U/mL) .....	241
Methyl viologen (1 M).....	241
MgCl <sub>2</sub> (1 M) .....	241
MgCl <sub>2</sub> (1 M) (anaerobic).....	241

NaCl (1 M) (anaerobic).....	241
NAD (40 mM).....	241
NAD (200 mM) (anaerobic) .....	241
NADH (40 mM).....	241
NADP (10 mM) .....	242
Oxaloacetate (10 mM) .....	242
Perchloric acid (4 N).....	242
Phosphoenolpyruvate (100 mM) Reference: (73).....	242
Phosphotransacetylase (3000 U/mL) .....	242
Phosphotransacetylase (1000 U/mL) .....	243
Potassium acetate (1 M) (pH = 7.0).....	243
Potassium bicarbonate (1 M) .....	243
Potassium hydroxide (8 N) .....	243
Potassium phosphate buffers (1 M) (pH = 5.8 to 8.0).....	243
Potassium phosphate buffer (pH = 7.0) (1 M) (anaerobic) .....	244
Potassium phosphate buffer (pH = 7.0) (0.1 M) with dithiothreitol (2 mM) (anaerobic).....	244
Propionate (1 M) (pH = 7) .....	244
Propionyl-CoA (5 mM).....	244
Pyruvate (100 mM) .....	244
Pyruvate (1 M) (anaerobic).....	244
Pyruvate kinase (100 U/mL).....	244
Sodium fluoride (100 mM) .....	245
Sodium vanadate (200 mM) (pH = 10.0).....	245
Succinate (0.25 M) (pH = 7.0) .....	245
Succinyl-CoA (5 mM) .....	245
Thiamine pyrophosphate (0.1 M) (anaerobic) .....	245
Trichloroacetic acid (30 % w/v).....	245
Triethanolamine buffer (1 M) (pH = 7.3 to 8.4).....	246
Tris buffers (1 M) (pH = 7.2 to 9.0).....	246
Tris (pH 7.6) (1 M) (anaerobic) .....	246
Vitamin B <sub>12</sub> (1 mg/mL).....	247
Water (anaerobic).....	247
Purification of ferredoxin from <i>Clostridium pasteurianum</i> .....	248

Preparation of reagents and solutions .....	248
Preparing medium for <i>C. pasteurianum</i> .....	249
Preparing DEAE-cellulose slurry.....	251
Cultivation of <i>C. pasteurianum</i> and preparation of cell pellets.....	252
Purification of ferredoxin.....	252
Assay of lactate dehydrogenase activity .....	258
Assay mixture .....	258
Calculation of activity.....	259
Assay of succinyl-CoA:acetate CoA-transferase activity with phosphotransacetylase and DTNB.....	260
Principle of measurement.....	260
Preparation of enzyme samples.....	260
Preparation of assay mixture.....	260
Measurement of activity.....	261
Calculation of activity.....	261
Assay of succinyl-CoA:acetate CoA-transferase activity with citrate synthase and malate dehydrogenase.....	263
Assay mixture .....	263
Calculation of activity.....	264
Assay of succinyl-CoA synthetase activity with hydroxylamine.....	265
Assay mixture .....	265
Assay of ferredoxin:NAD <sup>+</sup> oxidoreductase (Rnf) activity .....	267
Principle of measurement.....	267
Preparation of enzyme samples.....	267
Preparation of assay mixture.....	267
Measurement of activity.....	268
Calculation of activity.....	268
Notes .....	269
Assay of acetate with lactate dehydrogenase, pyruvate kinase, phosphotransacetylase, and acetate kinase .....	271
Preparation of samples .....	271
Assay mixture .....	271
Calculation of analyte concentration.....	272
Assay of acetyl-CoA with citrate synthase and DTNB.....	273
Preparation of samples .....	273

Assay mixture .....	273
Calculation of analyte concentration.....	274
Assay of acetyl-CoA with malate dehydrogenase and citrate synthase .....	275
Preparation of samples.....	275
Assay mixture .....	275
Calculation of analyte concentration.....	276
Assay of acetyl phosphate with malate dehydrogenase, citrate synthase, and phosphotransacetylase .....	277
Preparation of samples.....	277
Assay mixture .....	277
Calculation of analyte concentration.....	278
Measurement of path length of 96-well plates .....	279
NADH standard solution.....	279
Absorbance in cuvettes .....	279
Absorbance in well plates .....	279
Calculation of path length.....	279
<b>SECTION V: MOLECULAR TECHNIQUES AND BIOINFORMATICS .....</b>	<b>281</b>
Preventing contamination in molecular biology experiments.....	282
Colony PCR .....	283
Sequencing.....	287
Primers .....	287
PCR and sample submission .....	287
Sequence alignment and assembly .....	288
Agarose gel electrophoresis .....	293
Example experiment: Sequencing and taxonomic identification of bacteria using 16S rDNA gene...	294
Example experiment: Sequencing of the mannose phosphotransferase (PTS) operon .....	298
Notes .....	298
Example experiment: Finding the sequence of mannose phosphotransferase (PTS) genes with IMG 302	
Amplification of DNA from small numbers of rumen bacteria cells.....	308
Preparation of equipment and other materials .....	308
Preparation of solutions and reagents .....	308
Preparation of rumen bacteria .....	308
Amplification of genomic DNA .....	309
Amplification of 16S rDNA.....	310
Notes .....	310
Untargeted proteomics by LC-MS/MS .....	318
Preparing reagents.....	318
Preparing protein samples .....	319



Precipitating protein with trichloroacetic acid/acetone .....	319
Digesting with trypsin/Lys-C .....	319
Desalting with ZipTips .....	320
Drying samples .....	321
Submitting samples for LC-MS/MS analysis .....	323
Identifying proteins from LC-MS/MS files .....	323
Notes .....	331
<b>SECTION VI: CALORIMETRY .....</b>	<b>332</b>
Heat production of microbial cells by calorimetry .....	333
Use .....	333
Calibration .....	334
Installation .....	334
Notes .....	334
Example experiment: Measuring response of rumen microbes to 20 mM glucose .....	343
Day 1 .....	343
Day 2 .....	343
Day 3 and beyond .....	344
<b>SECTION VII: KINETICS OF SUGAR TRANSPORT .....</b>	<b>346</b>
Example experiment: Transport kinetics of 2-NBDG and radiolabeled sugars by bacteria .....	347
Apparatus and reagents .....	347
Inoculation of cultures .....	347
Incubation in 2-NBDG and radiolabeled sugars .....	348
Notes .....	349
Stocks for radiolabeled sugar transport experiments .....	352
Safe use of radioactive materials .....	355
Liquid scintillation counting .....	356
Notes .....	356
Swipe assays .....	357
Notes .....	357
Phosphotransferase assay of rumen bacteria .....	360
Reagents .....	360
Inoculation of cultures .....	360
Preparation of toluene-treated cells .....	360
Measurement of phosphotransferase activity .....	361
<b>SECTION VIII: MASS SPECTROMETRY .....</b>	<b>362</b>

Operation of Waters Radian Mass spectrometer.....	363
Turning on.....	363
Running samples.....	365
Placing into standby and shutting down .....	373
Preparing samples for the Radian mass spectrometer.....	376
Processing and exporting mass spectrum for the Radian mass spectrometer .....	378
Processing the mass spectrum.....	378
Measuring signal (ion count) for peaks in mass spectrum.....	382
Exporting data.....	383
Setting up a project, sample list, and analysis method for Radian mass spectrometer .....	385
Setting up a project .....	385
Setting up sample list.....	386
Setting up a method .....	387
Maintenance of Radian mass spectrometer.....	390
Gas ballasting the backing pump .....	390
Checking oil of backing pump.....	390
Changing oil of backing pump.....	391
Replacing oil mist filter.....	392
Cleaning the instrument air filter .....	393
Cleaning source enclosure and other parts.....	393
Other details of using the Radian mass spectrometer .....	394
Checking resolution and calibration.....	394
Resetting the embedded PC (EPC) .....	395
Service mode.....	396
Tuning.....	396
Selection of compressed gas and regulators for Radian mass spectrometer .....	400
<b>REFERENCES.....</b>	<b>401</b>

## **SECTION I: ANAEROBIC TECHNIQUE AND CULTURING**

Rumen microbes are challenging to work with because they are sensitive to O<sub>2</sub>; most are strictly anaerobic. Special equipment must be built for gassing cultures with O<sub>2</sub>-free gas, to exclude O<sub>2</sub>. This equipment not only dispenses O<sub>2</sub>-free gas, but it also scrubs commercial gas of trace impurities of O<sub>2</sub>. 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  $\frac{1}{4}$ " holes in paint-grade plywood board (48" L x 12" H x  $\frac{3}{4}$ " D actual dimensions) for bolts (20- $\frac{1}{4}$ ") (**Fig. 2**).
  - b. Drill  $\frac{3}{8}$ " 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  $\frac{1}{2}$ " 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 ( $\frac{1}{2}$ " diameter x 48" length) (see **Fig. 4B** and **6**).
- 3.) Construct O<sub>2</sub>-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- $\frac{2}{3}$  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  $\frac{3}{32}$ " drill bit (for later insertion of  $\frac{1}{8}$ " 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<sub>2</sub> (99.99%), N<sub>2</sub> (99.999%), and H<sub>2</sub> (99.999%) tanks near gassing station and install 50 psi regulators (**Fig. 1**). Install a flash arrestor on the H<sub>2</sub> regulator (**Fig. 9**) and compression fittings on all regulators (**Fig. 10**).
- 5.) Connect various parts of station together using  $\frac{1}{8}$ " 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<sub>2</sub>, opening 3-way valve (B-41S2) to until pressure on gauge (PGI-63C-PG30-LAQX) reaches 1 bar, then closing

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

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

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

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

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

## Use

The column is used to scrub all O<sub>2</sub> from CO<sub>2</sub> and N<sub>2</sub> gas used in culturing. Commercial CO<sub>2</sub> and N<sub>2</sub> gas contains trace O<sub>2</sub> that will kill or inhibit rumen microbes if O<sub>2</sub> is not removed.

1.) If needed, reduce copper turnings in column. Reduced copper is red and is effective in removing O<sub>2</sub>. Copper turns pale red, colorless, and then black as it is oxidized. It is ineffective in removing O<sub>2</sub> when oxidized.

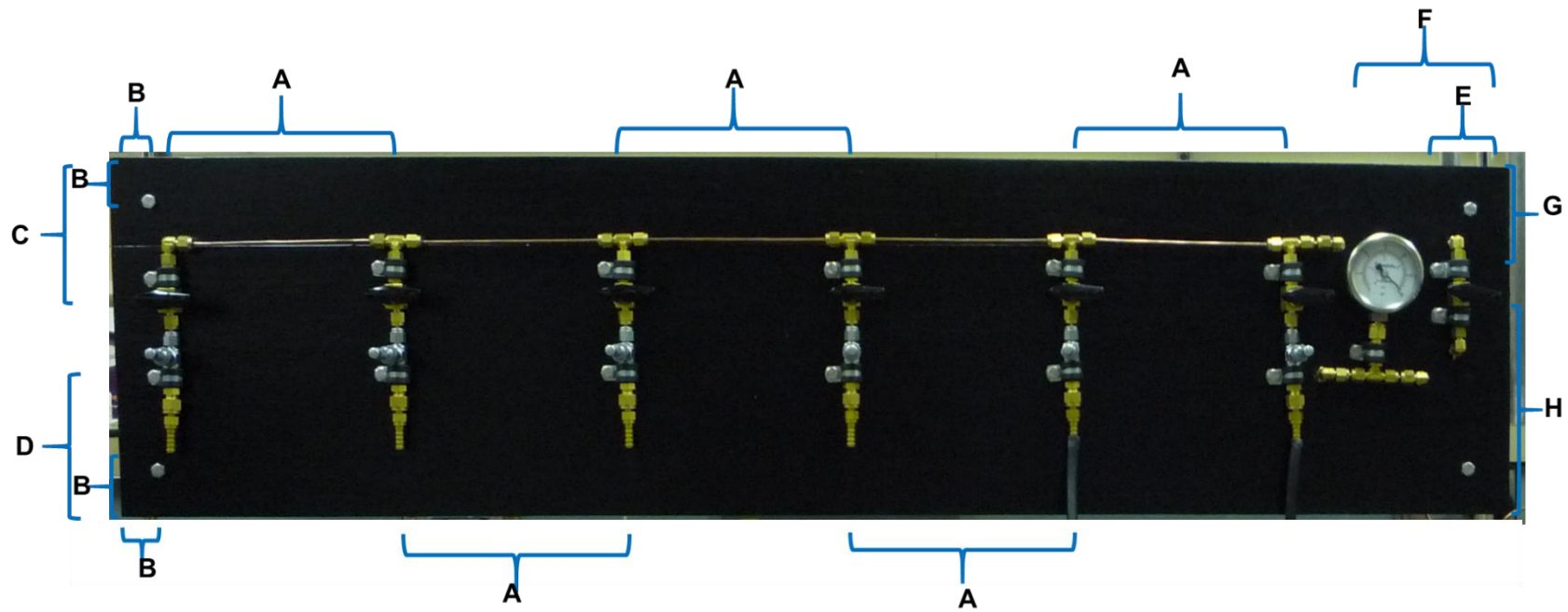
To reduce, first turn on CO<sub>2</sub> or N<sub>2</sub> and exhaust through a gassing probe for a few minutes (to flush system of most O<sub>2</sub>). Turn on H<sub>2</sub> to 0.5 bar. Combust H<sub>2</sub> by holding a lighter up to the gassing probe until a flame is lit. Allow H<sub>2</sub> to circulate until copper is red and reduced (may require several minutes).

2.) Turn on CO<sub>2</sub> or N<sub>2</sub>. 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).

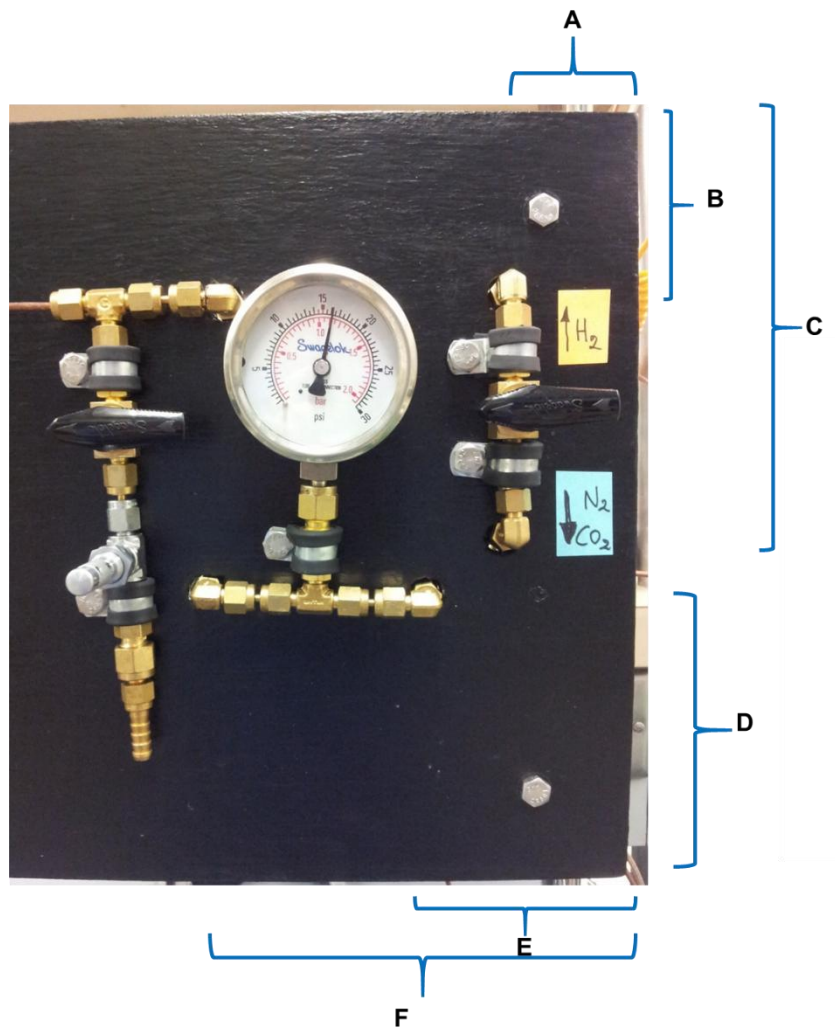
**Fig 1.** Overview of gassing station.



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



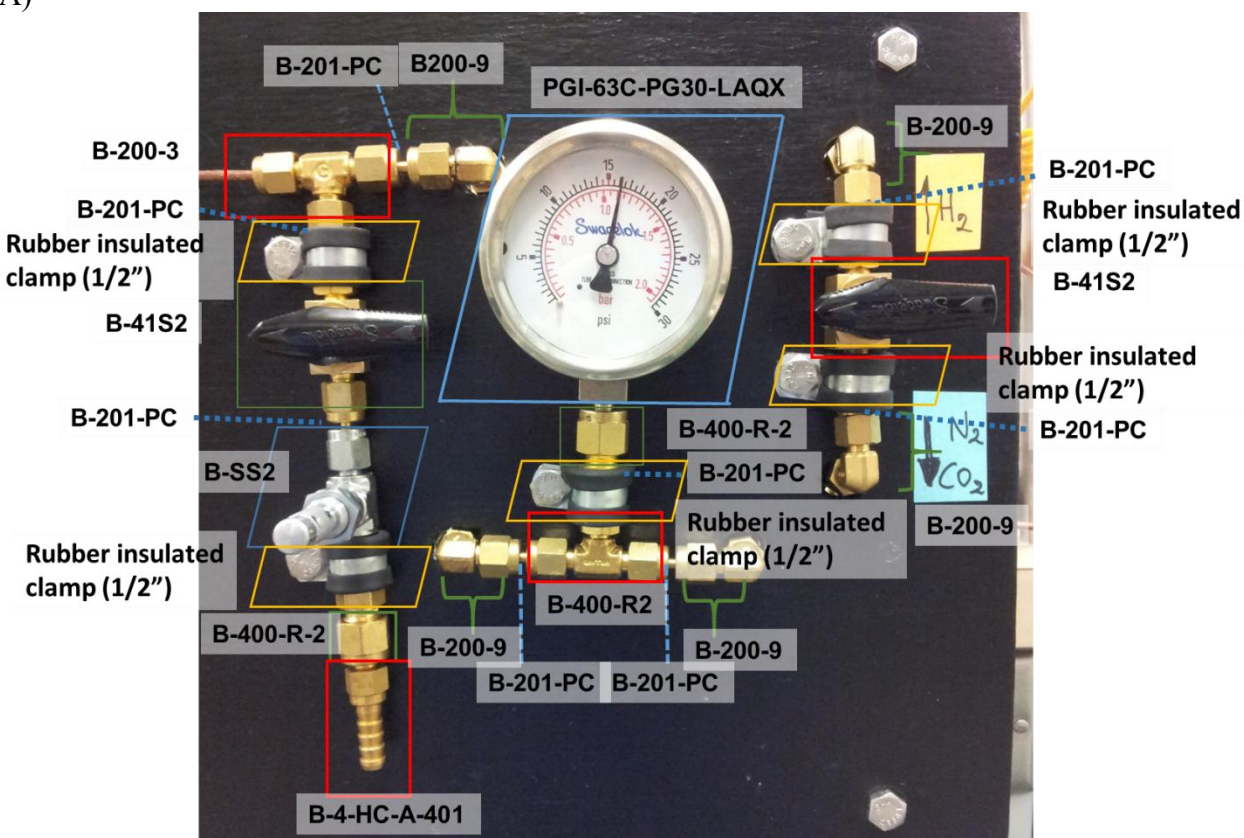
**Fig 3.** Placement of holes for Swagelok parts. A = 2", B = 2.75", C = 6.5", D = 4.75", E = 3.25", F = 6.75".





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

(A)



(B)

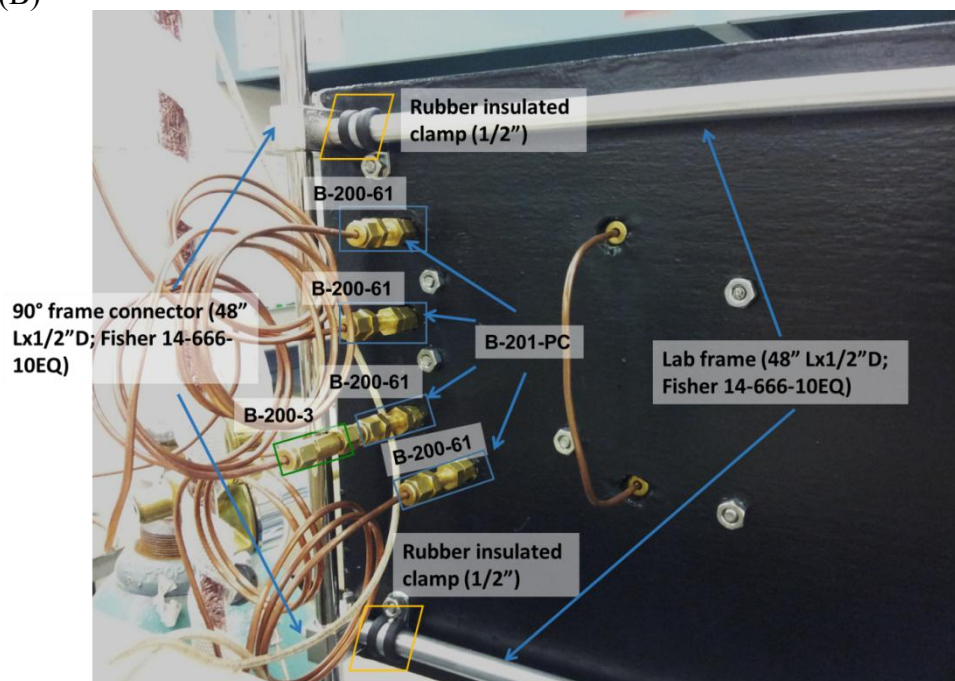
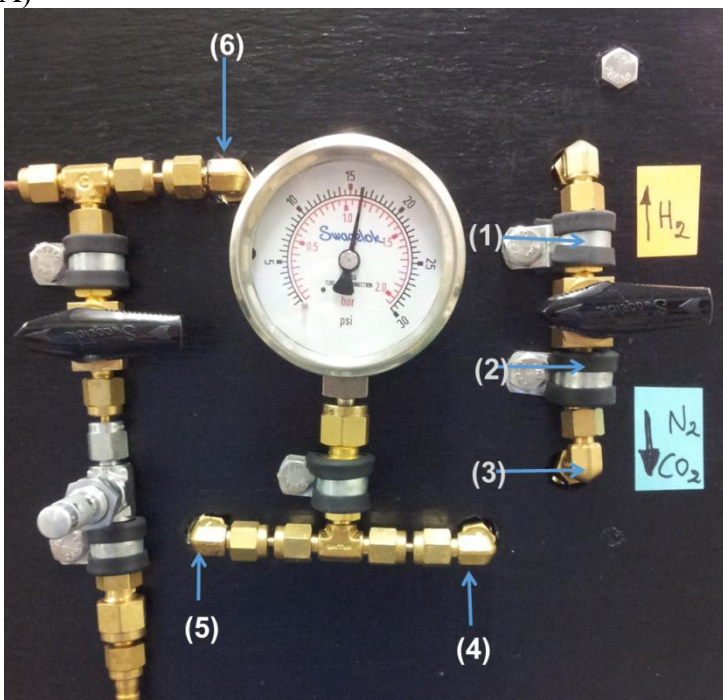
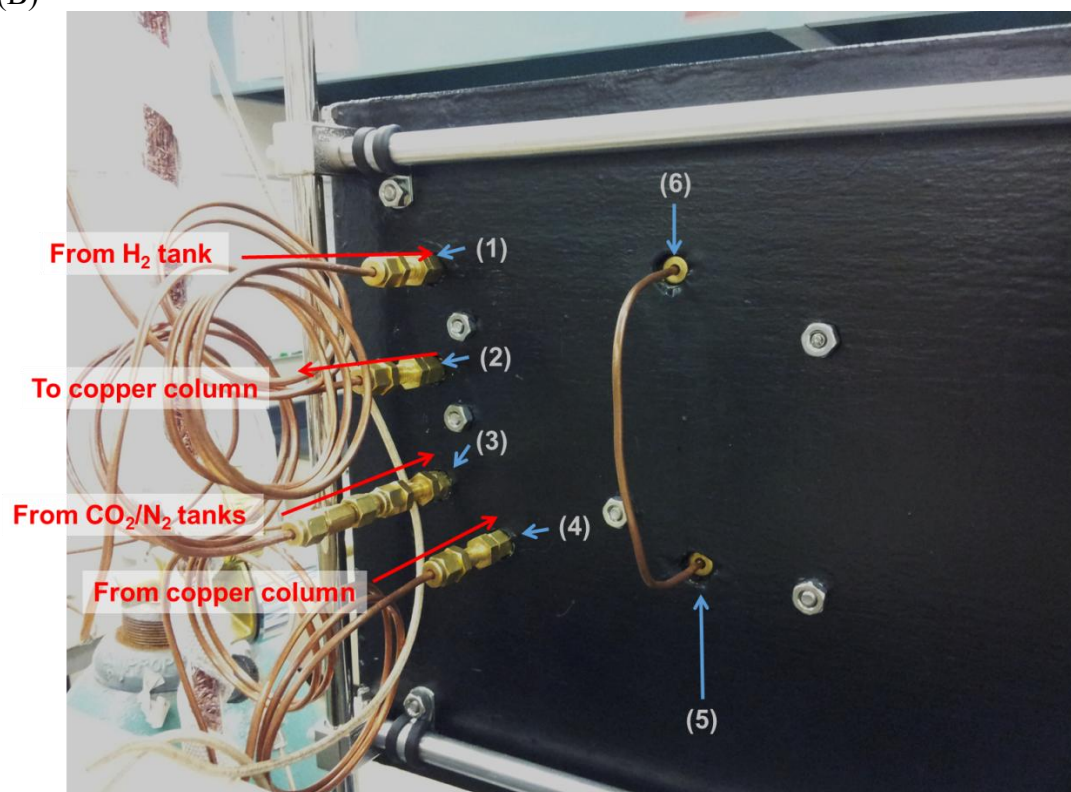


Fig 5. Connections between (A) front and (B) rear panels.

(A)

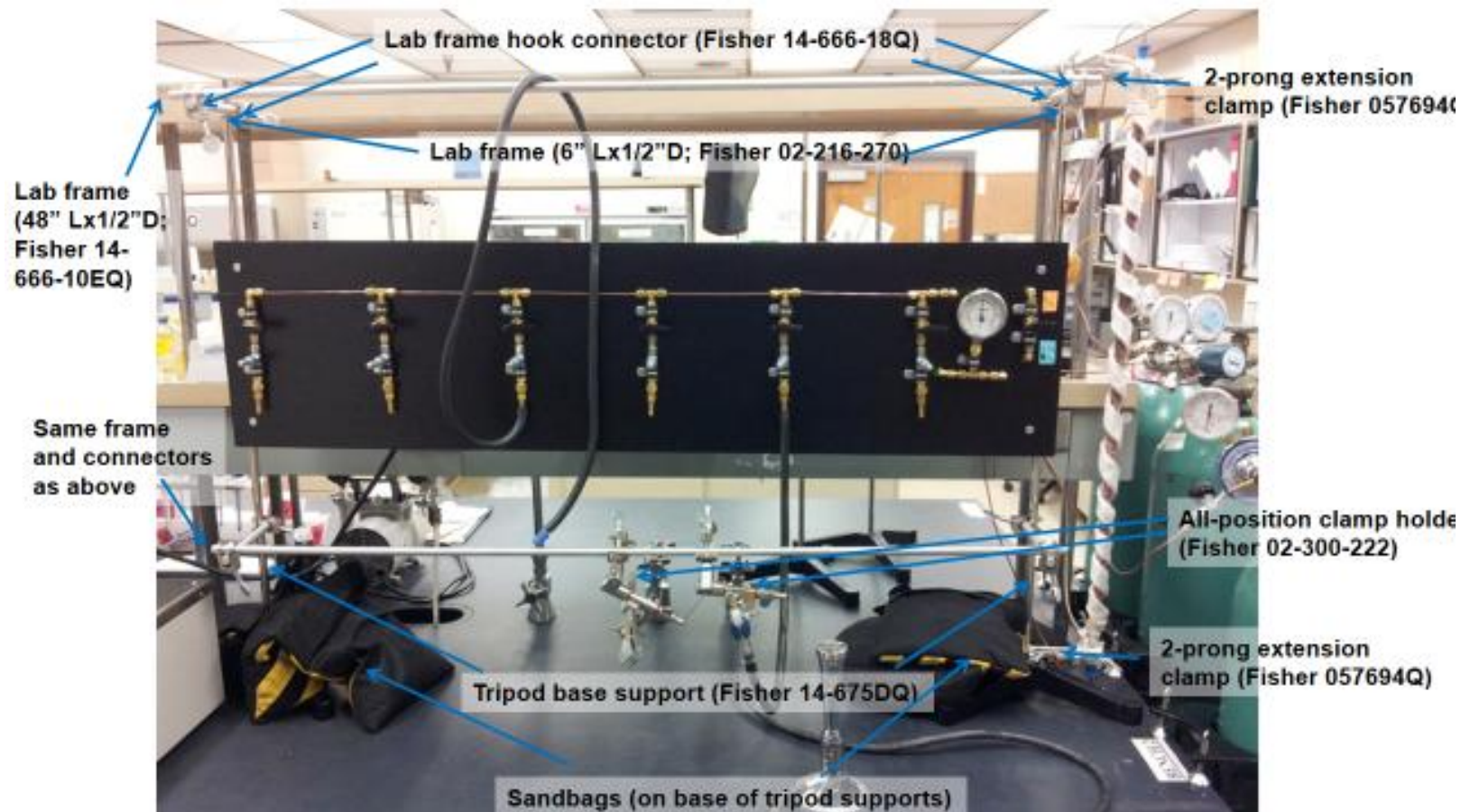


(B)

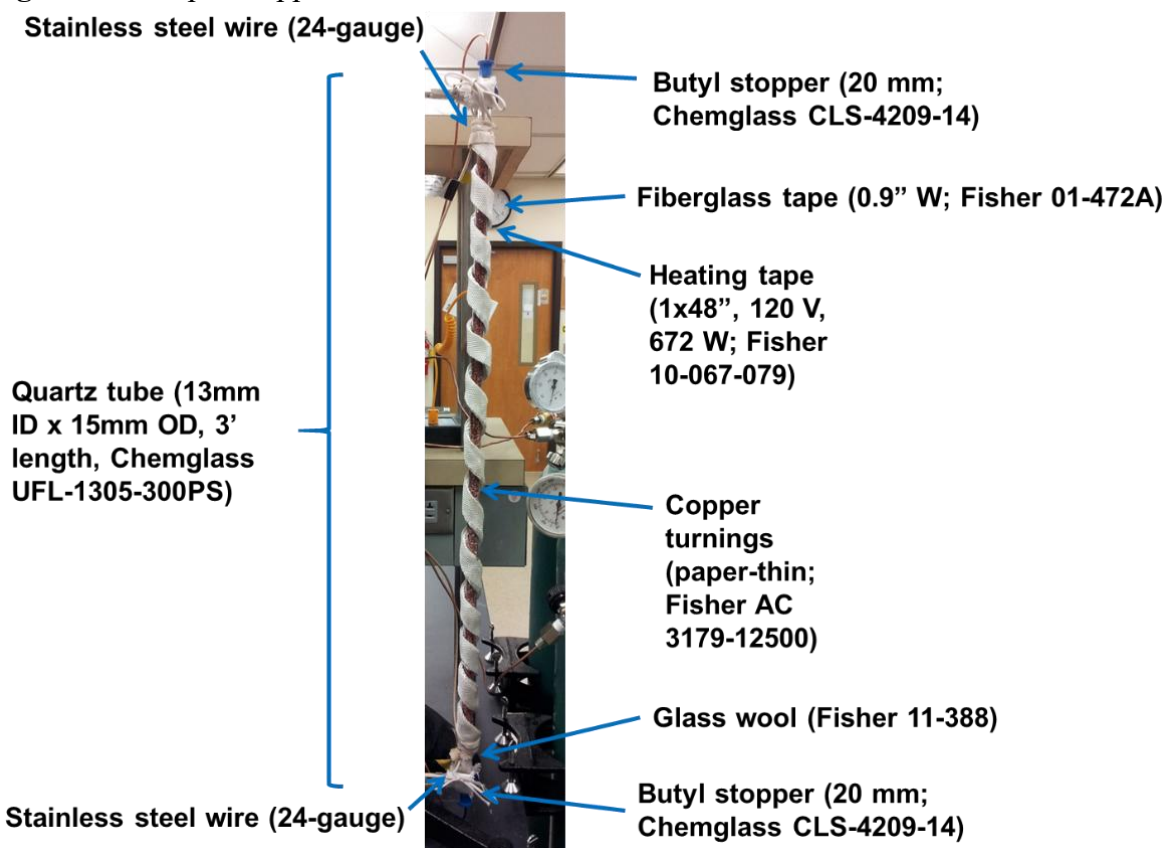




**Fig 6.** Frame components. For components visible from rear only, see Fig 4B.



**Fig. 7.** Close-up of copper column.



**Fig 8.** Packing column with copper turnings.



**Fig 9.** Flash arrestor and compression fitting on H<sub>2</sub> tank.

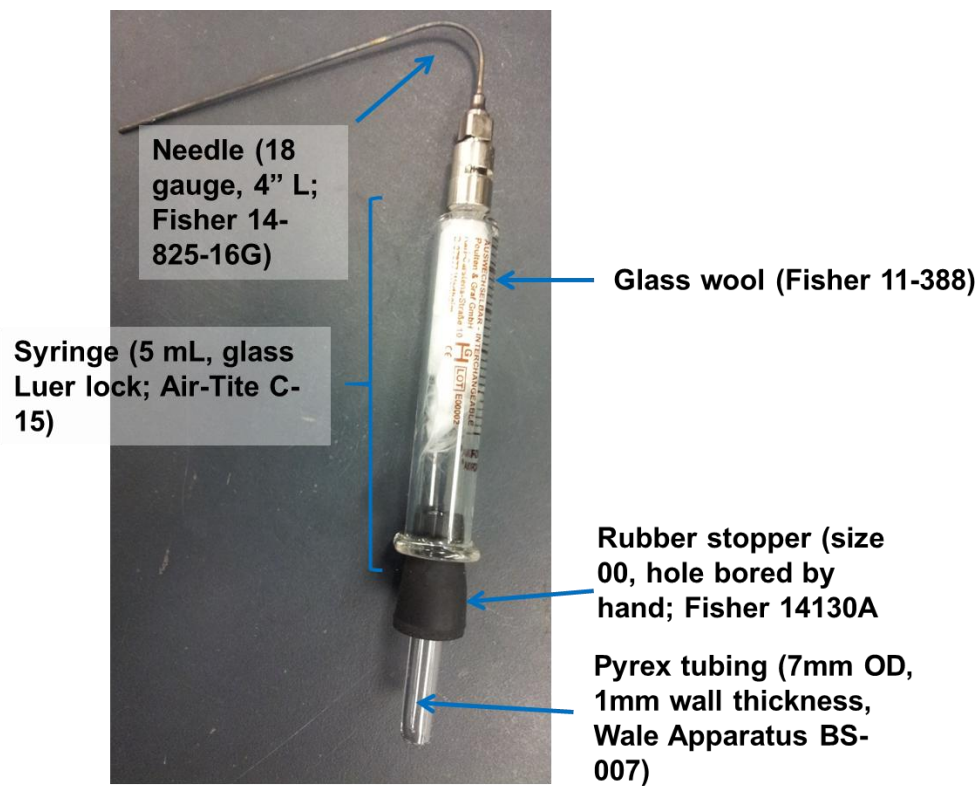


**Fig 10.** Compression fitting on N<sub>2</sub> tank (identical for CO<sub>2</sub> tank).





**Fig 11.** Gassing probe.

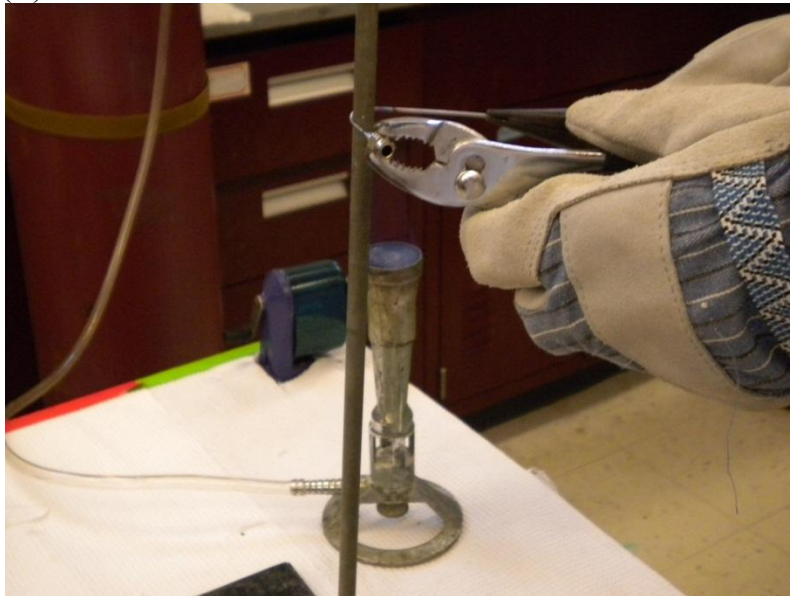


**Fig 12.** Bending needle of gassing probe. (A) Flaming needle above hub. (B) Bending needle around metal stand.

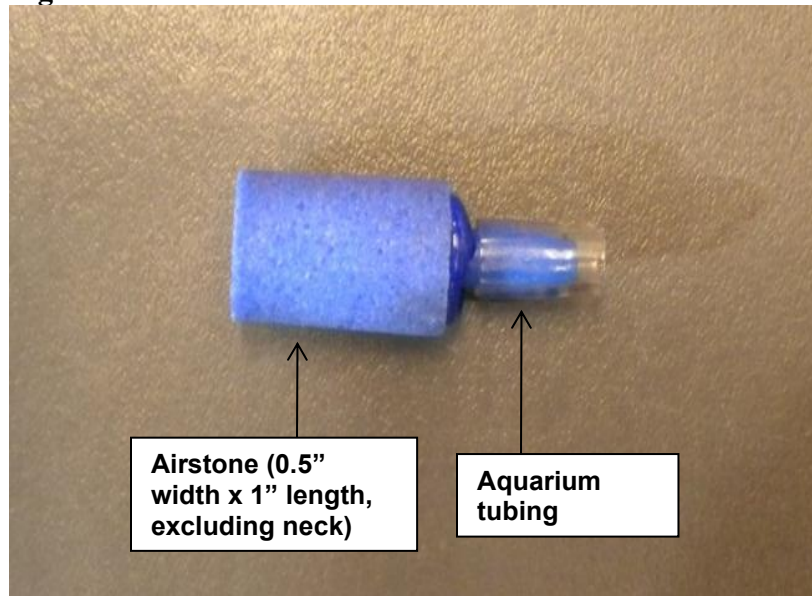
(A)



(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<sub>2</sub> by inserting it into gassing probe (see *Making buffer and media*). Withdraw 0.3 mL CO<sub>2</sub> (same volume as culture removed in next step).
  - (d) Withdraw ~0.3 mL culture from stock tube after injecting 0.3 mL CO<sub>2</sub>.
  - (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<sub>2</sub>. Agar may be individually weighed in tubes. Seal with rubber bung and aluminum lid then autoclave at 121°C/15 lb pressure for 20 min. Cool.
- 2.) Flush bottle plate with CO<sub>2</sub>. Insert needle of bottle plate gassing probe (**Fig. 2**) into rubber bung of plate, turn on CO<sub>2</sub> to the probe, and insert a 22-gauge needle into rubber bung to exhaust gas. Continue flushing for several minutes, then remove needles and probe. Autoclave at 121°C/15 lb pressure for 20 min.

- 3.) Melt the agar medium prepared in #1 by placing Balch tube in 100°C water bath. Transfer tubes to  $\geq 50$  °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<sub>2</sub>, 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<sub>2</sub> 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 ( $\geq 0.5$  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  $39^{\circ}\text{C}$ . 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  $\text{CO}_2$ . Agar may be individually weighed in tubes. Seal with rubber bung and aluminum lid then autoclave at  $121^{\circ}\text{C}/15$  lb pressure for 20 min. Cool.
- 2.) Before use, re-melt agar in roll tubes by placing in  $100^{\circ}\text{C}$  water bath. Transfer tubes to  $45^{\circ}\text{C}$  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  $45^{\circ}\text{C}$  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  $39^{\circ}\text{C}$  for 1 to 3 days. Roll tube after incubation is shown in **Fig. 5**.
- 6.) Assess culture purity.
  - (a) Count colonies in  $10^{-1}$  to  $10^{-9}$  dilutions. Determine if colonies decrease in accordance with dilution. Rough counts, particularly at low dilutions, are often sufficient.
  - (b) Examine colonies under dissecting microscope for colony morphology (color, size, shape). Determine if only 1 colony morphology appears. Be aware that colonies growing in agar will be smaller and less diffuse than colonies growing on surface of agar, even in pure culture. **Fig. 6** compares morphology of colonies from mixed and pure cultures.

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

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

### **Qualitative dilutions**

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

- 1.) Follow #1 to #3 of *Quantitative dilutions* above to prepare a  $10^{-1}$  dilution. Do not remove needle from  $10^{-1}$  dilution tube.
- 2.) Draw and expel liquid agar into syringe repeatedly. To avoid introducing bubbles, expel liquid from syringe only when tube is upright (liquid is at bottom).
- 3.) With the syringe fully depressed (agar only in the deadspace of the syringe and needle), insert the needle into a new roll tube. Mix and repeat for a total of 5-7 serially-diluted tubes. The same syringe and needle can be used throughout.
- 4.) Assess colonies and cells for purity according to *Colony picking and subculture*. Because quantitative (10-fold dilutions) are not used, do not assess colonies numbers.

### **Picking colonies**

- 1.) Prepare roll tube for picking colony. Remove rubber bung aseptically by removing aluminum cap, flaming bung with ethanol, and twisting off bung with curved-end hemostats. Before use, autoclave hemostat and flame sterilize after dipping in ethanol. Immediately insert gassing probe (autoclaved and flame-sterilized) and clamp tube into place (**Fig. 7**). Be careful not to touch agar with probe.
- 2.) Pick colony. Attach pipette bulb to colony picker, and insert colony picker into roll tube (careful not to touch agar) (**Fig. 8**). For best control, hold colony picker with 2 hands and rest elbows on table. Squeeze bulb to force out air; stab pipette tip into agar region containing colony; cut agar with pipette tip by twisting; release pipette bulb to create suction; and remove picker. Small agar plug will be lodged in colony picker (as far as several centimeters from tip).
- 3.) Eject plug with colony into another roll tube. Before picking colony, remove bung from recipient roll tube, and insert sterile gas probe. Keep upright in 45°C water bath until plug is ejected. Re-insert bung into tube after flame sterilizing. Mix contents by rocking tube back-and-forth. Do not yet roll tube.
- 4.) Prepare quantitative or qualitative dilutions of the tube in #3. Roll the tubes.

### **Growth on slants**

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

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

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

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

(b) Load a syringe with 0.5 mL ADS after flushing needle with CO<sub>2</sub> (see *Growth on broth*), then inject the 0.5 mL ADS into stock slant. Here and before every injection, flame sterilize the rubber bung of the tube using ethanol. Gently tilt tube back and forth to dislodge bacteria on agar surface.

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

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

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

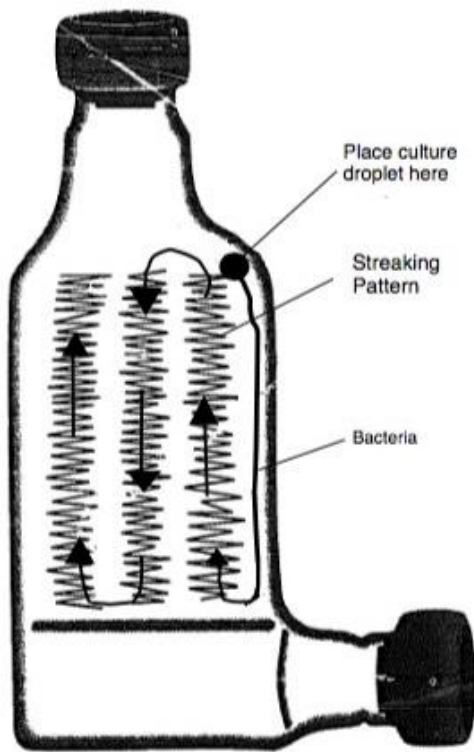
### **Preparing colony picker**

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

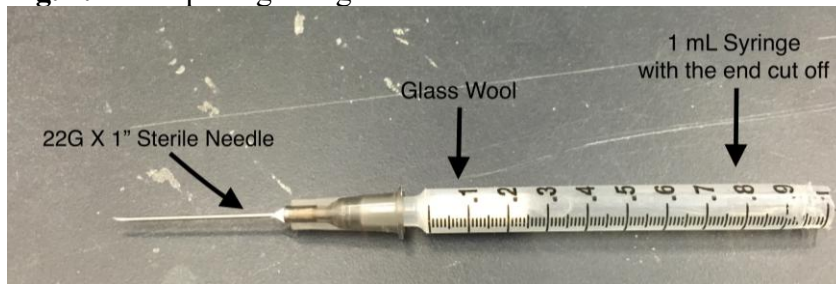
2.) Pack wide end with glass wool (to filter out particulates from gas entering pipette). Use needle from gassing probe to aid packing.

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

**Fig. 1.** Bottle plate and streaking pattern.



**Fig. 2.** Bottle plate gassing Probe

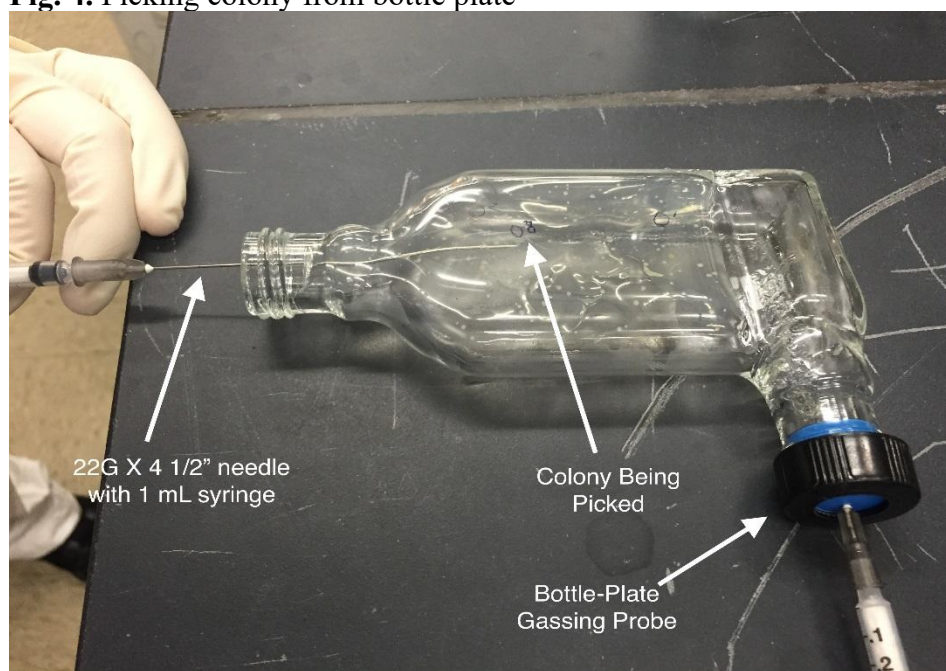


**Fig. 3.** Colony picker.

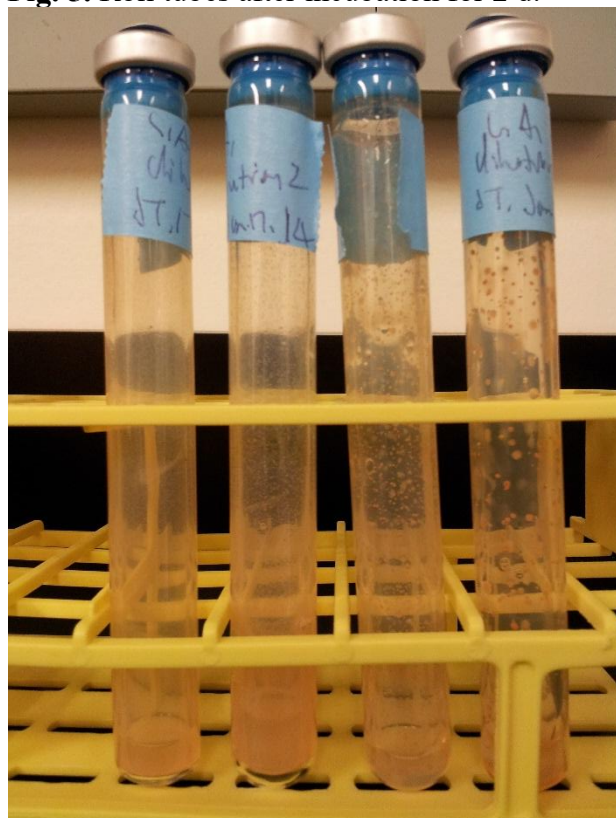




**Fig. 4.** Picking colony from bottle plate



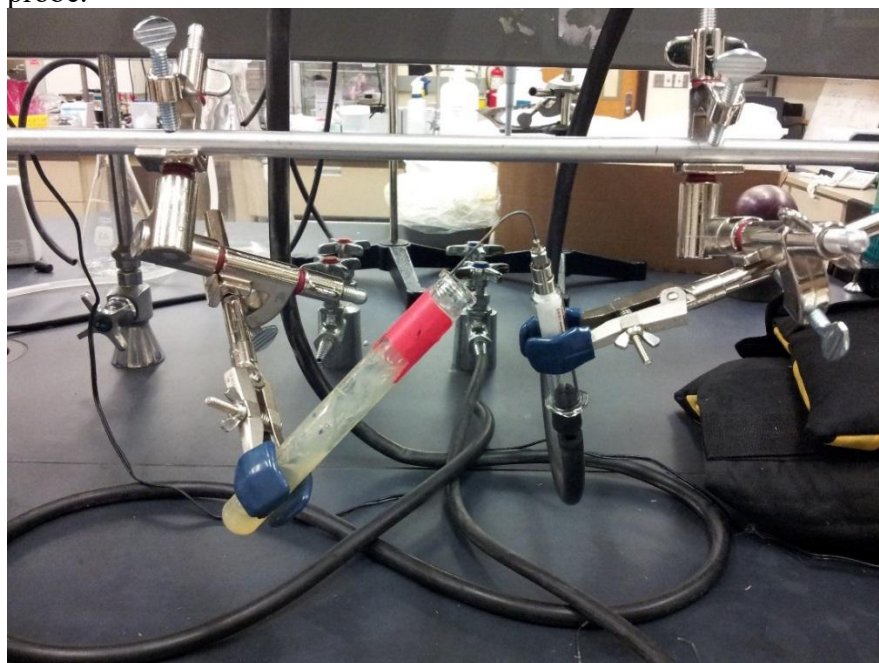
**Fig. 5.** Roll tubes after incubation for 2 d.



**Fig. 6.** Colonies of mixed and pure cultures.



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



**Fig. 8.** Picking colony.



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



## **Measuring optical density for monitoring growth of bacterial cultures**

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

### **Notes**

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

## Isolation of pure cultures of bacteria from rumen fluid

From (3, 4)

### Dilution and inoculation

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

### Notes

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

## Ordering and growing bacteria from culture collections

Adapted from DSMZ website

([https://www.dsmz.de/fileadmin/Bereiche/Microbiology/Dateien/Kultivierungshinweise/Kultivierungshinweise\\_neu\\_CD/Opening\\_14.pdf](https://www.dsmz.de/fileadmin/Bereiche/Microbiology/Dateien/Kultivierungshinweise/Kultivierungshinweise_neu_CD/Opening_14.pdf))

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

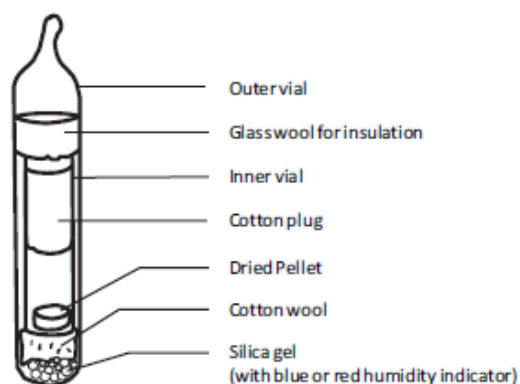
## Notes

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

- (ii) If no growth occurs in primary or other cultures, contact supplier for replacement vial.
- (iii) The first culture that is cryopreserved (usually the primary culture) should be kept but not used unless all subsequent cultures die or are compromised.

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

(A)



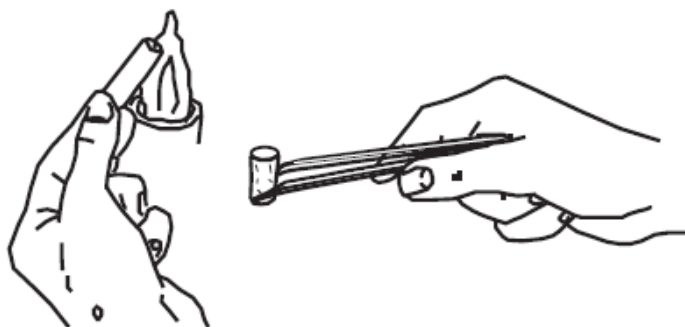
(B)



(C)



(D)





## **Cryopreservation of bacteria**

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

### **Freezing**

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

### **Revival**

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

### **Notes**

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

## Culturing methanogens

Procedure is similar to *Culturing bacteria*, except tubes and bottles must be pressurized with 80% H<sub>2</sub> /20% CO<sub>2</sub> (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<sub>2</sub>, not 100% CO<sub>2</sub>.
- 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<sub>2</sub> for flushing syringe.
- 3.) Pressurize tubes with 80% H<sub>2</sub>.
  - (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<sub>2</sub> to 1 bar and allow to circulate for a few minutes. To ensure that H<sub>2</sub> 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<sub>2</sub>. 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<sub>2</sub>.
- 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<sub>2</sub>, not 100% CO<sub>2</sub>. Bottle plate is still flushed with CO<sub>2</sub>.

### ***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<sub>2</sub>. 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<sub>2</sub> 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 ref. (1, 7–10)

### Schedule

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

### Feeding

- 1.) From freezer, remove substrate tubes. Place in rack in 39°C water bath and thaw.
- 2.) Remove protozoal tubes from 39°C incubator. Place in rack in 39°C water bath.
- 3.) After following all steps from *Constructing and using O<sub>2</sub>-scrubbing copper column*, light Bunsen burner. Flame metal needle of gassing probes until red-hot. Flame needle (**Fig. 1**) across its entire length of needle.
- 4.) Remove stopper from substrate tube and insert needle of gassing probe (**Fig. 2A**). Flame stopper and lip of culture tube. Hold culture tube with left hand, placing end of tube against body (waist) for support. Loosen stopper with thumb and forefinger of left hand while continuing to hold tube with remaining fingers of left hand. Hold gassing probe with right hand. While removing stopper, slide needle into tube, keeping it between stopper and inner wall of tube.
- 5.) While gassing, place culture tube containing substrate in clamp (**Fig. 3**). Place stopper upside down (to avoid contaminating tube upon re-insertion) near Bunsen burner. Place 1 mL, blunt-tip glass pipette in tube. Before each use, pipette should be sterilized with 80% ethanol or placed in 100°C oven then cooled. Using pipette bulb, flush pipette with CO<sub>2</sub> by placing the pipette in the upper part of the feeding tube and running CO<sub>2</sub> in and out of the pipette.
- 6.) Remove stopper from protozoal tube then insert needle of gassing probe, following #4. Mix then aspirate substrate (between 0.1 to 0.5 mL, depending on feeding schedule) with blunt-tip pipette. While continuing to hold tube open (**Fig. 2B**), dispense substrate into protozoal tube.
- 7.) Replace stopper in protozoal tube essentially by reversing steps of #4. Place stopper into tube while removing gassing probe (**Fig. 2C**). After one hand is free, flame the stopper and lip of the tube, then firmly seat stopper by twisting it firmly into place; inverting the tube to wet the stopper can help. Replace stopper on feeding tube using same procedure.
- 8.) Place protozoal tubes in 39°C incubator at a 10° angle (**Fig. 4**). Replace substrate tube in -20°C freezer.

## Transferring

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

## Preparing blunt-tip pipette

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

## Other recommendations

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

## Making protozoal substrate

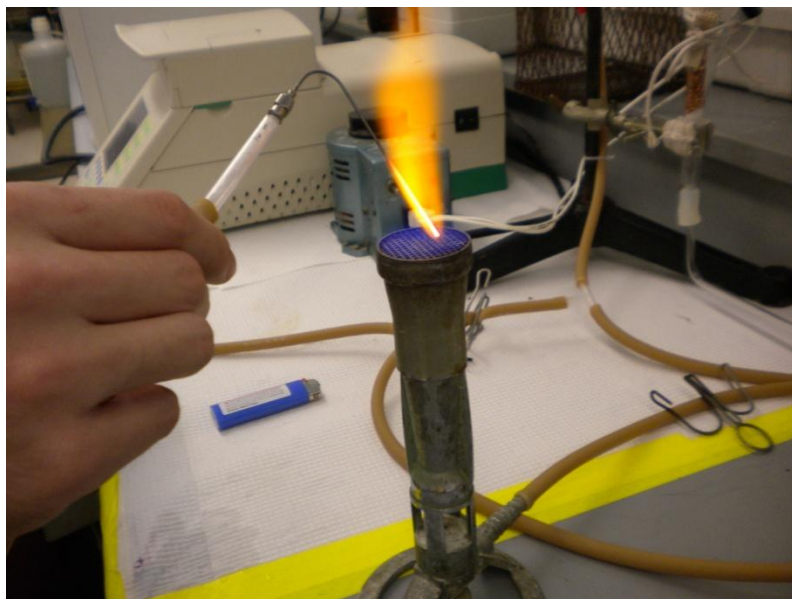
- 1.) Fill 500-mL Pyrex bottle with 100 mL dH<sub>2</sub>O. Bubble dH<sub>2</sub>O with CO<sub>2</sub> for 15 to 20 min to help remove O<sub>2</sub> from dH<sub>2</sub>O.
- 2.) Make a 1.5% w/v ground wheat and 1% w/v ground orchardgrass suspension. Add 1.5 g ground wheat and 1 g ground orchardgrass to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

### **Growing large volumes of protozoa**

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

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

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



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

(A)



(B)

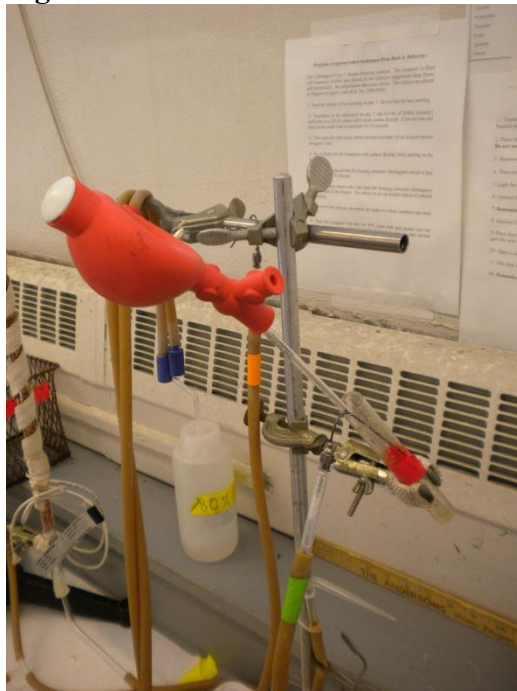




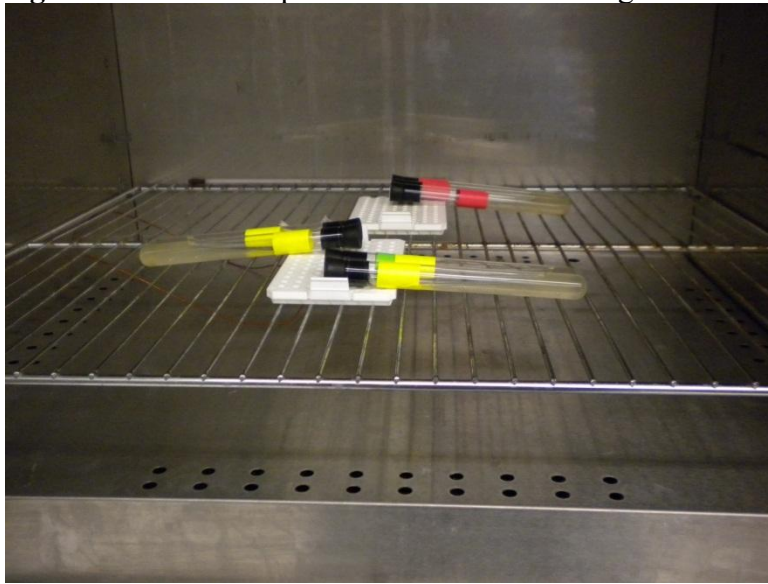
(C)



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



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



## **Cryopreservation of rumen protozoa**

Adapted from Burk Dehority (personal communication) and ref. (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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>. Repeat for more cryovials.
- 4.) Place vials in freezing container (Stratogene Cryo 1). Place container in -80°C freezer.
- 5.) After 24 h, remove vials from freezing container. Keep vials in -80°C freezer until ready for use. Thaw freezing container for next batch of protozoa.

### **Revival**

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

### **Notes**

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

## Collecting rumen fluid

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

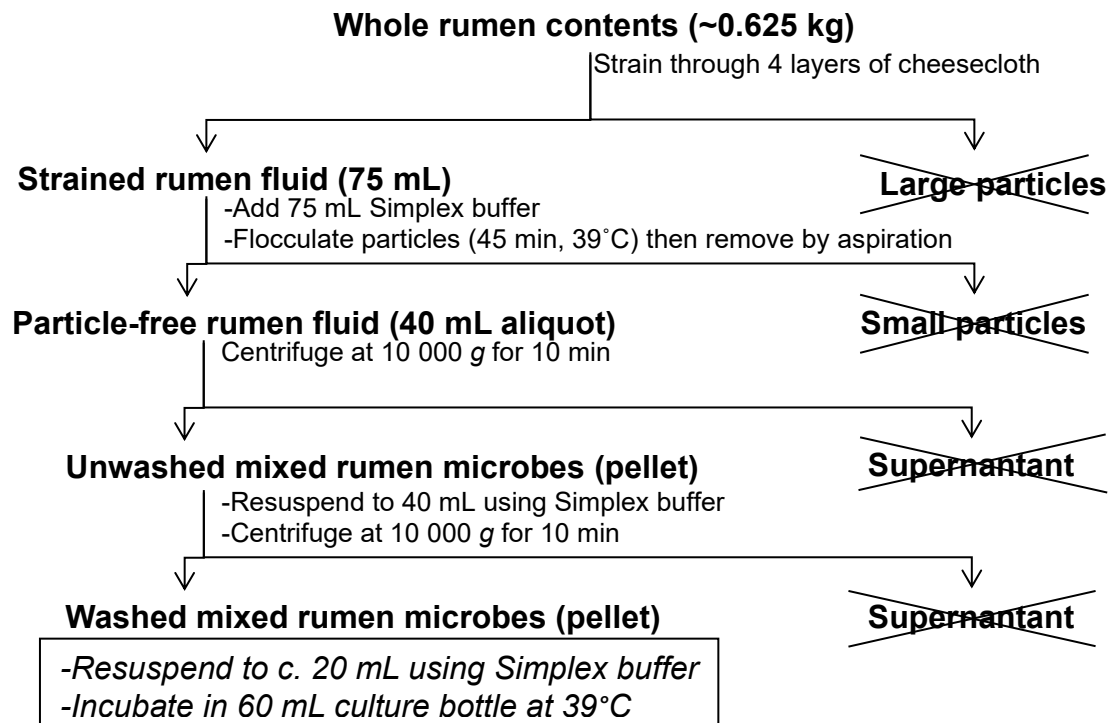
- (i) Wait up to 90 min for particles to flocculate. If many particles still settle at bottom of funnel, collect fluid on another day. If no improvement is achieved after several attempts, modify the cow's diet (increase concentrate and select higher-quality forage). Some cows never achieve perfect flocculation, 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.



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





## Preparing and culturing mixed bacteria from rumen fluid

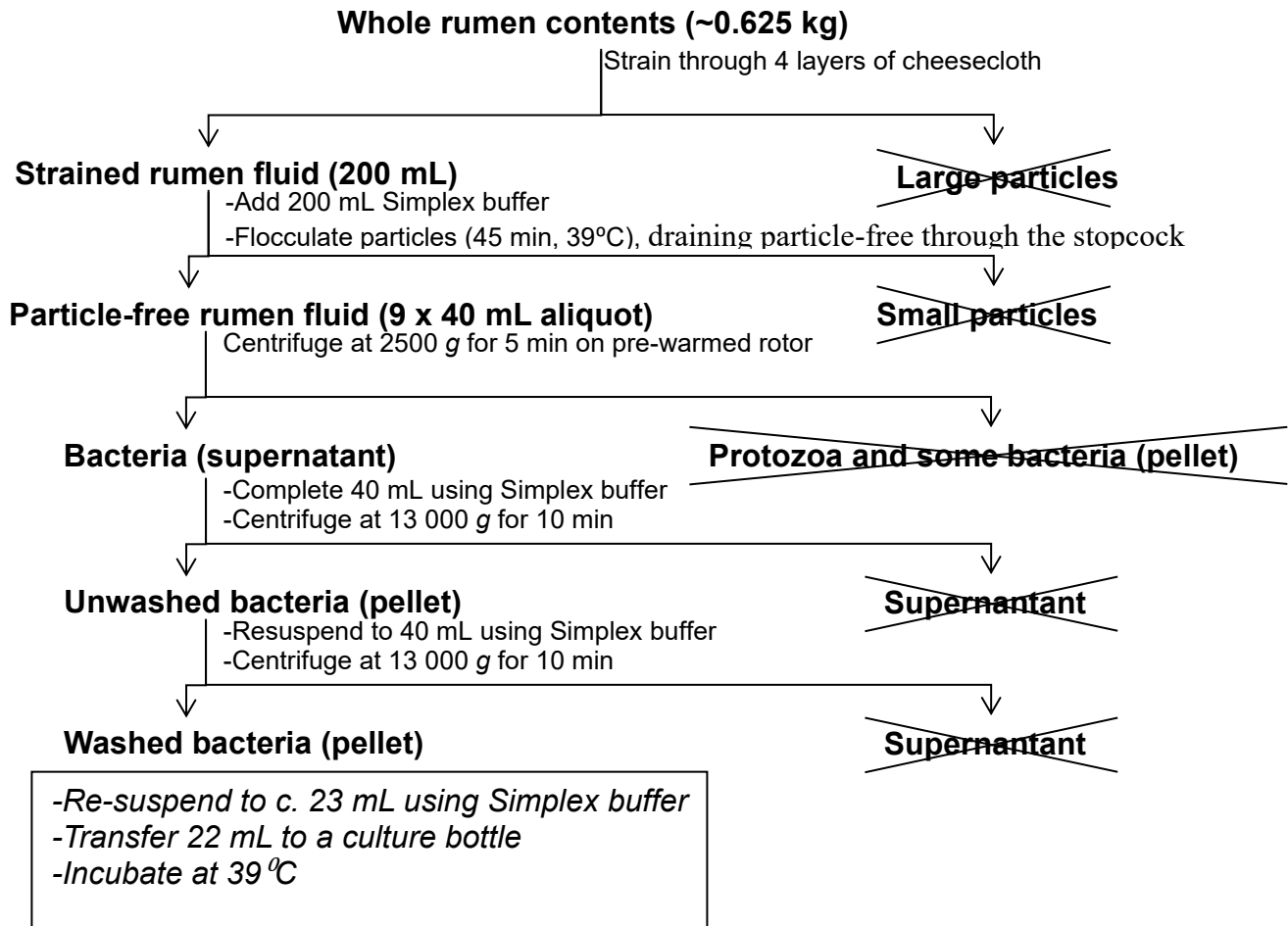
Adapted from ref. (11, 12)

- 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<sub>2</sub>-free CO<sub>2</sub>. Do this step quickly to minimize exposure of supernatant to air.
  - (e) Bring tubes to 40-mL volume using Simplex buffer.
- 3.) Centrifuge supernatant in #2 according to **Fig. 1**. After removing supernatant, pool the pellets of multiple tubes together into a single 40-mL tube. Do this by resuspending one pellet in 10 mL of Simplex buffer, transferring the suspension to the next tube, resuspending the next pellet in the suspension, and repeating sequentially for all tubes. Bring to 40-mL volume using Simplex buffer.
- 7.) Centrifuge and resuspend pellet in 23 mL using Simplex buffer according to Fig. 1. Transfer 22 mL aliquot to a culture bottle and incubate in 39°C incubator.

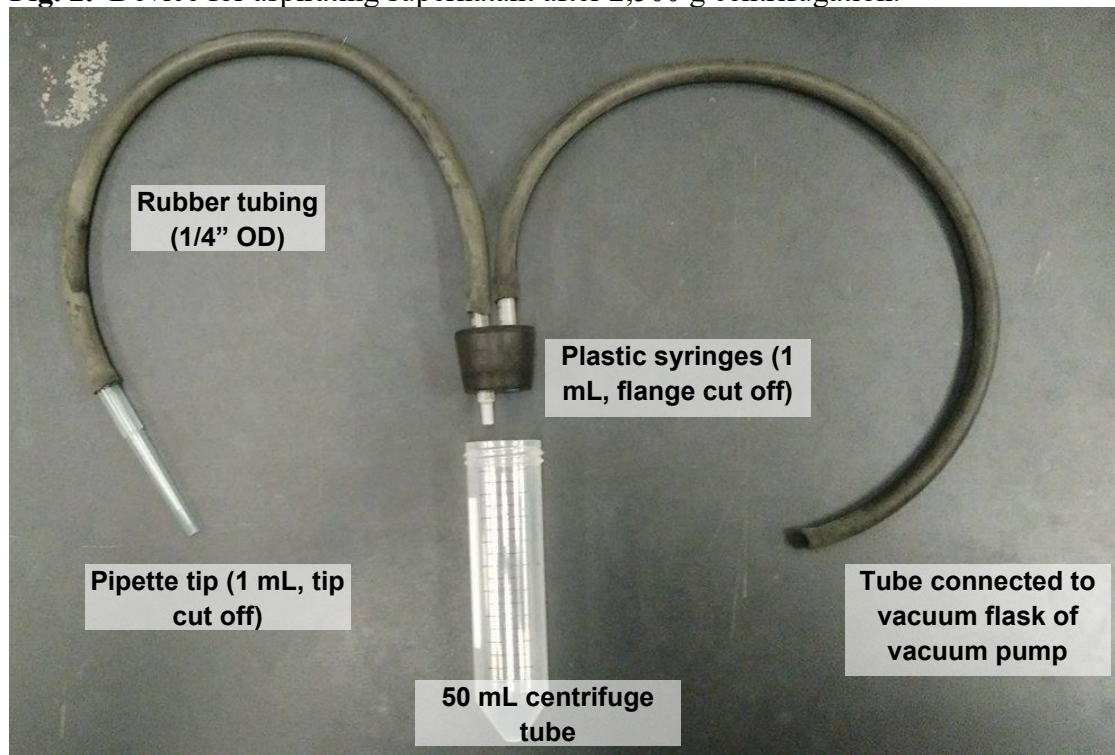
## Notes

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

**Fig. 1.** Flowchart for preparation of rumen bacteria.



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



## Preparing and culturing mixed protozoa from rumen fluid

Adapted from ref. (11, 12)

- 1.) Follow **Fig. 1** along with steps #1-3 from *Preparing and culturing mixed rumen microbes from rumen fluid*.
- 2.) Place 20  $\mu\text{m}$  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  $\text{CO}_2$  to maximum setting.
- 3.) Turn on  $\text{CO}_2$  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  $\text{CO}_2$ . 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  $39^\circ\text{C}$  and under  $\text{CO}_2$  in interim.
- 8.) Bring volume of suspension to 23 mL with Simplex buffer, transfer 22 mL to culture bottle, and incubate at  $39^\circ\text{C}$ .

## Notes

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

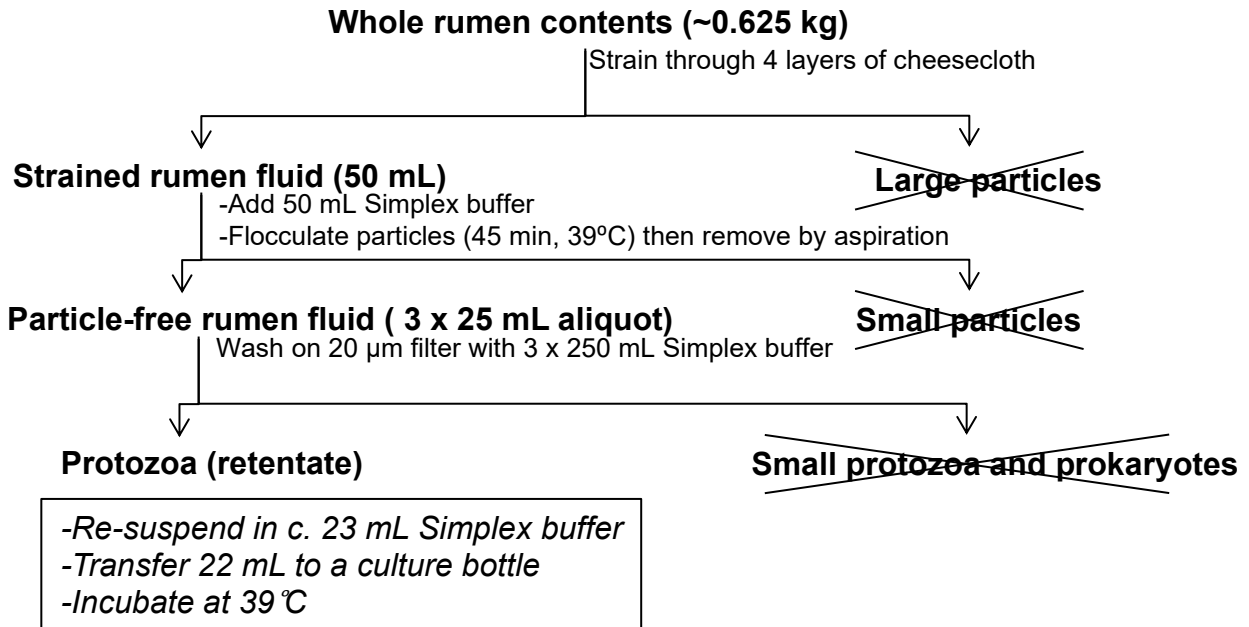
(ii) Mean (SD) recovery of protozoal cells was 70.77 (8.28) % of particle-free rumen fluid ( $n = 4$ ). Contamination of protozoa with bacteria was 0.77 (0.38) g/100 g microbial protein ( $n = 8$  [3 cows with  $n = 2$  or 3 each] using  $1.79 \cdot 10^{-13}$  g protein/prokaryotic cell measured on separate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

Incomplete recovery of protozoa owed to small *Entodinium* sp. (those  $<20 \mu\text{m}$ ) passing through the 20- $\mu\text{m}$  filter. Recovery of larger, non-*Entodinium* species was nearly complete (94.9 [15.6]%). In preliminary experiments, a smaller pore filter (10  $\mu\text{m}$ ) 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).

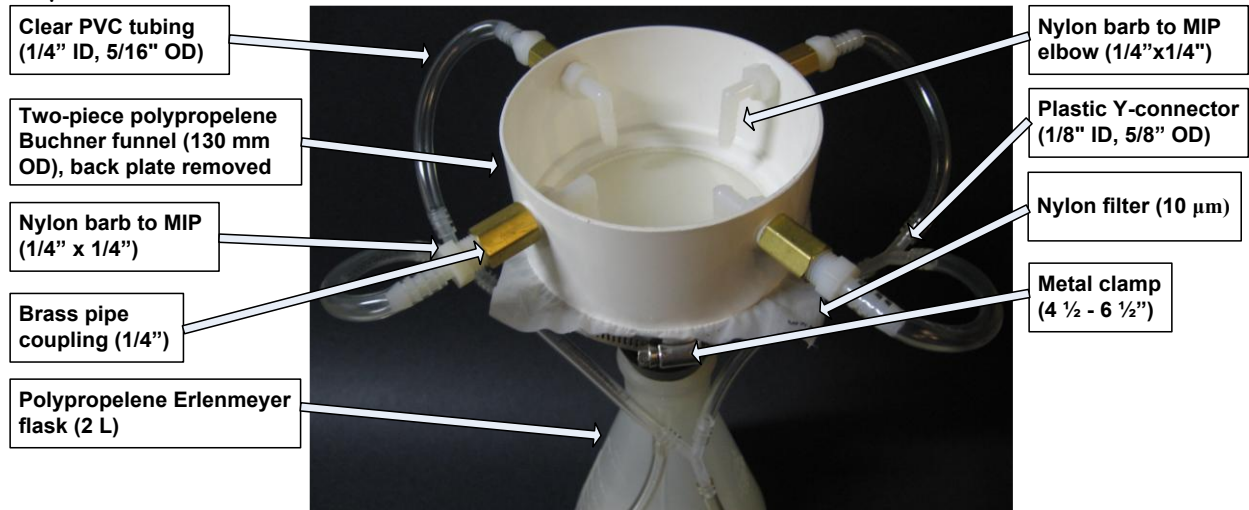
(iii) Metabolic activity of protozoa is normal. To verify that filtration indeed does not alter metabolic activity of protozoa, we compared heat production of (i) particle-free rumen fluid that was not filtered and (ii) particle-free rumen fluid that was filtered. Fluid that was filtered was done so 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.

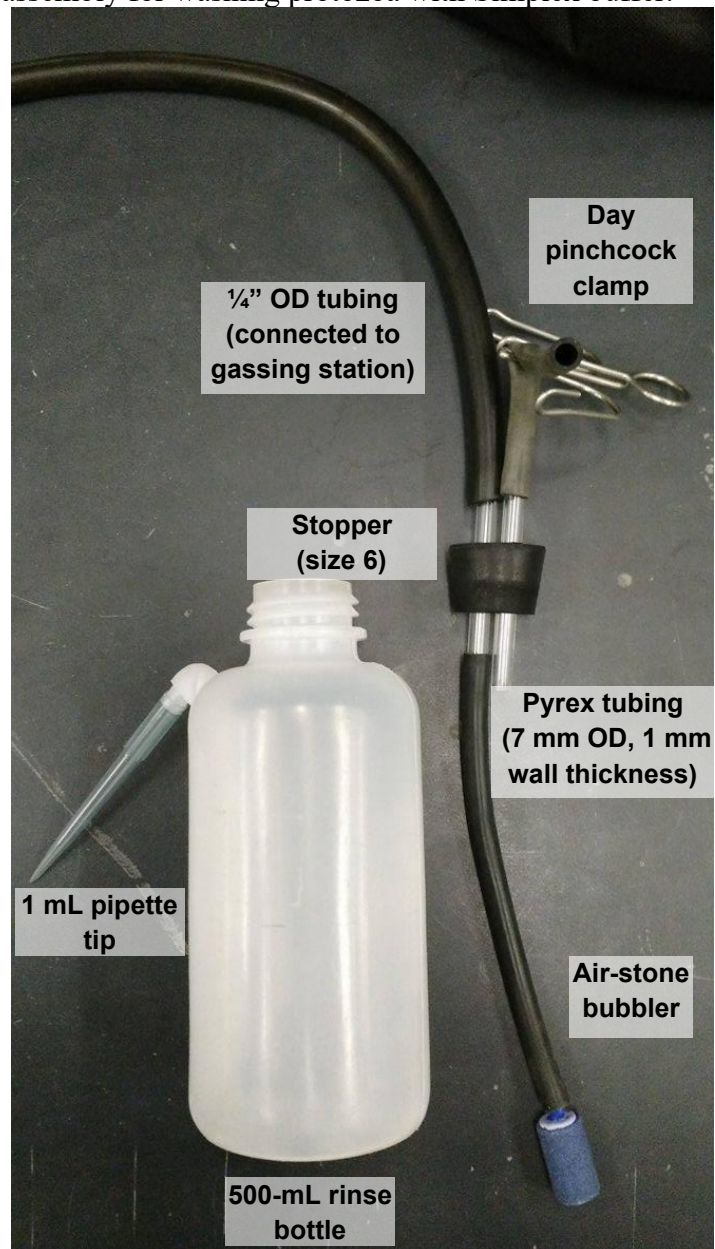
**Fig. 1.** Flowchart for preparation of rumen protozoa.



**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 ref. (13)

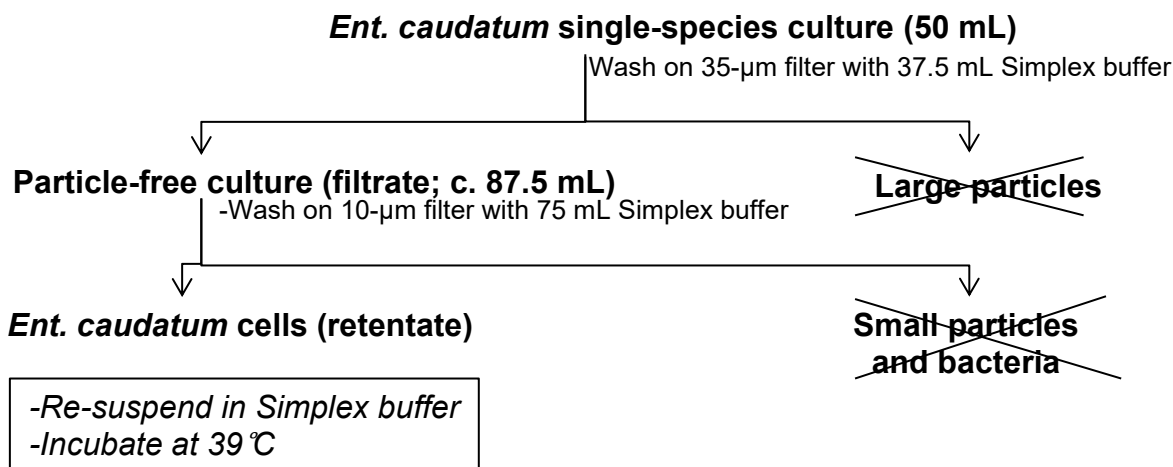
Follow **Fig. 1** or **Fig. 2** along with steps #1-3 from *Preparing and culturing protozoa from rumen fluid*.

### 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  $\mu\text{m}$  for *Ent. caudatum*) are invariably retained.

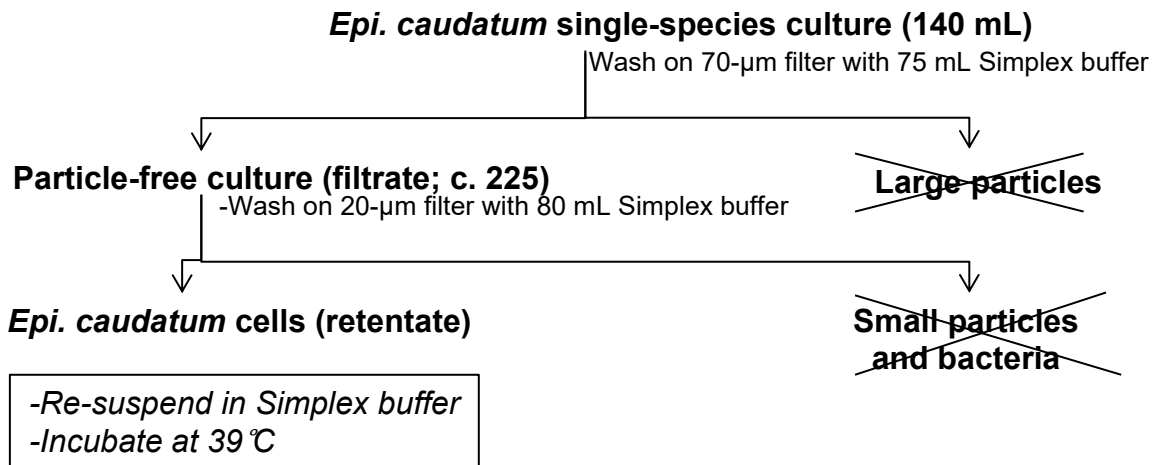
(i) Mean (SD) recovery of *Entodinium caudatum* cells was 95.5 (31.3 SD) % ( $n = 3$ ). Contamination of protozoa with bacteria was 1.89 (0.18 SD) g/100 g microbial protein ( $n = 3$ ), using  $1.34 \cdot 10^{-10}$  ( $2.18 \cdot 10^{-11}$  SD) mg/prokaryotic cell ( $n = 8$ ) measured on separate samples.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

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





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







## Making buffer and media

Adapted from ref. (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  $\leq 600$  mL.

1.) Add buffer/media components (dH<sub>2</sub>O, salts, rumen fluid, resazurin,) to 1-L round bottom flask in quantities directed in *Buffer and media recipes*. Do not include NaCHO<sub>3</sub> or cysteine·HCl at this step.

2.) Boil buffer/media to remove most O<sub>2</sub> (**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<sub>3</sub> in quantity directed in individual procedures. Buffer/media must be cool before adding NaHCO<sub>3</sub> because NaHCO<sub>3</sub> will boil off as CO<sub>2</sub> 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<sub>2</sub> by drawing plunger in and out several times, and fill syringe with volume of CO<sub>2</sub> equal to amount of cysteine to be withdrawn. Puncture bung of culture bottle with syringe needle, inject CO<sub>2</sub>, 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<sub>2</sub>, 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<sub>2</sub> by placing pipette in the upper part of the round-bottom flask tube and drawing CO<sub>2</sub> 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  $\leq 95$  mL aliquots of buffer/media, dispense into 160 mL culture bottles. Flush culture bottle with CO<sub>2</sub> by placing it under a gassing probe clamped to a metal stand. Flush volumetric pipette with CO<sub>2</sub> by drawing placing the pipette in the upper part of the round-bottom flask tube and drawing CO<sub>2</sub> 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  $\leq 600$  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  $\leq 1.8$  L.

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

2.) Add buffer/media components (salts, resazurin, rumen fluid, dH<sub>2</sub>O) to 3-L round bottom flask in amounts directed in individual procedures. Do not include NaCHO<sub>3</sub> 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<sub>2</sub> tubing and bubble solution with CO<sub>2</sub>.

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<sub>3</sub> 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<sub>2</sub> for 4 min (**Fig. 7A**).

(ii) Flush dispensing tube (**Fig. 7B**). Raise 3-L tubing assembly so end is above liquid (but CO<sub>2</sub> is still flowing into 3-L flask), remove clamp from dispensing tube, and clamp vent tubing on 1-L tubing assembly. Flush tubing 60-90 sec.

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

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

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

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

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

### ***Assembly of boiling and dispensing apparatus***

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

2.) Prepare tubing assemblies. Follow **Fig. 9** and **Fig. 10**. Cut tubing and attach to syringes. Insert glass tubing into dispensing tubing for 1-L flask (to weigh down and stabilize tubing during dispensing). Attach air stones to CO<sub>2</sub> 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<sub>2</sub> tubing to gassing station.

### **Preparing buffer or media by distillation**

Procedures above remove O<sub>2</sub> mostly by boiling. To remove even more O<sub>2</sub>, water can be distilled under O<sub>2</sub>-free CO<sub>2</sub>. 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.

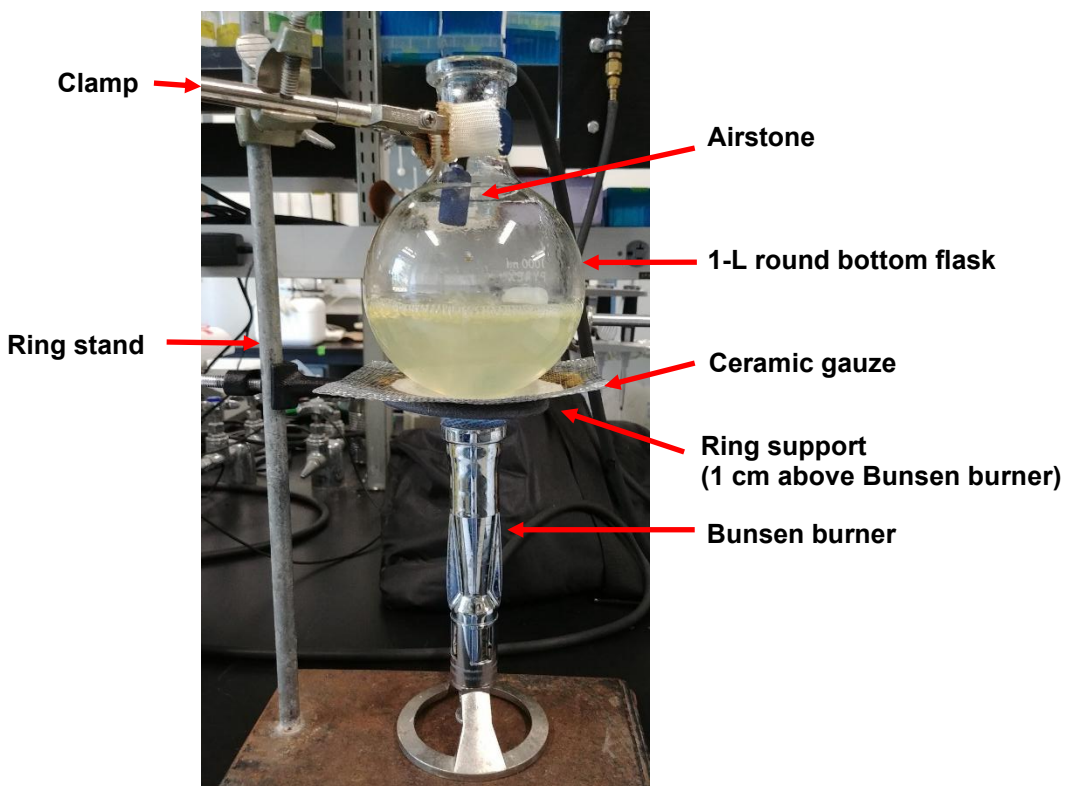
- 1.) Assemble distillation unit as shown (**Fig. 11**).
- 2.) Add buffer/media components (minus the dH<sub>2</sub>O) to the 500-mL Erlenmeyer flask in quantities directed in ***Buffer and media recipes***. NaCHO<sub>3</sub> can be included, but do not include cysteine at this step.
- 3.) Add 600 mL of dH<sub>2</sub>O 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<sub>2</sub>).
- 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.

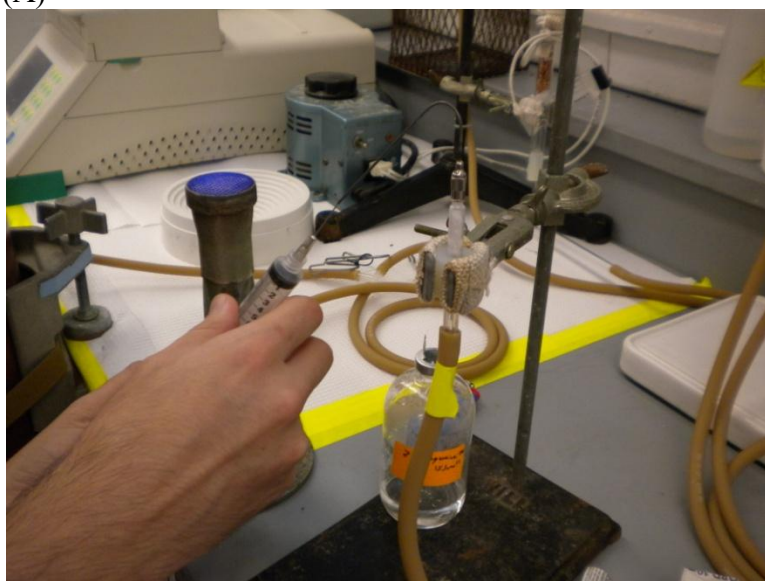


**Fig. 1.** Technique for gassing and boiling buffer/media.

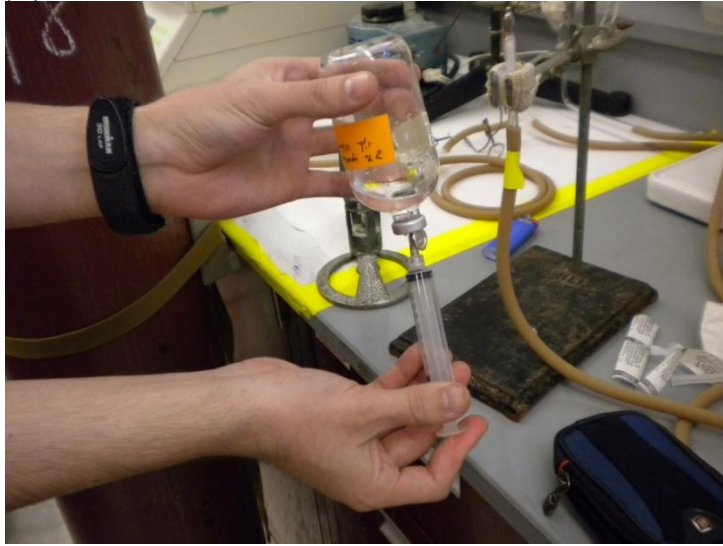


**Fig. 2.** Technique for withdrawing cysteine from culture bottle. (A) Flushing syringe with  $\text{CO}_2$  via gassing probe clamped to stand. (B) Withdrawing cysteine.

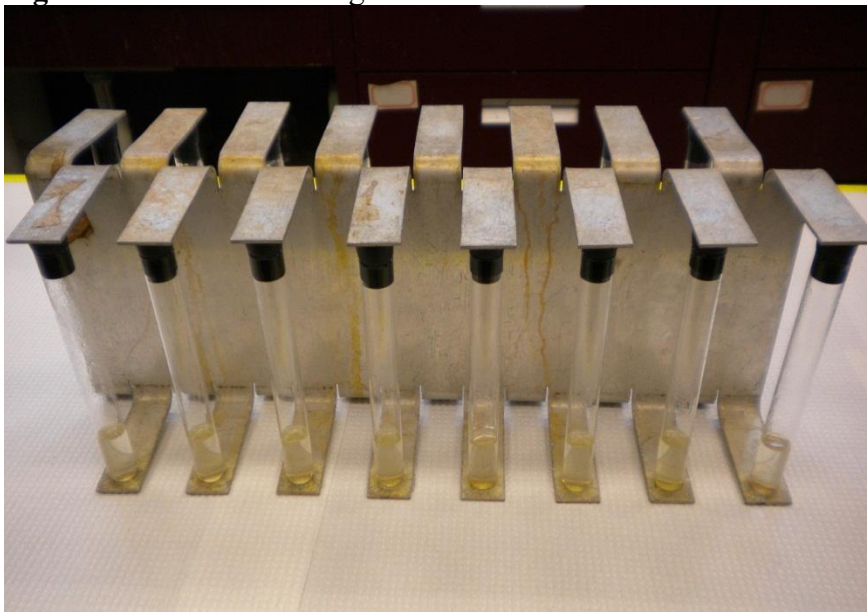
(A)



(B)



**Fig. 3.** Rack for autoclaving culture tubes.

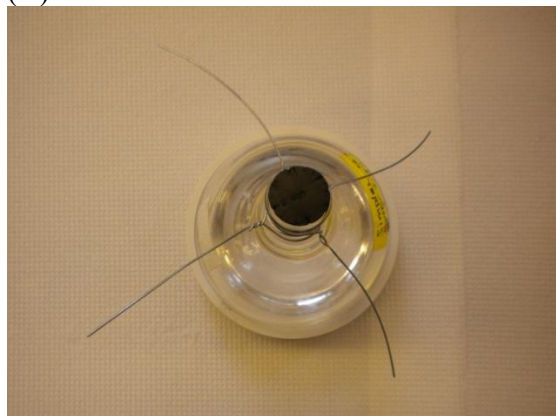


**Fig. 4.** Wiring stopper in place. (A). Securing 1 wire around neck of flask. (B) Repeating 3 more times to give 4 secured wires. Wires are positioned for securing around stopper; they are at 12, 3, 6, and 9 o'clock. (C) Securing 2 wires around stopper. (D) Securing remaining 2 wires around stopper.

(A)



(B)



(C)

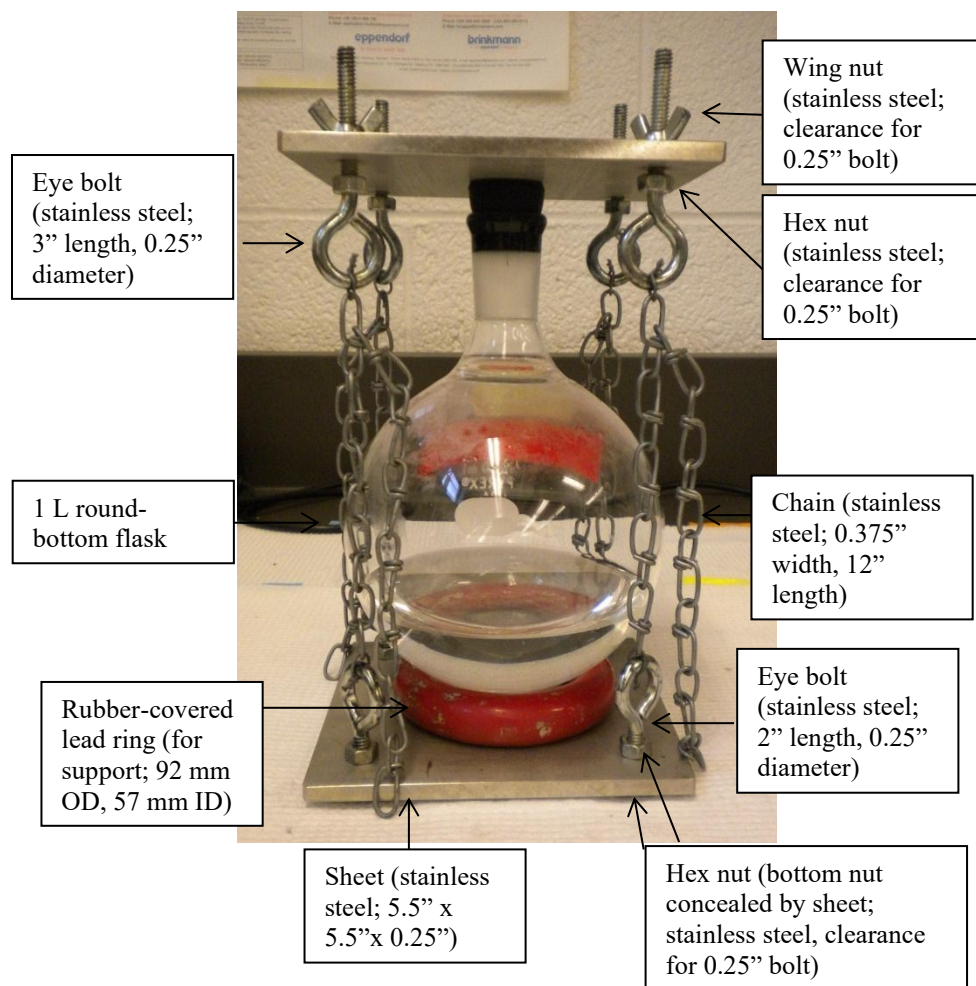


(D)



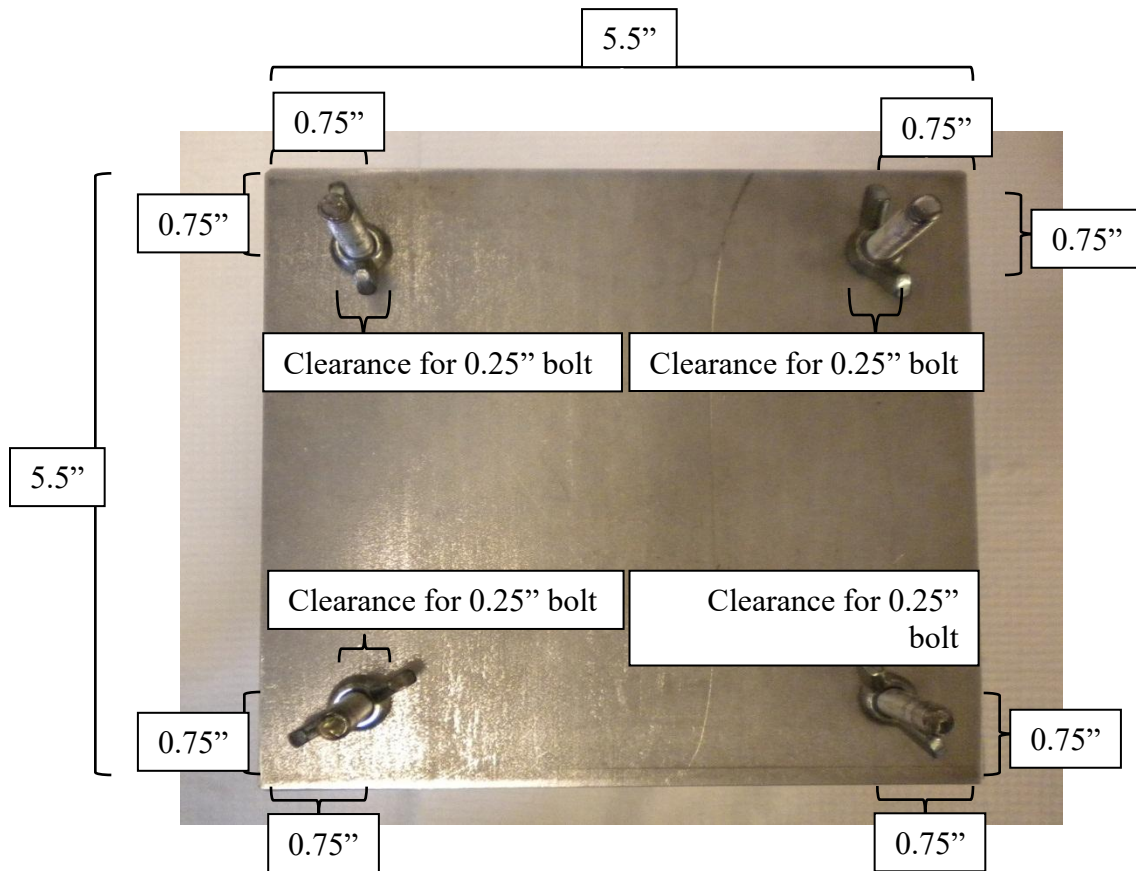
**Fig. 5.** Press for holding stopper in place during autoclaving. (A) Press with round-bottom flask in place. (B) Dimensions of steel sheet.

(A)

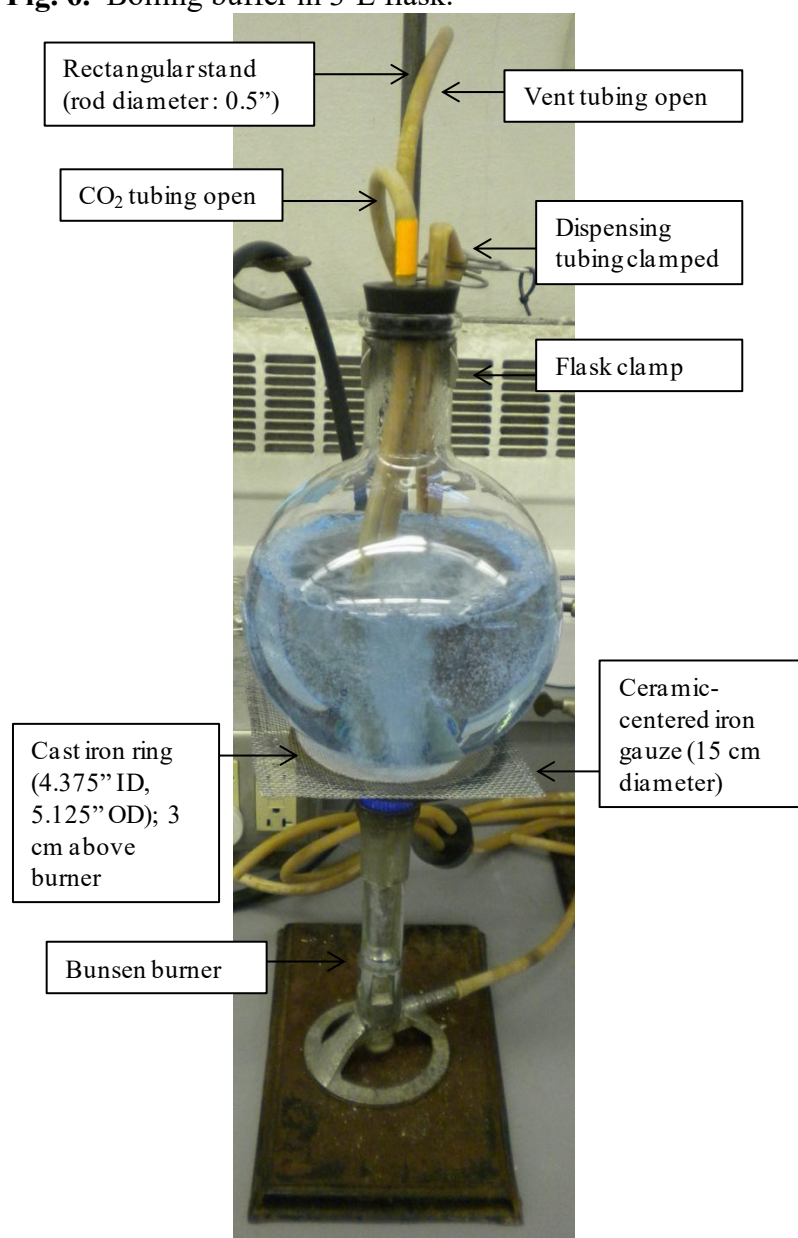




(B)

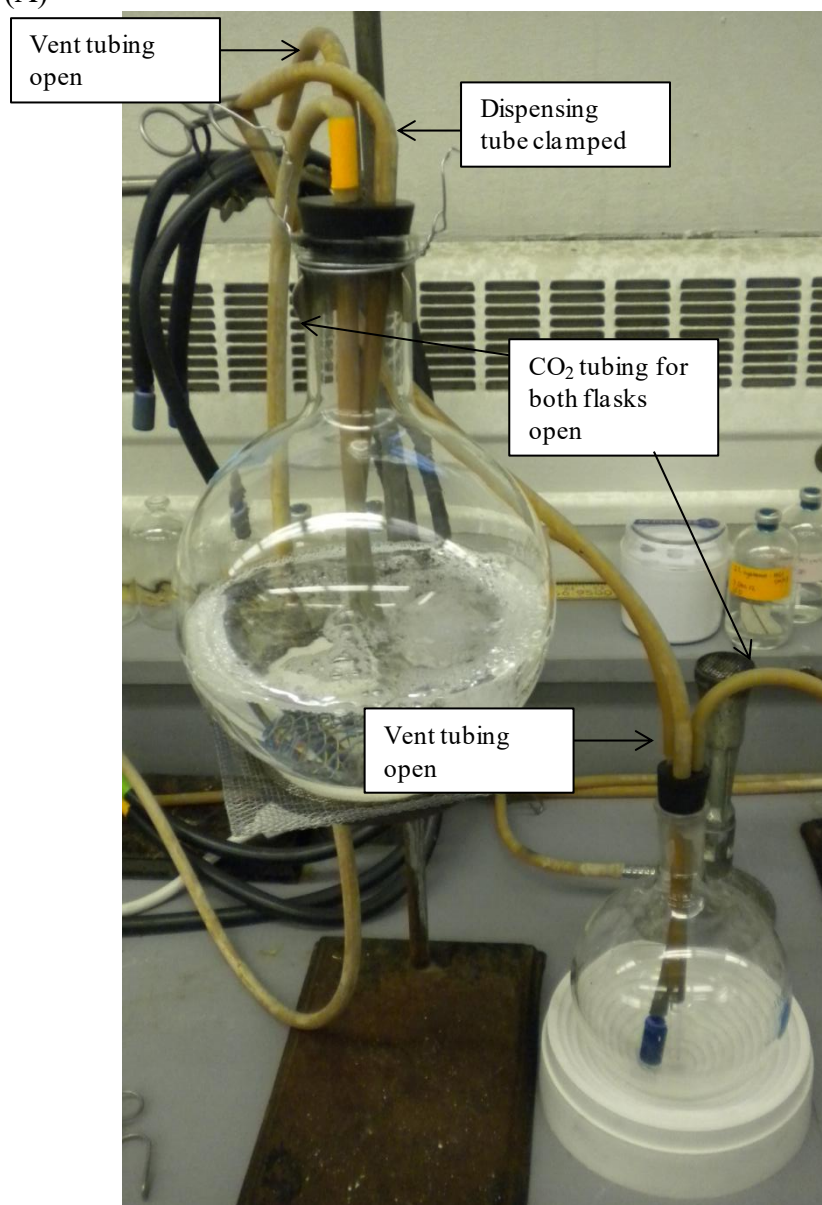


**Fig. 6.** Boiling buffer in 3-L flask.



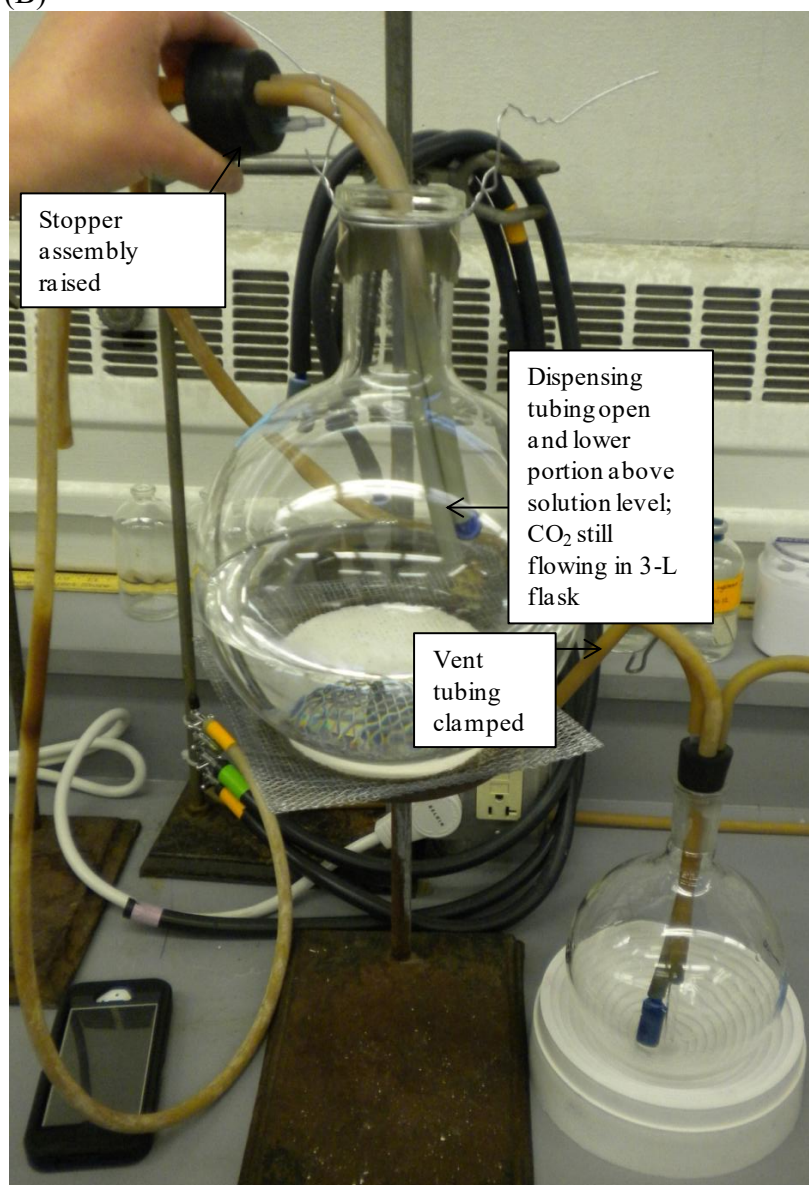
**Fig. 7.** Dispensing buffer into 1-L flask. (A) Flushing 1-L flask. (B) Flushing dispensing tube. (C) Dispensing buffer.

(A)

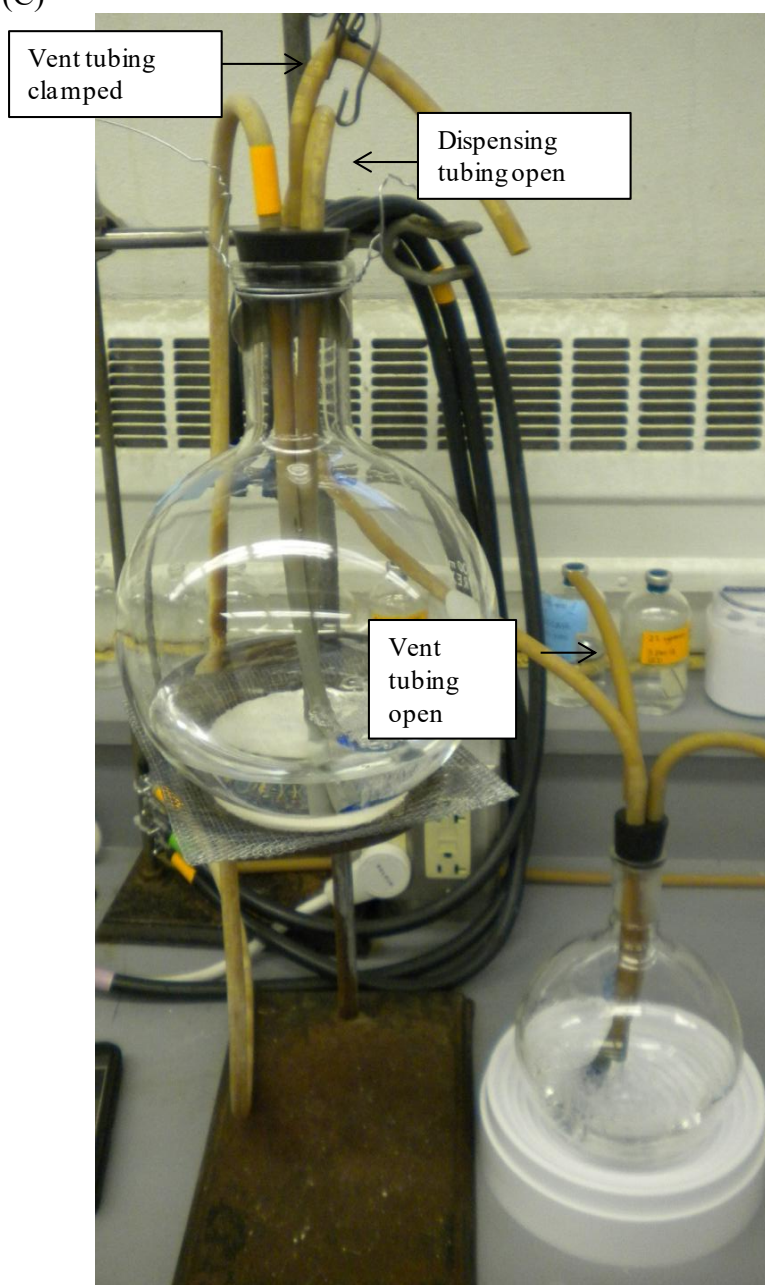




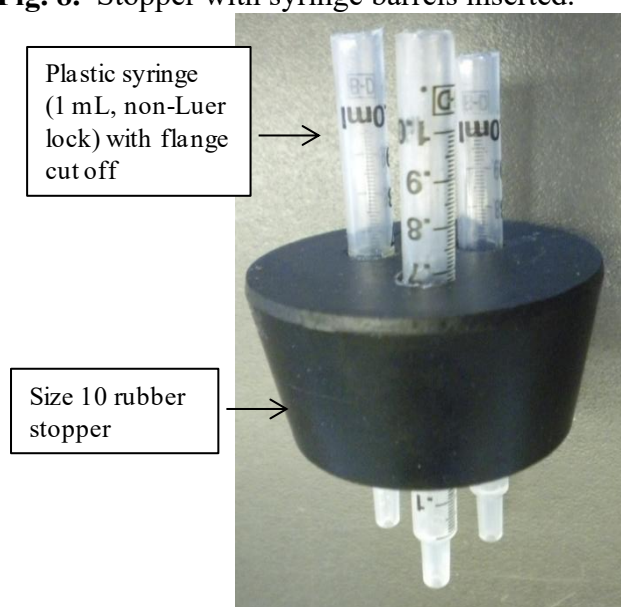
(B)



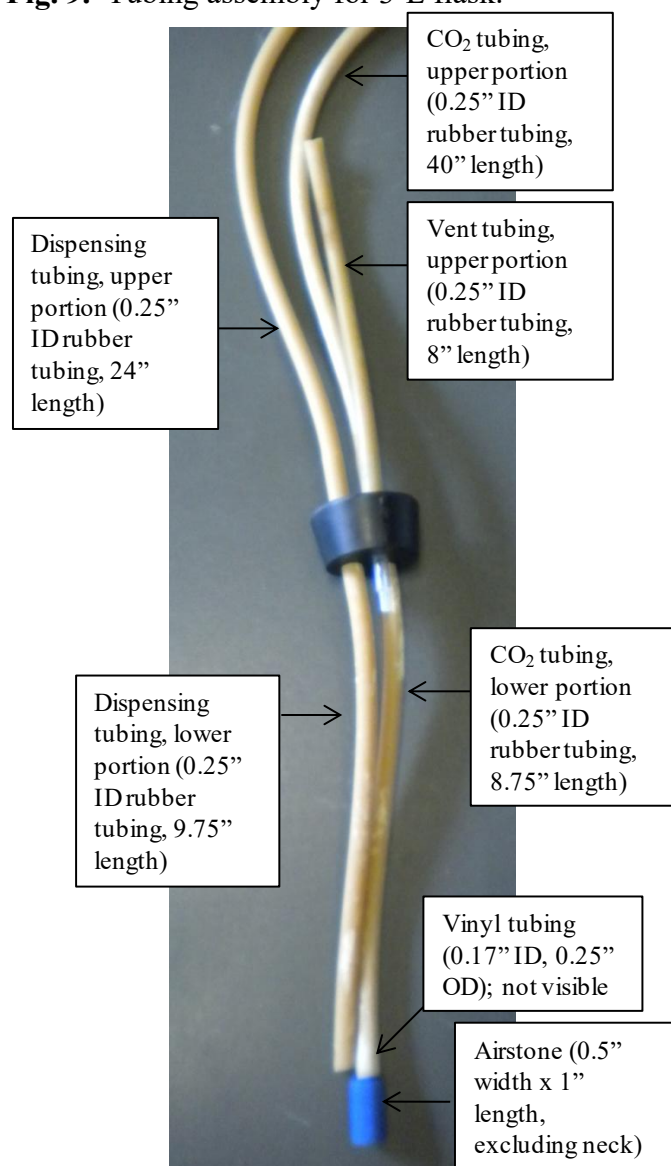
(C)



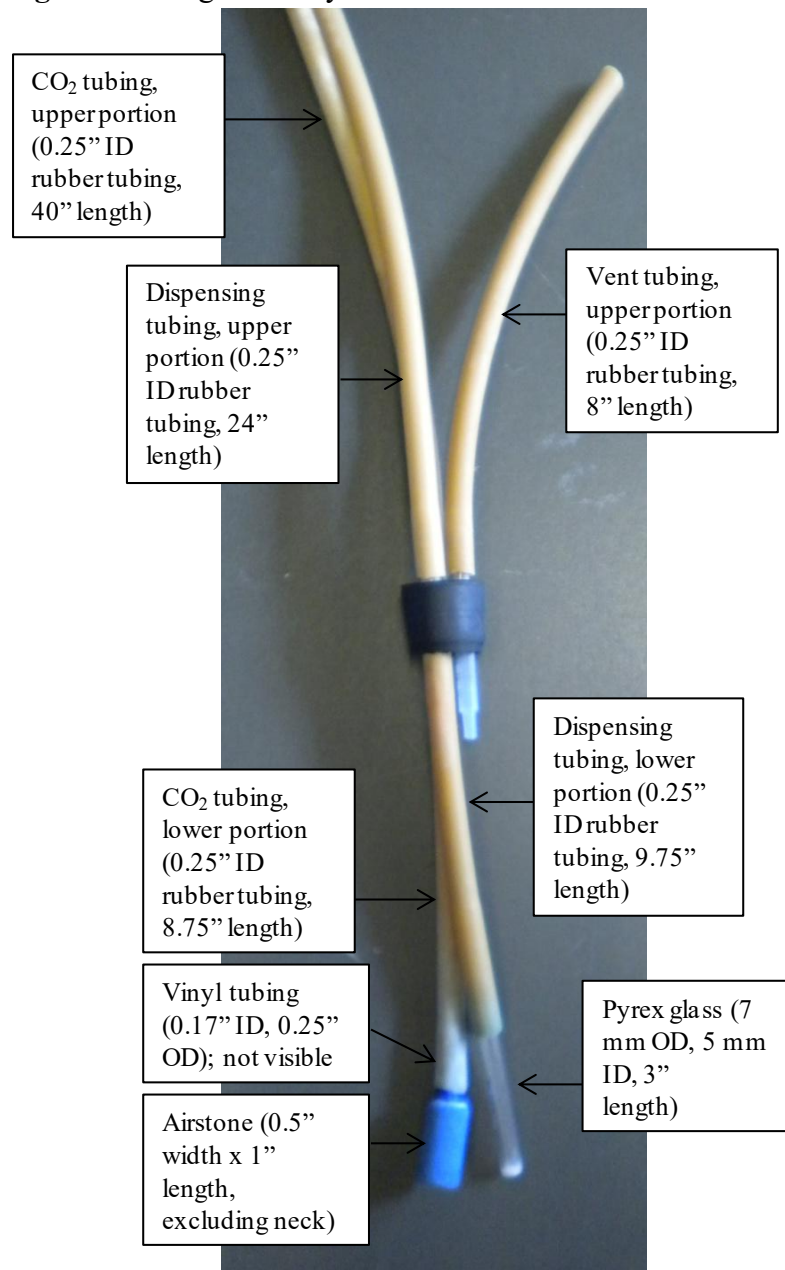
**Fig. 8.** Stopper with syringe barrels inserted.



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

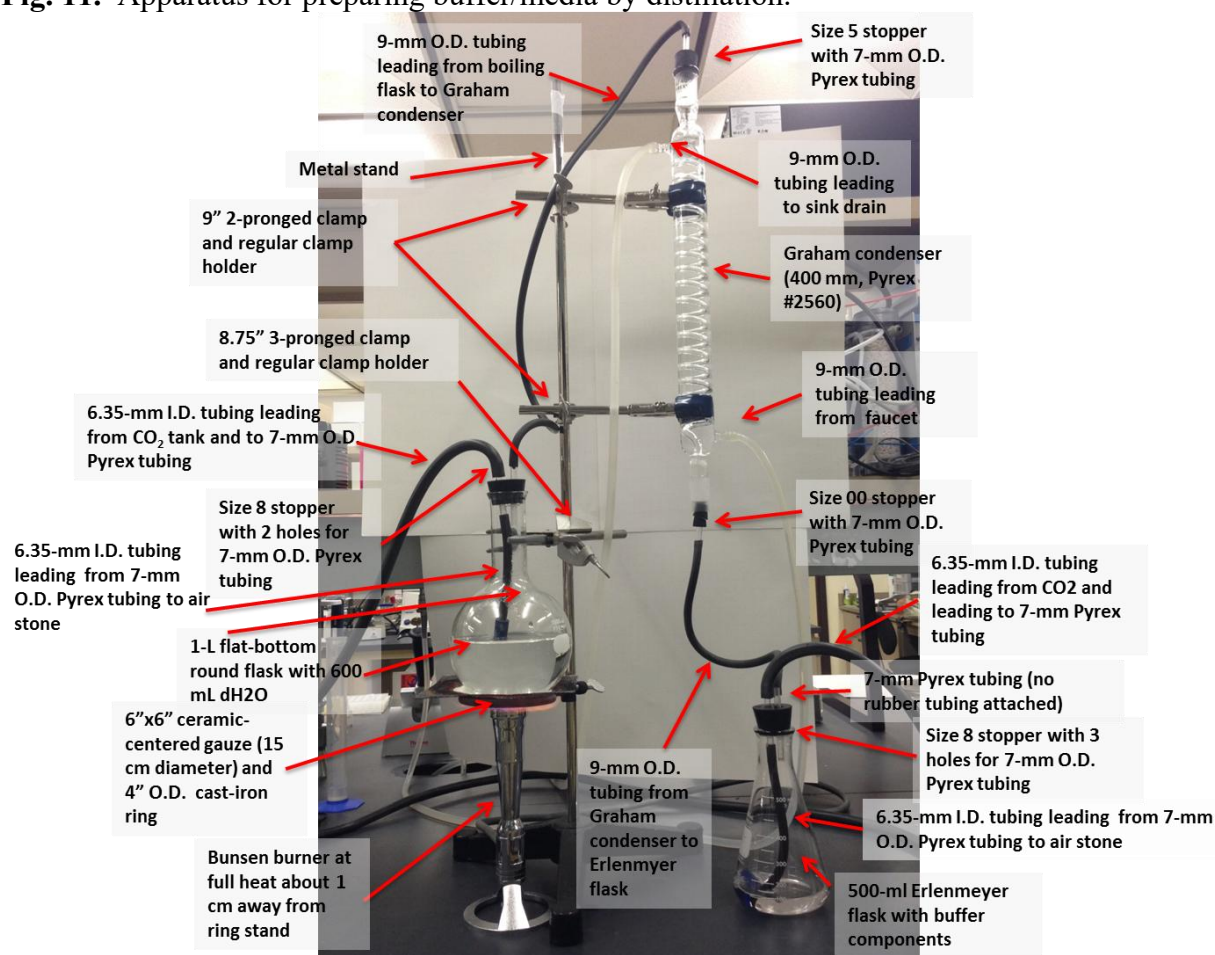


**Fig. 10.** Tubing assembly for 1-L flask.





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



## Buffer and media recipes

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

### 6SG330 media

Adapted from ref. (15)

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

- 350 mL dH<sub>2</sub>O
- 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<sub>2</sub>HPO<sub>4</sub>
- 0.135 g KH<sub>2</sub>PO<sub>4</sub>
- 0.135 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.27 g NaCl
- 0.0563 g MgSO<sub>4</sub>
- 0.036 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na<sub>2</sub>CO<sub>3</sub>

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

- 2.0.1667 g L-cysteine HCl·H<sub>2</sub>O

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 ref. (16)

Add the following ingredients to a 250 mL beaker

- 1.4911 g FeCl<sub>2</sub>·4H<sub>2</sub>O 7.5 mM

0.0618 g  $\text{H}_3\text{BO}_4$  1 mM  
0.0682 g  $\text{ZnCl}_2$  0.5mM  
0.0169 g  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  0.1mM  
0.0989 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.5 mM  
0.1189 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.5mM  
0.0238 g  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  0.1mM  
4.13 mL  $\text{HCl}$  50mM

Dissolve in  $\text{dH}_2\text{O}$  and bring to 1 L using volumetric flask. Transfer to 1 L Pyrex bottle. Store at  $4^\circ\text{C}$  under dark (wrap bottle in aluminum foil).

### **Alkaline trace element solution**

From ref. (16)

Add the following ingredients to a 250 mL beaker

0.0173 g  $\text{Na}_2\text{SeO}_3$  0.1mM  
0.0255 g  $\text{Na}_2\text{WO}_4$  0.1 mM  
0.0206 g  $\text{Na}_2\text{MoO}_4$  0.1 mM  
0.4 g  $\text{NaOH}$  10mM

Dissolve in  $\text{dH}_2\text{O}$  and bring to 1 L using volumetric flask. Transfer to 1 L Pyrex bottle. Store at  $4^\circ\text{C}$  under dark (wrap bottle in aluminum foil).

### **Anaerobic dilution solution (ADS)**

Modified from ref. (3, 17)

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

350 mL  $\text{dH}_2\text{O}$   
0.135 g  $\text{K}_2\text{HPO}_4$   
0.135 g  $\text{KH}_2\text{PO}_4$   
0.27 g  $(\text{NH}_4)_2\text{SO}_4$   
0.27 g  $\text{NaCl}$   
0.027 g  $\text{MgSO}_4$   
0.0358 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$   
3 drops of 0.1% resazurin

Approximately 50 mL  $\text{dH}_2\text{O}$  will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

0.9 g  $\text{Na}_2\text{CO}_3$

Bubble overnight (until color turns pink). Then add

0.1667 g L-cysteine  $\text{HCl} \cdot \text{H}_2\text{O}$

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<sub>2</sub>O instead of 350 mL dH<sub>2</sub>O. 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<sub>2</sub>HPO<sub>4</sub>
- 0.3 g cellobiose
- 0.3 g maltose
- 0.3 g starch
- 3 drops of 0.1% resazurin

Boil under CO<sub>2</sub> 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 ref. (18) and Michael Flythe (personal communication)

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

- 350 mL dH<sub>2</sub>O
- 1.2 g glucose
- 0.0876 g K<sub>2</sub>HPO<sub>4</sub>
- 0.0876 g KH<sub>2</sub>PO<sub>4</sub>
- 0.1440 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.1440 g NaCl
- 0.0147 g MgSO<sub>4</sub>
- 0.0192 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 3 drops of 0.1% resazurin
- 0.3 mL hemin solution
- 3.0 mL Russell vitamin solution

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na<sub>2</sub>CO<sub>3</sub>

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

0.2 g cysteine HCl·H<sub>2</sub>O

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- $\alpha$ -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<sub>2</sub>O

0.3375 g KH<sub>2</sub>PO<sub>4</sub>

0.309 g NaCl

0.309 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

0.03 g MgCl<sub>2</sub>·6H<sub>2</sub>O

0.0225 g CaCl<sub>2</sub>·2H<sub>2</sub>O

0.00943 g MnCl<sub>2</sub> 4H<sub>2</sub>O

0.00686 g FeSO<sub>4</sub> 7 H<sub>2</sub>O

0.00324 g ZnCl<sub>2</sub>

0.00072 g CoCl<sub>2</sub> 6H<sub>2</sub>O

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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g NaCHO<sub>3</sub>

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

12.0.1667 g L-cysteine HCl·H<sub>2</sub>O

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

### **Chen media**

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

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

350 mL dH<sub>2</sub>O

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

0.0876 g K<sub>2</sub>HPO<sub>4</sub>

0.0876 g KH<sub>2</sub>PO<sub>4</sub>

0.144 g Na<sub>2</sub>SO<sub>4</sub>

0.144 g NaCl

0.0146 g MgSO<sub>4</sub>

0.0192 g CaCl<sub>2</sub>·2H<sub>2</sub>O

1.5 mL Pfennigs heavy metal solution

3 mL Russell vitamin solution

3 drops of 0.1% resazurin

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na<sub>2</sub>CO<sub>3</sub>

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 ref. (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 ref. (3)

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

### **3% (w/v) cysteine HCl**

Add ~100 mL dH<sub>2</sub>O to a 1-L round-bottom flask, bubble with CO<sub>2</sub>, and boil for 5 to 7 min. Transfer 75 mL dH<sub>2</sub>O to 160-mL culture bottle that is being flushed with gassing probe. Add

2.507 g L-cysteine HCl·H<sub>2</sub>O

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<sub>2</sub>O to a 1-L round-bottom flask, bubble with CO<sub>2</sub>, and boil for 5 to 7 min. Transfer 75 mL dH<sub>2</sub>O 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<sub>2</sub>). Add after autoclaving medium.

### **Glucose (10% w/v) (anaerobic)**

Add 1 g glucose to a Balch tube. Flush with N<sub>2</sub> for 10 min. While continuing to flush, add 9.25 mL water (anaerobic). Stopper tube and shake to dissolve glucose. Filter sterilize into a sterile Balch tube previously flushed with N<sub>2</sub> and autoclaved.

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

### **Hemin solution**

Adapted from ref. (22, 23)

Add following to 100 mL volumetric flask

0.28 g KOH

25 mL 95% ethanol

0.1 g hemin

Dissolve, then and bring to volume with dH<sub>2</sub>O. 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.

### **Isaacson buffer**

Adapted from ref. (24)

Add the following to a 20-L carboy

- 20 L dH<sub>2</sub>O
- 8 g Trypticase peptone
- 2.51 g of (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>
- 19.464 g K<sub>2</sub>HPO<sub>4</sub>
- 19.33 g KH<sub>2</sub>PO<sub>4</sub>
- 19.192 g NaCl
- 2.588 g CaCl<sub>2</sub>
- 1.95 g MgSO<sub>2</sub>
- 126.82 g NaHCO<sub>3</sub>
- 13.354 g urea
- 200 mL Pfennig heavy metal solution
- 4 L of clarified rumen fluid

Bubble solution overnight with CO<sub>2</sub>. Redox should remain below 0 mV.

For a carboy already containing buffer, new buffer can be added in small increments (5 L/h) without affecting redox.

### **LH media**

Adapted from ref. (25)

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

- 350 mL dH<sub>2</sub>O
- 10 g sodium lactate (60% w/w)
- 6 g trypticase peptone (BD 211921)
- 0.6 g yeast extract (BD 212750)
- 0.135 g K<sub>2</sub>HPO<sub>4</sub>
- 0.135 g KH<sub>2</sub>PO<sub>4</sub>
- 0.27 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.27 g NaCl
- 0.027 g MgSO<sub>4</sub>
- 0.0358 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 1.5 mL Pfennigs heavy metal solution
- 6 mL hemin solution (0.05%)
- 3 mL VFA solution
- 3 drops of 0.1% resazurin

Adjust pH to 6.7 to 6.8 with NaOH

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.911 g NaHCO<sub>3</sub>

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

- 0.1705 g L-cysteine HCl·H<sub>2</sub>O

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

Immediately before inoculation, add 0.205 mL of 2.5%  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  to each tube.

The composition of the media follows ref. (25), except 1.5 mL Pfennings heavy metal solution is added. Ref. (25) 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  $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ ), 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  $\text{MgSO}_4$
- 0.26 g  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$
- 2.0 g  $\text{KH}_2\text{PO}_4$  g/L

Bring to volume with  $\text{dH}_2\text{O}$ .

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

- 150 mL mineral mix M
- 30 mL clarified rumen fluid
- 15 mL 1.5%  $\text{CH}_3\text{COONa}$
- 128 mL  $\text{dH}_2\text{O}$
- 2 drops 0.1% resazurin

Approximately 50 mL  $\text{dH}_2\text{O}$  will be lost during boiling, giving 273 mL final volume. After boiling and cooling, add

- 25 mL of 6%  $\text{NaHCO}_3$

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  $\text{KH}_2\text{PO}_4$
- 4 g NaCl
- 0.212 g  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$
- 0.154  $\text{MgSO}_4$

Bring to volume with  $\text{dH}_2\text{O}$ .

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](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· $H_2O$

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<sub>2</sub>S  
Do not add Na<sub>2</sub>S until tubes are stoppered (to prevent release of toxic H<sub>2</sub>S gas). Autoclave.

### **Modified Methanobacterium medium for Methanobrevibacter ruminantium (DSMZ 1523)**

Adapted from DSMZ  
([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium1523.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium1523.pdf)) and ref. (26)

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 *Methanobrevibacter 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<sub>2</sub>O  
3 g trypticase peptone (BD 211921)  
3 g yeast extract (BD 212750)  
1.5. Glucose  
0.15g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
0.012g K<sub>2</sub>HPO<sub>4</sub>  
0.012g KH<sub>2</sub>PO<sub>4</sub>  
0.0024g MgSO<sub>4</sub>  
0.00318g CaCl<sub>2</sub> 2H<sub>2</sub>O  
0.024g NaCl  
3 drops of 0.1% resazurin  
0.93 ml VFA solution  
3 mL of Vitamin K<sub>3</sub>-Hemin solution

Adjust pH to 6.7 with NaOH  
Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add  
1.2 g NaHCO<sub>3</sub>

After boiling, color is golden. Bubble until the redox reaches approximately -150 mV. Then add  
0.15 g L-cysteine HCl·H<sub>2</sub>O

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

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

- 350 mL dH<sub>2</sub>O
- 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<sub>2</sub>HPO<sub>4</sub>
- 0.012 g KH<sub>2</sub>PO<sub>4</sub>
- 0.00293 g MgSO<sub>4</sub>
- 0.003g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 0.024 g NaCl
- 0.3 mL Tween80
- 3 ml hemin solution (0.05%)
- 0.06 mL vitamin K1 solution
- 3 drops of 0.1% resazurin

Adjust pH to 6.7 with HCl.

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g NaHCO<sub>3</sub>

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

- 0.15 g L-cysteine HCl·H<sub>2</sub>O

Continue bubbling until color turns amber. Dispense and autoclave.

### **Mucin medium for *Akkermansia muciniphila***

From ref. (27)

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

- 350 mL dH<sub>2</sub>O
- 0.12 g KH<sub>2</sub>PO<sub>4</sub>
- 0.159 g Na<sub>2</sub>HPO<sub>4</sub>
- 0.09 g NH<sub>4</sub>Cl
- 0.09 g NaCl
- 0.03 g MgCl<sub>2</sub>·6 H<sub>2</sub>O
- 0.044 g CaCl<sub>2</sub>·2 H<sub>2</sub>O

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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add  
1.2 g NaCHO<sub>3</sub>

After boiling, the color is a turbid pink. Bubble until the redox reaches approximately -150mV. Then add  
0.1667 g L-cysteine HCl·H<sub>2</sub>O

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

Immediately before inoculation, add 0.9 mL of 2.5% Na<sub>2</sub>S·9H<sub>2</sub>O 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<sub>2</sub>O

Attach the flask to a metal stand, and boil under CO<sub>2</sub> for 8 to 10 minutes (until meat browns). Stir contents gently while boiling. Approximately 50 mL dH<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>, transfer approximately 1.4 g of meat particles to a Balch tubes also kept under CO<sub>2</sub>. 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 ref. (26)

Add ~100 mL dH<sub>2</sub>O to a 1-L round-bottom flask, bubble with CO<sub>2</sub>, and boil for 5 to 7 min. Transfer 75 mL dH<sub>2</sub>O 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 ref. (23, 28–30) and Michael Flythe (personal communication)

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

350 mL dH<sub>2</sub>O

1.2 g glucose

0.0876 g K<sub>2</sub>HPO<sub>4</sub>

0.0876 g KH<sub>2</sub>PO<sub>4</sub>

0.1440 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

0.1440 g NaCl

0.0147 g MgSO<sub>4</sub>

0.0192 g CaCl<sub>2</sub>·2H<sub>2</sub>O

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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na<sub>2</sub>CO<sub>3</sub>

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

0.2 g cysteine HCl·H<sub>2</sub>O

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

The composition of the medium follows ref. (28), but this reference did not provide a complete description. Composition of buffer salts is from ref. (28), with carbonate added as in ref. (23, 30). Composition and inclusion rate of vitamins is from ref. (23). Composition of minerals is from ref. (23), and their inclusion rate is the maximum recommended by the Russell “spell book” (M. Flythe, personal communication).

### **Minimal media plus VFA**

Adapted from Minimal media for *Streptococcus bovis*

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

350 mL dH<sub>2</sub>O

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  $HCl \cdot H_2O$

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

### **Mucin purified by ethanol**

Adapted from ref. (27)

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)

Obtain high purity  $Na_2S \cdot 9 H_2O$  ( $\geq 99.99\%$ ; Sigma 431648-50G). Lower purity will not support growth of methanogens, likely due to toxic oxidation products. After opening, store  $Na_2S \cdot 9 H_2O$  under  $N_2$  in Pyrex bottle with stopper.

Flush 160-mL culture bottle with N<sub>2</sub>, then add

1 drop 10% NaOH

75 mL water (anaerobic)

1.875 g Na<sub>2</sub>S·9 H<sub>2</sub>O

Add the NaOH first to make solution basic and loss of H<sub>2</sub>S. Do not flush bottle with CO<sub>2</sub>, which will acidify the solution and increase loss of Na<sub>2</sub>S as H<sub>2</sub>S (toxic).

Immediately seal with bung and aluminum seal (to minimized exposure to any H<sub>2</sub>S), 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<sub>2</sub>O

1.5 g cellobiose

3.0 g trypticase peptone (BD 211921)

0.75 g yeast extract (BD 212750)

0.135 g K<sub>2</sub>HPO<sub>4</sub>

0.135 g KH<sub>2</sub>PO<sub>4</sub>

0.27 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

0.27 g NaCl

0.027 g MgSO<sub>4</sub>

0.027 g CaCl<sub>2</sub>·2H<sub>2</sub>O

3 drops of 0.1% resazurin

45 mL rumen fluid (supernatant from 16,000 g centrifugation)

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.8 g Na<sub>2</sub>CO<sub>3</sub>

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 ref. (18) and Michael Flythe (personal communication)

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

- 350 mL dH<sub>2</sub>O
- 1.2 g glucose
- 0.3 g trypticase peptone (BD 211921)
- 0.15 g yeast extract (BD 212750)
- 0.0876 g K<sub>2</sub>HPO<sub>4</sub>
- 0.0876 g KH<sub>2</sub>PO<sub>4</sub>
- 0.1440 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.1440 g NaCl
- 0.0147 g MgSO<sub>4</sub>
- 0.0192 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 3 drops of 0.1% resazurin

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na<sub>2</sub>CO<sub>3</sub>

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

- 0.2 g cysteine HCl·H<sub>2</sub>O

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

### **PC plus VFA media**

Adapted from ref. (18)

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

- 350 mL dH<sub>2</sub>O
- 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<sub>2</sub>HPO<sub>4</sub>
- 0.0876 g KH<sub>2</sub>PO<sub>4</sub>
- 0.1440 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.1440 g NaCl
- 0.0147 g MgSO<sub>4</sub>
- 0.0192 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 0.93 mL VFA solution
- 3 drops of 0.1% resazurin

Adjust pH to 6.7 with NaOH

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

1.2 g Na<sub>2</sub>CO<sub>3</sub>

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

0.2 g cysteine HCl·H<sub>2</sub>O

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

### **Pfennigs heavy metal solution**

Adapted from ref. (33)

Add the following 1-L volumetric flask

0.5 g EDTA disodium salt

0.2 g FeSO<sub>4</sub>·7 H<sub>2</sub>O

0.2 g MnCl<sub>2</sub>·4 H<sub>2</sub>O

0.01 g ZnSO<sub>4</sub>·7 H<sub>2</sub>O

0.03 g H<sub>3</sub>BO<sub>4</sub>

0.02 g CoCl<sub>2</sub>·6 H<sub>2</sub>O

0.001 g CuCl<sub>2</sub>·2 H<sub>2</sub>O

0.002 g NiCl<sub>2</sub>·6 H<sub>2</sub>O

0.003 g NaMoO<sub>4</sub>·2 H<sub>2</sub>O

Bring to 1 L with dH<sub>2</sub>O. Store at 4°C.

### **Phosphate buffer for phophotransferase assays**

Adapted from ref. (28, 34, 35)

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

3.054 KH<sub>2</sub>PO<sub>4</sub>

1.155 Na<sub>2</sub>HPO<sub>4</sub>

0.0305 g MgCl<sub>2</sub>·6H<sub>2</sub>O

3 drops of 0.1% resazurin

Adjust pH to 7.2.

After boiling and cooling, bubble until color turns pink. Approximately 50 mL dH<sub>2</sub>O 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 ref. (36)

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

- 350 mL dH<sub>2</sub>O
- 3g trypticase peptone (BD211921)
- 3g Yeast extract (BD 212750)
- 0.15g K<sub>2</sub>HPO<sub>4</sub>
- 0.15g KH<sub>2</sub>PO<sub>4</sub>
- 0.3g NaCl
- 0.15g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.015g MgSO<sub>4</sub>
- 0.03gCaCl<sub>2</sub>·2H<sub>2</sub>O
- 0.6ml glycerol (0.2%)
- 3 drops of 0.1% resazurin

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1 g NaHCO<sub>3</sub>

After boiling, color is orange. Continue bubbling until stops decreasing and stabilizes. Then add  
0.1667 g L-cysteine HCl·H<sub>2</sub>O

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

### **Reinforced Clostridial Medium (RCM)**

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

- 350 mL dH<sub>2</sub>O
- 3 g peptone (BD 211677)
- 0.9 g yeast extract (BD 212750)
- 3 g beef extract (LP0029)
- 1.5 g glucose
- 0.3 g Starch
- 1.5 g NaCl
- 0.9 g Sodium Acetate (NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)

Adjust pH to 6.8 with NaOH.

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume.

After boiling and cooling, continue bubbling until redox no longer decreases (requires 1 h) and add

- 0.15 g L-cysteine HCl·H<sub>2</sub>O

Continue bubbling until redox no longer decreases (requires 1 h or more). Dispense and autoclave.

### **Rumen bacteria medium (DSMZ 330)**



From DSMZ ([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium330.pdf](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium330.pdf))

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

- 350 mL dH<sub>2</sub>O
- 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<sub>2</sub>HPO<sub>4</sub>
- 0.0684 g KH<sub>2</sub>PO<sub>4</sub>
- 0.0684 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.1368 g NaCl
- 0.0139 g MgSO<sub>4</sub>
- 0.0182 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na<sub>2</sub>CO<sub>3</sub>

After boiling, color is amber. Bubble until redox stops decreasing and stabilizes. Then add  
2.0.1667 g L-cysteine HCl·H<sub>2</sub>O

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

- 0.09 mL 2.5% Na<sub>2</sub>S·9H<sub>2</sub>O

to individual tubes.

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

From ref. (3)

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

- 270 mL dH<sub>2</sub>O
- 0.45 g glucose
- 0.45 g cellobiose
- 0.15 soluble starch
- 0.1349 g K<sub>2</sub>HPO<sub>4</sub>
- 0.1349 g KH<sub>2</sub>PO<sub>4</sub>

0.2697 NaCl  
0.2697 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>  
0.02697 MgSO<sub>4</sub>  
0.0357 g CaCl<sub>2</sub>·2H<sub>2</sub>O  
3 drops of 0.1% resazurin.

120 mL rumen fluid (supernatant from 1000 g centrifugation)

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

0.9 g NaCHO<sub>3</sub>

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

0.1667 g L-cysteine HCl·H<sub>2</sub>O

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 ref. (23) and Micheal Flythe (personal communication)

Add following to 1-L Pyrex bottle

0.1 g pyridoxamine dihydrochloride (stored at -20°C) (Fisher 436250010)  
0.1 g pyridoxal hydrochloride (stored at 4°C) (Fisher 352710010)  
0.1 g pyridoxine HCl (Sigma P6280-10G)  
0.2 g riboflavin (Sigma R4500-25G)  
0.2 g thiamine HCl (Sigma T1270-100G)  
0.2 g nicotinamide (Sigm 72340-250G)  
0.2 g calcium pantothenate (Sigma 21210-100G-F)  
0.1 g lipoic acid (stored at 4°C) (Fisher 138720050)  
0.01 g 4-aminobenzoic acid (Sigma 100536-250G)  
0.005 g folic acid (stored at 4°C) (Fisher 216630100)  
0.005 g biotin (stored at 4°C) (Fisher BP232-1)  
0.005 g coenzyme B<sub>12</sub> (stored at 4°C) (Sigma V6629-1G)

Prepare 1 L 0.1 M KH<sub>2</sub>PO<sub>4</sub> (pH = 6). Add 1.1994 g K<sub>2</sub>HPO<sub>4</sub> and 11.8123 g KH<sub>2</sub>PO<sub>4</sub> to a 1 L volumetric flask and bring to volume with dH<sub>2</sub>O.

To the Pyrex bottle containing the vitamins, add the 1 L 0.1 M KH<sub>2</sub>PO<sub>4</sub> (pH = 6) and a stir bar. Mix by stirring (solution will not completely dissolve). Store at 4°C and protected from light

(wrap Pyrex storage bottle in aluminum foil). Stir before use. Solution is stable no longer than 1 yr.

### **Schaefer's vitamin solution + THF**

Adapted from Paul Weimer (personal communication)

Add following to 1-L Pyrex bottle

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

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

### **Selenomonas ruminantium medium (DSMZ 181)**

Adapted from DSMZ

([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium181.pdf](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium181.pdf)).

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

- 350 mL dH<sub>2</sub>O
- 1.5 g Trypticase
- 0.6 g Yeast extract
- 0.3 g glucose
- 0.3 g KH<sub>2</sub>PO<sub>4</sub>
- 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<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 1.2 g Na<sub>2</sub>CO<sub>3</sub>

Bubble until color changes. Then add

- 0.1667 g L-cysteine HCl·H<sub>2</sub>O

Continue bubbling until colorless. Dispense and autoclave

### **Selenite-tungstate solution**

Adapted from DSMZ

([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium385.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium385.pdf))

Add following to a 1-L Pyrex bottle

0.5 g NaOH

0.0019 g Na<sub>2</sub>SeO<sub>3</sub>

0.0040 g Na<sub>2</sub>WO<sub>4</sub>·2 H<sub>2</sub>O

Dissolve by gentle swirling. Store at 4°C.

### **Seven vitamins solution**

Adapted from DSMZ

([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium503.pdf](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<sub>2</sub>O

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 ref. (37)

### **Small volumes**

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

350 mL dH<sub>2</sub>O

1.905 g K<sub>2</sub>HPO<sub>4</sub>

1.5 g KH<sub>2</sub>PO<sub>4</sub>

0.195 NaCl

0.027 MgSO<sub>4</sub>·7H<sub>2</sub>O

0.0045 g CaCl<sub>2</sub>·2H<sub>2</sub>O

3 drops of 0.1% resazurin.

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

2.25 g NaCHO<sub>3</sub>

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).

Ref. (37) calls 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 ( $\text{K}_2\text{HPO}_4$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{NaCl}$ ,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) is also higher than that in ref. (37).

### **Large volumes**

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

- 2.1 L  $\text{dH}_2\text{O}$
- 11.430 g  $\text{K}_2\text{HPO}_4$
- 9.0 g  $\text{KH}_2\text{PO}_4$
- 1.170 g  $\text{NaCl}$
- 0.162 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- 0.027 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- 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  $\text{NaCHO}_3$

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).

### **Weller and Pilgrim buffer**

Adapted from ref. (38)

Add the following to a 20-L carboy:

- 20 L  $\text{dH}_2\text{O}$
- 32.25 g  $\text{Na}_2\text{HPO}_4$
- 100 g  $\text{NaHCO}_3$
- 12 g  $\text{KCl}$
- 32 g  $\text{KHCO}_3$
- 8 g urea

Bubble solution overnight with  $\text{CO}_2$ . Redox should remain below 0 mV.

For a carboy already containing buffer, new buffer can be added in small increments (5 L/h) without affecting redox.

## **Titanium (III) nitrilotriacetate**

Adapted from ref. (39)

Add ~500 mL dH<sub>2</sub>O to a 1-L round-bottom flask, bubble with N<sub>2</sub>, and boil for 5 to 7 min.

Prepare saturated Na<sub>2</sub>CO<sub>3</sub> by adding approximately

20 g Na<sub>2</sub>CO<sub>3</sub>

to 160-mL culture bottle flushed with N<sub>2</sub> gas from a gassing probe. Transfer 75 mL of boiled dH<sub>2</sub>O. Seal with rubber bung and aluminum cap. Autoclave.

Transfer 45 mL dH<sub>2</sub>O to a 125-mL Erlenmeyer flask flushed with N<sub>2</sub> 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<sub>3</sub>

Keep pH above 2 by adding TiCl<sub>3</sub> slowly and alternating with saturated Na<sub>2</sub>CO<sub>3</sub> (otherwise precipitate will form). Adjust to pH = 7 with saturated Na<sub>2</sub>CO<sub>3</sub>. Approximately 3.8 mL Na<sub>2</sub>CO<sub>3</sub> will be used in total. Solution begins as green, but it will turn violet-blue as TiCl<sub>3</sub> is progressively added.

Adjust to 75 mL volume with boiled dH<sub>2</sub>O. Filter through 0.2 µm membrane into 160-mL culture bottle (previously autoclaved and flushed with N<sub>2</sub>). 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](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf))

Add following to 500 mL beaker

0.07 g ZnCl<sub>2</sub>

0.1 g MnCl<sub>2</sub>·4H<sub>2</sub>O

0.006 g HBO<sub>3</sub>

0.19 g CoCl<sub>2</sub>·6H<sub>2</sub>O

0.002 g CuCl<sub>2</sub>·2H<sub>2</sub>O

0.024 g NiCl<sub>2</sub>·6H<sub>2</sub>O

0.036 g Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O

Some components stick to weigh paper and must be washed into the beaker with a small volume of dH<sub>2</sub>O.

Add the following ingredients to another 500 mL beaker

10 mL 7.7 M HCl

1.5 g FeCl<sub>2</sub>·4H<sub>2</sub>O

Dissolve FeCl<sub>2</sub> completely by gently swirling.

Combine contents of the two beakers, dilute in ~250 mL dH<sub>2</sub>O 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](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium159.pdf))

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

- 260 mL dH<sub>2</sub>O
- 90 mL rumen fluid, clarified
- 2 g glucose
- 0.135 g K<sub>2</sub>HPO<sub>4</sub>
- 0.135 g KH<sub>2</sub>PO<sub>4</sub>
- 0.27 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>
- 0.27 g NaCl
- 0.026 g MgSO<sub>4</sub>
- 0.036 g CaCl<sub>2</sub>·2H<sub>2</sub>O
- 3 drops of 0.1% resazurin

Adjust pH to 7.0 with NaOH

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

- 5 g NaHCO<sub>3</sub>

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

### ***Treponema ruminis* medium**

Adapted from DSMZ

([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium212a.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium212a.pdf))

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

- 335 mL dH<sub>2</sub>O
- 1.2 g glucose
- 0.3 g cellobiose
- 0.3 g maltose
- 0.3 g soluble starch
- 0.15 g sodium pyruvate
- 0.3 g trypticase peptone (BD 211921)
- 0.15 g K<sub>2</sub>HPO<sub>4</sub>

0.12 g NH<sub>4</sub>Cl  
0.84 g Na<sub>2</sub>SO<sub>4</sub>  
0.12 g NaCl  
0.099 g MgCl<sub>2</sub>·6 H<sub>2</sub>O  
0.015 g CaCl<sub>2</sub>·2 H<sub>2</sub>O  
0.3 mL trace element solution SL-10  
0.3 mL selenite-tungstate solution  
0.3 mL seven vitamins solution  
0.93 ml VFA solution  
15 mL clarified rumen fluid

Adjust pH to 7.0 with NaOH

Approximately 50 mL dH<sub>2</sub>O will be lost during boiling, giving 300-mL final volume. After boiling and cooling, slowly add

0.45 g Na<sub>2</sub>CO<sub>3</sub>

Continue bubbling until redox no longer decreases. Then add

0.12 g dithiothreitol

Continue bubbling until redox no longer decreases. 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<sub>3</sub>- Hemin solution**

Adapted from ATCC

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

#### **Part A**

100 mg menadione (vitamin K<sub>3</sub>)

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 ddH<sub>2</sub>O. 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 ref. (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<sub>12</sub> (stored at 4°C)
- 0.1 g 4-aminobenzoic acid
- 0.1 g pantothenic acid

Dissolve in dH<sub>2</sub>O 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 ref. (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- $\alpha$ -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**

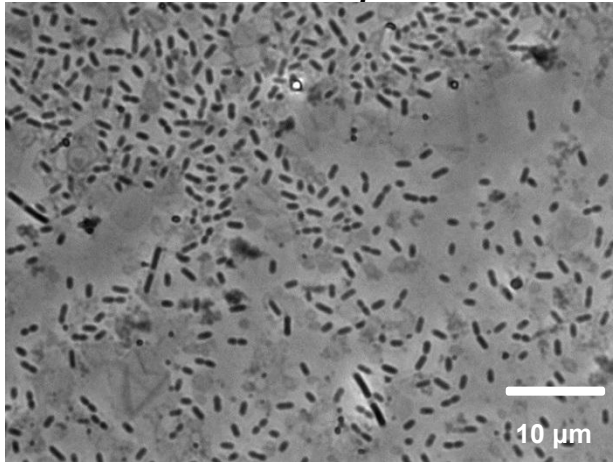
Strain	Medium <sup>1</sup>
<i>Akkermansia muciniphila</i> BAA-835	Mucin medium > PYG (Anaerobe Systems)
<i>Anaerovibrio lipolytica</i> 5S	Prins semi-defined medium > PC + VFA + glycerol
<i>Butyrivibrio fibrisolvens</i> D1	PC+VFA = PC – VFA = minimal media + VFA > rumen bacteria medium
<i>Butyrivibrio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

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

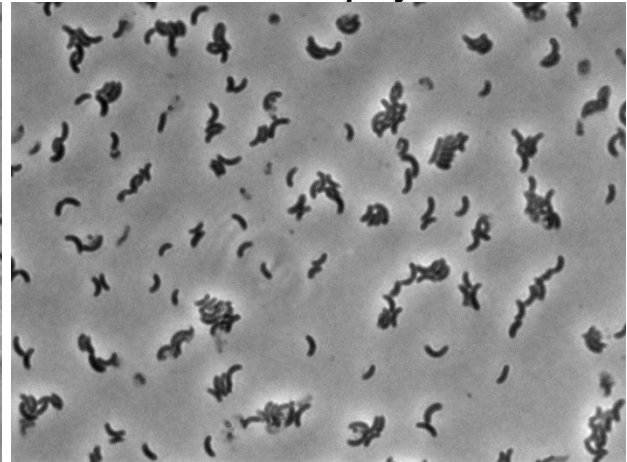
<sup>2</sup>These media should be avoided for long-term maintenance because they may cause biosynthetic mutants (loss of the wild-type function)

Images of pure cultures of bacteria and methanogens

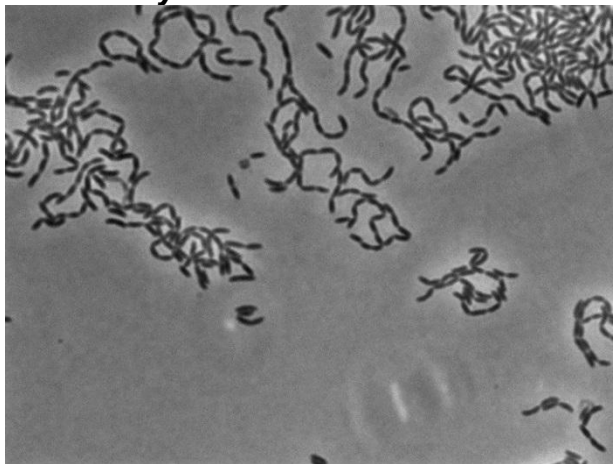
*Akkermansia muciniphila* BAA-835



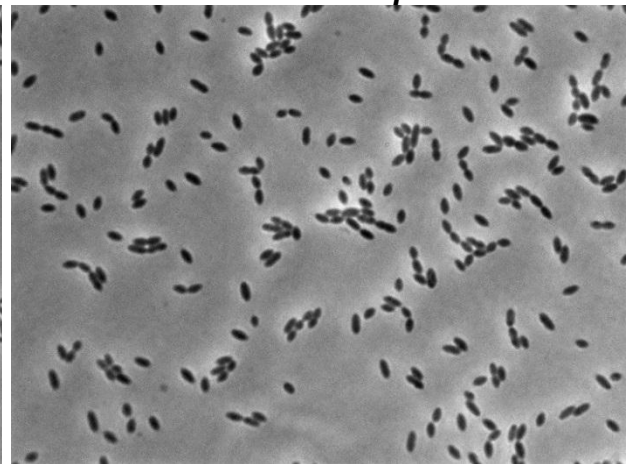
*Anaerovibrio lipolytica* 5S



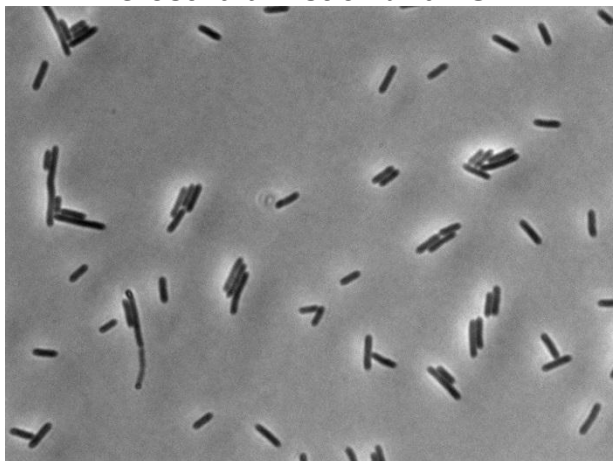
*Butyrivibrio fibrisolvens* D1



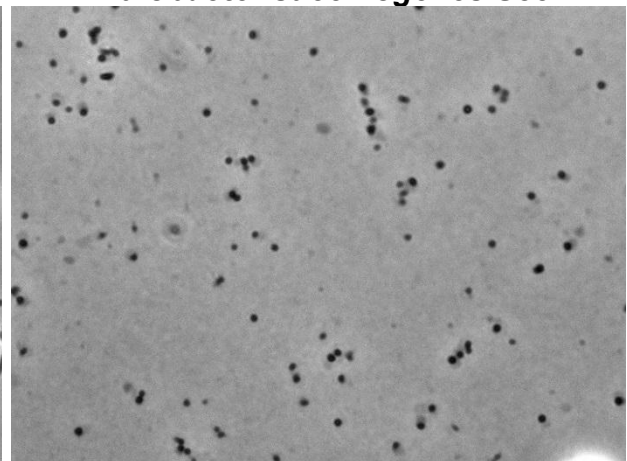
*Clostridium aminophilum* F



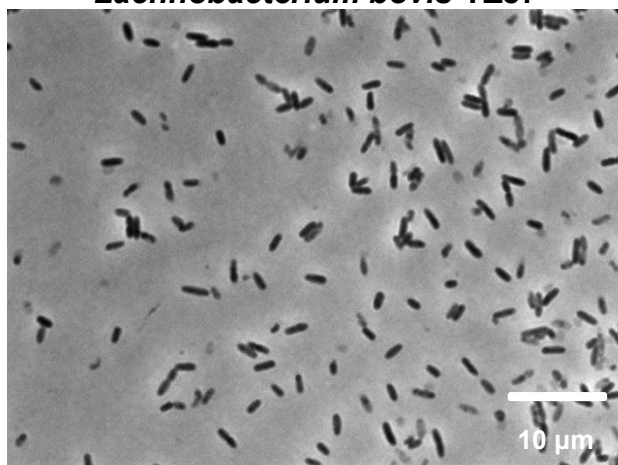
*Clostridium sticklandii* SR



*Fibrobacter succinogenes* S85



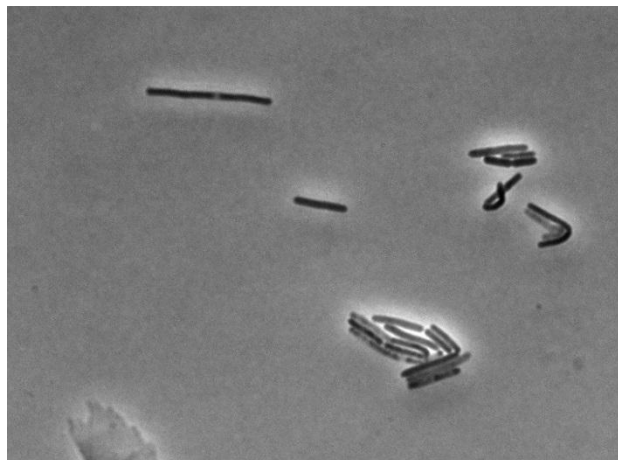
***Lachnobacterium bovis* YZ87**



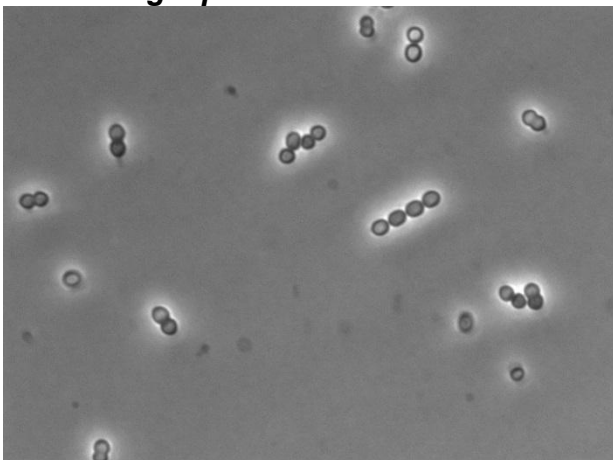
***Lachnospira multipara* D32**



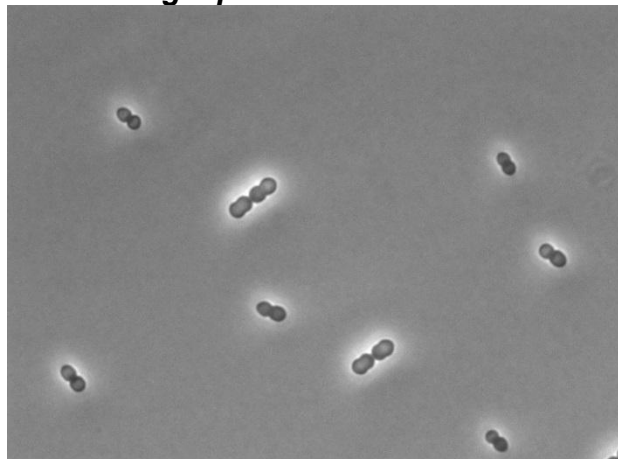
***Lactobacillus ruminis* RF1**



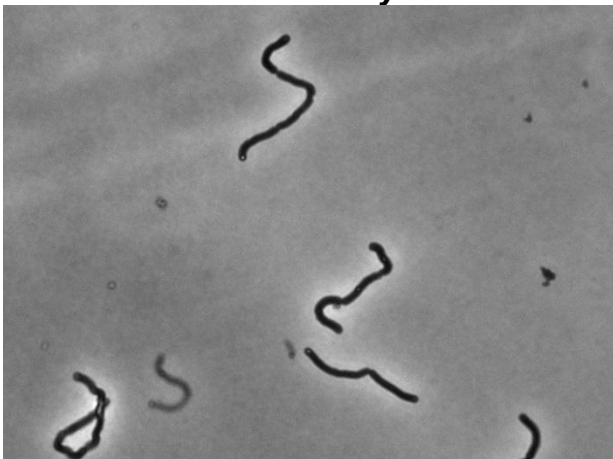
***Megasphaera elsdenii* B159**



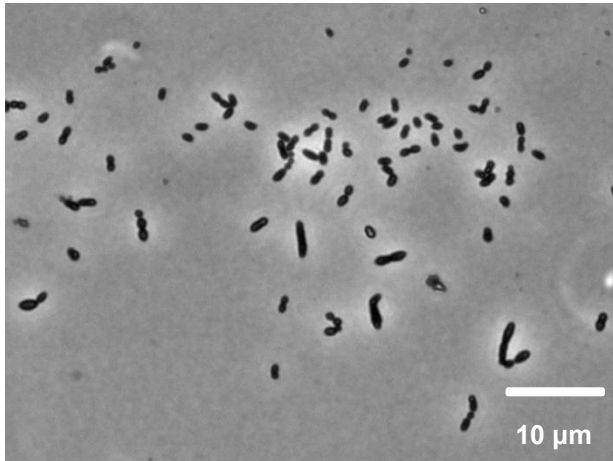
***Megasphaera elsdenii* T81**



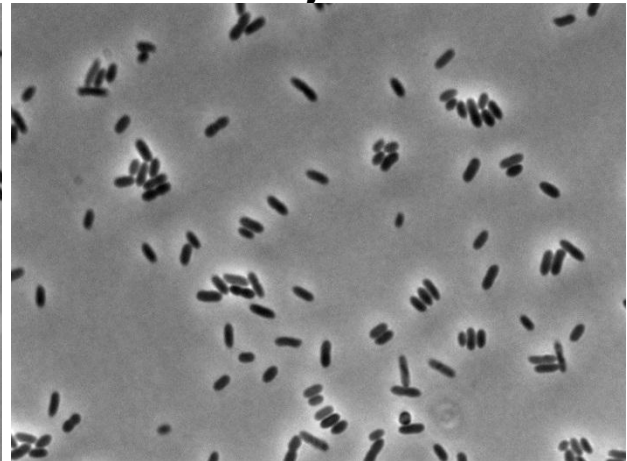
***Methanobacterium bryantii* M.o.H.**



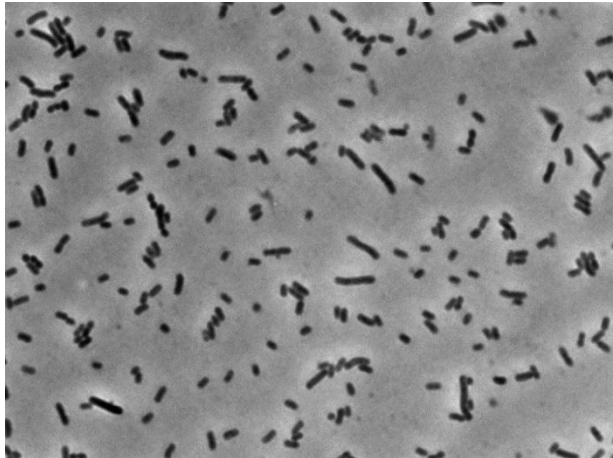
***Methanobrevibacter ruminantium* M1**



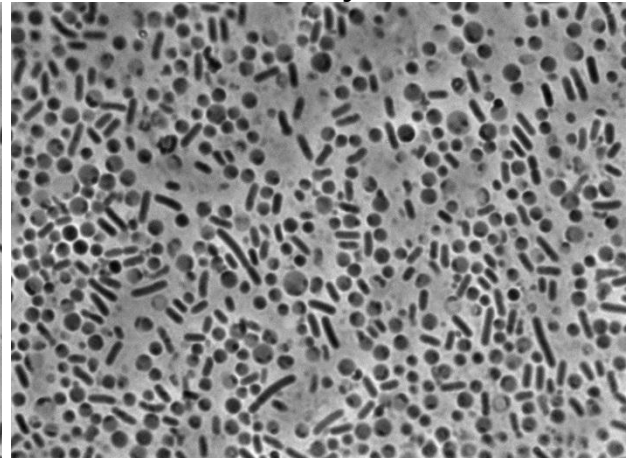
***Mitsuokella jalaludinii* M9**



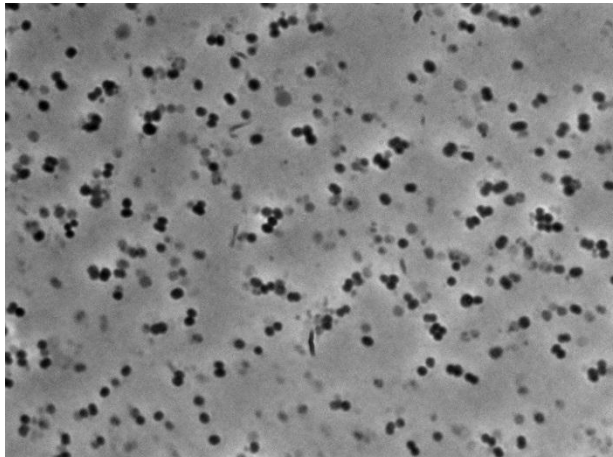
***Prevotella albensis* M384**



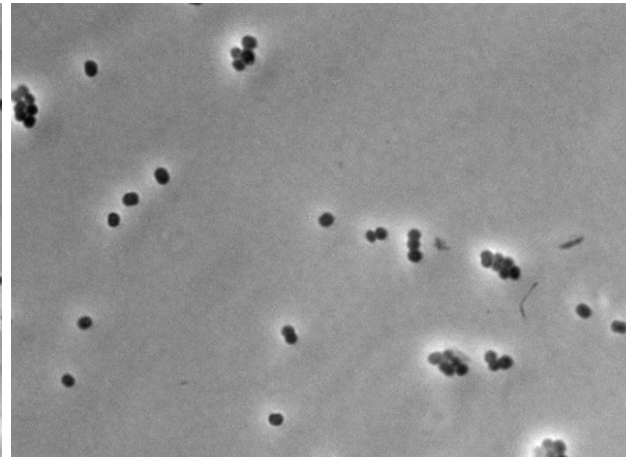
***Prevotella bryantii* B<sub>14</sub>**



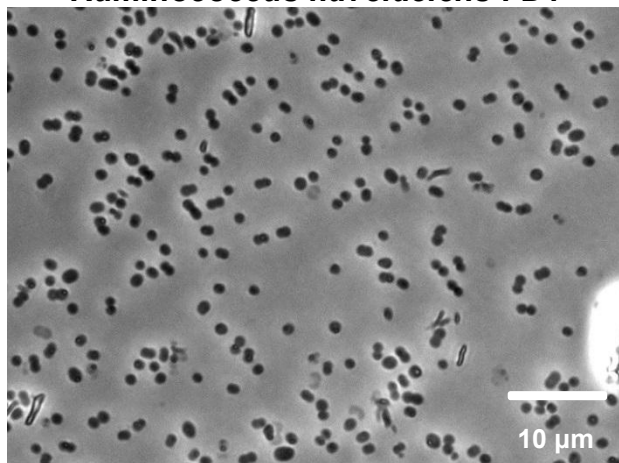
***Ruminococcus albus* 7**



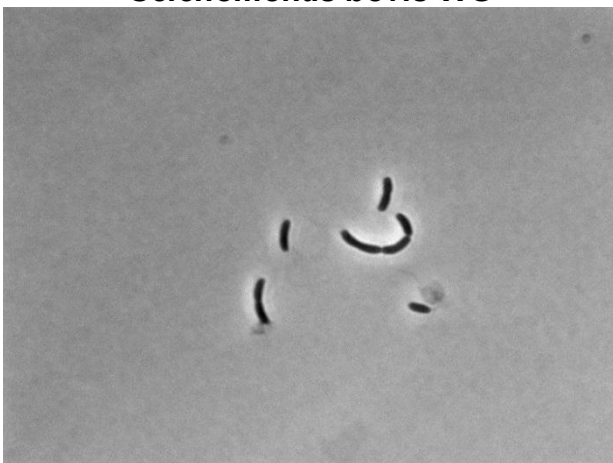
***Ruminococcus albus* 8**



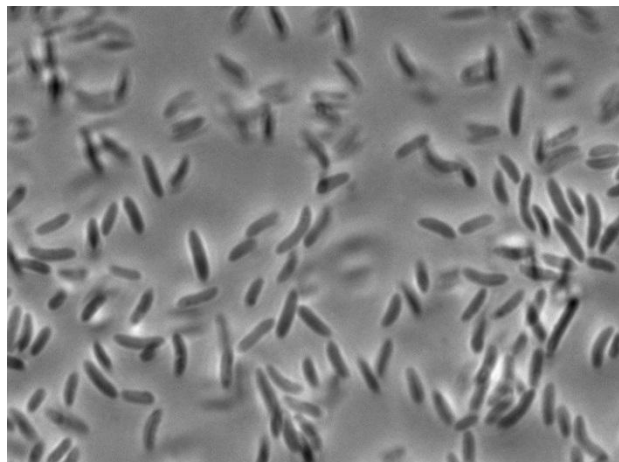
***Ruminococcus flavefaciens* FD1**



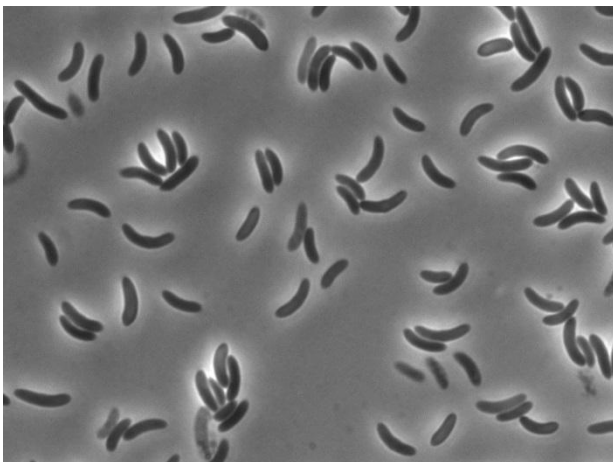
***Selenomonas bovis* WG**



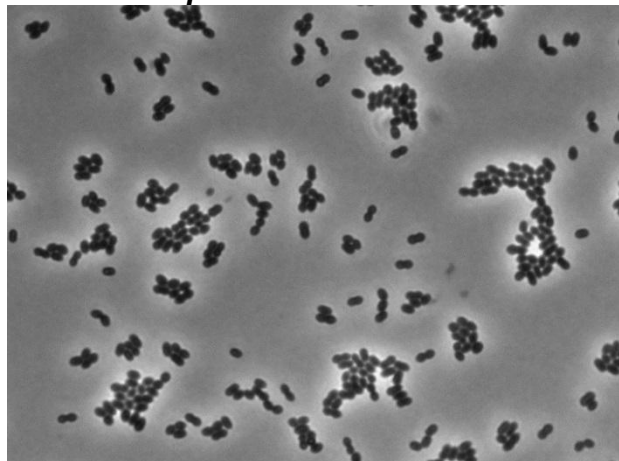
***Selenomonas ruminantium* GA-192**



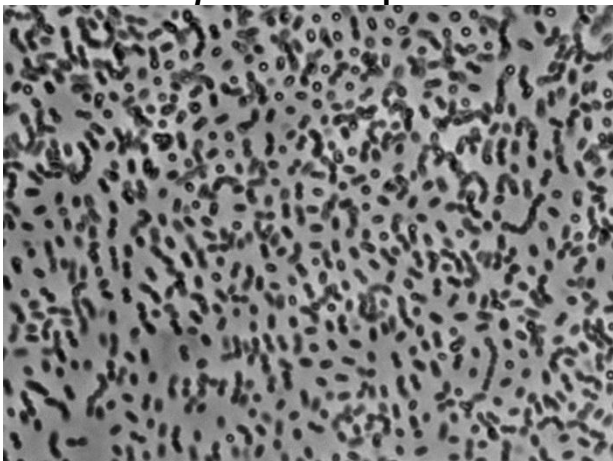
***Selenomonas ruminantium* HD4**



***Streptococcus bovis* JB1**

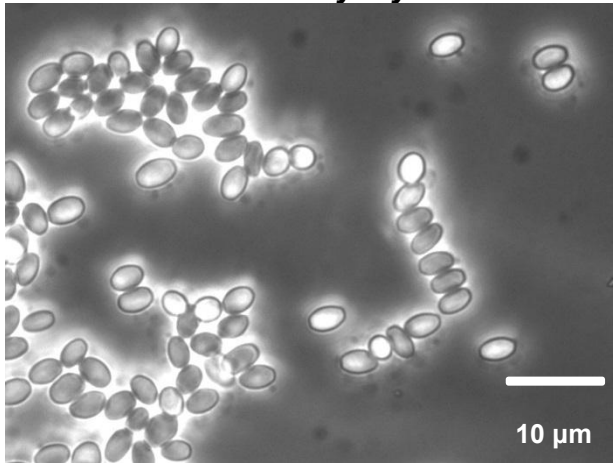


***Streptococcus* sp. C1A1**

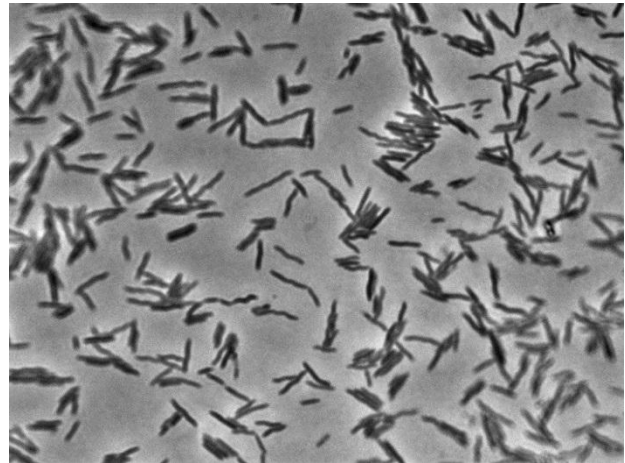




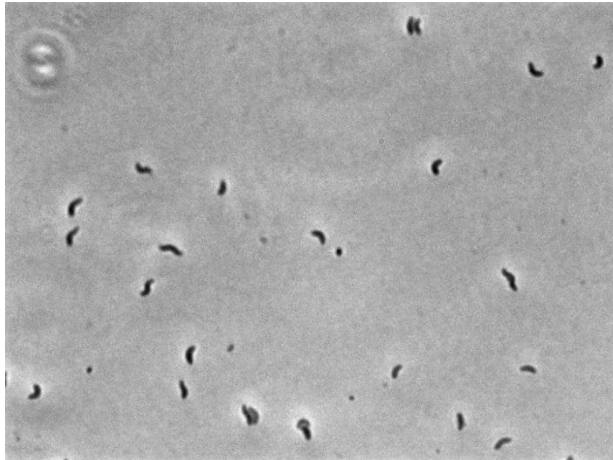
***Succinimonas amylolytica* 13846**



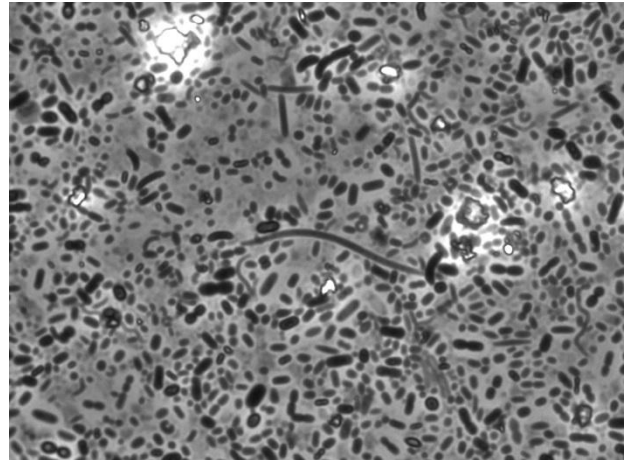
***Succinivibrio dextrinosolvens* 24**



***Succinivibrio dextrinosolvens* 226**



**Mixed rumen bacteria**



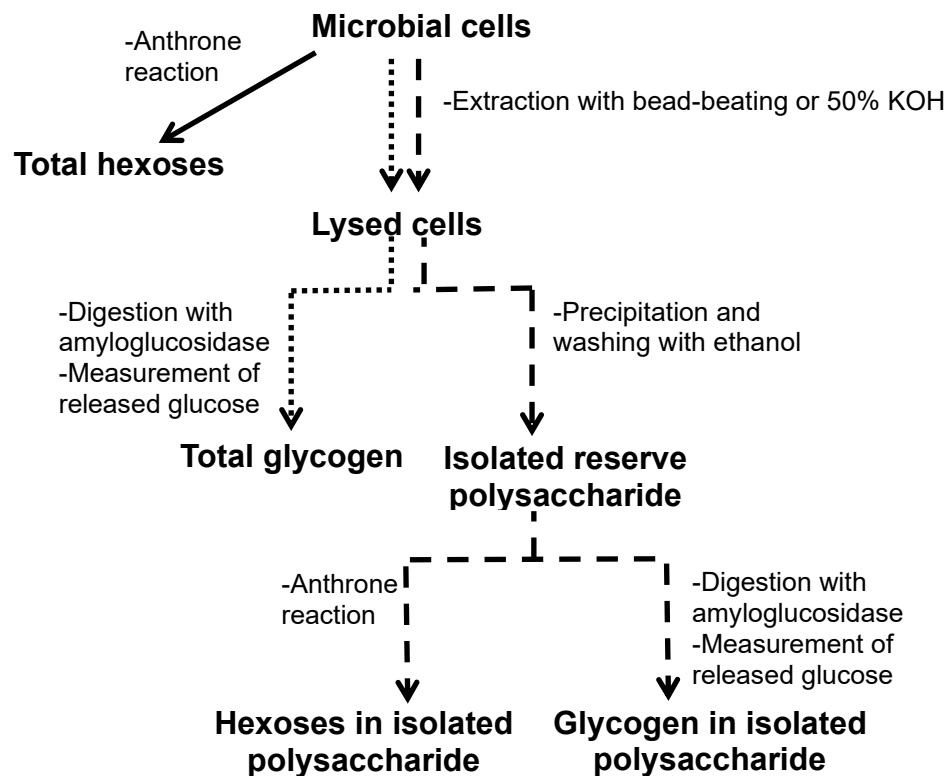
## **SECTION II: ANALYSIS OF MICROBIAL SAMPLES**

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

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



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



**Procedure in manual**

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

**Table 1.** Comparison of methods to measure glycogen.

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

## Preparing cells and culture supernatant for chemical analysis

### Collecting cells and supernatant

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

2.) Store at  $-20^{\circ}\text{C}$ .

### Resuspending cell pellet with dH<sub>2</sub>O

1.) Immediately prior to chemical analysis, resuspend cell pellets to 1 mL final volume using dH<sub>2</sub>O. With micropipette, add dH<sub>2</sub>O to the 1 mL graduation on centrifuge tube. Less than 1 mL dH<sub>2</sub>O should be added because the pellet occupies some volume.

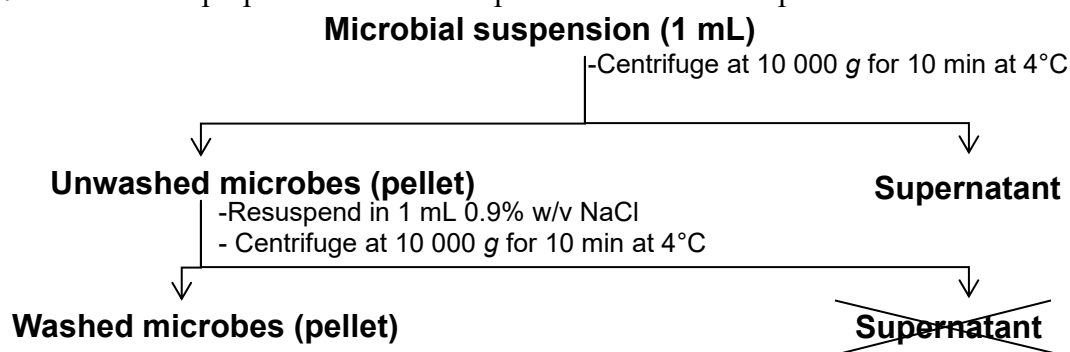
2.) After resuspension, immediately boil for 15 min to inactivate degradative enzymes.

### 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<sub>2</sub>O is added during resuspension of cell pellet. This requires the weighing the empty tube (i.e., before preparing cell pellet). The increase in mass should be 1 g (assuming density of resuspended cell pellet is 1 g/L).

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



### Wet, dry, and organic matter content of microbial cells

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

## Protein content of microbial cells

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

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

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

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

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

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

3.) Prepare standards in 2 mL tubes using 2 000 µg/mL BSA stock from Pierce BCA assay kit according to the following:

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

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

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

4.) Dilute samples and standards 1:5 using dH<sub>2</sub>O 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  $\mu$ L sample or standard in triplicate. Then add 200  $\mu$ L working reagent to each well; an 8-chambered pipette is helpful.

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

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

10.) Calculate protein concentration. For diluted standards, protein concentrations are ( $\mu$ g/mL) 360 for A, 270 for B, 180 for C, 135 for D, 90 for E, 45 for F, 22.5 for G, 4.5 for H, and 0 for I. For samples, make sure to multiply concentration by 5 (dilution factor for dH<sub>2</sub>O in step #4 of the main procedure), then multiply by either 3 (dilution factor for expected protein mass <2700  $\mu$ g/tube in step #3 of the main procedure), 8.93 (2700 to 8100  $\mu$ g/tube), or 25 (8100 to 22500  $\mu$ g/tube).

## Notes

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

(ii) Protein mass for microbial cells is (mean [SD])  $1.29 (0.28) \cdot 10^{-8}$  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<sub>2</sub>O that gives (i) final concentration of NaOH of 0.2 N; (ii) cell protein between 300 to 900  $\mu$ g/tube (which will give readings that fall within standard curve if actual protein mass is between 1/3 to 2x of expected mass); and (iii) a final volume of 0.5 mL (similar to standards).

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

(v) Cell pellets and standards are diluted 1:5 with dH<sub>2</sub>O 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 ref. (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<sub>2</sub>O. 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<sub>2</sub>O. Chill acetaldehyde to 4°C before opening because boiling point is 21°C. Likewise, chill dH<sub>2</sub>O 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<sub>2</sub>SO<sub>4</sub>. 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<sub>2</sub>O.

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 <sub>2</sub> O (μL)	1 N perchloric acid (μL)
0	0	1000	1000
0.025	125	875	1000
0.05	250	750	1000
0.075	375	625	1000
0.1	500	500	1000

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

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

### Measurement of RNA using orcinol

- 1.) Prepare FeCl<sub>3</sub> solution. Dissolve 0.540 g FeCl<sub>3</sub> in 1 L concentrated HCl. Solution is stable indefinitely.

Prepare aqueous orcinol. Dissolve 0.5 g orcinol in 50 mL dH<sub>2</sub>O. 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<sub>3</sub> 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 <sub>2</sub> O (µL)	1 N perchloric acid (µL)
0	0	1000	1000
0.025	250	750	1000
0.05	500	500	1000
0.075	750	250	1000
0.1	1000	0	1000

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

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



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

## Notes

- (i) The diphenylamine reaction is specific for DNA, but orcinol reaction is not nearly as specific for RNA. Both DNA and hexoses can interfere in the orcinol reaction. This procedure already attempts to minimize interference with hexoses by using a short (10 min) incubation time [after the recommendation of ref. (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 ref. (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 ref. (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 ref. (46) and procedure from lab of Dr. Konrad Dabrowski

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

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

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  $\text{dH}_2\text{O}$  by running homogenizer for 10 s in beaker filled with  $\text{dH}_2\text{O}$ . Repeat similarly with solvent. Blot homogenizer tip dry with Kimwipe.

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

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

6.) Add 2 mL  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$  solution. Fill tube with  $\text{N}_2$  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  $\text{N}_2$ . Place boiling tube in warm (c. 40° C) water bath. Place tubing from  $\text{N}_2$  tank into upper part of boiling tube (1-2 cm away from liquid surface), and turn on  $\text{N}_2$  gas so that surface is slightly disturbed. A commercial nitrogen evaporator, which has  $\text{N}_2$  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<sub>2</sub> 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<sub>2</sub>. 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<sub>2</sub> 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 ref. (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<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>O to a 1 L Berzelius beaker. Stir and heat gently to dissolve. Transfer quantitatively to 500 mL volumetric flask and bring to volume. Store stock at 4°C. Stock is stable for months (at least 6 months in our experience).
- 4.) Prepare standards in 2-mL microcentrifuge tubes.

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

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

- 5.) Dilute cell pellets in a separate, boil-proof, 2-mL centrifuge tube with dH<sub>2</sub>O 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<sub>2</sub>O 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 ref. (41) for more details.

(iii) After being thawed and resuspended in dH<sub>2</sub>O, pellets should not be re-frozen/thawed because doing so lowers the concentration of hexoses. Incubating at 100°C partially protects against losses from freeze-thawing.

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

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

Old  $\text{H}_2\text{SO}_4$  can also cause formation of white precipitate because  $\text{H}_2\text{SO}_4$  tends to accumulate water over time (it is hygroscopic). Use only relatively fresh  $\text{H}_2\text{SO}_4$ , and change to a new bottle of  $\text{H}_2\text{SO}_4$  if most tubes form white precipitate. Avoid using  $\text{H}_2\text{SO}_4$  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  $4^\circ\text{C}$ . Before storage, the headspace in its container should be flushed with  $\text{N}_2$ .

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

(viii) Volumes shown here are 1/5 that recommended by ref. (45) because the small boiling tube used here is 1/5 the volume of the large tube recommended by ref. (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 ref. (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  $\mu$ L dH<sub>2</sub>O. Add 120  $\mu$ L, 50% KOH to tube. Heat at 100°C for 3 h.
- 4.) Neutralize 50% KOH with 6 N HCl, referring to titration in #2 for the volume of 6 N HCl needed.

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

### **Amyloglucosidase digestion and measurement of glucose**

1.) Add 56 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 disadvantage is that it measures less polysaccharide than the anthrone method and probably does not measure all polysaccharide that can indeed serve as reserve material (see *Notes of Total hexoses of microbial cells using the anthrone reaction*).

Unlike the anthrone reaction, the procedure above probably does not detect all glycogen in microbes. Ref. (47) 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<sub>2</sub>O, pellets should not be re-frozen/thawed because doing so lowers the concentration of glycogen. Incubating at 100°C partially protects against losses from freeze-thawing.

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

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



## Isolation of reserve polysaccharide and measurement of glycogen and total hexoses

Adapted from ref. (45, 51, 52)

### Extraction

#### ***Bead-beating***

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

2.) Prepare 25% w/v trichloroacetic acid (TCA). Add 25 g TCA pellets (in refrigerator) to 100 mL volumetric flask then bring to volume. Store at 4°C, and chill on ice before use.

2.) Follow **Fig. 1**. Also refer to *Total glycogen content of microbial cells using amyloglucosidase* because that procedure is similar to the present, except in the present procedure (i) bead-beating is done on the pellet directly, not pellet suspended in NaAc buffer, (ii) protein is precipitated using TCA and (iii) glycogen is precipitate and washed with ethanol.

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

#### **50% KOH**

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

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

2.) Follow **Fig. 2**. Also refer to *Total glycogen content of microbial cells using amyloglucosidase* because that procedure is similar to the present, except in the present procedure (i) no neutralization of KOH is needed and (ii) glycogen is precipitate and washed with ethanol.

### Measurement of hexoses and glycogen

1.) Follow *Total hexoses of microbial cells using the anthrone reaction* to measure hexose content of the isolated polysaccharide.

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

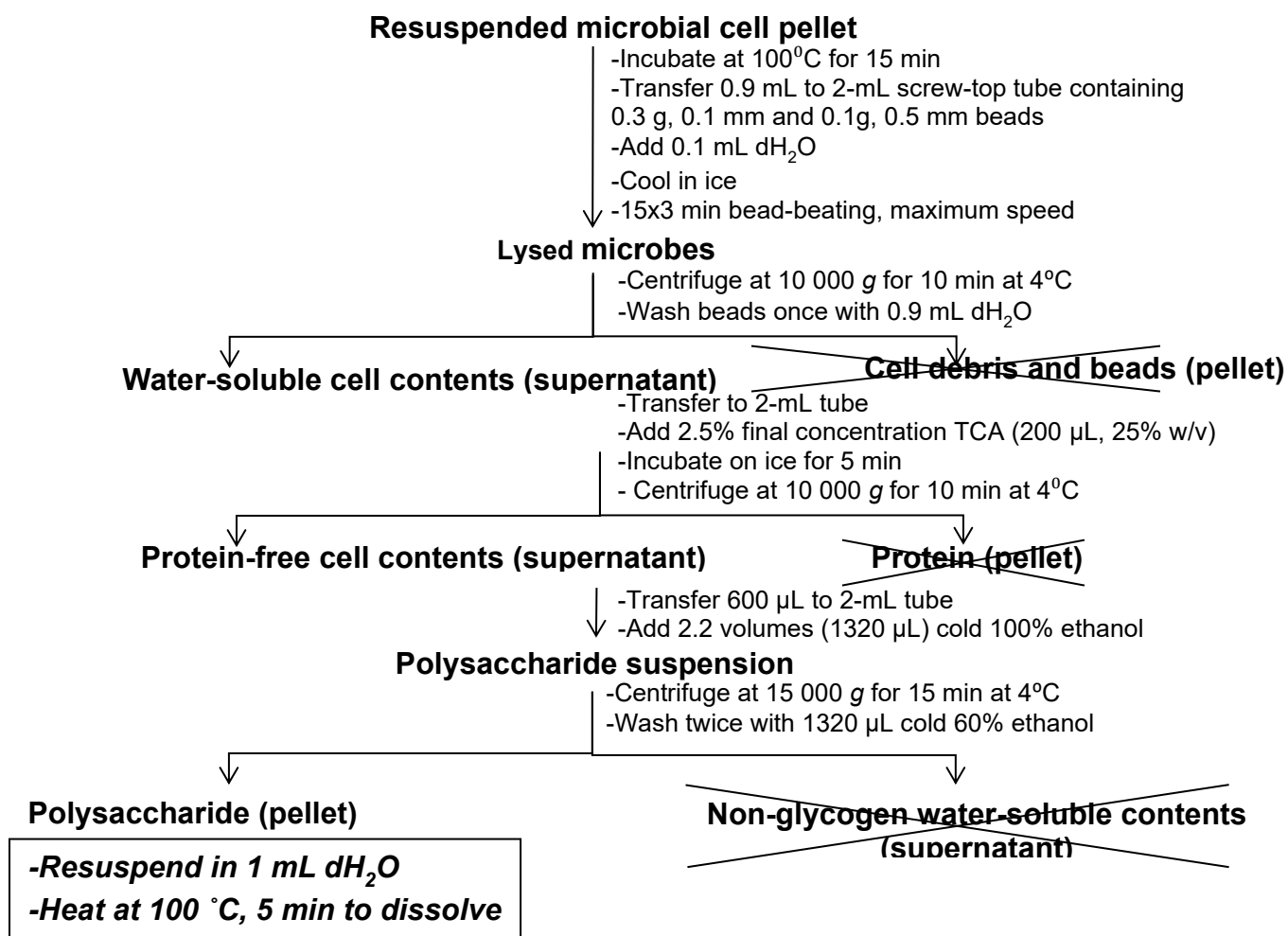
## Notes

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

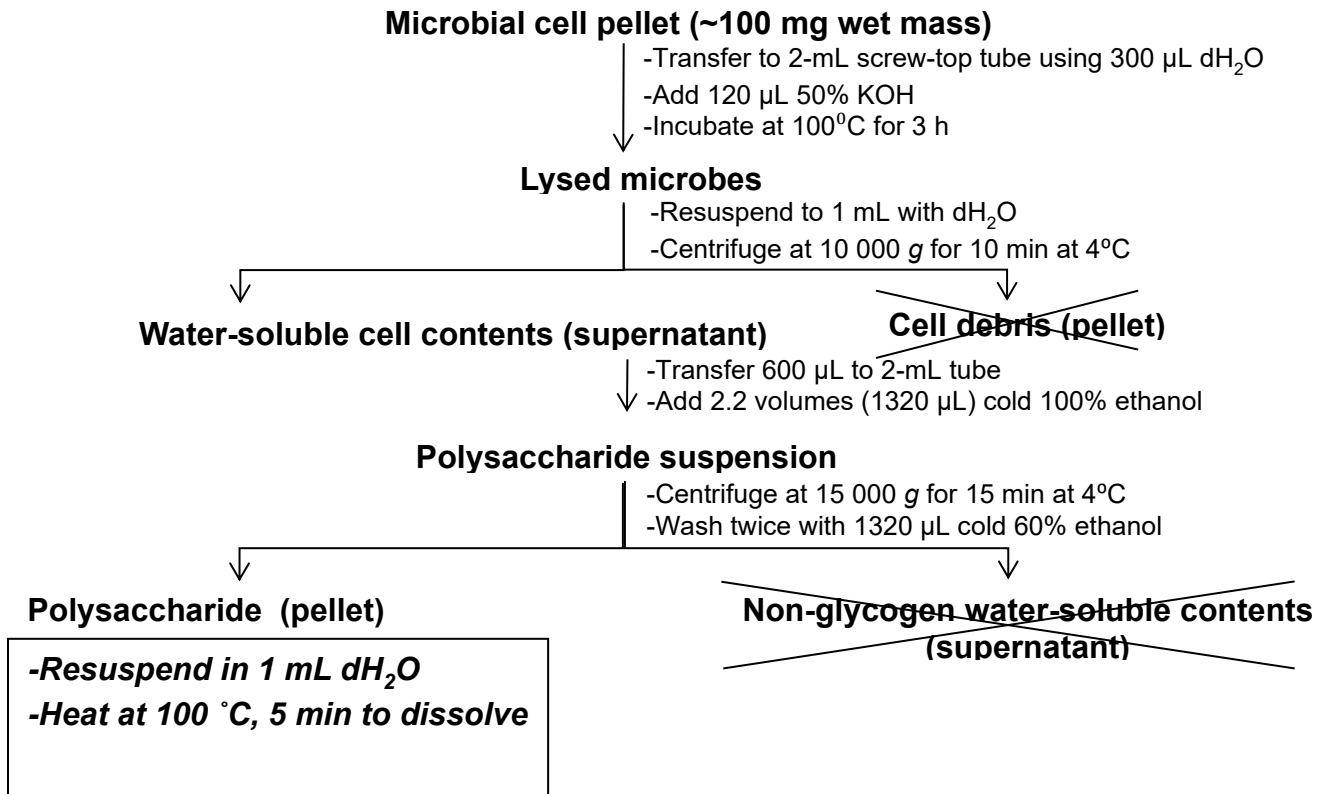
(ii) Measurement of reserve polysaccharide is higher for extraction by bead-beating vs. extraction with 50% KOH (Appendix II). Bead-beating requires special equipment (bead-beater) while 50% KOH does not.

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

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



(B)



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

Based on ref. (53–56)

### 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<sub>2</sub>O. For maltose, repeat but with 0.3 g maltose monohydrate. For maltotriose hydrate, add 0.02915 g maltotriose hydrate to 10 mL dH<sub>2</sub>O (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<sub>2</sub>O (assuming 94% maltoheptaose; adjust for actual purity). For beef liver glycogen, add 0.0314 g to 10 mL dH<sub>2</sub>O (assuming 90.04% DM and 94% glucose; adjust for actual DM and purity).

### Plate preparation

- 1.) Prepare solvent (1-propanol:acetone:dH<sub>2</sub>O; 2:2:1 by volume).
- 2.) Cut plates (20 x 20 cm, Merck Si 60 F<sub>254</sub>, 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**

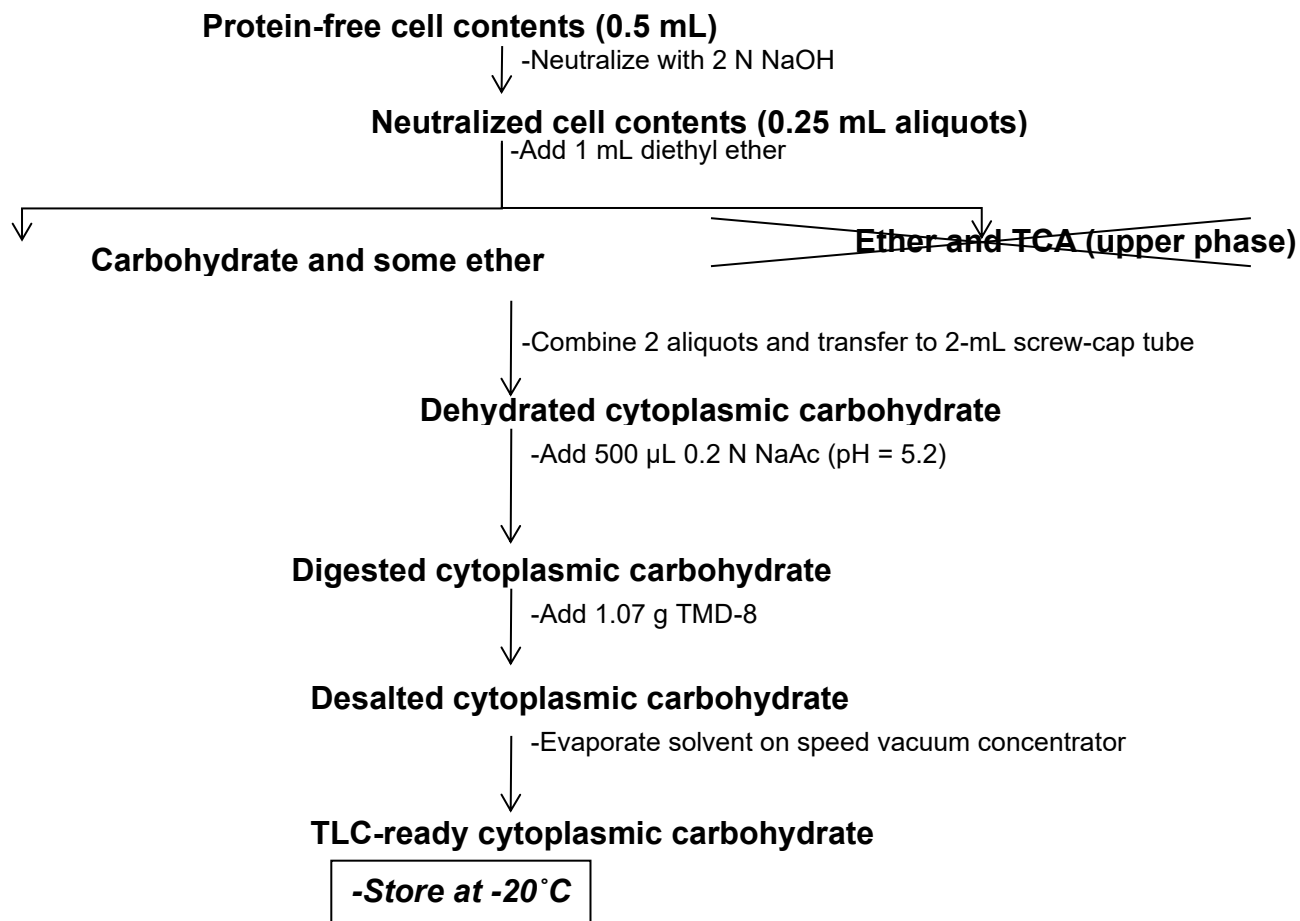
- 1.) Prepare 2 L detection reagent (H<sub>2</sub>SO<sub>4</sub>:ethanol; 9:1 by volume).
- 2.) Apply standards and samples on starting line. Use a micropipette to apply 1 µL. Apply approximately 3 µg glucose equivalents. Dry samples (using hair dryer as needed).
- 3.) Develop plate (**Fig. 3**). Fill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

### **Notes**

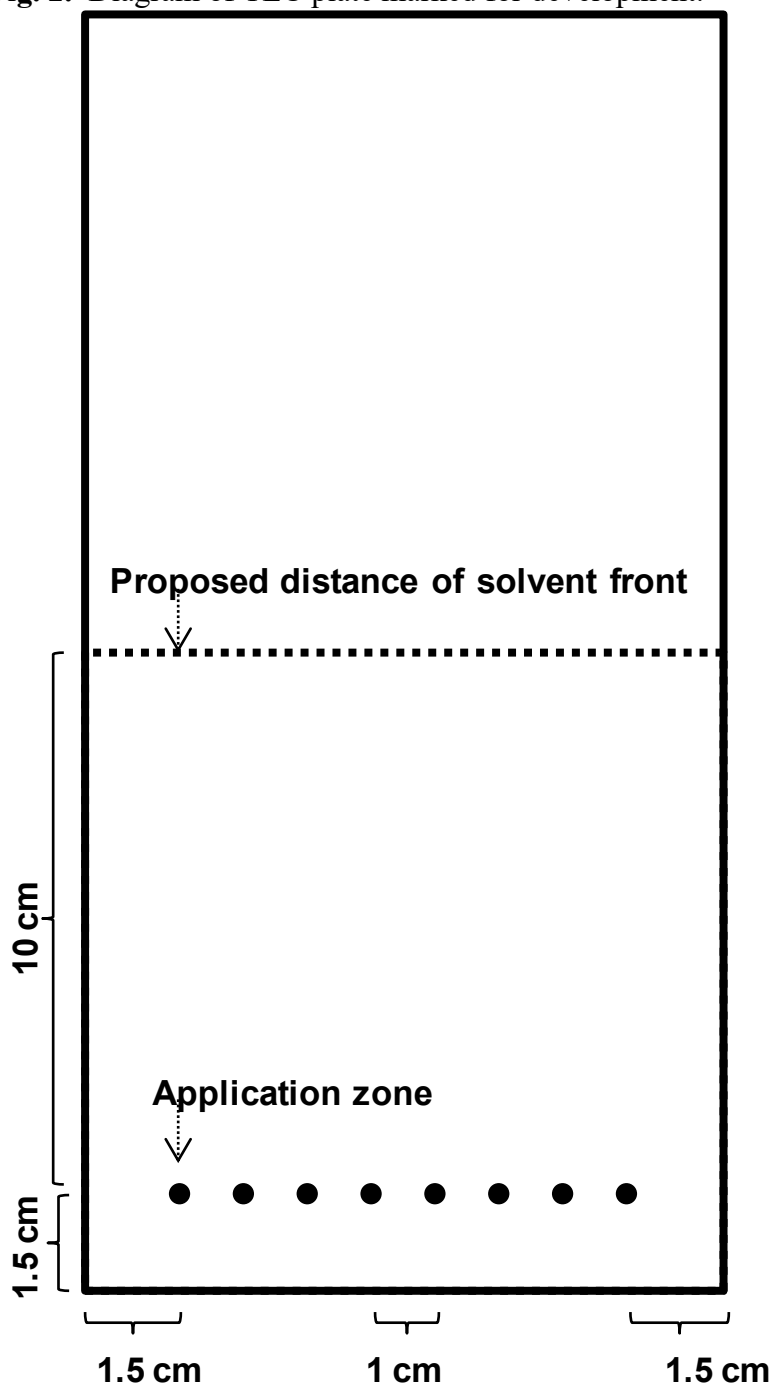
(i) Handle the TLC plate carefully to avoid contamination or damage to front of plate. Wear gloves at all times. Handle plate by grasping it with tweezers at the top when possible; when hands must be used, grasp plate only by the edges. Place plate on clean surface (e.g., aluminum foil) and cover plate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

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

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

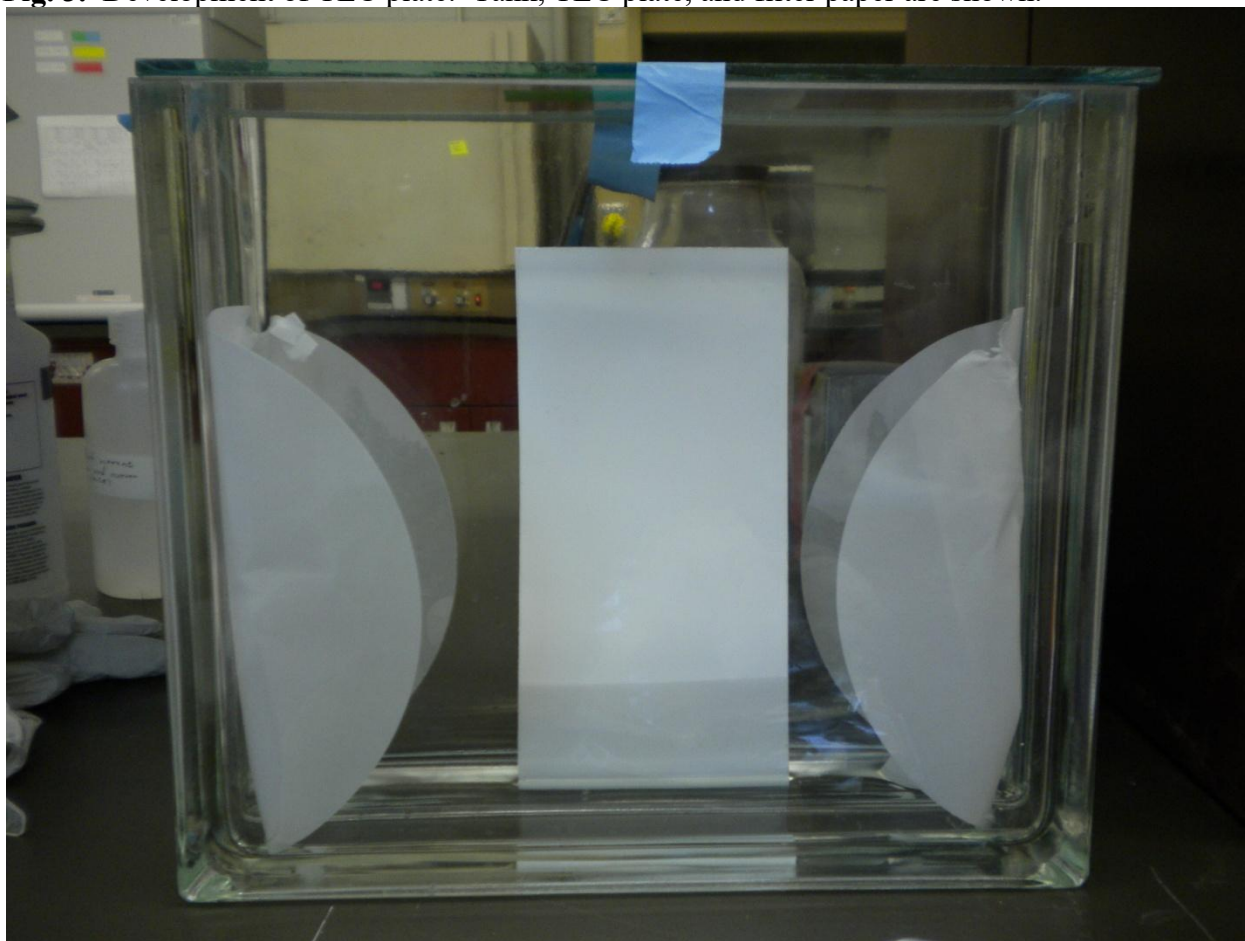


**Fig. 2.** Diagram of TLC plate marked for development.





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



## Iodine absorbance spectrum of reserve polysaccharide

Adapted from ref. (57)

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  $dH_2O$ . 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  $\mu L$  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  $\mu L$  reserve polysaccharide and 97.5  $\mu L$  saturated  $CaCl_2$  to each well of a microplate. Include blank wells with 30  $\mu L$   $dH_2O$  and 97.5  $\mu L$  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  $A_1(\text{reserve polysaccharide})$  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  $\mu L$  2x iodine reagent. Read absorbances from 380 to 700 nm in 10 nm increments. This set of absorbances is called  $A_2(\text{reserve polysaccharide})$  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  $[A_2(\text{reserve polysaccharide}) - A_1(\text{reserve polysaccharide})] - [A_2(dH_2O) - A_1(dH_2O)]$ .

### 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  $A_1(\text{reserve polysaccharide})$  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  $\text{CaCl}_2$  is made adding  $\text{CaCl}_2$  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 ref. (58, 59)

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

2.) Prepare glucose oxidase-peroxidase (GOP) reagent (9.1 g Na<sub>2</sub>HPO<sub>4</sub>, 5 g KH<sub>2</sub>PO<sub>4</sub>, 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<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> in dH<sub>2</sub>O (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<sub>2</sub>O. 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<sub>2</sub>O 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 <sub>2</sub> O (μL)
0	0	2000
50	100	1900
100	200	1800
150	300	1700
200	400	1600
350	700	1300
500	1000	1000

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

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

6.) Dilute samples from #5 with dH<sub>2</sub>O 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<sub>2</sub>O.

## Notes

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

(ii) *N*-ethylmaleimide and phenol are toxic and should be handled under the hood. 4-aminoantipyrene is light-sensitive. It should be stored in an amber jar or clear covered with aluminum foil. Jar should be sealed and contain 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, ref. (58) 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 ref. (58) 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 ref. (60)

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

- 1.) Prepare supernatant according to *Preparing cells and culture supernatant for chemical analysis*.
- 2.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve a concentration of D-lactic acid of  $\leq 0.03$  g/L (0.33 mM) and L-lactic acid of  $\leq 0.03$  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  $\mu$ L supernatant and 200  $\mu$ L dH<sub>2</sub>O.
- 4.) Make lactic acid standards (Table 1). Make fresh (long-term stability is unknown).
- 5.) Make solution 2. Add 6 mL dH<sub>2</sub>O 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  $\mu$ L solution 1, 20  $\mu$ L solution 2, and 2  $\mu$ L solution 3. Include one extra reaction to avoid running out. Make fresh daily.
- 8.) Pipette 122  $\mu$ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction. Pop any bubbles that form by using tip of disposable Pasteur pipetted heated over Bunsen burner.
- 9.) Pipette 100  $\mu$ L sample or standard into wells. Use one well per reaction. Cover plate with aluminum foil, and incubate at room temperature or 39°C.
- 10.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 5 min (until absorbances stabilize), then read one final time. This set of absorbances is called A<sub>1</sub>.
- 11.) Add 2  $\mu$ L solution 4 to each well. Incubate at room temperature.
- 12.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 180 min (until absorbances stabilize), then read one final time. This set of absorbances is called A<sub>2</sub>.

13.) Add 2  $\mu$ L solution 5 to each well. Incubate at room temperature.

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

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

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

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

### Notes

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

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

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

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

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

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

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

**Table 1.** Lactic acid standards\*

Standard	D-lactic acid solution (Bottle 6)	L-lactic acid solution (Bottle 7)	dH <sub>2</sub> O
		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.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve formic acid concentration of  $\leq 0.2$  g/L (4.3 mM). If supernatant has 0.4 g/L, for example, dilute by combining 50  $\mu$ L supernatant and 50  $\mu$ L dH<sub>2</sub>O.
- 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<sub>2</sub>O. Make standards by diluting stock (Table 1). Store at -20°C or use fresh.
- 5.) Make solution 1. Add 4 mL dH<sub>2</sub>O 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<sub>2</sub>O 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  $\mu$ L dH<sub>2</sub>O, 20  $\mu$ L solution 1, and 20  $\mu$ L solution 2. Include one extra reaction to avoid running out. Prepare immediately before use.
- 9.) Pipette 240  $\mu$ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 10.) Pipette 10  $\mu$ L of sample or standard into wells. Cover with aluminum foil, and incubate at room temperature.
- 11.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 3 min (until absorbances stabilize), then read one final time. This set of absorbances is called A<sub>1</sub>.

This set of absorbances is called A<sub>1</sub>.

12.) Add 5  $\mu\text{L}$  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  $\text{dH}_2\text{O}$  in #2.

**Table 1.** Formic acid standards

Standard	Formic acid stock	dH <sub>2</sub> O
	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.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve succinic acid concentration of  $\leq 0.4$  g/L (3.4 mM). If supernatant has 0.8 g/L, for example, dilute by combining 50  $\mu$ L supernatant and 50  $\mu$ L dH<sub>2</sub>O.
- 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<sub>2</sub>O. 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<sub>2</sub>O 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<sub>2</sub>O 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  $\mu$ L dH<sub>2</sub>O, 20  $\mu$ L solution 1, 20  $\mu$ L solution 2, 20  $\mu$ L solution 3, and 2  $\mu$ L solution 4. Include one extra reaction to avoid running out. Prepare immediately before use.
- 9.) Pipette 262  $\mu$ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 10.) Pipette 10  $\mu$ L of sample or standard into wells. Cover plate with aluminum foil, and incubate at room temperature.
- 11.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 5 min (until absorbances stabilize), then read one final time. This set of absorbances is called A<sub>1</sub>.

12.) Add 2  $\mu\text{L}$  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  $\text{dH}_2\text{O}$  in #2.

**Table 1.** Succinic acid standards.

Standard	Succinic acid stock	dH <sub>2</sub> O
	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.) Inactivate enzymes in supernatant. Heat tubes with supernatant in 100°C water bath for 10 min, then cool on ice.
- 3.) Dilute supernatant to achieve ethanol acid concentration of  $\leq 0.12$  g/L (2.6 mM). If supernatant has 0.24 g/L, for example, dilute by combining 50  $\mu$ L supernatant and 50  $\mu$ L dH<sub>2</sub>O.
- 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<sub>2</sub>O 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  $\mu$ L dH<sub>2</sub>O, 20  $\mu$ L solution 1, 20  $\mu$ L suspension 2, and 5  $\mu$ L solution 3. Include one extra reaction to avoid running out. Make fresh daily.
- 8.) Pipette 245  $\mu$ L working reagent into wells of 96-well UV-transparent plate. Use one well per reaction.
- 9.) Pipette 10  $\mu$ L of sample or standard into wells. Cover plate with aluminum foil, and incubate at room temperature.
- 10.) Measure absorbance of microplate at 340 nm following *Measuring sample absorbance for microplate assays* (pre-reading the plate is not needed). Read at intervals for approximately 10 min (until measurements stabilize), then read one final time. This set of absorbances is called A<sub>1</sub>.
- 11.) Add 2  $\mu$ L solution 4 to each well. Mix by stirring with pipette tip until white precipitate clears. Incubate at room temperature.
- 12.) Read absorbance of microplate at 340 nm. Read at intervals for approximately 50 min (until measurements stabilize), then read one final time. This set of absorbances is called A<sub>2</sub>.

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  $\text{dH}_2\text{O}$  in #2.



**Table 1.** Ethanol standards.

Standard	Ethanol stock	dH <sub>2</sub> O
	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 ref. (61, 62)

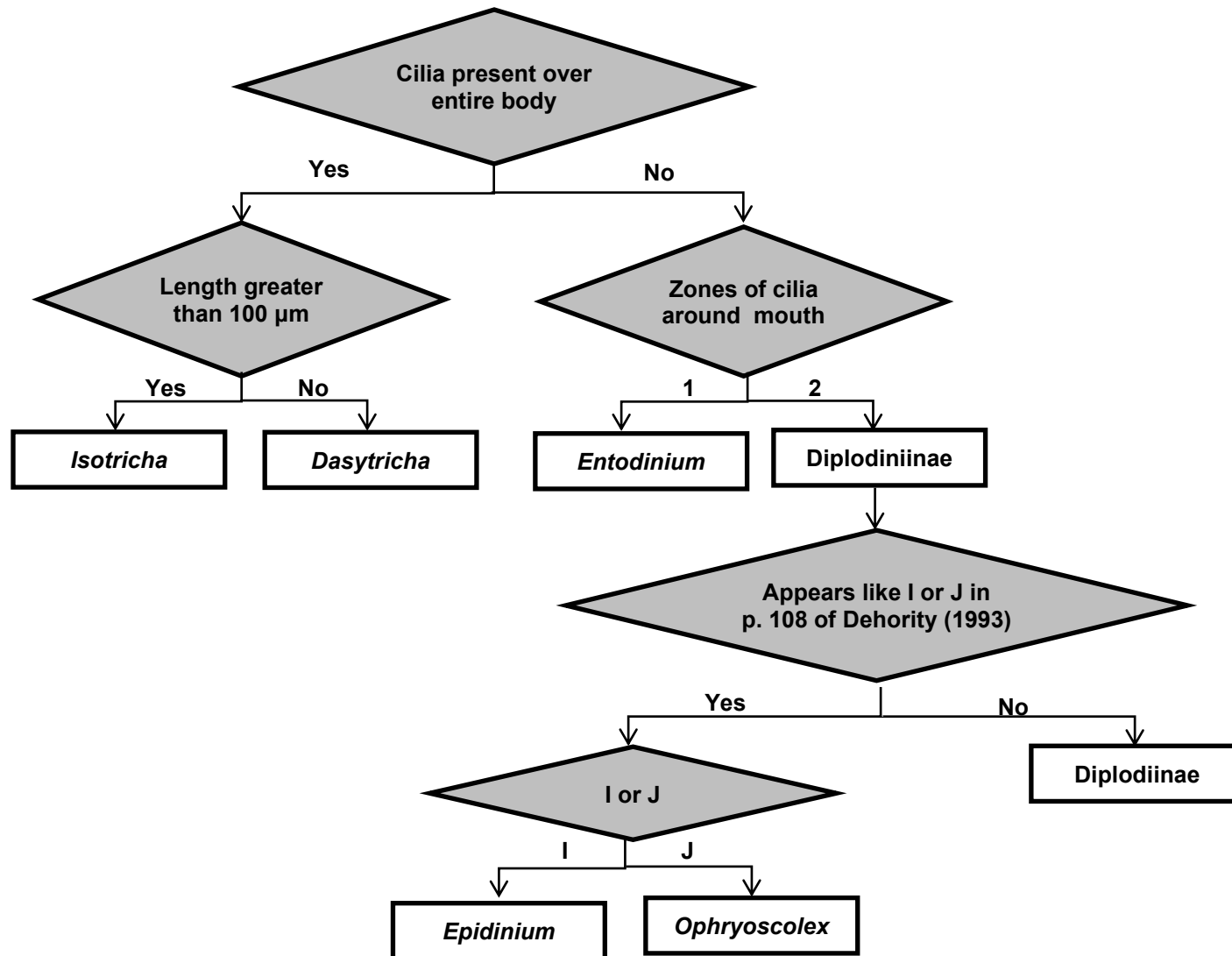
- 1.) Prepare 50% formalin by diluting one volume of 37% formaldehyde with an equal volume of dH<sub>2</sub>O. Prepare Brilliant green dye by diluting 2 g and 2 mL of glacial acetic acid to 100 mL with dH<sub>2</sub>O. Prepare 30% v/v glycerol by diluting 300 mL glycerol to 1000 mL with dH<sub>2</sub>O.
- 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. Ref. (61) 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<sup>5</sup> 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 ref. (61, 62) 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<sup>2</sup>),  $G$  is the number of fields (100),  $A_g$  is the area of each field (0.3128 mm<sup>2</sup>),  $V$  is the volume of the sample (1 mL), and  $d$  is dilution (e.g., 40). Note count is per milliliter of formalized sample and stained sample, not original sample.

## Notes

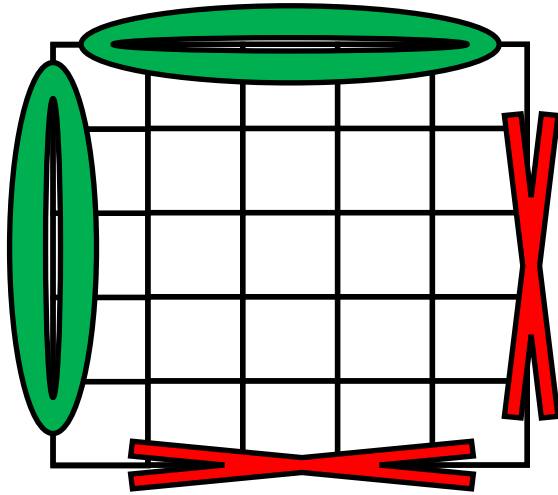
- (i) Because glycerol is viscous and mixes with difficulty, dilute glycerol in a 1000-mL graduated cylinder, and mix the glycerol by transferring it back and forth between the cylinder and the glass bottle in which it is to be stored. Store at 4°C.

- (ii) To store at samples at 4°C for short periods (a few weeks), the sample need be fixed in only 1% final concentration of formalin.
- (iii) The dilution needs to be adjusted to give approximately 200 to 300 cells per 100 fields. The 200 to 300 cell range ensures enough cells are present to give reasonable accuracy but not so many are present that counting becomes tedious.
- (iv) To obtain a representative sample, culture tubes of sample need to be mixed well (a) by flipping the tube end-over-end, (b) drawing sample in and out of the pipette repeatedly, and (c) sampling quickly. (iii) To avoid shearing large protozoa, the opening of pipette tips should be enlarged by cutting off 1-2 mm of their pointed end.
- (v) Glycerol is used to dilute samples because its viscosity prevents settling during mixing. The slow settling also means that one must wait 1 to 2 minutes to count protozoa after loading the counting chamber, to allow protozoa to settle at the bottom.
- (vi) To help evenly count 50 fields, mark with pencil on the microscope stage 10 evenly-spaced lines in the horizontal direction and 5 lines in the vertical direction. These lines will form an imaginary grid with 50 points. Count 50 fields by aligning a corner of the slide at each of 50 points of the imaginary grid.

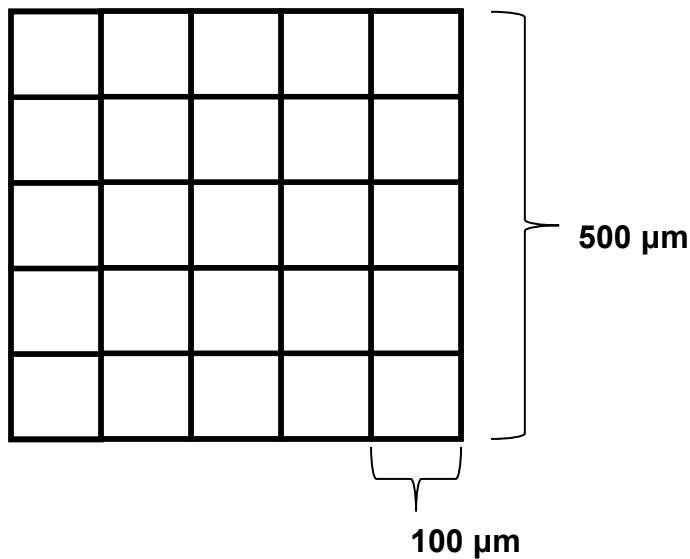
**Fig. 1.** Decision chart for classifying rumen protozoa



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



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



## Concentration of bacteria

Modified from ref. (63, 64) and Life Technologies DAPI counterstaining protocol

### Preparing DAPI stock and other reagents

- 1.) Prepare DAPI stock. Add 2 mL dH<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O (sterilized through 0.2-µm filter and autoclaved). Make fresh daily.

### Filtering and counting cells

- 1.) If cells are clumped (e.g., in centrifuged samples), incubate sample in Triton-X (0.1% final concentration) overnight to help unclump cells. If cells are not clumped (e.g., in uncentrifuged samples), proceed to #2.
- 2.) Dilute sample with diluent water (see composition below). Prepare 10<sup>4</sup> dilution when expecting 10<sup>9</sup> cells/mL.
- 3.) Connect filter holder (25-mm diameter; Kimble 953705-0000) to vacuum flask. Connect flask to vacuum pump. Place filter (black Nuclepore, 0.2 µm pore, 25 mm diameter; Whatman 110656) shiny side up on filter holder and pre-wet with 1 drop of diluent water.
- 4.) Perform staining in the filter holder. Bring all solutions and sample to room temperature. Add diluent water (1.48 mL), diluted sample (0.5 mL), and DAPI stain (20 µL) directly to the filter and in that exact order. Incubate for 7 min, occasionally mixing contents with pipette.
- 5.) Vacuum with slight pressure (<30 mmHg) to filter sample. Just before all sample passes the filter, apply rinse water (two, 1-mL aliquots). If filtration takes more than 1 to 2 min, dilute the sample.
- 6.) Place 1 drop of immersion oil to acetone-cleaned glass slide. Apply filter sample-side up. Place another drop of immersion oil on top of filter, and apply coverglass.
- 7.) Examine at 1000x with Zeiss AxioScope A.1 epifluorescent microscope and filter for DAPI. Count at least 400 cells within a minimum of 20 fields of view.
- 8.) Calculate #cells/mL for the formalized sample as

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

where  $N$  is the number of cells counted,  $A_t$  is the effective area of the filter ( $25 \text{ mm}^2$ ),  $G$  is the number of fields,  $A_g$  is the area of each field ( $0.00346 \text{ mm}^2$ ),  $V$  is the volume of the sample ( $0.5 \text{ mL}$ ), 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  $491 \text{ mm}^2$ , but not all filter is exposed because filter holder shields clamps down on edges of filter and shields this area from sample. The effective area reported here is that given by the manufacturer.

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

## Preparing wet mounts of bacteria for imaging

Adapted from ref. (65)

- 1.) Prepare 2% w/v noble agar by adding 1 g noble agar (BD 214220) to 50 mL ddH<sub>2</sub>O 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 CHROMATOGRAPHY**

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

## Operation of gas chromatograph

### Risks of operation

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

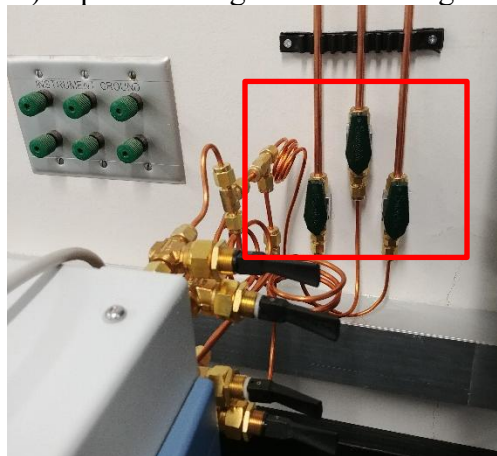
- Any component is heated above 240°C. This is the maximum temperature of the WAX column.
- The carrier gas is off, and the oven or detector is heated above 100°C. The columns are destroyed by a combination of oxygen and heat. It is safe to heat only after the carrier gas is on and has been flushed of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

### Turning on and placing into standby mode

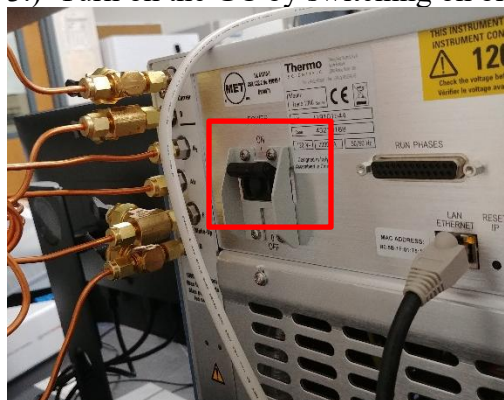
1.) Turn on compressed gases (nitrogen, air, hydrogen). Turn on cylinder valve, then turn on diaphragm at regulator to 100 psi.



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

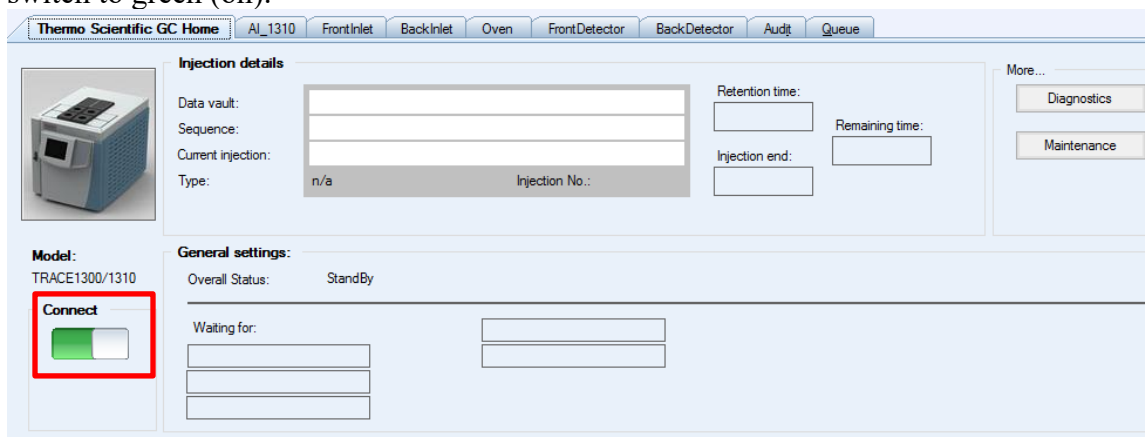


3.) Turn on the GC by switching on breaker on back of instrument.

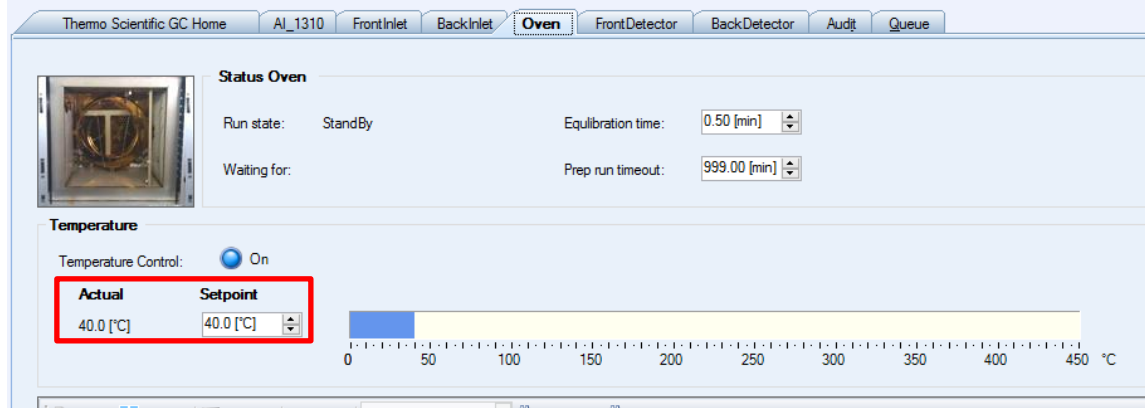


4.) Start Chromeleon 7 software on GC computer.

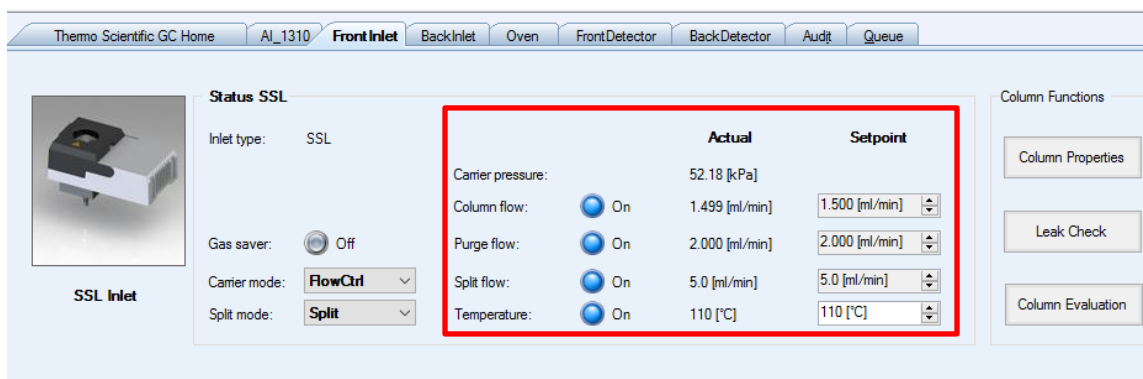
5.) Connect to Chromeleon to instrument. In Thermo Scientific GC Home tab, toggle Connect switch to green (on).



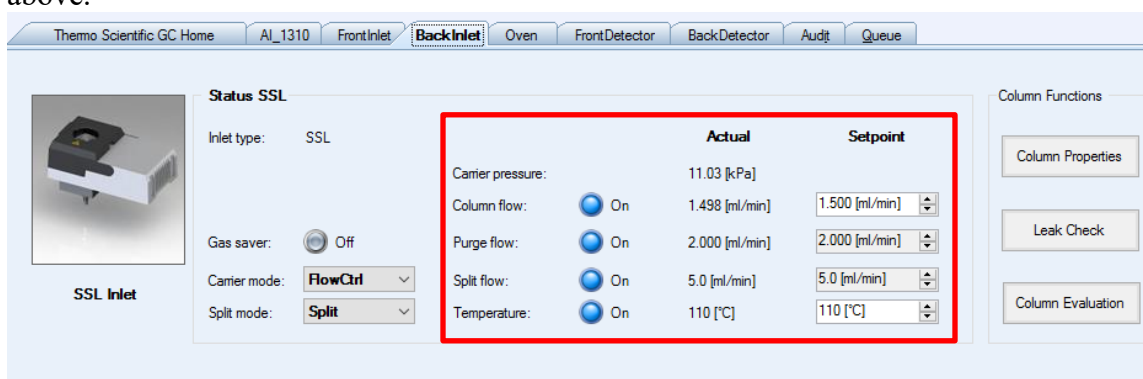
6.) Verify that oven is at safe temperature. In Oven tab, check that Actual and Setpoint temperatures are well below 100°C (e.g., 40°C). If above, immediately adjust the set point.



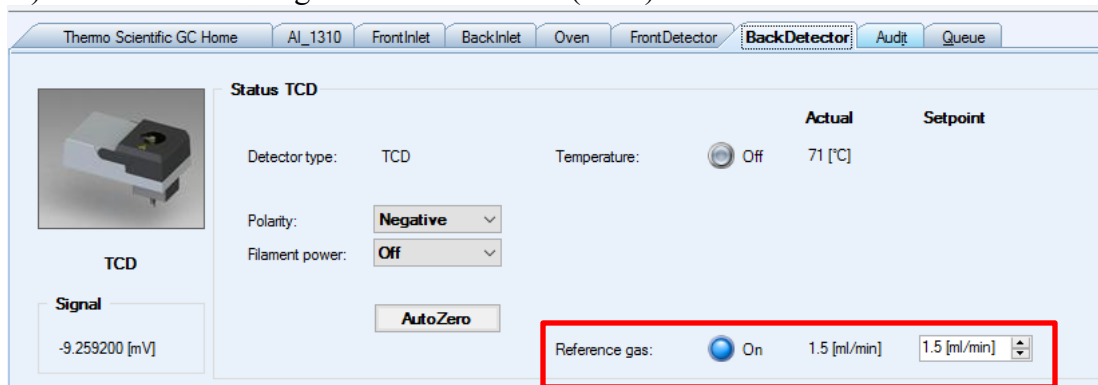
7.) Turn on carrier gas and temperature for front inlet. Go to FrontInlet tab, and turn Column flow to On. Repeat for Purge flow, Split flow, and Temperature. Choose setpoints shown (minimum needed to purge system and prevent condensation of water).



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).



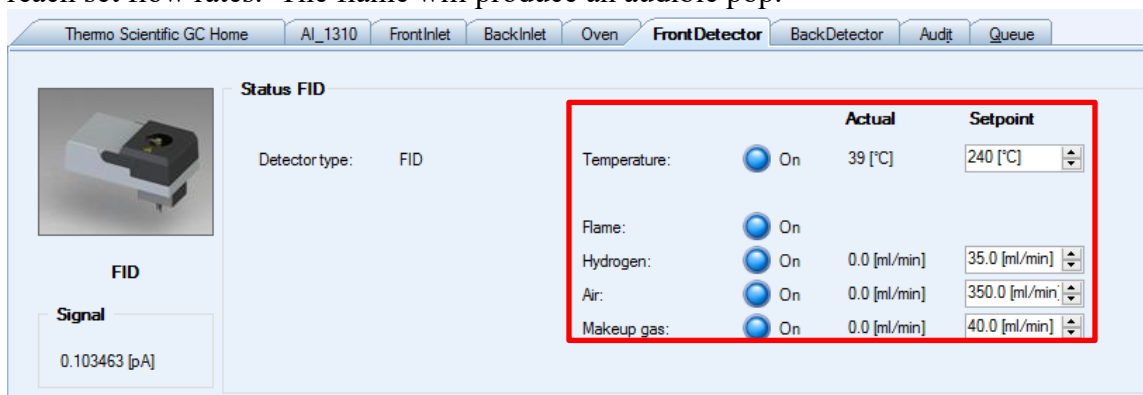
10.) Continue to monitor the compressed gases, and adjust back to 100 psi as needed.

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

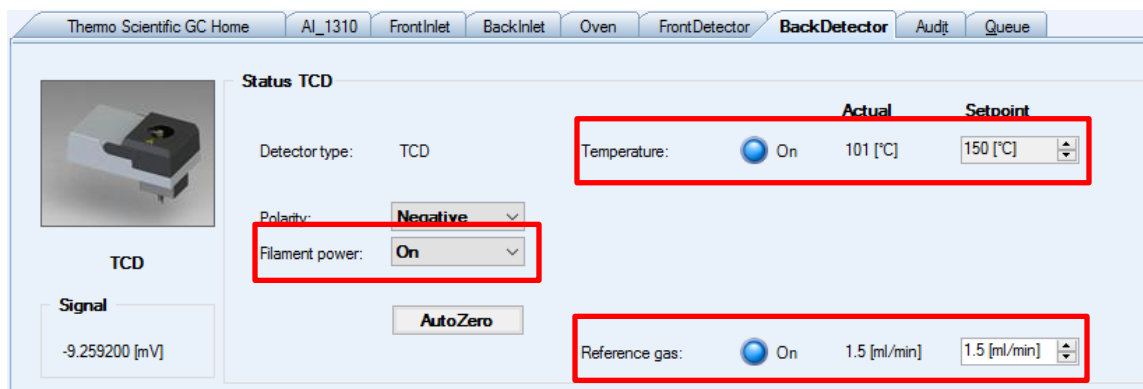
There are two detectors (front and back). Only the detector that is needed should be turned on. The front detector is needed for VFA analysis, and the back detector is needed for gas analysis.

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.



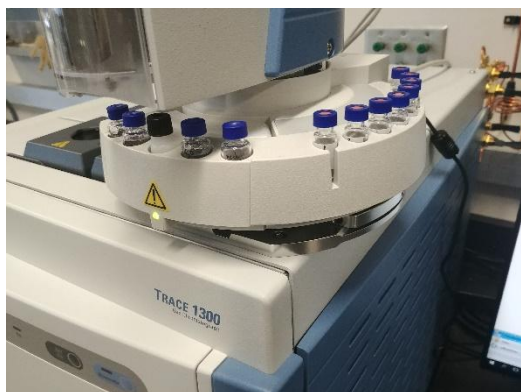
Allow signal from detector to stabilize. Signal will decrease quickly (from 1200 to 130 pA) in 3 h, when decrease more slowly (to 50 pA) overnight.

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.

Thermo Scientific GC Home | AI\_1310 | FrontInlet | BackInlet | Oven | **FrontDetector** | BackDetector | Audit | Queue

**Status FID**

Detector type: FID

		Actual	Setpoint
Temperature:	<input type="radio"/> Off	182 [°C]	
Flame:	<input type="radio"/> Off		
Hydrogen:	<input type="radio"/> Off	0.0 [ml/min]	
Air:	<input type="radio"/> Off	0.0 [ml/min]	
Makeup gas:	<input type="radio"/> Off	0.0 [ml/min]	

FID

Signal: 0.138137 [pA]

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.

Thermo Scientific GC Home | AI\_1310 | FrontInlet | BackInlet | Oven | FrontDetector | **BackDetector** | Audit | Queue

**Status TCD**

Detector type: TCD

		Actual	Setpoint
Temperature:	<input type="radio"/> Off	71 [°C]	

Polarity: Negative

Filament power: Off

AutoZero

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

TCD

Signal: -9.259200 [mV]

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

Thermo Scientific GC Home AI\_1310 **FrontInlet** BackInlet Oven FrontDetector BackDetector Audit Queue

**Status SSL**

Inlet type: SSL

Gas saver: ☐ Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	52.18 [kPa]	
Column flow:	1.499 [ml/min]	1.500 [ml/min]
Purge flow:	2.000 [ml/min]	2.000 [ml/min]
Split flow:	5.0 [ml/min]	5.0 [ml/min]
Temperature:	110 [°C]	110 [°C]

Column Functions

Column Properties

Leak Check

Column Evaluation

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

Thermo Scientific GC Home AI\_1310 FrontInlet **BackInlet** Oven FrontDetector BackDetector Audit Queue

**Status SSL**

Inlet type: SSL

Gas saver: ☐ Off

Carrier mode: **FlowCtrl**

Split mode: **Split**

	Actual	Setpoint
Carrier pressure:	11.04 [kPa]	
Column flow:	1.499 [ml/min]	1.500 [ml/min]
Purge flow:	2.000 [ml/min]	2.000 [ml/min]
Split flow:	5.0 [ml/min]	5.0 [ml/min]
Temperature:	110 [°C]	110 [°C]

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

Thermo Scientific GC Home AI\_1310 FrontInlet BackInlet **Oven** FrontDetector BackDetector Audit Queue

**Status Oven**

Run state: Ready

Waiting for:

Equilibration time: 0.50 [min]

Prep run timeout: 999.00 [min]

**Temperature**

Temperature Control: ☒ On

Actual	Setpoint
40.0 [°C]	40.0 [°C]

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

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

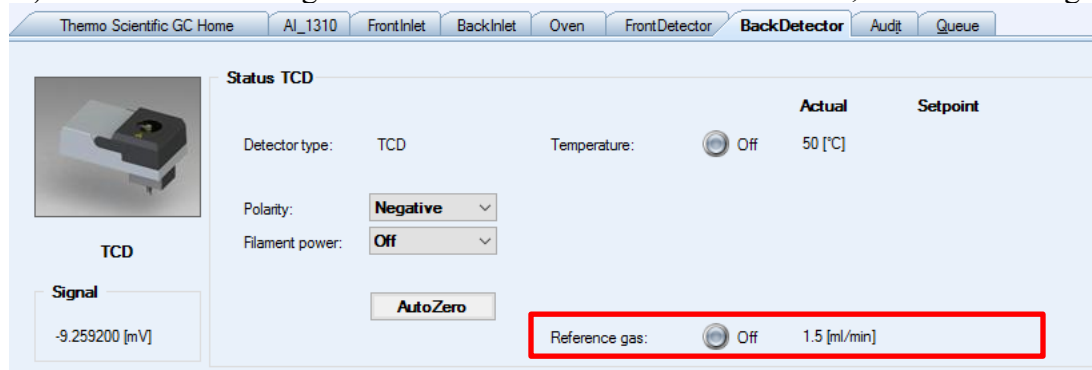
8.) Keep in standby mode (if using within next day or so) or proceed with turning off.



## Turning off

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

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



Thermo Scientific GC Home | AI\_1310 | FrontInlet | BackInlet | Oven | FrontDetector | **BackDetector** | Audit | Queue

**Status TCD**

Detector type: TCD      Temperature: ☒ Off      Actual: 50 [°C]      Setpoint:

Polarity: **Negative** ▼

Filament power: **Off** ▼

**AutoZero**

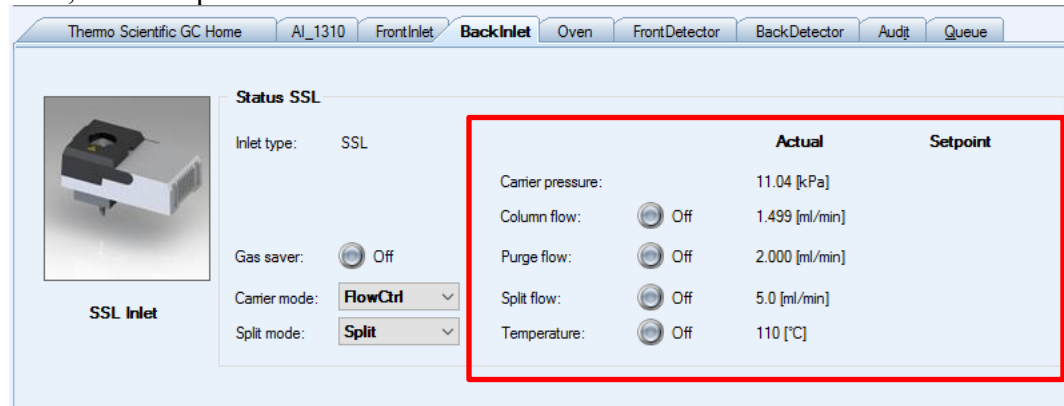
**Reference gas:** ☒ Off      1.5 [ml/min]

**TCD**

**Signal**

-9.259200 [mV]

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



Thermo Scientific GC Home | AI\_1310 | FrontInlet | **BackInlet** | Oven | FrontDetector | BackDetector | Audit | Queue

**Status SSL**

Inlet type: SSL

Gas saver: ☒ Off

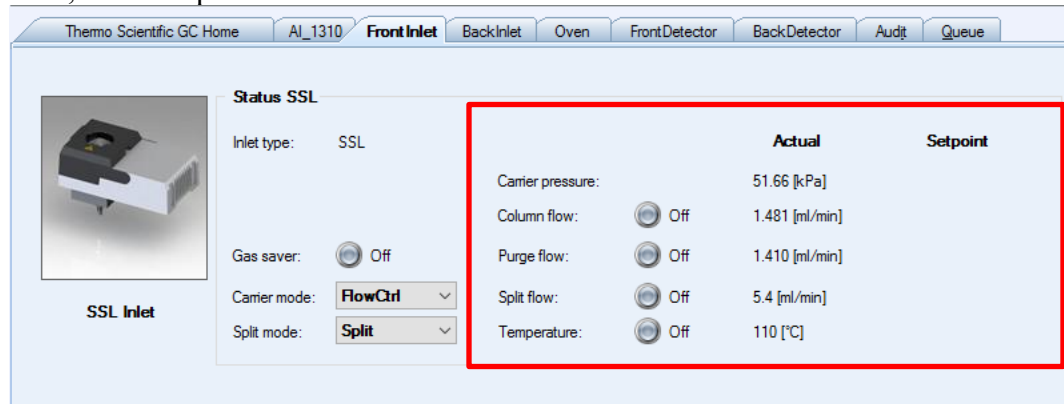
Carrier mode: **FlowCtrl** ▼

Split mode: **Split** ▼

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

**SSL Inlet**

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



Thermo Scientific GC Home | AI\_1310 | **FrontInlet** | BackInlet | Oven | FrontDetector | BackDetector | Audit | Queue

**Status SSL**

Inlet type: SSL

Gas saver: ☒ Off

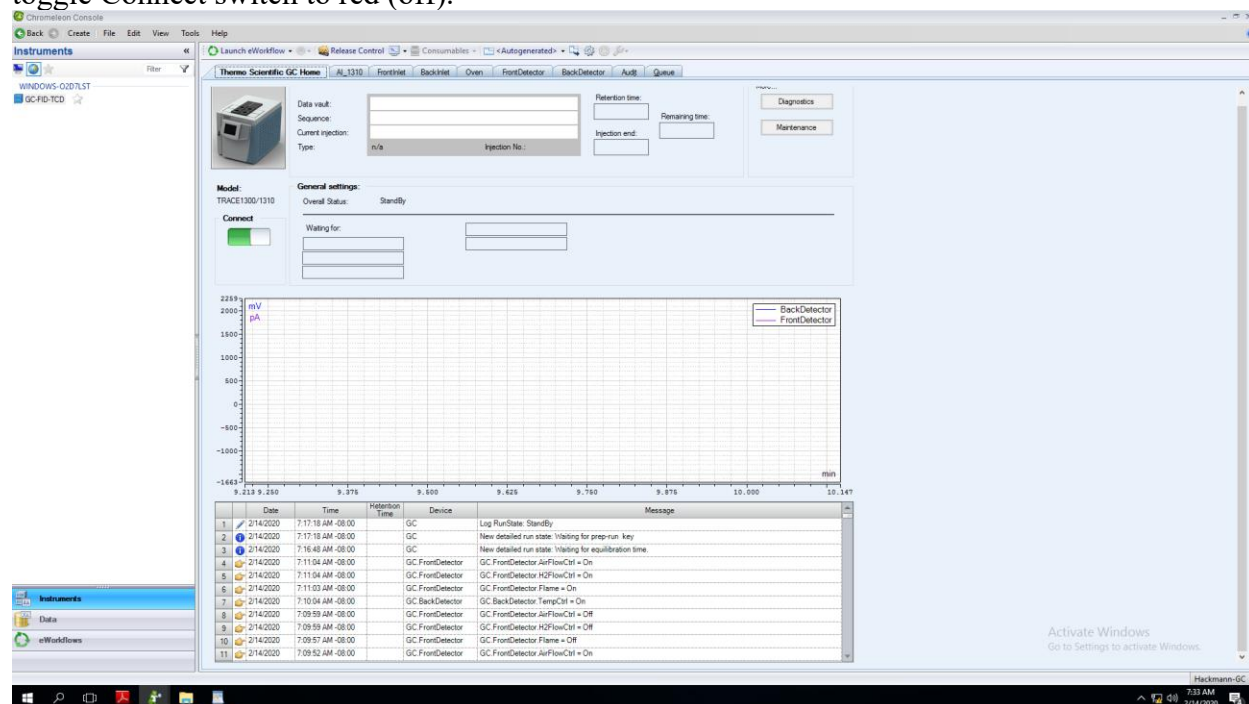
Carrier mode: **FlowCtrl** ▼

Split mode: **Split** ▼

	Actual	Setpoint
Carrier pressure:	51.66 [kPa]	
Column flow:	<input checked="" type="radio"/> Off      1.481 [ml/min]	
Purge flow:	<input checked="" type="radio"/> Off      1.410 [ml/min]	
Split flow:	<input checked="" type="radio"/> Off      5.4 [ml/min]	
Temperature:	<input checked="" type="radio"/> Off      110 [°C]	

**SSL Inlet**

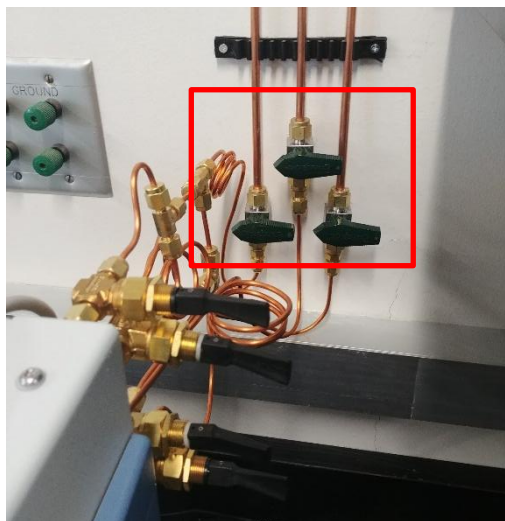
5.) Optional: disconnect Chromleon from instrument. In Thermo Scientific GC Home tab, toggle Connect switch to red (off).



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



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



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

## Maintenance of gas chromatograph

### Replacing injector septum

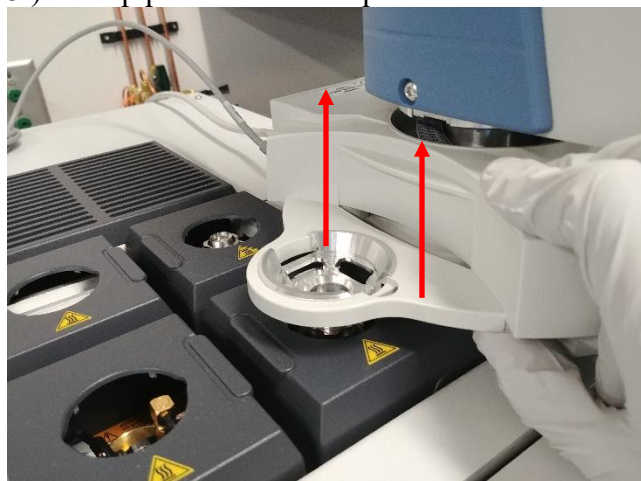
Injector septum will get dirty and should be replaced after a certain number of injections..

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

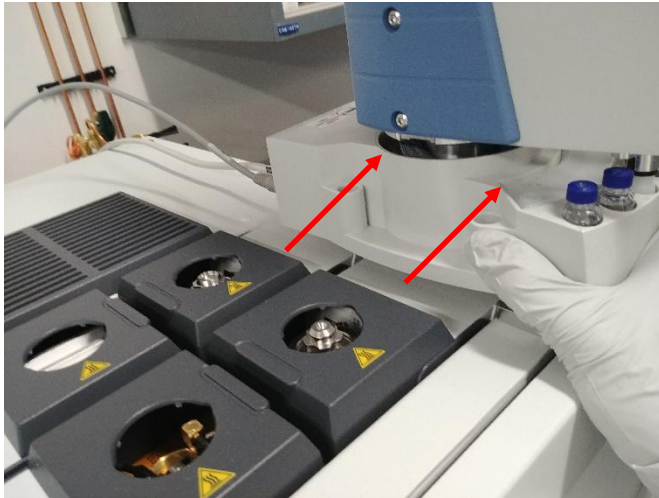
2.) Unplug autosampler.



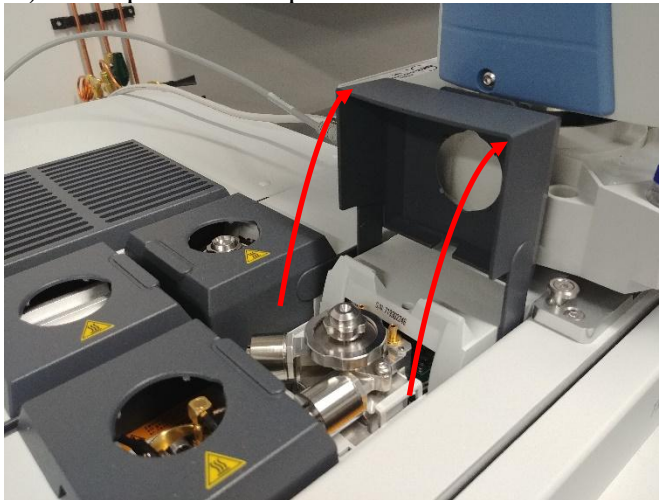
3.) Lift up plate on autosampler.



4.) Push autosampler away from injector.



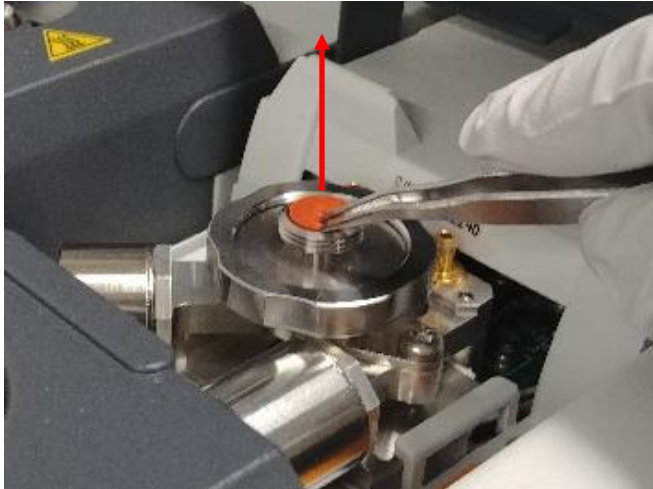
5.) Lift up module flap cover.



6.) Unscrew septum cap and lift up.



8.) Remove old septum.



9.) Put in new septum.

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

### **Replacing injector liner**

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

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

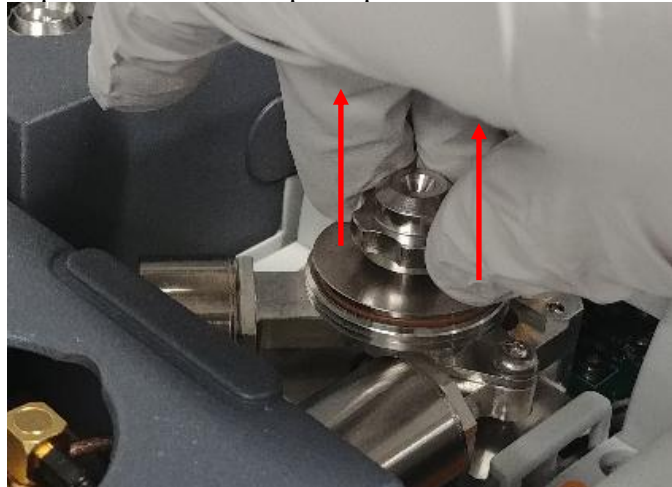
2.) Follow steps 2 through 5 ***Replacing injector septum***.

3.) Unscrew and lift up ring nut.

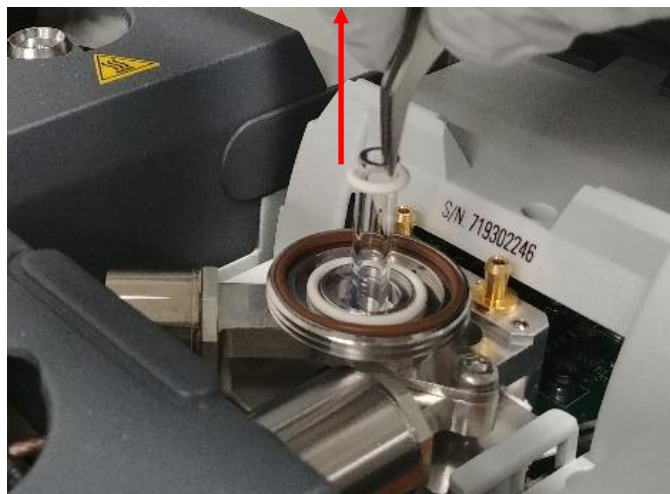




4.) Pull up the septum holder, using septum cap as a grip. If the septum holder is stuck, it may require extra effort to pull up.



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



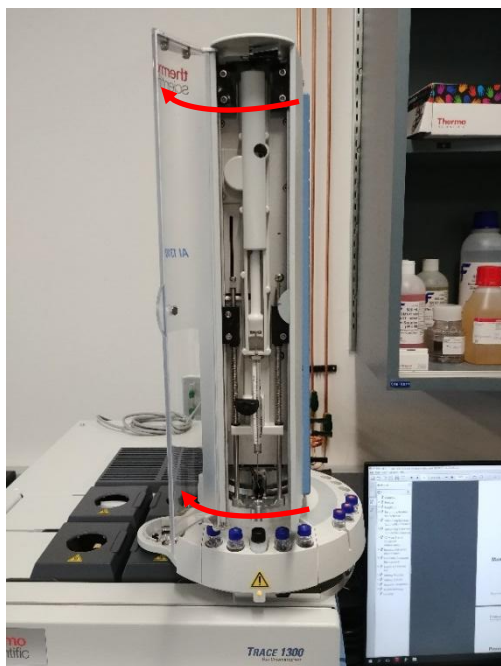
6.) Replace injector liner. Make sure to transfer white o-ring from old liner to new one.

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

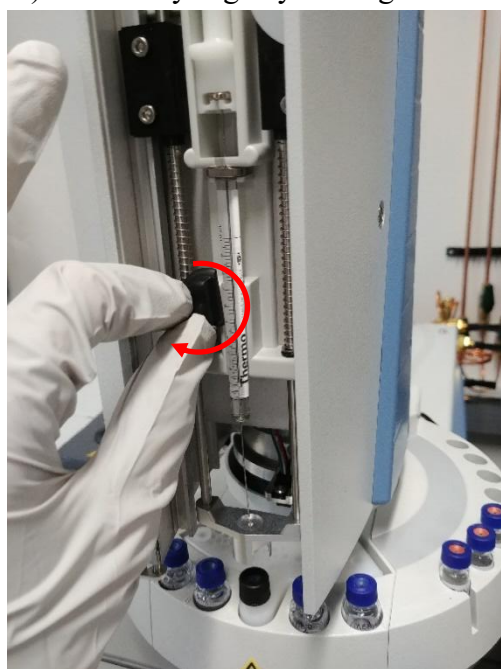
### **Replacing autosampler syringe**

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

1.) Open door of autosampler.

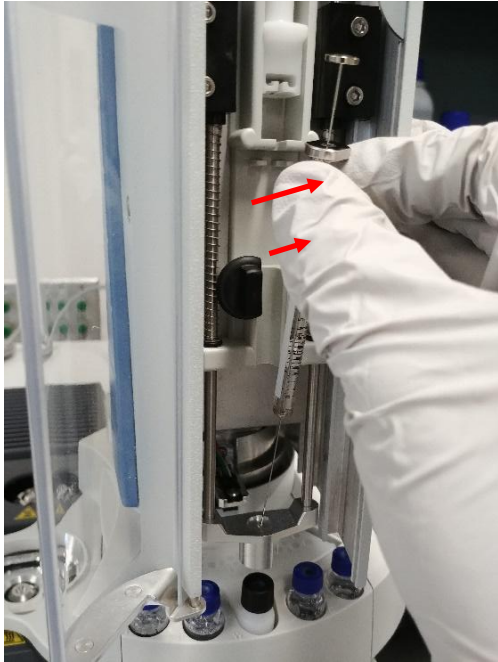


2.) Unlock syringe by turning black half circle clockwise.

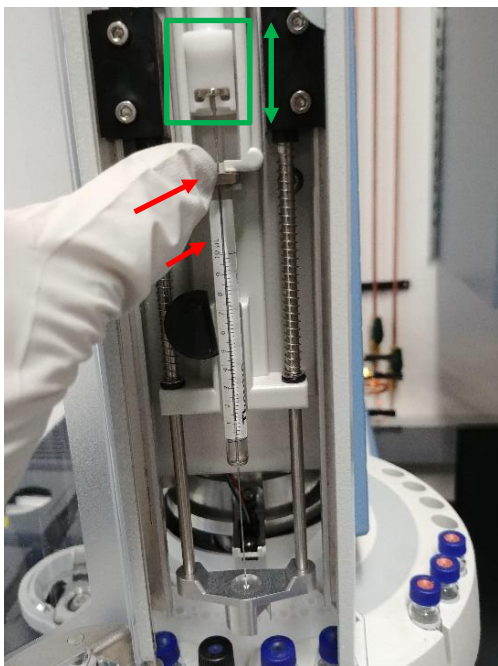




3.) Remove syringe. Grip syringe by top of barrel, and pull out and up gently. Make sure the plunger comes out with barrel, and it does not get caught in the plunger head guide. Likewise, make sure that needle comes out, and it does not get caught by the vial capture device.

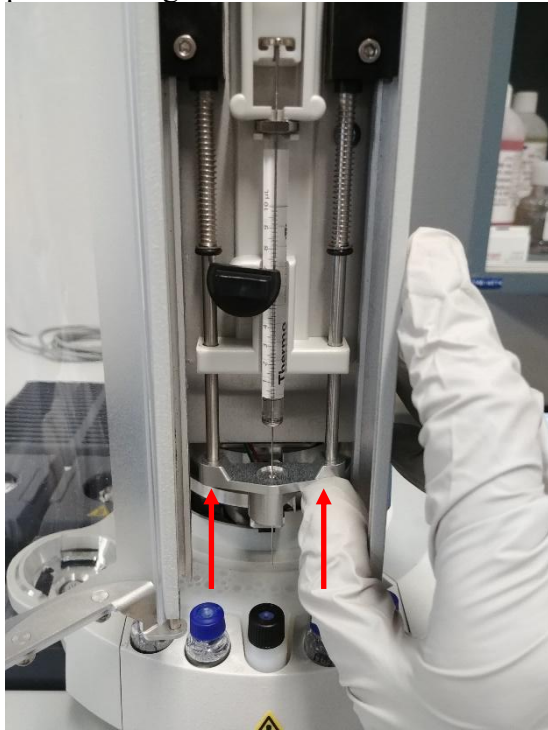


4.) Replace syringe. Slide needle through hole of vial capture device. Line up plunger in plunger head guide, sliding guide up or down by hand as needed. Push back into original position by pushing top of barrel.



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

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



## **Selection of compressed gas and regulators for gas chromatography**

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

If choosing different gases from above, verify that N<sub>2</sub> and H<sub>2</sub> have purity 99.999% or higher. They should have maximum of 1 ppm each for H<sub>2</sub>O, O<sub>2</sub>, and THC

Air should have maximum of 1 ppm for THC. Maximum of 1 ppm for H<sub>2</sub>O 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<sub>2</sub>O. Store in autoclaved culture bottle with bung (to prevent volatilization). Solution is stable indefinitely at 4°C.

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

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

Record the actual masses added. Bring to volume with ddH<sub>2</sub>O.

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

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

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

4.) Prepare wash samples in autosampler vials. To a 2-mL vial, add 100 μL formic acid, 400 μL ddH<sub>2</sub>O, and 500 μL of methanol.

5.) Prepare blank samples autosampler vials. To a 2-mL vial, add 500 μL ddH<sub>2</sub>O 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  $\mu\text{L}$  supernatant, 50  $\mu\text{L}$  internal standard, 100  $\mu\text{L}$  formic acid, and 500  $\mu\text{L}$  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.

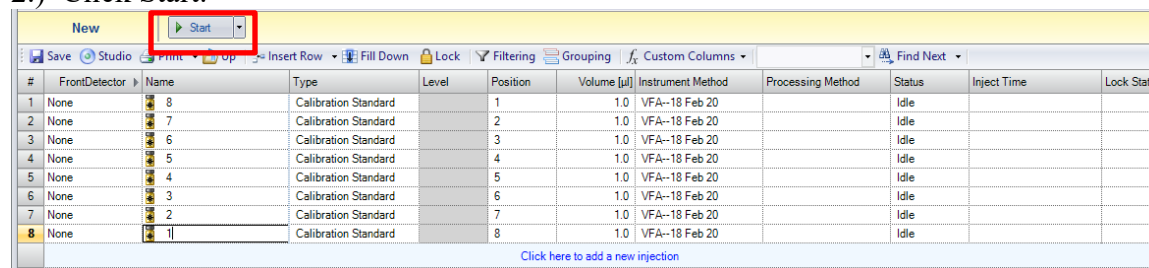
3.) Set up autosampler according to *Setting up autosampler* in *Operation of gas chromatograph* procedure. Fill wash vial A with 2 mL of ddH<sub>2</sub>O and 2 mL methanol (1:1 ratio). Fill vial C with 4 mL ddH<sub>2</sub>O and vial D with 4 mL methanol.

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.

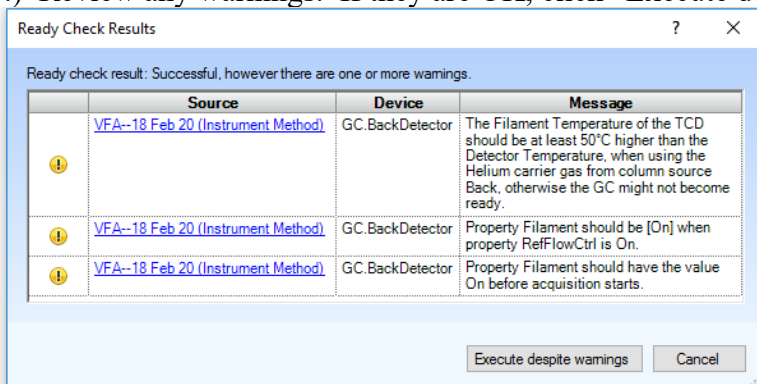
2.) Click Start.



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

Click here to add a new injection

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

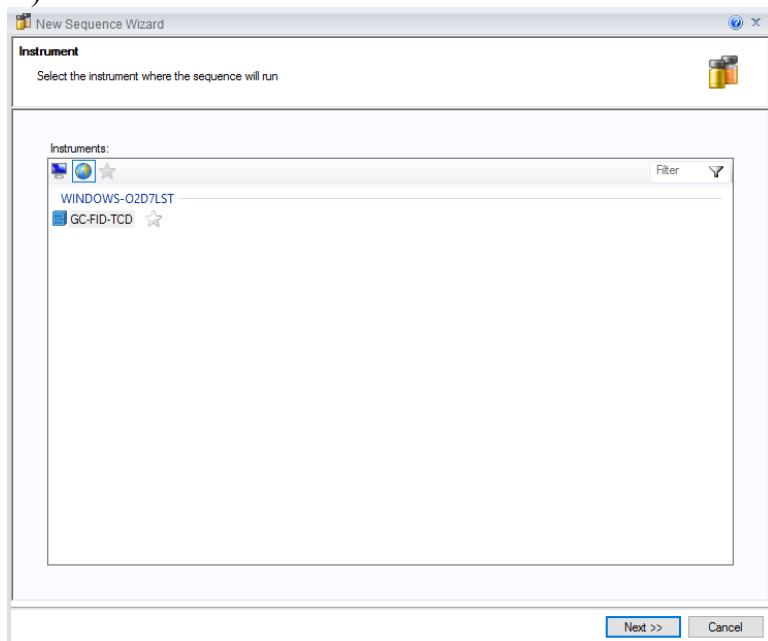
1.) Place the instrument back into standby mode according to *Placing back into standby mode*.

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

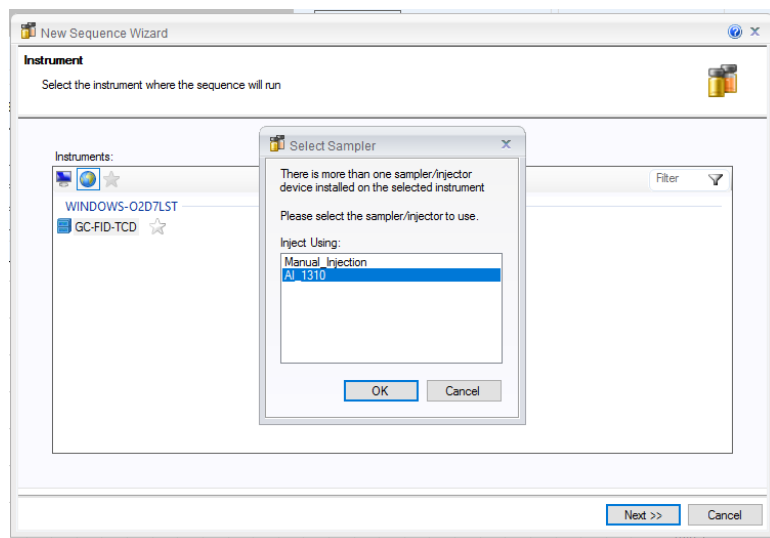
### Setting up a sequence

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

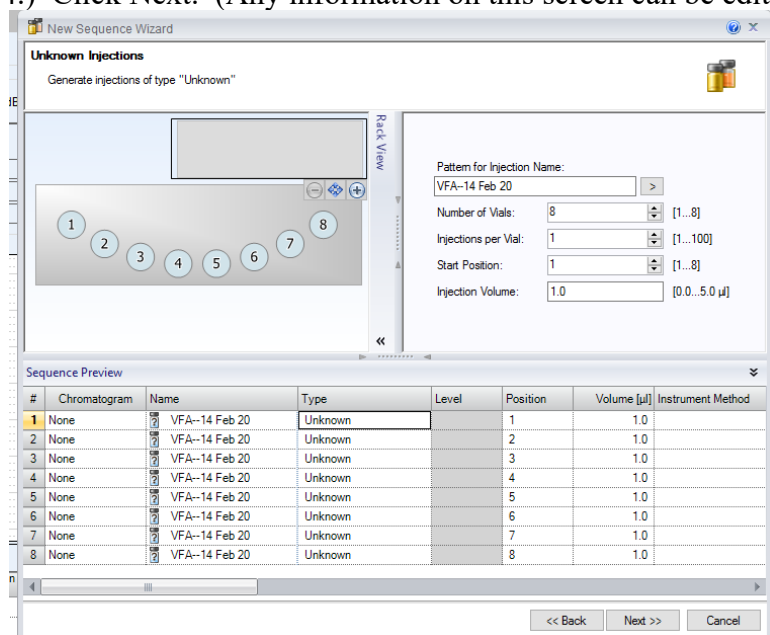
2.) Choose GC-FID-TCD.



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*.

New Sequence Wizard

**Methods & Reporting**  
Specify methods and reporting preferences

Method Selection

Instrument Method:

Processing Method:

Defaults

Report Template:    
☐ Save only a link to the selected Report Template

View Settings:    
☐ Save only a link to the selected View Settings

Channel:

<< Back **Next >>** Cancel

## 6.) Choose Automatically.

New Sequence Wizard

**Features**  
Specify sequence features

Update Visible Views for MS Data

☒ Automatically ☐ On-demand

<< Back **Next >>** Cancel

## 7.) Enter comments (optional).



New Sequence Wizard

**General Sequence Settings**

Add a detailed comment for your new sequence

Comment:

<< Back Finish Cancel

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

Save Sequence

Look in: Instrument Sequence

Name	Type	Date Modified	Comment
Fatty Acid_1	Sequence	2/13/2020 3:18:47 PM -08:00	
Fatty Acid_std	Sequence	2/14/2020 3:46:15 PM -08:00	
Methanol_1	Sequence	2/14/2020 3:30:18 PM -08:00	
Standby--14 Feb 20	Sequence	2/14/2020 3:31:45 PM -08:00	
test-Bo 14 Feb 20	Sequence	2/14/2020 4:32:38 PM -08:00	test
test-tjh 18 Feb 20	Sequence	2/18/2020 8:24:37 AM -08:00	test
VFA-tjh--14 Feb 20	Sequence	2/14/2020 8:55:37 AM -08:00	
VFA-tjh--14 Feb 20 b	Sequence	2/14/2020 12:42:23 PM -08:00	
Zhao et al. VFA-14 Feb 20	Sequence	2/14/2020 1:41:45 PM -08:00	

Object name: VFA standards--18 Feb 20

Channel:

Object type: Sequence

Save Cancel

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

New

Start

Save

Studio

Print

Up

Insert Row

Fill Down

Lock

Filtering

Grouping

Custom Columns

Find Next

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

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

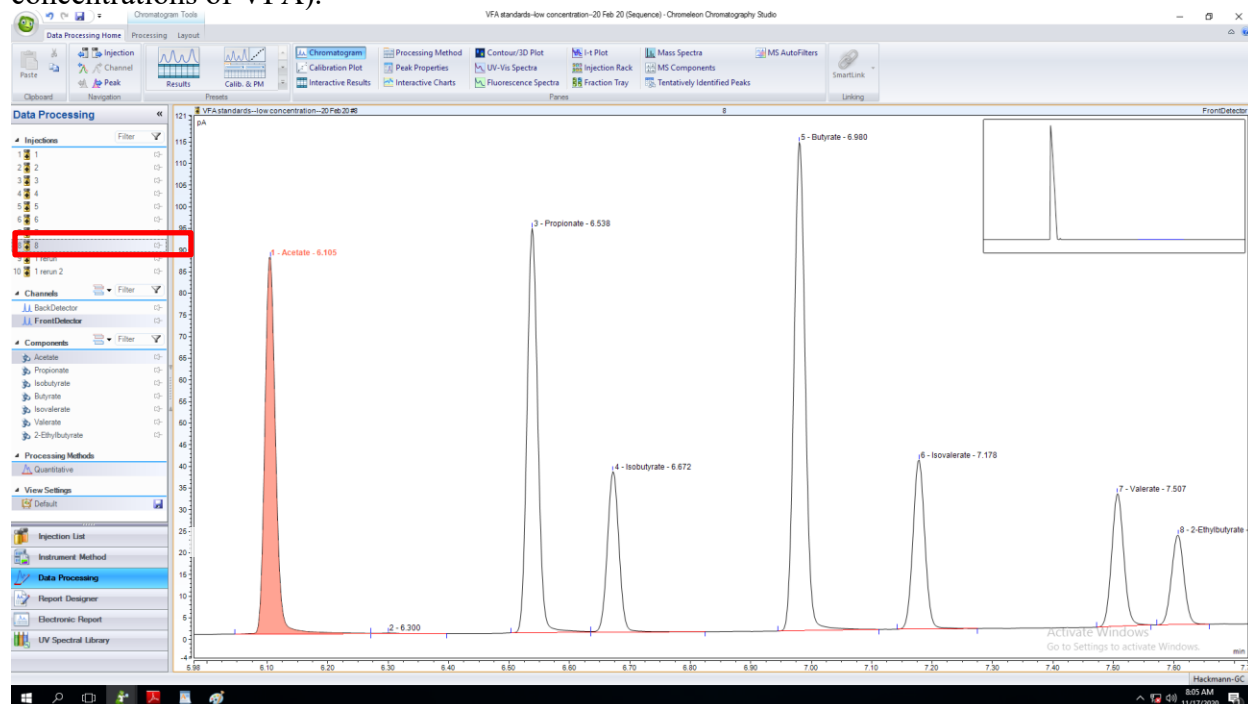
## Constructing a standard curve and quantifying VFA concentrations

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

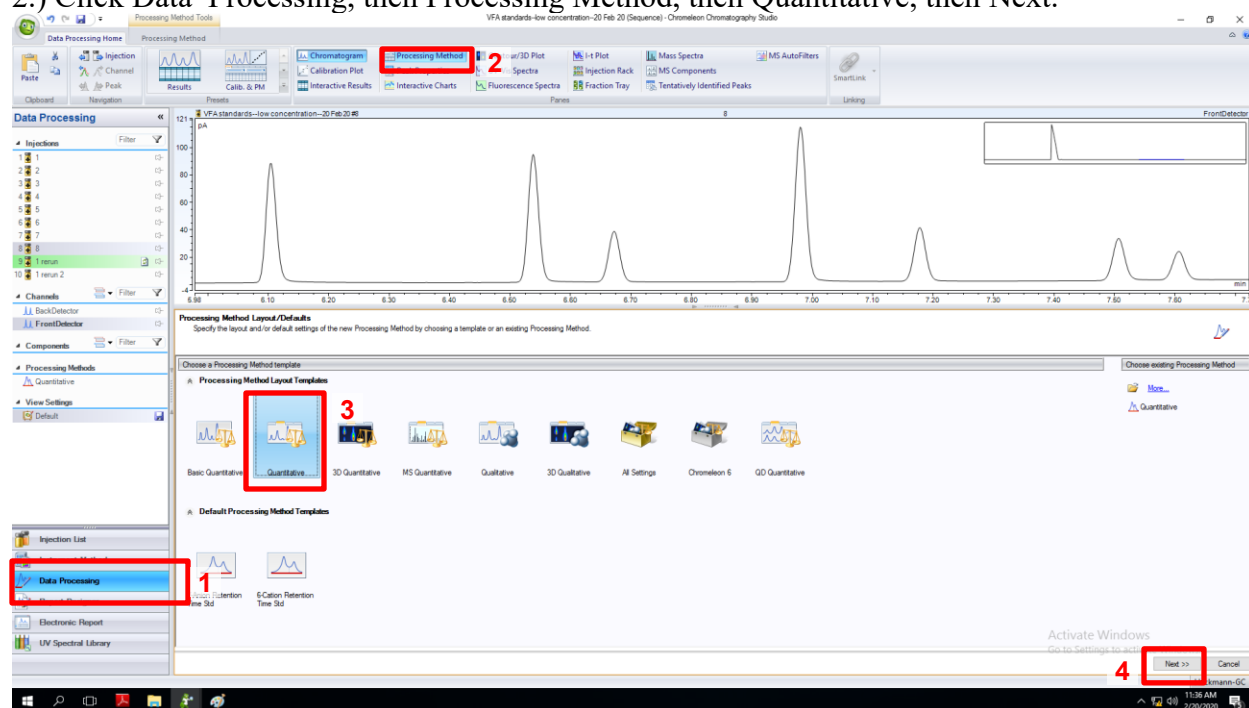
The screenshot shows the Chromeleon 7 software interface. The 'Data' window on the left lists various instrument data files. The 'Studio' window on the right displays a table of calibration standards and a chromatogram plot.

#	Name	Type	Level	Position	Volume [μl]	Instrument Method	Processing Method	Status	Inject Time
1	1	Calibration Standard	1	1	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 9:37:16 AM
2	2	Calibration Standard	2	2	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 9:51:34 AM
3	3	Calibration Standard	3	3	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:06:00 AM
4	4	Calibration Standard	4	4	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:20:31 AM
5	5	Calibration Standard	5	5	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:35:01 AM
6	6	Calibration Standard	6	6	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 10:49:34 AM
7	7	Calibration Standard	7	7	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:04:05 AM
8	8	Calibration Standard	8	8	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:18:39 AM
9	1 run	Calibration Standard	2	2	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:33:12 AM
10	1 run 2	Calibration Standard	2	2	1.0	VFA-low split ratio-2	Quantitative	Finished	2/20/2020 11:47:44 AM

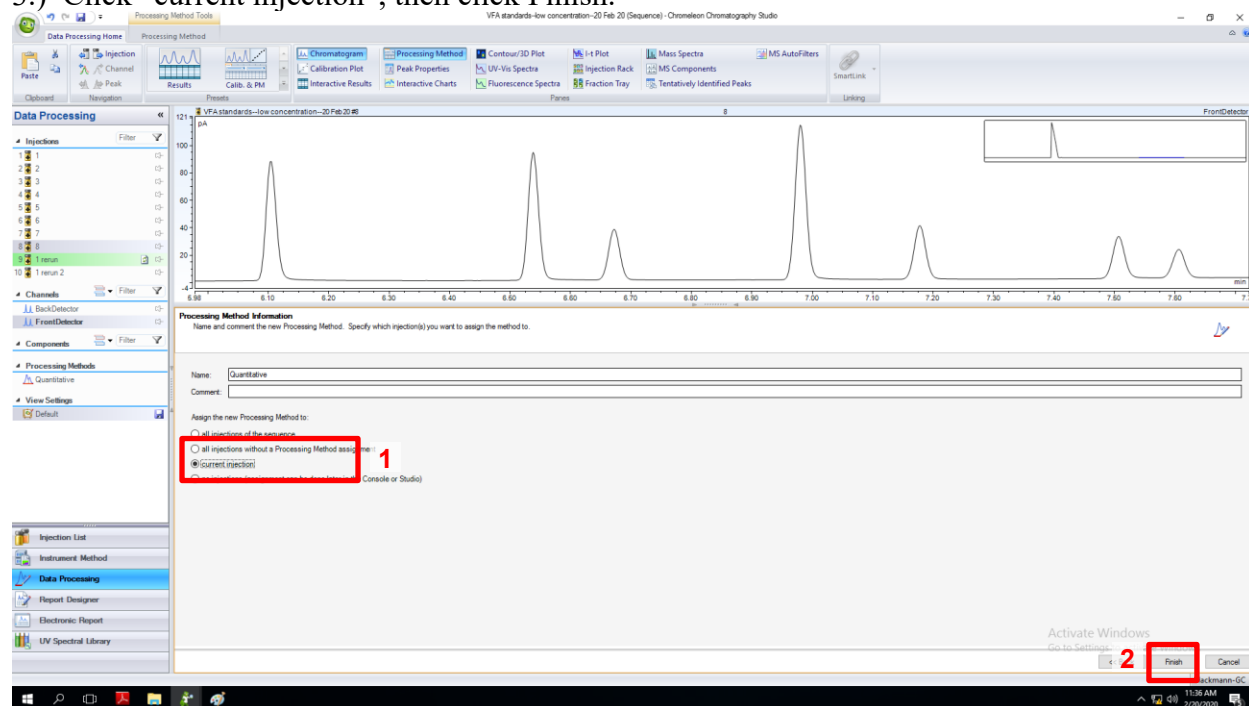
2.) In the window that appears, click on an injection of the last standard (the one with the highest concentrations of VFA).



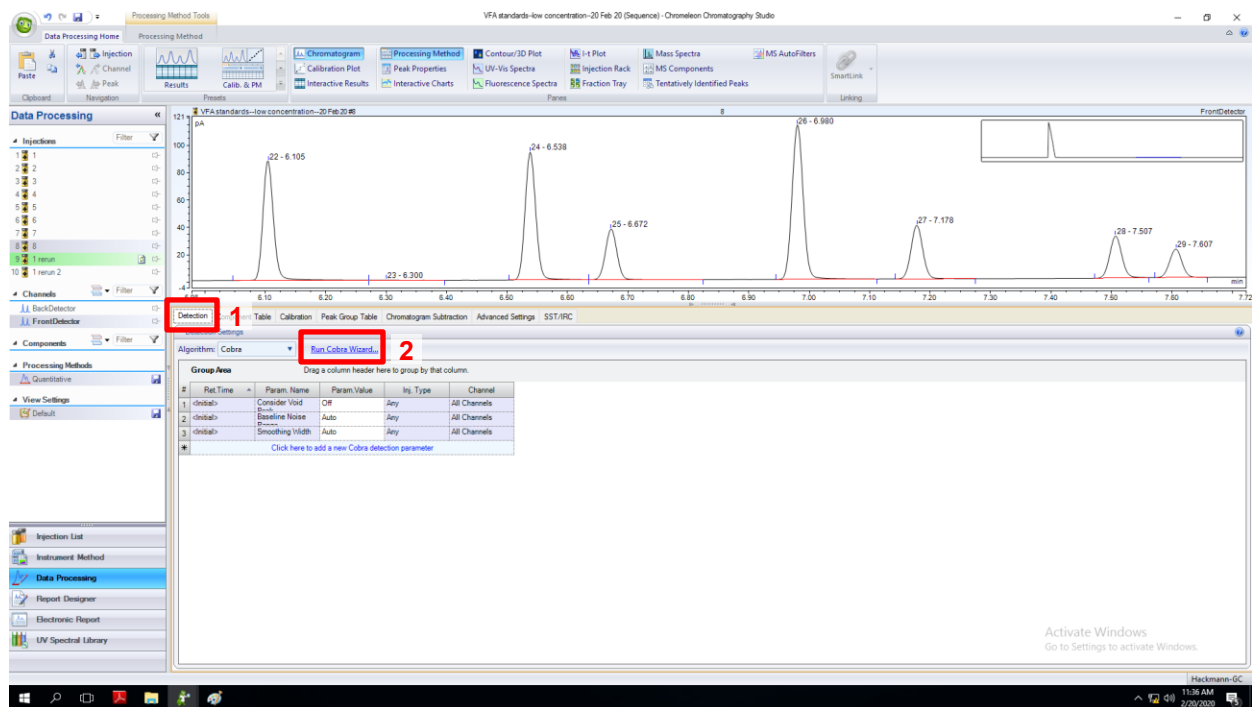
2.) Click Data Processing, then Processing Method, then Quantitative, then Next.



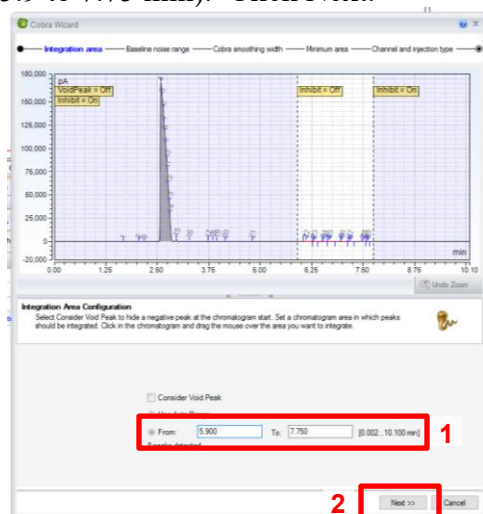
3.) Click “current injection”, then click Finish.



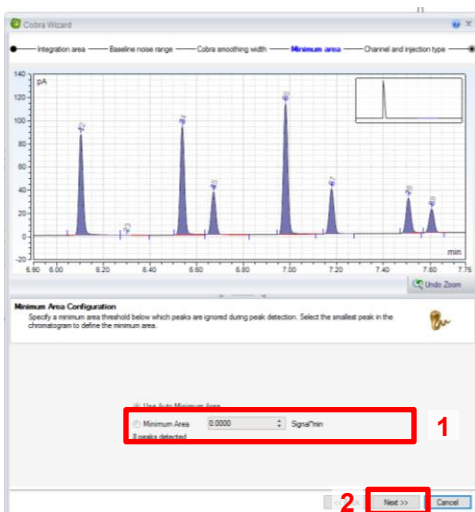
4.) Click Detection, then Click Run Cobra Wizard.



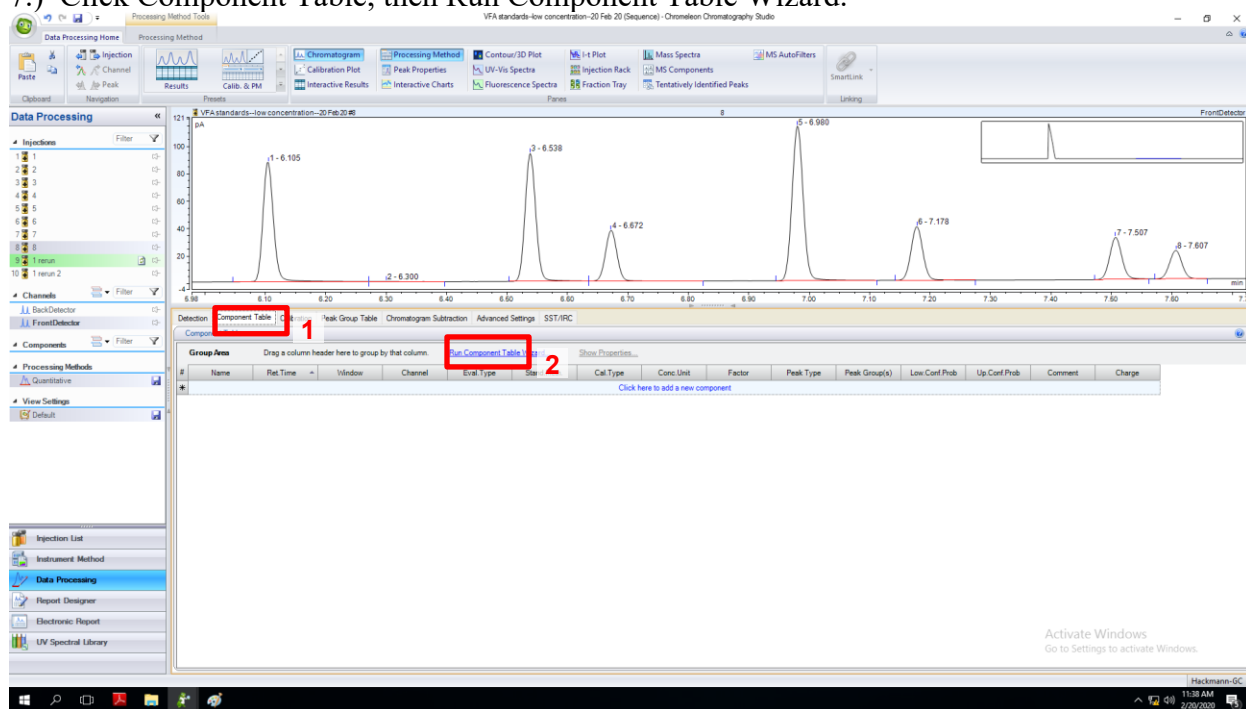
5.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next.



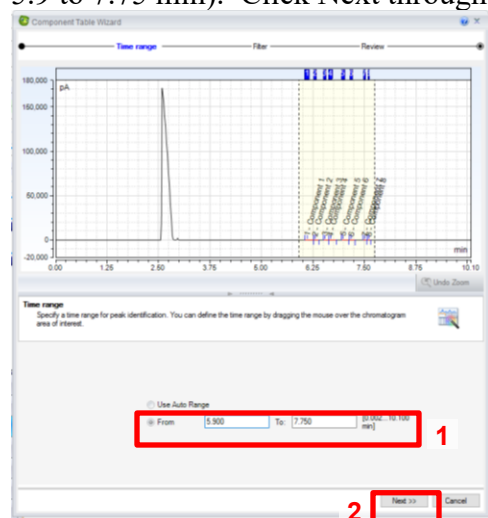
6.) Continue to click Next until reaching window below. If unexpected, small peaks are detected, enter a value of Minimum Area to suppress these small peaks. Otherwise, click Next, then click Finish.



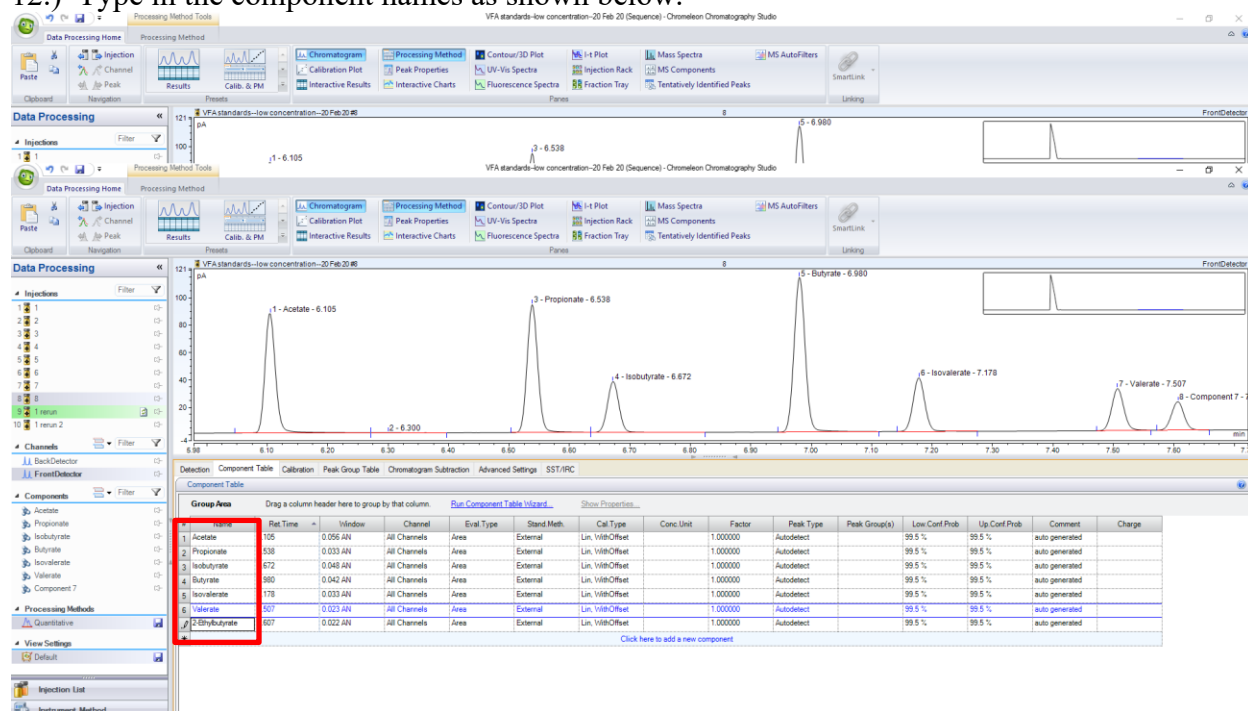
7.) Click Component Table, then Run Component Table Wizard.



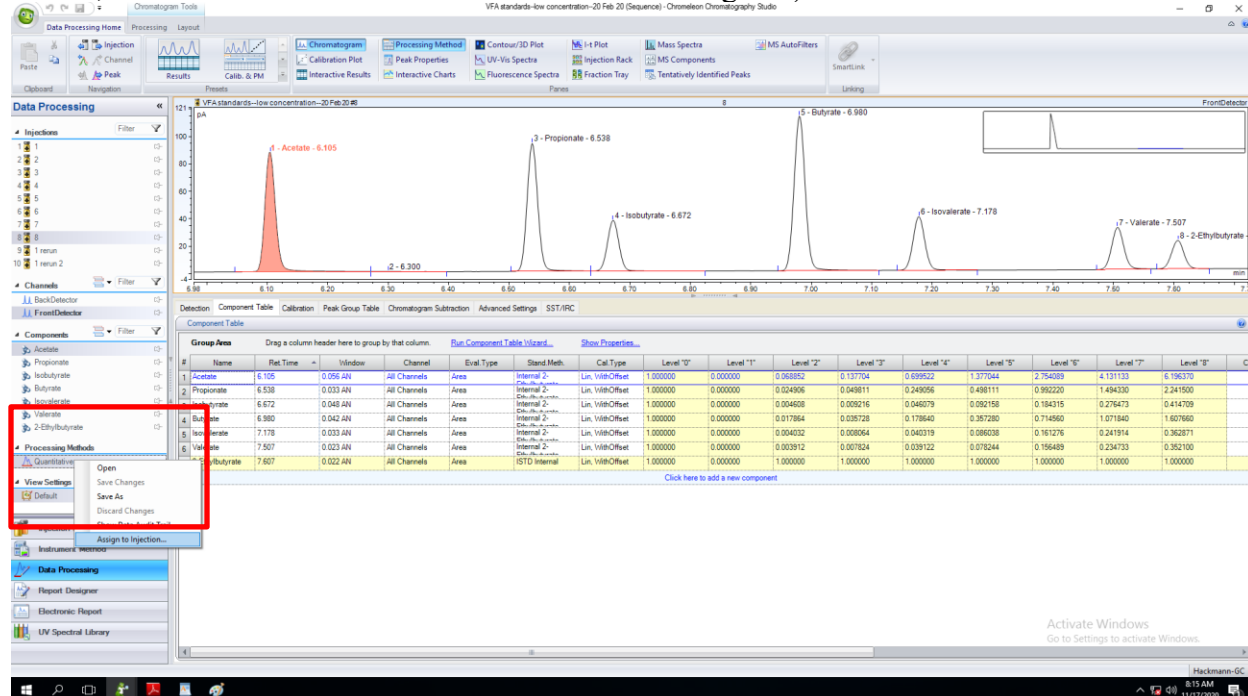
11.) In the window that appears, type in the time range where peaks appear (from approximately 5.9 to 7.75 min). Click Next through all subsequent windows, then click Finish.



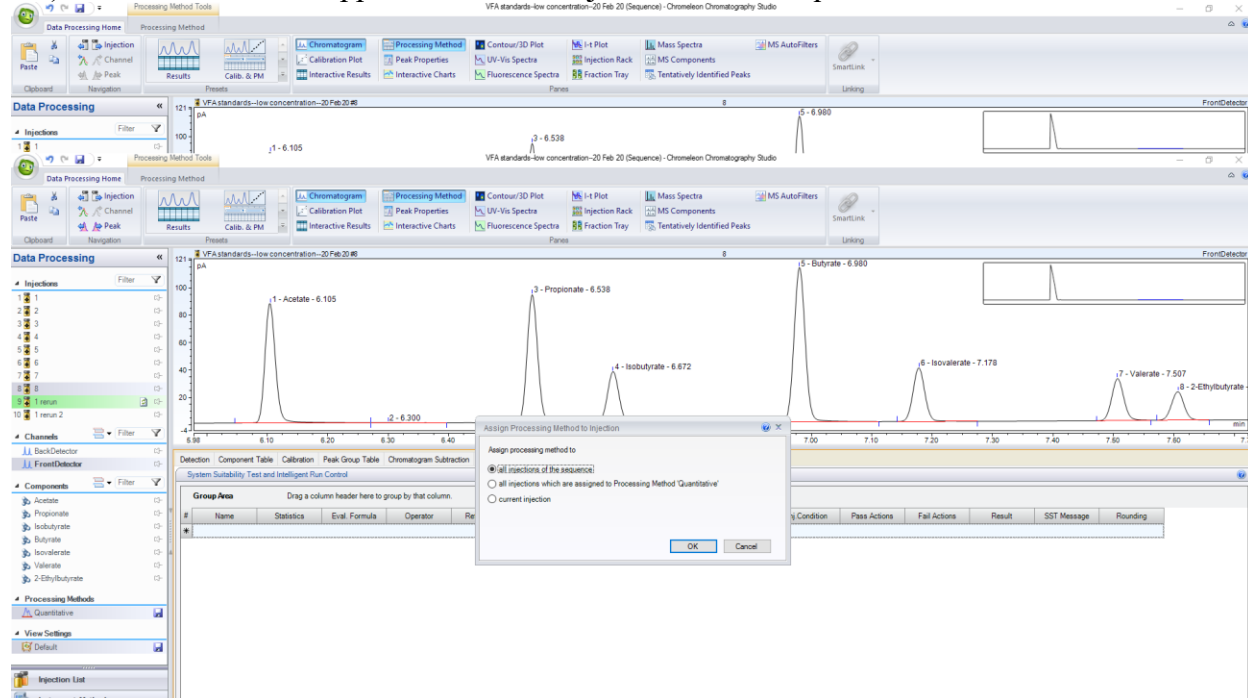
12.) Type in the component names as shown below.



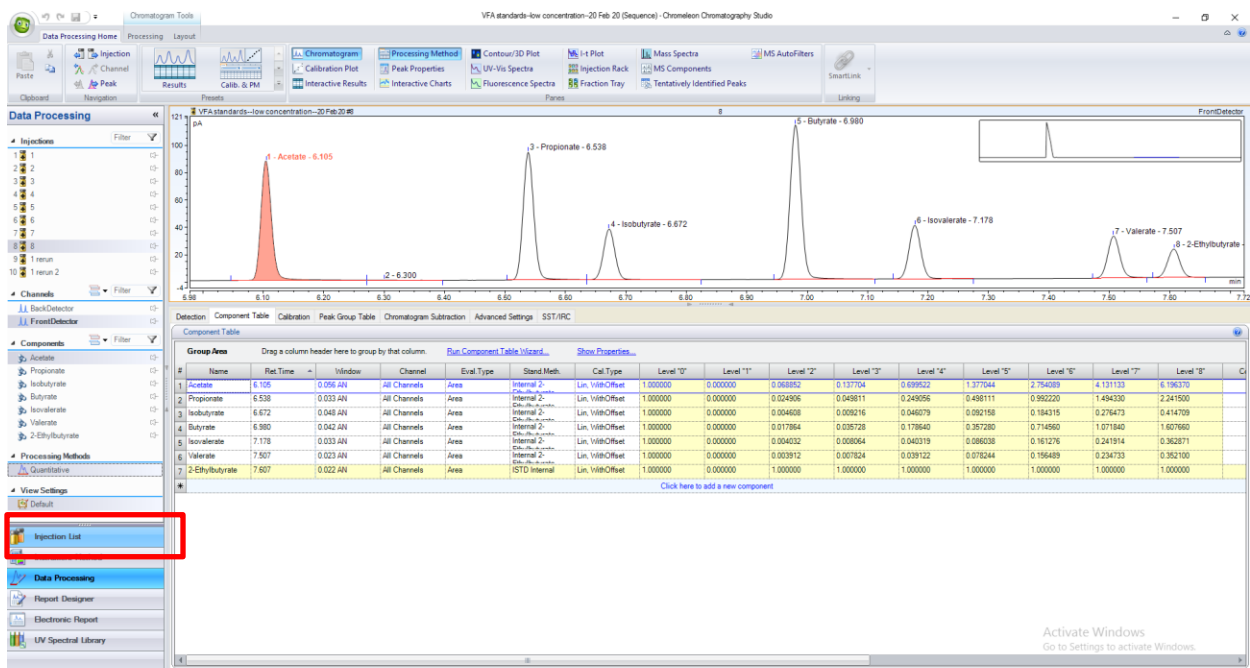
13.) Right click Processing Method, then click Assign to Injection. (If Processing Method is not visible, scroll down to the bottom of the Data Processing tab.)



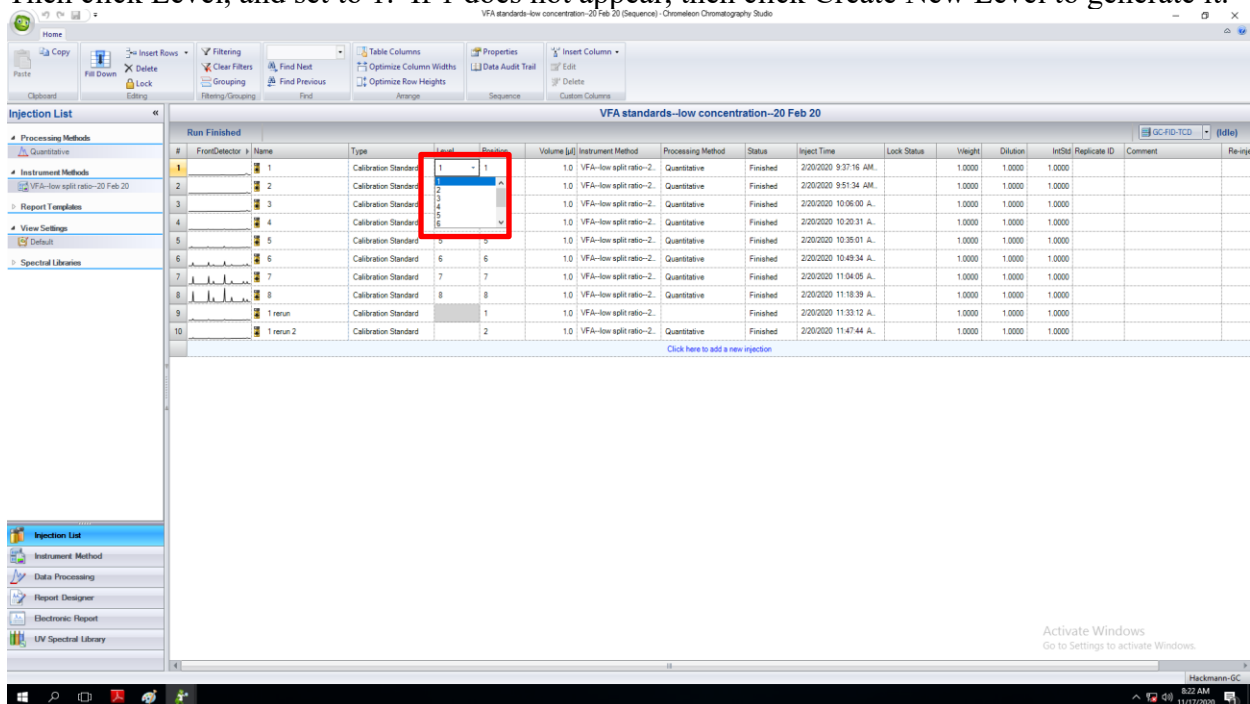
14.) In the window that appears, click “all injections of the sequence”, then click OK.



15.) Click Injection List.

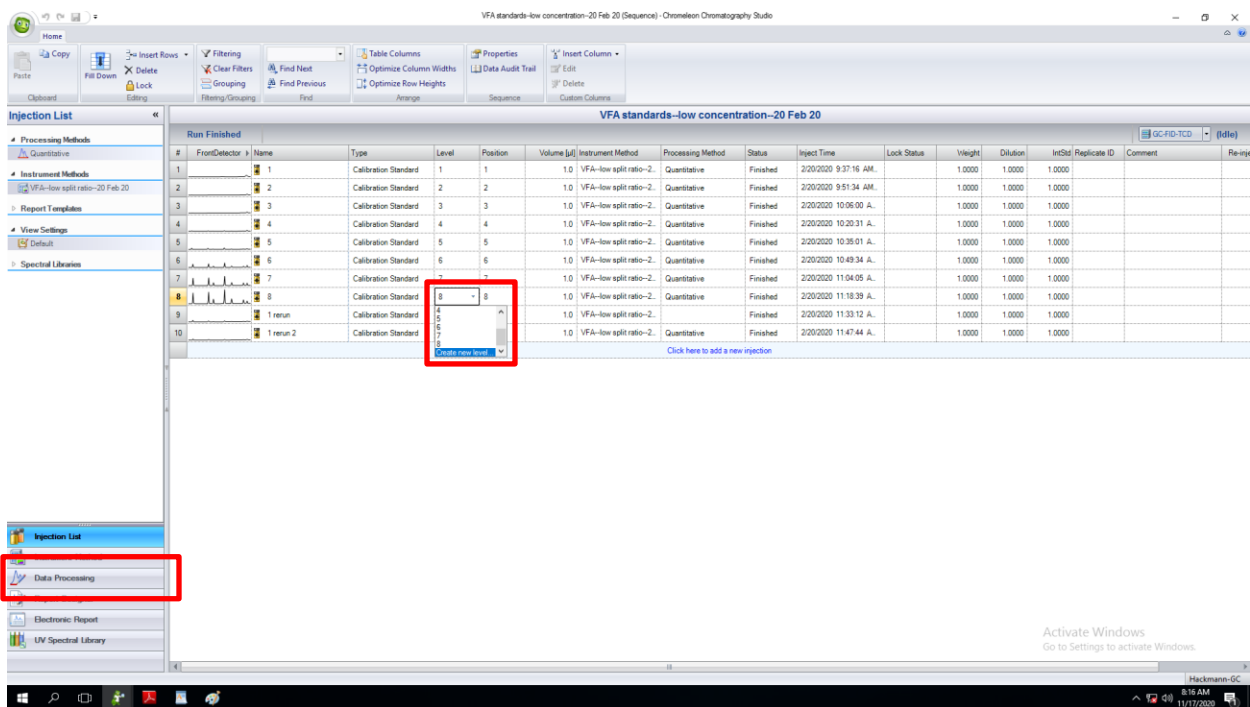


16.) Click on the injection for the first standard (the one with the lowest concentrations of VFA). Then click Level, and set to 1. If 1 does not appear, then click Create New Level to generate it.

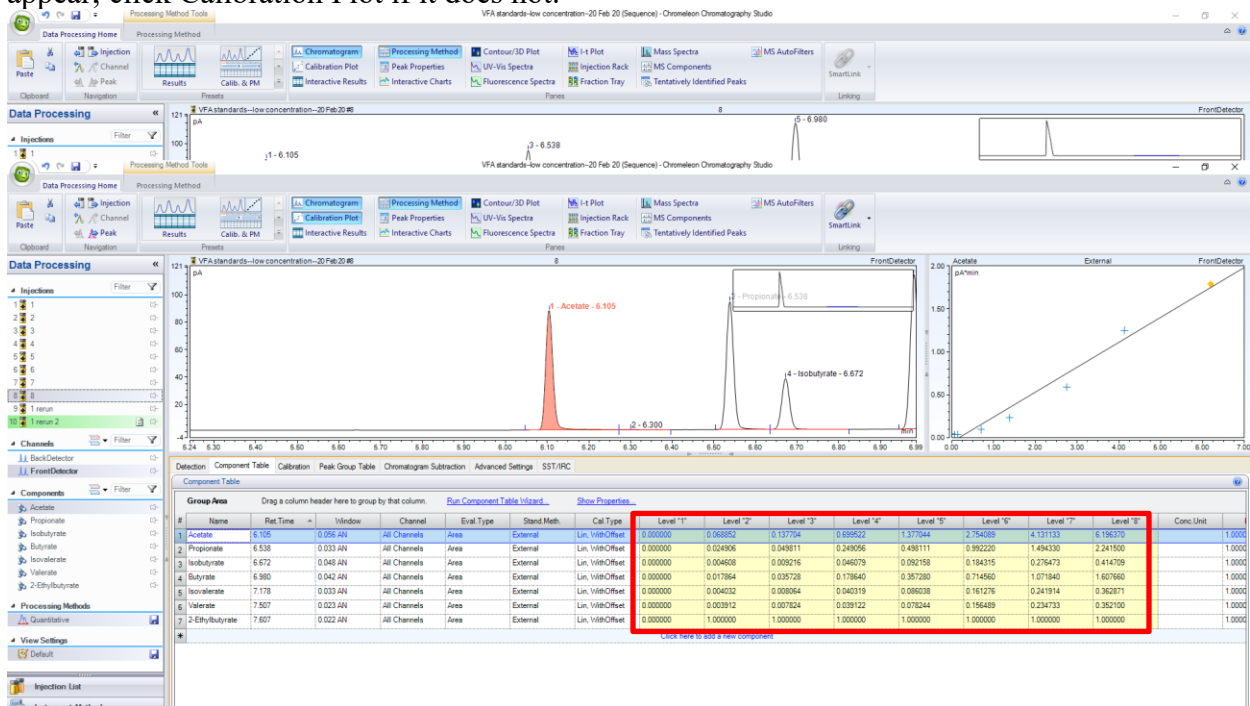


17.) Repeat for the second standard, but set Level to 2. Repeat similarly for all other standards. Go back by clicking Data Processing.

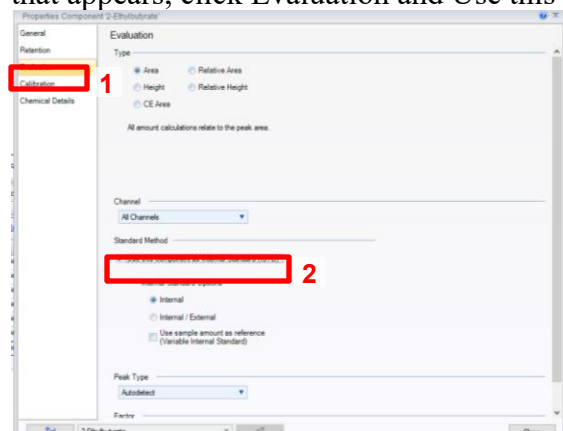




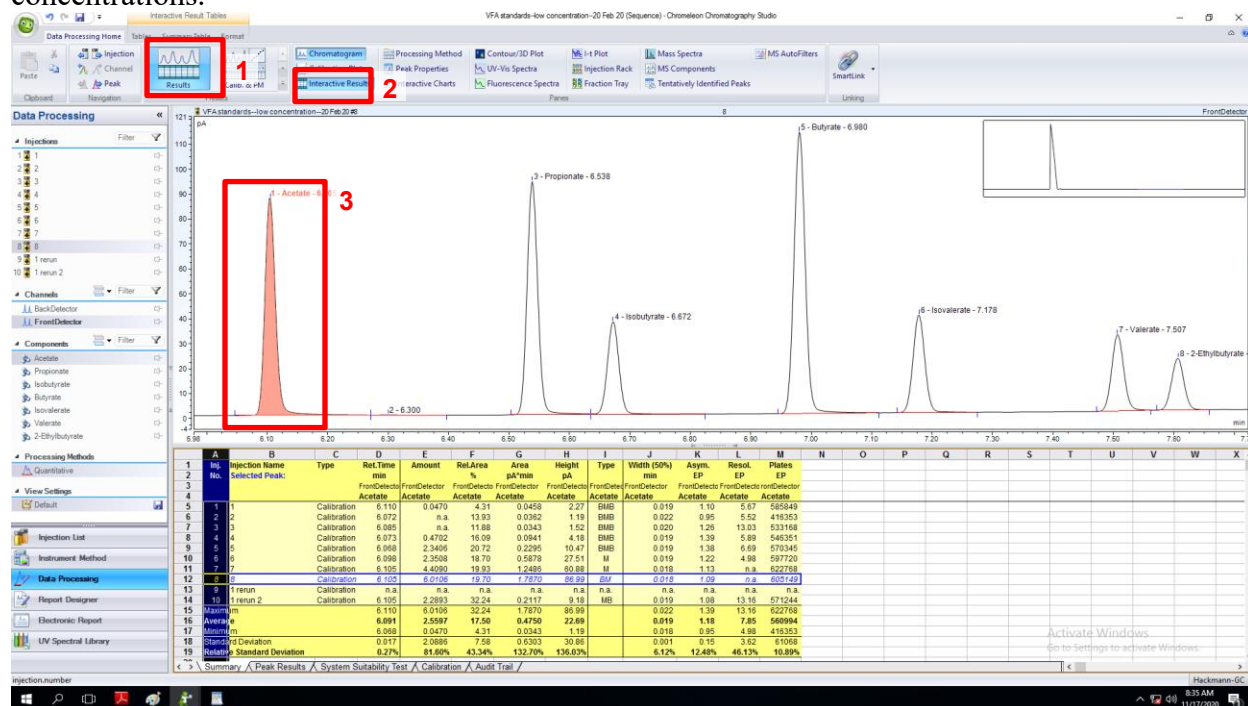
18.) Under Level, type in concentrations of standards (in mmol/L). Standard curve should appear; click Calibration Plot if it does not.



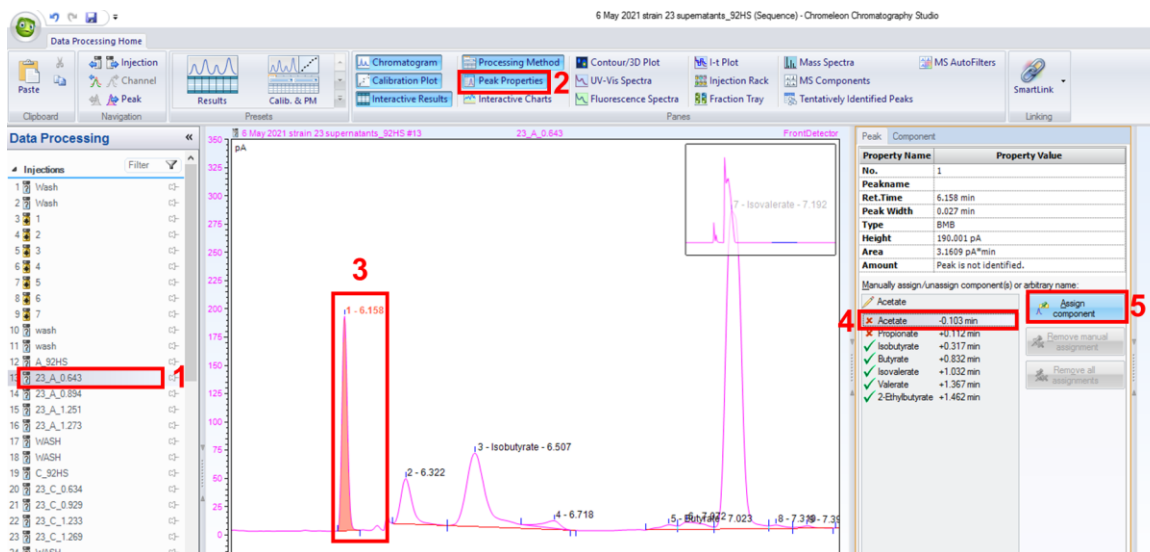
20.) Optional: set 2-ethylbutyrate as an internal standard by double clicking on it. In window that appears, click Evaluation and Use this component as Internal Standard (ISTD).



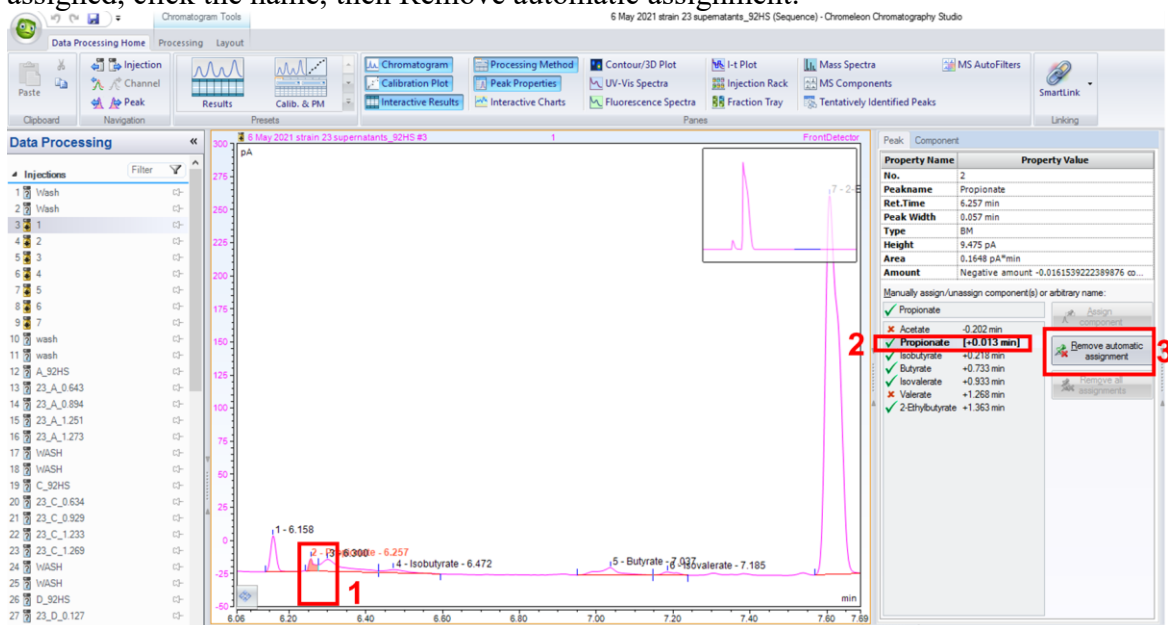
21.) Click Results, then Interactive Results, and then the acetate peak in the chromatogram. The concentration of VFA is reported under Amount. Click the remaining peaks to see their concentrations.



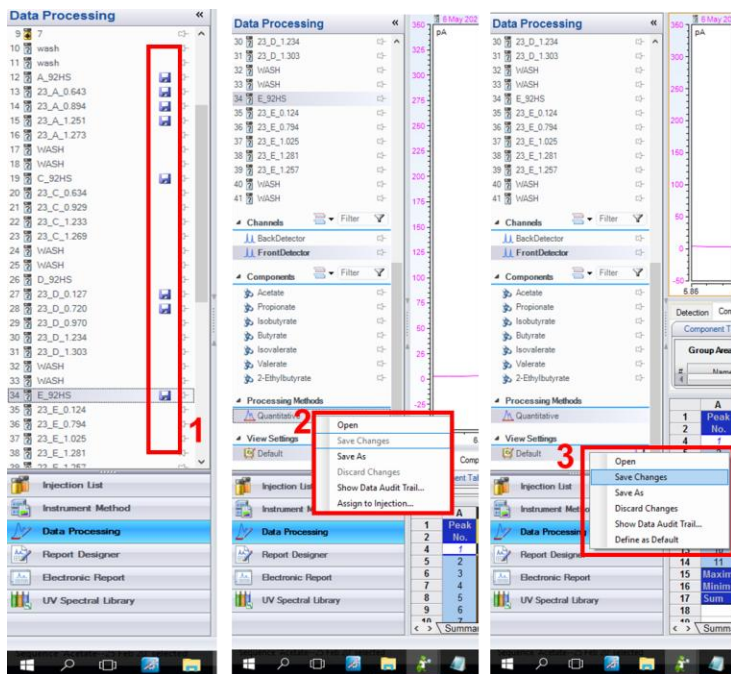
22.) Optional: Rename any peaks that are misidentified by the software. Click the sample, Peak Properties, the misidentified peak, the correct name, then Assignment component.



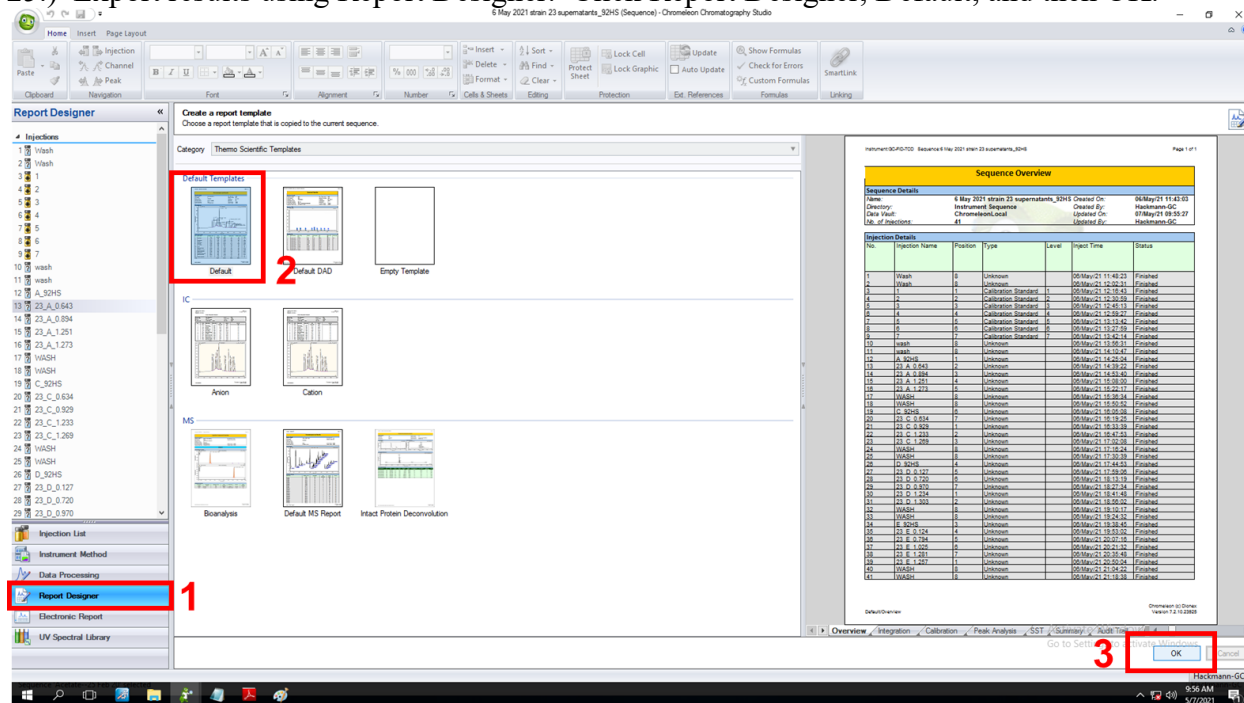
22.) Optional: Remove any peak assignments that are spurious. Click the peak that is wrongly assigned, click the name, then Remove automatic assignment.



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 FrontDetector in Channels; click Acetate in Components; then click Summary.



Report Designer

1 Injections

2 Channels

3 Components

4 Report Templates

5 Summary

Summary							
Sequence Details							
Name		6 May 2021 strain 23 supernatants_52HS		Created On		06/May/21 11:43:03	
Directory		Instrument Sequence		Created By		Hackmann.GC	
Data Vault		ChromleonLocal		Updated On		07/May/21 09:55:27	
No. of Injections		41		Updated By		Hackmann.GC	
By Component							
No.	Injection Name	Ret. Time min	Area pA*min	Height pA	Amount	Rel. Area %	Peak Type
1	Wash	6.073	0.954	44.956	0.052	33.60	BM
2	Wash	6.078	0.280	16.394	n.a.	23.58	BMB
3	1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	2	6.077	2.606	152.005	1.291	13.33	BMB
5	3	6.075	12.264	687.438	8.471	24.97	BMB
6	4	6.147	25.646	1439.320	18.421	26.15	BMB
7	5	6.062	49.406	2324.202	36.085	29.40	BMB
8	6	6.055	72.208	3091.417	53.038	30.11	BMB
9	7	6.055	83.593	3473.729	61.503	30.20	BMB
10	wash	6.085	0.271	15.342	n.a.	19.80	BMB
11	wash	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	A_52HS	6.082	0.719	41.749	n.a.	1.97	BMB
13	23 A_0.634	6.158	3.161	190.001	1.703	7.87	BMB
14	23 A_0.894	6.155	4.346	266.065	2.584	10.38	BMB
15	23 A_1.251	6.083	6.427	369.465	4.131	14.91	BMB
16	23 A_1.273	6.082	7.682	432.976	5.065	16.68	BMB
17	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
18	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
19	C_52HS	6.088	0.699	39.899	n.a.	2.12	BMB
20	23 C_0.634	6.087	2.856	164.573	1.476	7.82	BMB
21	23 C_0.929	6.030	0.025	0.971	n.a.	0.06	BMB
22	23 C_1.233	6.083	6.868	389.040	4.459	16.81	BMB
23	23 C_1.269	6.082	7.712	428.590	5.087	18.48	BMB
24	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
26	D_52HS	6.087	0.788	43.680	n.a.	2.45	BM
27	23 D_0.127	6.087	1.514	84.660	0.479	4.65	BM
28	23 D_0.720	6.088	3.021	170.356	1.600	8.48	BM
29	23 D_0.970	6.088	4.014	225.171	2.338	10.92	BM
30	23 D_1.234	6.085	5.917	336.114	3.752	14.85	BMB
31	23 D_1.303	6.087	7.041	397.043	4.588	17.03	BMB
32	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
33	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

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.

Export

1 Apply to

2 Destination Path

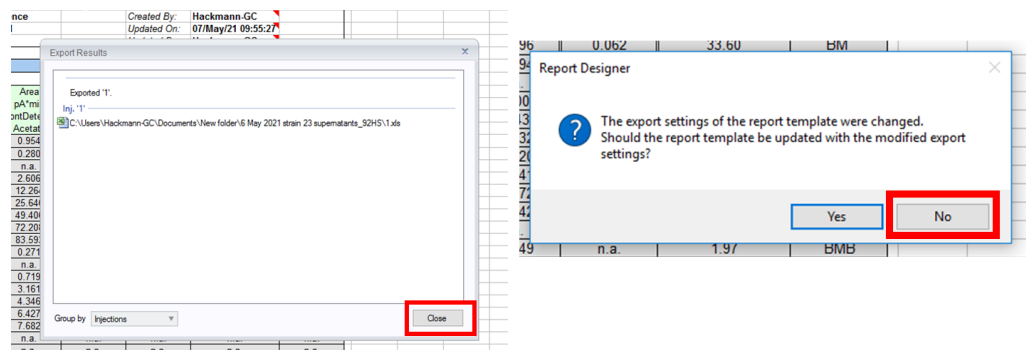
3 Export Formats and Filenames

4 OK

5 OK

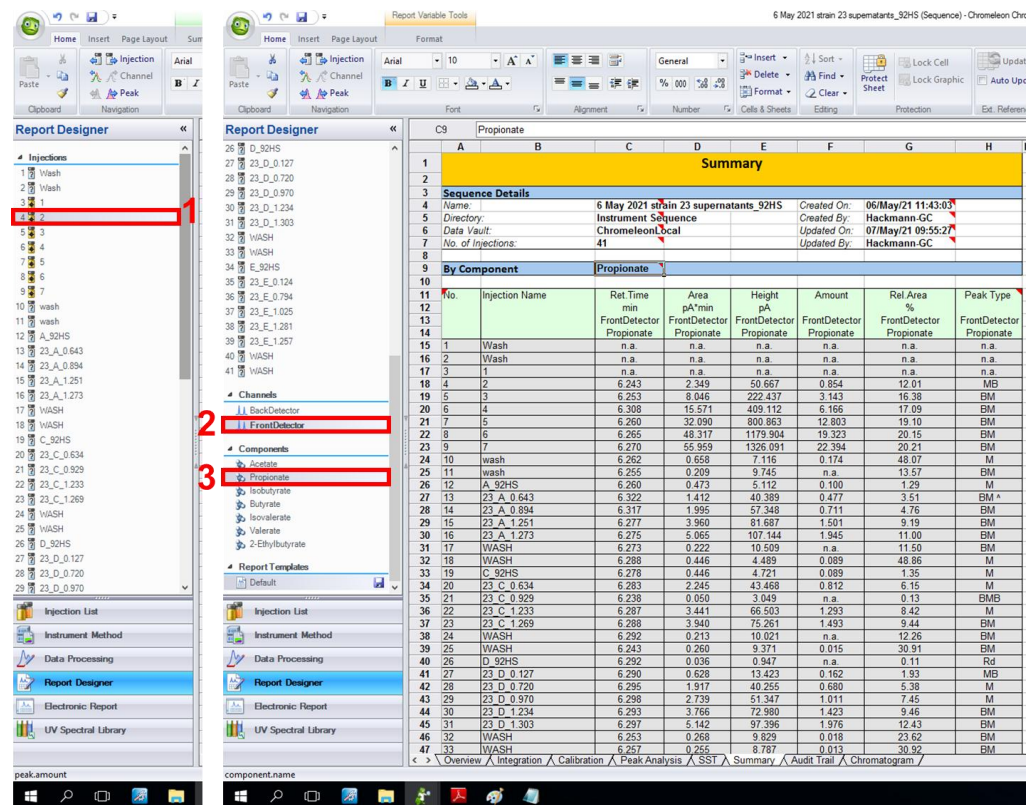
6 OK

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.

Report Variable Tools

6 May 2021 strain 23 supernatant\_92HS (Sequence) - Chromatogram Chromatography Study

Home Insert Page Layout Format

Font Arial 10

General % 000 23

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

Protection Lock Sheet Lock Graphic Auto Update Edit References

Report Designer

CS Acetate

7 No. of Injections 41 Updated By Hackmann GC

8 By Component Acetate

9

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Channels

BackDetector

FrontDetector

Components

Acetate

Propionate

Isobutyrate

Butyrate

Isovalerate

Valerate

2-Ethylbutyrate

Report Templates

Default

Injection List

Instrument Method

Data Processing

Report Designer

Electronic Report

UV Spectral Library

component.name

Overview Integration Calibration Peak Analysis SST Summary Audit Trail Chromatogram

Activate Windows  
Go to Settings to activate Windows.

Save

Cancel

Save Modifications

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

Modified Objects Type Status

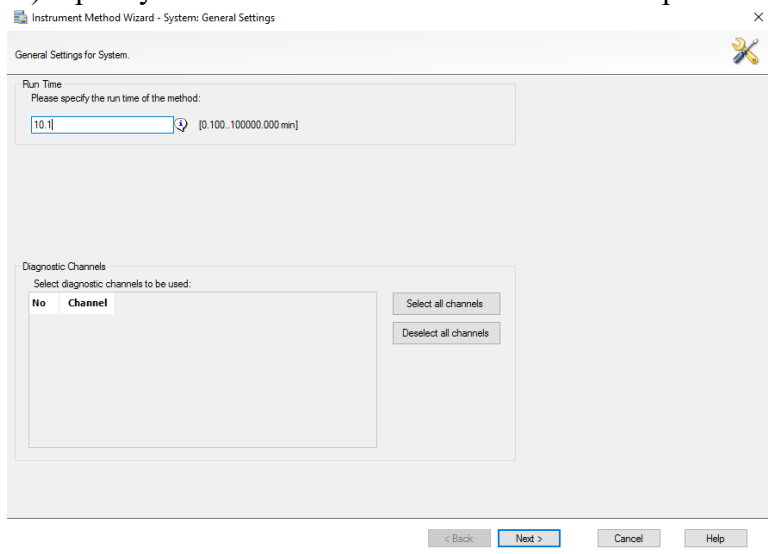
6 May 2021 st. Sequence

Default Report Template Created

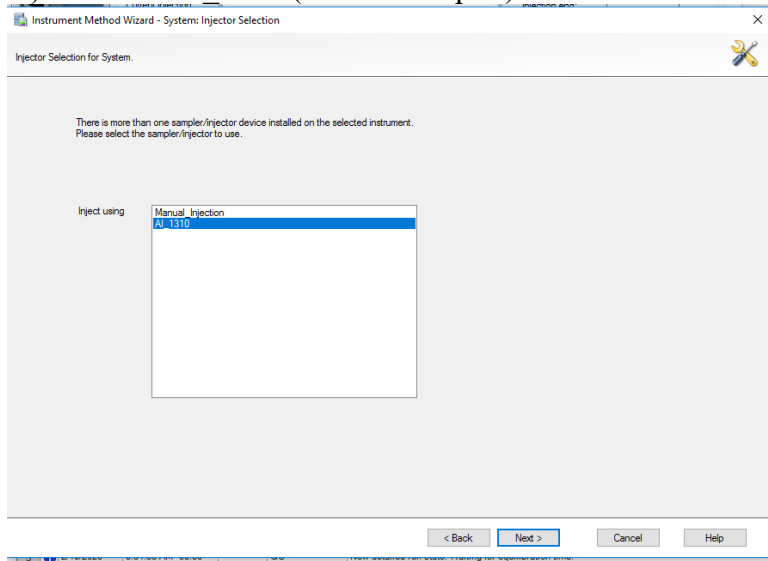
No.	Injection Name	Ret Time min	Area pA*min	Height pA	Amount FrontDetector Acetate	Rel Area % FrontDetector Acetate	Peak Type FrontDetector Acetate
1	Wash	6.073					
2	Wash	6.078					
3		n.a.					
4		6.077					
5		6.075					
6		6.147					
7		6.062					
8		6.055					
9		6.055					
10	wash	6.085					
11	wash	n.a.					
12	A 92HS	6.082					
13	23 A 0.043	6.158					
14	23 A 0.084	6.155					
15	23 A 1.251	6.083					
16	23 A 1.273	6.082					
17	WASH	n.a.					
18	WASH	n.a.					
19	C 92HS	6.088					
20	23 C 0.634	6.087					
21	23 C 0.929	6.030					
22	23 C 1.233	6.083					
23	23 C 1.269	6.082					
24	WASH	n.a.					
25	WASH	n.a.					
26	D 92HS	6.087	0.788	43.680	n.a.	2.45	BM
27	23 D 0.127	6.087	1.514	84.660	0.479	4.65	BM
28	23 D 0.720	6.088	3.921	170.356	1.560	8.48	BM
29	23 D 0.970	6.088	4.914	225.171	2.338	10.92	BMB
30	23 D 1.234	6.085	5.917	336.114	3.752	14.85	BMB
31	23 D 1.363	6.087	7.041	397.043	4.588	17.03	BMB
32	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
33	WASH	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
34	E 92HS	6.115	0.781	44.164	n.a.	2.30	BMBH
35	23 E 0.124	6.055	0.032	1.148	n.a.	0.09	BMB
36	23 E 0.784	6.088	3.409	192.269	1.888	9.12	BMB
37	23 E 1.025	6.090	4.560	255.607	2.744	11.87	BMB
38	23 E 1.281	6.087	6.536	362.310	4.213	16.41	BMB
39	23 E 1.257	6.087	7.211	402.886	4.714	17.10	BMB

## 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.

Instrument Method Wizard - AI\_1310: Inject Options

Inject Options for AI\_1310.

Sampling Parameters

Draw speed: Slow

Fill strokes: 3

Air volume: 1.00

Sample depth: Bottom

Injection Parameters

☐ Cold needle injection

GC type: TRACE\_1300\_1310

Pre injection delay time: 0.0

Post injection delay time: 4.0

< Back Next > Cancel Help

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

Instrument Method Wizard - AI\_1310: Sampler Wash Program

Sampler Wash Program for AI\_1310.

Pre Injection Washing Parameters

Pre injection washing cycles: 8

Pre injection washing vial(s): A

Sample Washing Parameters

Sample wash cycles: 0

Post Injection Washing Parameters

Post injection washing cycles: 3

Post injection washing vial(s): C\_D

< Back Next > Cancel Help

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

will shut off carrier gas to the back column.

Instrument Method Wizard - GC (TRACE1300/1310): GC component selection

GC component selection for GC (TRACE1300/1310)

Please select the components to be used:

Available inlets: ☒ FrontInlet  
☒ BackInlet

Available columns: ☒ FrontInlet (carrier settings)  
☒ BackInlet (carrier settings)

Available detectors: ☒ FrontDetector  
☒ BackDetector

☐ Send parameters only (don't run the GC)  
☒ Specify oven parameters

Choose either current values or default values:  
☐ Use default values for all TRACE 1300 GC modules

< Back Next > Cancel Help

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

Instrument Method Wizard - GC Inlets (TRACE1300/1310): FrontInlet Flow/Pressure Options

FrontInlet Flow/Pressure Options for GC Inlets (TRACE1300/1310)

Flow(m/min)

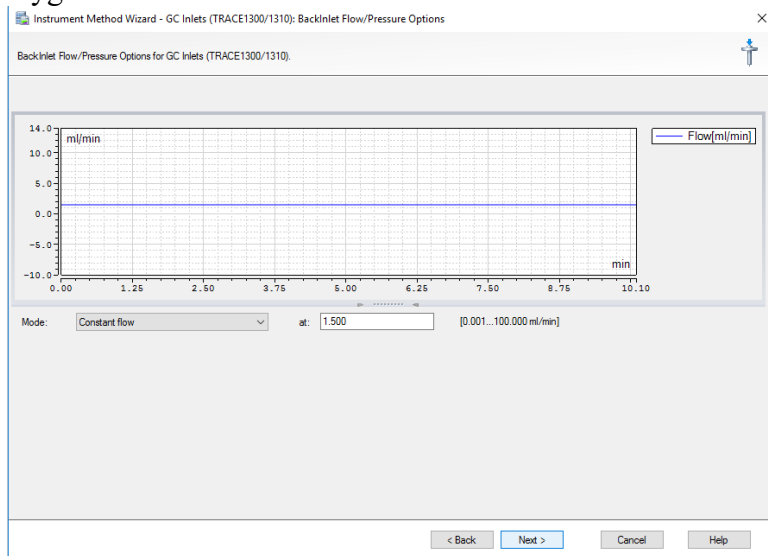
min

Mode: Constant flow at: 2.5 [0.001...100.000 ml/min]

< Back Next > Cancel Help

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.

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

BackInlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

☒ Enable temperature control

Temperature: 110 [0.400 °C]

Utilises

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

☒ Split flow control

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

Split ratio: 3

Splitless time: 1.00 [0.00...999.99 min]

☒ Purge flow control

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

☒ Constant septum purge

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

Surge pressure: 5.00 [5.00...1000.00 kPa]

Surge duration: 0.00 [0.00...999.99 min]

☐ Vacuum compensation

☐ Enable gas saver mode

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

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

< Back Next > Cancel Help

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

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

GC Oven Settings for GC (TRACE1300/1310).

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

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

Ready delay: 0.00 [0.00...99.99 min]

Mode: Ramped temperature

Temperature [°C] vs Time [min] graph showing a ramp from 20°C to 235°C over 10.10 minutes.

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

Insert new row

Delete row(s)

< Back Next > Cancel Help

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

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

Column Configuration Settings for GC (TRACE1300/1310).

FrontColumn

☒ Validate column configuration

Description:

Length:  [0.10..200.00 m]

Diameter:  [0.050..0.999 mm]

Film thickness:  [0.00..20.00 µm]

< Back   Next >   Cancel   Help

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

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

FrontDetector Options for GC Detectors (TRACE1300/1310).

Detector type: Flame Ionization Detector (FID)

Signal Settings

Data collection rate:  [1..600 Hz]

Detector Settings

☒ Detector temperature control

Detector temperature:  [0..450 °C]

☒ Flame

☒ Enable flameout retry

Ignition threshold:  [0.0..10.0 pA]

Peak width:

Gas Settings

☒ Air flow control

Air flow:  [5.0..500.0 ml/min]

☒ Makeup gas flow control

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

☒ Hydrogen flow control

Hydrogen flow:  [1.0..100.0 ml/min]

< Back   Next >   Cancel   Help

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

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

FrontDetector Time Program for GC Detectors (TRACE1300/1310).

No	Time	Timed Autozero	Timed Flame Ctrl
1	{Initial Time}	<input type="checkbox"/>	
2	0.001	<input checked="" type="checkbox"/>	

Add  
Insert  
Remove  
Sort

< Back Next > Cancel Help

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.

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

BackDetector Options for GC Detectors (TRACE1300/1310).

**Detector type:** Thermal Conductivity Detector (TCD)

Signal Settings

Data collection rate: 10 [1...300 Hz]  
Polarity: Negative

Detector Settings

☐ Detector temperature control  
Detector temperature: 50 [0...400 °C]  
Column source: Back  
☐ Enable filament  
Filament temperature: 50 [50...450 °C]  
Peak width: Standard

Gas Settings

☒ Reference gas flow control  
Reference gas flow: 1.5 [0.5...5.0 ml/min]

< Back Next > Cancel Help

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

Instrument Method Wizard - GC Detectors (TRACE1300/1310): BackDetector Time Program

BackDetector Time Program for GC Detectors (TRACE1300/1310).

No	Time	Timed Autozero	Timed Polarity
1	{Initial Time}	<input type="checkbox"/>	

Add  
Insert  
Remove  
Sort

< Back Next > Cancel Help

## 17.) Enter comments for method (optional).

Instrument Method Wizard - Completion

You now have entered all data required to create an instrument method.

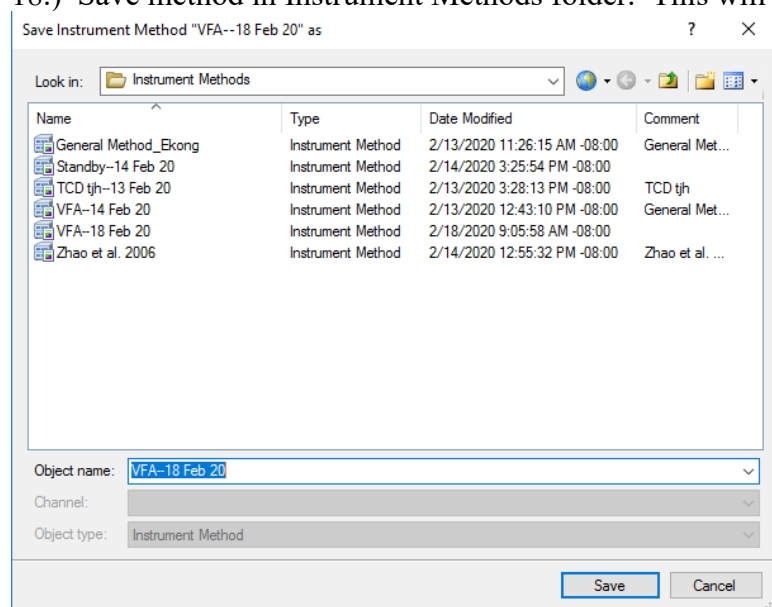
Comment

Description

Press Finish to open the instrument method in the Chromatography Studio.

< Back Finish Cancel Help

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.

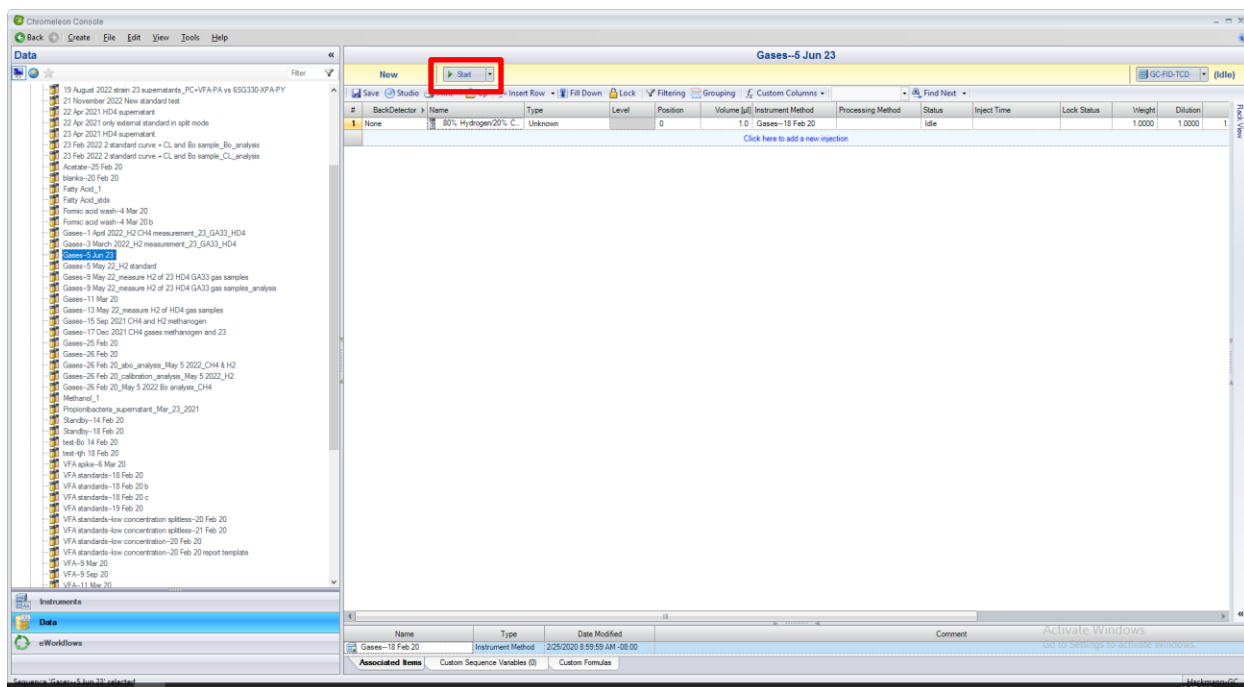
- 1.) Obtain a bacterial culture in a Balch tube (or a similar sample).
- 2.) Prepare a syringe to sample the gas. Attach a needle with a beveled tip (Hamilton 7779-01) to a 1-mL gas-tight with sample lock valve (Hamilton 81356).
- 3.) Flush the syringe and needle with CO<sub>2</sub>. Insert syringe into gassing probe dispensing CO<sub>2</sub>, 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<sub>2</sub> gas can be used as standard for H<sub>2</sub>.

### Preparing GC for analysis of samples and standards

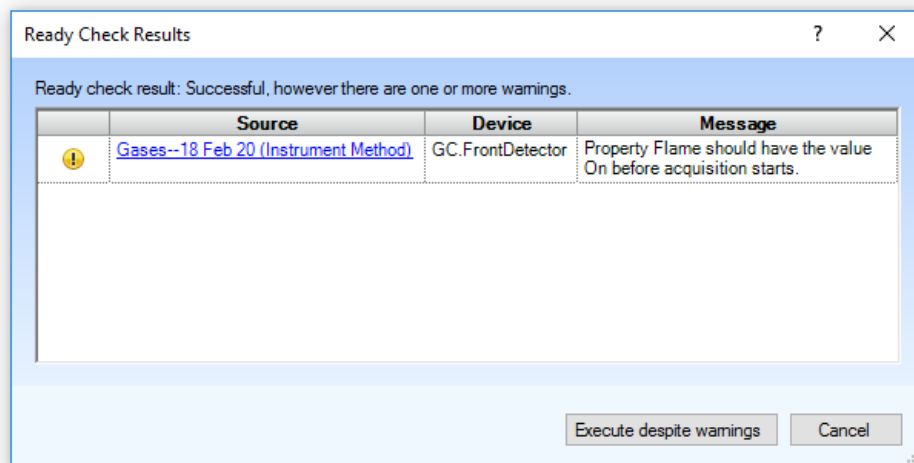
- 1.) Start GC and set up for gas analysis according to *Turning on GC and placing into standby mode*.
- 2.) Turn on TCD detector according to *Turning on detectors*.
- 3.) In Chromeloen software, create a sequence (list of samples to be analyzed). Follow *Setting up a sequence*.

### Analyzing samples and standards

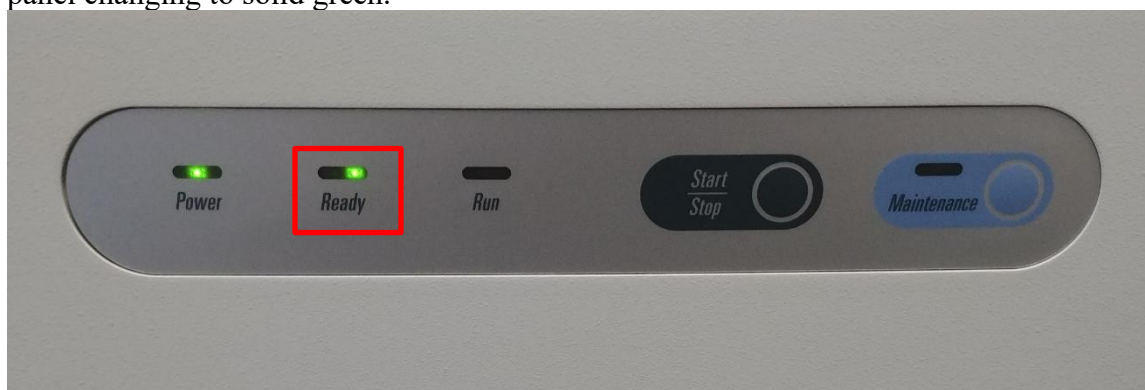
- 1.) Open the sequence.
- 2.) Click Start.



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

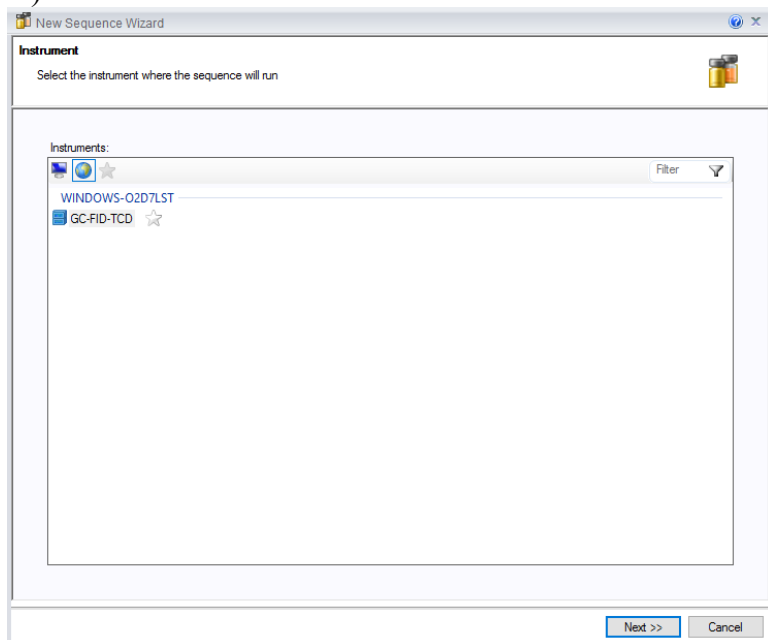
1.) Place the instrument back into standby mode according to *Placing back into standby mode*.

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

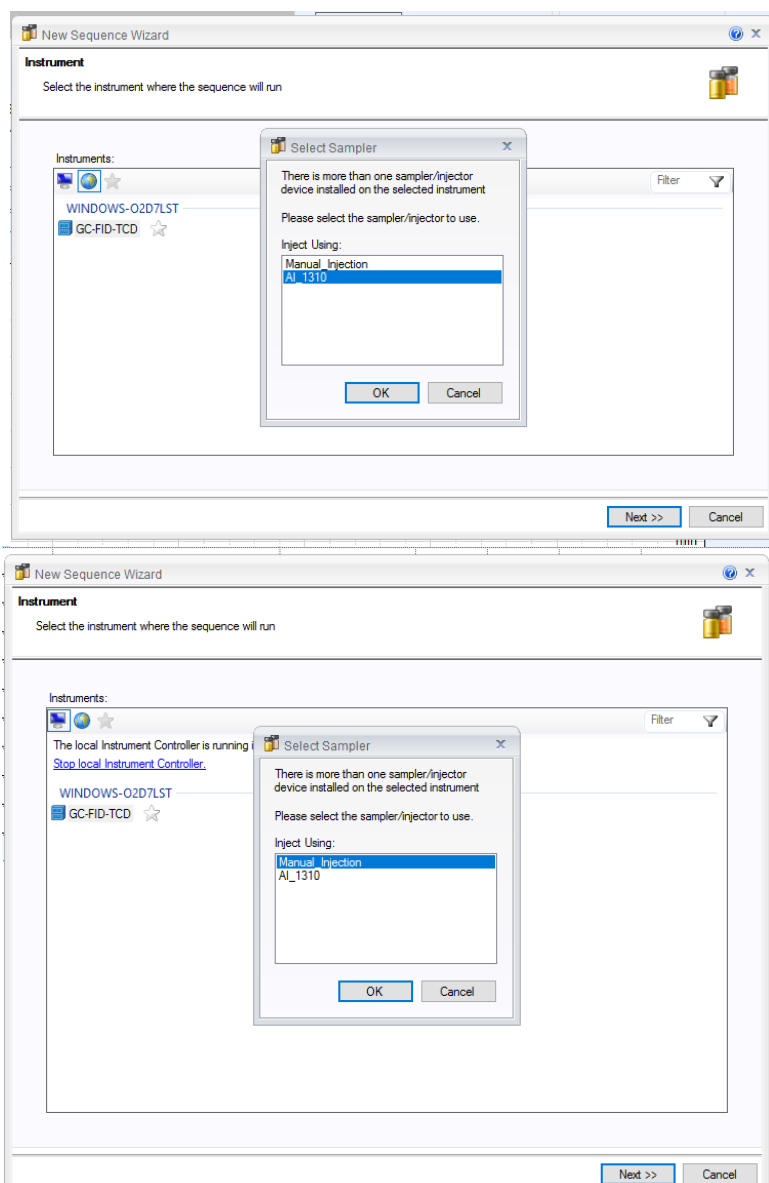
## Setting up a sequence

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.)

New Sequence Wizard

**Unknown Injections**  
Generate injections of type "Unknown"

No rack layout available.

Rack View

Pattern for Injection Name:  
Gases--5 Jun 23

Number of Vials: 1 [1...1000]

Injections per Vial: 1 [1...100]

Start Position: 0 [0...999]

Injection Volume: 1.0 [0.0...2000.0 µl]

Sequence Preview

#	Chromatogram	Name	Type	Level	Position	Volume [µl]	Instrument Method
1	None	Gases--5 Jun 23	Unknown		0	1.0	

<< Back Next >> Cancel

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

New Sequence Wizard

**Methods & Reporting**  
Specify methods and reporting preferences

Method Selection

Instrument Method: chrom://windows-o2d7st/ChromeleonLocal/Instrument Methods/Gases--18 Feb 20.instmeth Browse...

Processing Method: Browse...

Defaults

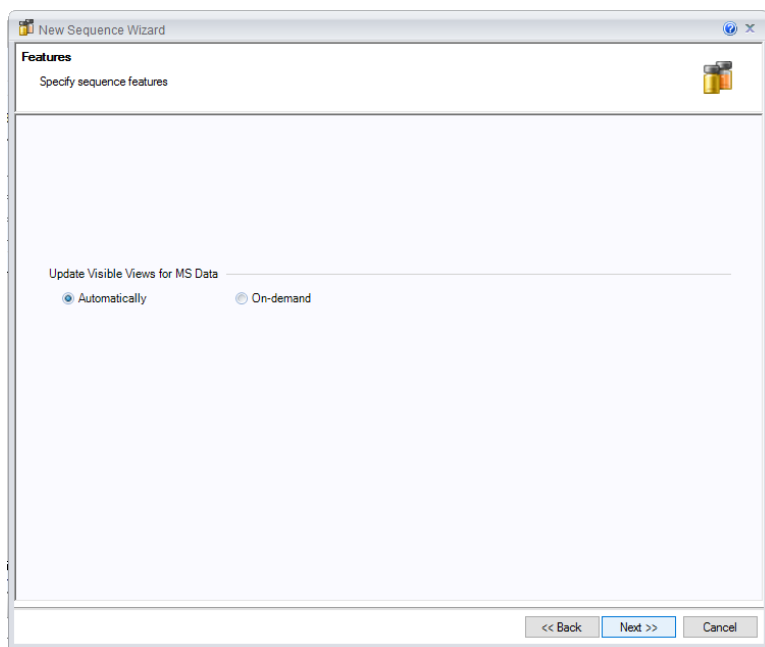
Report Template: Browse...  
☐ Save only a link to the selected Report Template

View Settings: Browse...  
☐ Save only a link to the selected View Settings

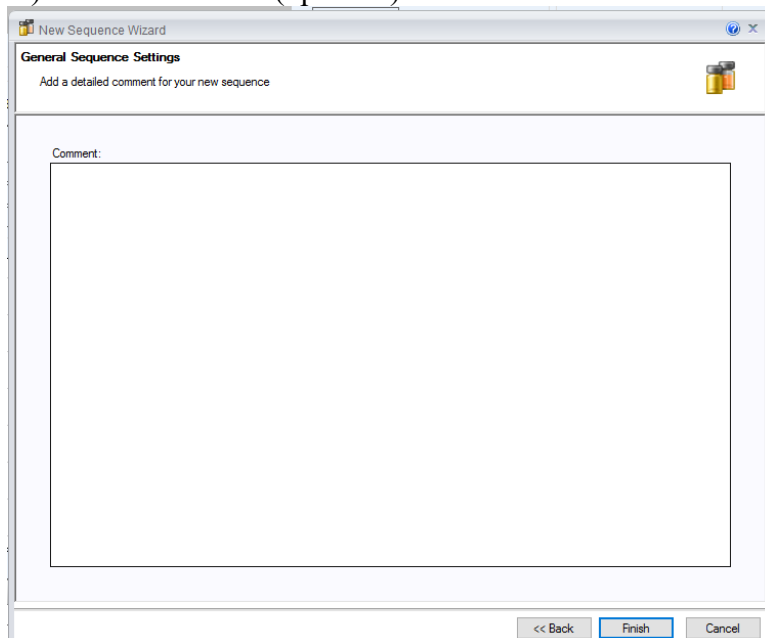
Channel: BackDetector

<< Back Next >> Cancel

6.) Choose Automatically.

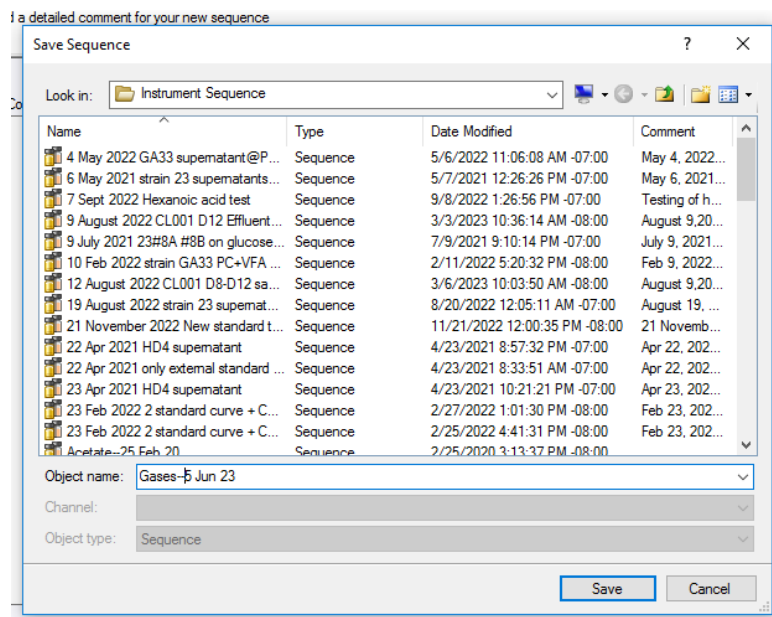


7.) Enter comments (optional).

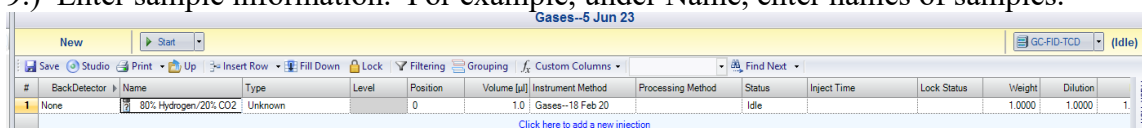


8.) Save the sequence. Save in Instrument Sequence folder so that it is easy to find.





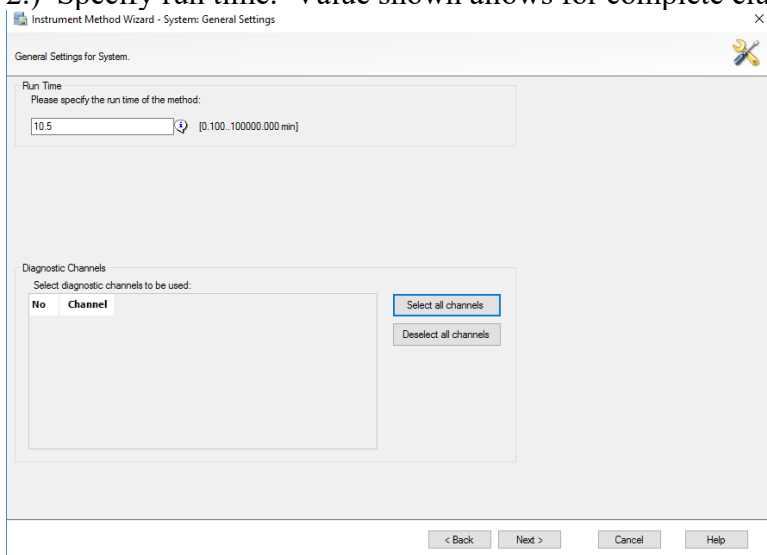
9.) Enter sample information. For example, under Name, enter names of samples.



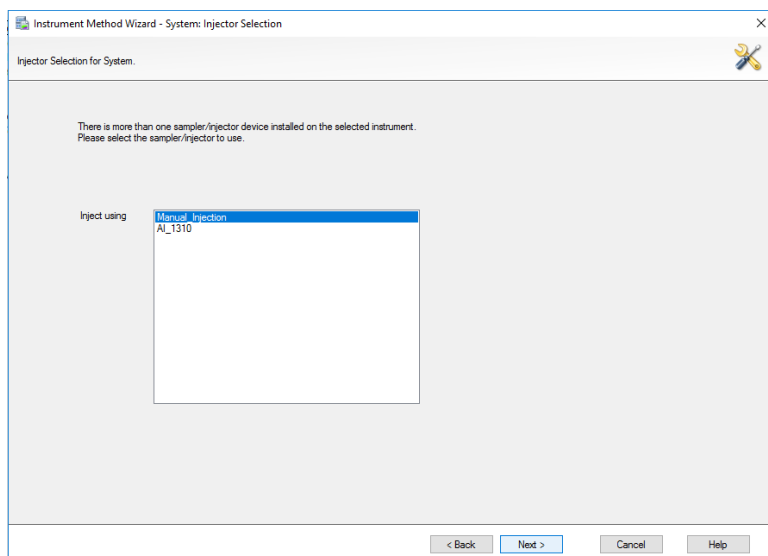
## Setting up a method

1.) Go to Create -> Instrument Method.

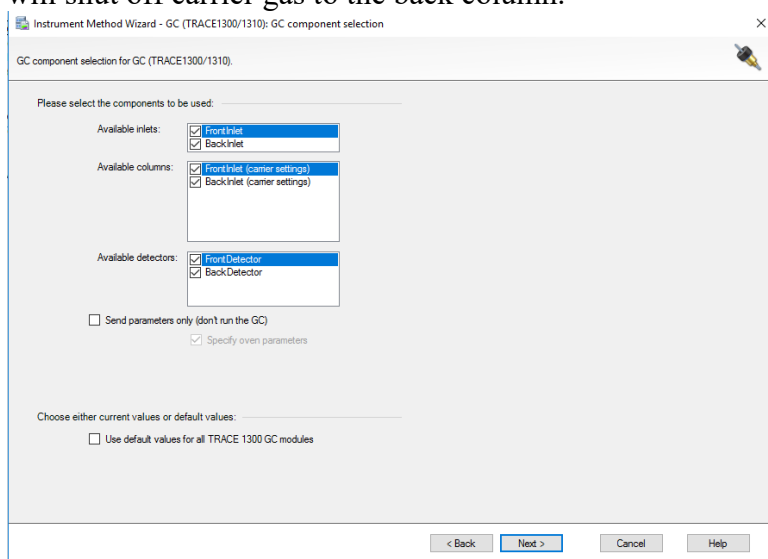
2.) Specify run time. Value shown allows for complete elution of gases.



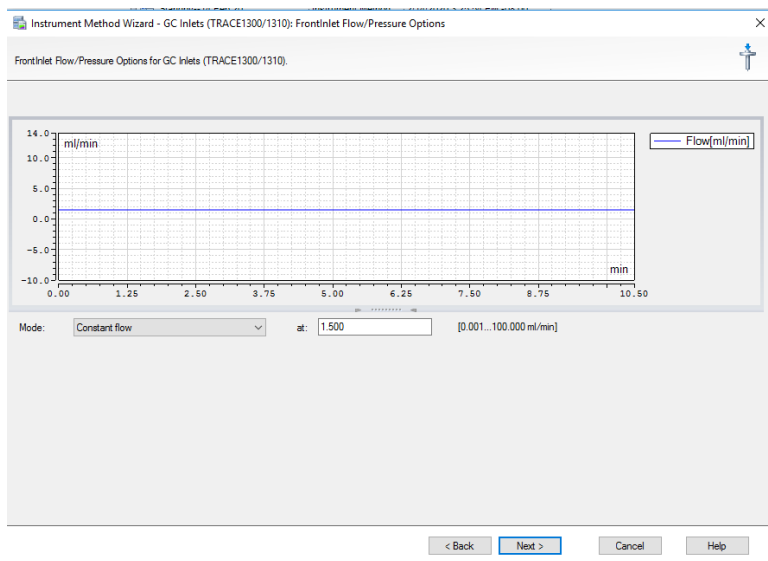
3.) Choose Manual\_Injection



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



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



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

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

FrontInlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

☒ Enable temperature control

Temperature: 110 [0...400 °C]

Utilities

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

☒ Split flow control

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

Split ratio: 3

Splitless time: 0.80 [0.00...999.99 min]

☒ Purge flow control

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

☒ Constant septum purge

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

Surge pressure: 5.00 [5.00...1000.00 kPa]

Surge duration: 0.00 [0.00...999.99 min]

☐ Vacuum compensation

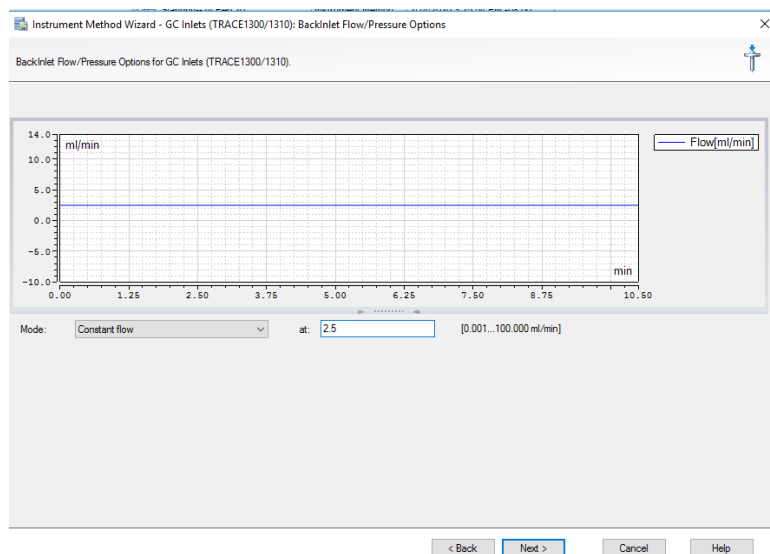
☐ Enable gas saver mode

Gas Saver Flow: 25.0 [5.0...500.0 ml/min]

Gas saver time: 2.00 [0.00...999.99 min]

< Back Next > Cancel Help

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



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

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

Backinlet Options for GC Inlets (TRACE1300/1310).

Temperature Settings

☒ Enable temperature control

Temperature: 150 [0...400 °C]

Utilises

Vapour volume calculator

Column flow calculator

Inlet Parameters

Operating mode: Split

☒ Split flow control

Split flow: 75 [5.0...1250.0 ml/min]

Split ratio: 30

Splitless time: 1.00 [0.00...999.99 min]

☒ Purge flow control

Purge flow: 3 [0.500...50.000 ml/min]

☒ Constant septum purge

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

Surge pressure: 5.00 [5.00...1000.00 kPa]

Surge duration: 0.00 [0.00...999.99 min]

☐ Vacuum compensation

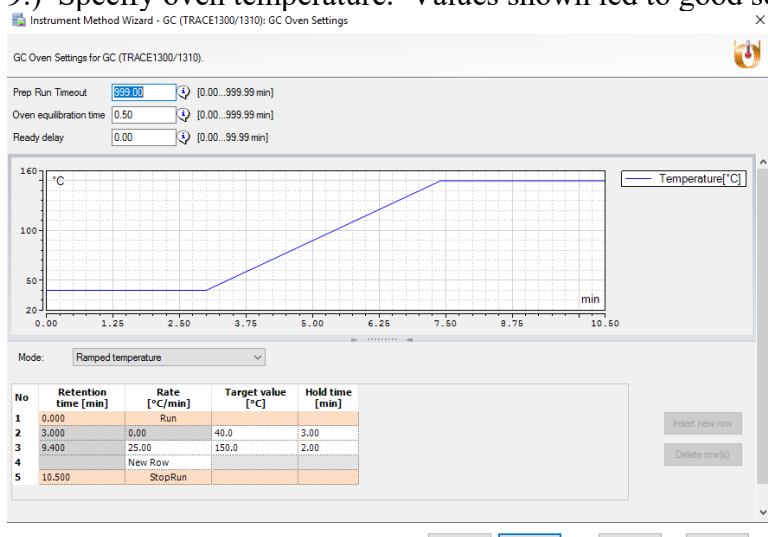
☒ Enable gas saver mode

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

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

< Back Next > Cancel Help

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



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

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

Column Configuration Settings for GC (TRACE1300/1310).

FrontColumn

☒ Validate column configuration

Description: [Empty]

Length: 30.00 [0.10...200.00 m]

Diameter: 0.320 [0.050...0.999 mm]

Film thickness: 1.00 [0.00...20.00 µm]

BackColumn

☒ Validate column configuration

Description: TG-BOND Q

Length: 30.00 [0.10...200.00 m]

Diameter: 0.530 [0.050...0.999 mm]

Film thickness: 20.00 [0.00...20.00 µm]

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

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

FrontDetector Options for GC Detectors (TRACE1300/1310)

**Detector type:** Flame Ionization Detector (FID)

**Signal Settings**

Data collection rate: 10 [1...600 Hz]

**Detector Settings**

☐ Detector temperature control  
Detector temperature: 240 [0...450 °C]

☐ Flame

☒ Enable flameout retry  
Ignition threshold: 1.0 [0.0...10.0 pA]

Peak width: Standard

**Gas Settings**

☐ Air flow control  
Air flow: 350.0 [5.0...500.0 ml/min]

☐ Makeup gas flow control  
Makeup gas flow: 40.0 [1.0...50.0 ml/min]

☐ Hydrogen flow control  
Hydrogen flow: 35.0 [1.0...100.0 ml/min]

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

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

FrontDetector Time Program for GC Detectors (TRACE1300/1310)

No	Time	Timed Autozero	Timed Flame Ctrl
1	{Initial Time}	<input type="checkbox"/>	

Add  
Insert  
Remove  
Sort

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

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

BackDetector Options for GC Detectors (TRACE1300/1310).

**Detector type:** Thermal Conductivity Detector (TCD)

**Signal Settings**

Data collection rate: 10 [1...300 Hz]

Polarity: Negative

**Detector Settings**

☒ Detector temperature control

Detector temperature: 150 [0...400 °C]

Column source: Back

☒ Enable filament

Filament temperature: 200 [50...450 °C]

Peak width: Standard

**Gas Settings**

☒ Reference gas flow control

Reference gas flow: 1.5 [0.5...5.0 ml/min]

< Back Next > Cancel Help

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

Instrument Method Wizard - GC Detectors (TRACE1300/1310): BackDetector Time Program

BackDetector Time Program for GC Detectors (TRACE1300/1310).

No	Time	Timed Autozero	Timed Polarity
1	(Initial Time)	<input type="checkbox"/>	
2	0.010	<input checked="" type="checkbox"/>	

Add  
Insert  
Remove  
Sort

< Back Next > Cancel Help

15.) Enter comments for method (optional).

Instrument Method Wizard - Completion

You now have entered all data required to create an instrument method.

Comment

Description

Press Finish to open the instrument method in the Chromatography Studio.

< Back Finish Cancel Help

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

Save Instrument Method "VFA--18 Feb 20" as

Look in: Instrument Methods

Name	Type	Date Modified	Comment
General Method_Ekong	Instrument Method	2/13/2020 11:26:15 AM -08:00	General Met...
Standby--14 Feb 20	Instrument Method	2/14/2020 3:25:54 PM -08:00	
TCD tjh--13 Feb 20	Instrument Method	2/13/2020 3:28:13 PM -08:00	TCD tjh
VFA--14 Feb 20	Instrument Method	2/13/2020 12:43:10 PM -08:00	General Met...
VFA--18 Feb 20	Instrument Method	2/18/2020 9:05:58 AM -08:00	
Zhao et al. 2006	Instrument Method	2/14/2020 12:55:32 PM -08:00	Zhao et al. ...

Object name: VFA--18 Feb 20

Channel:

Object type: Instrument Method

Save Cancel



## **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 ref. (66, 67)

See also YouTube videos at

[https://www.youtube.com/watch?v=FDMMY4krU\\_I&list=PLv\\_wI4iYQb1Azhpo8l0ZW2VBKosj9rVGJ](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<sub>600</sub> over time.
- 2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD<sub>600</sub> = 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 “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.

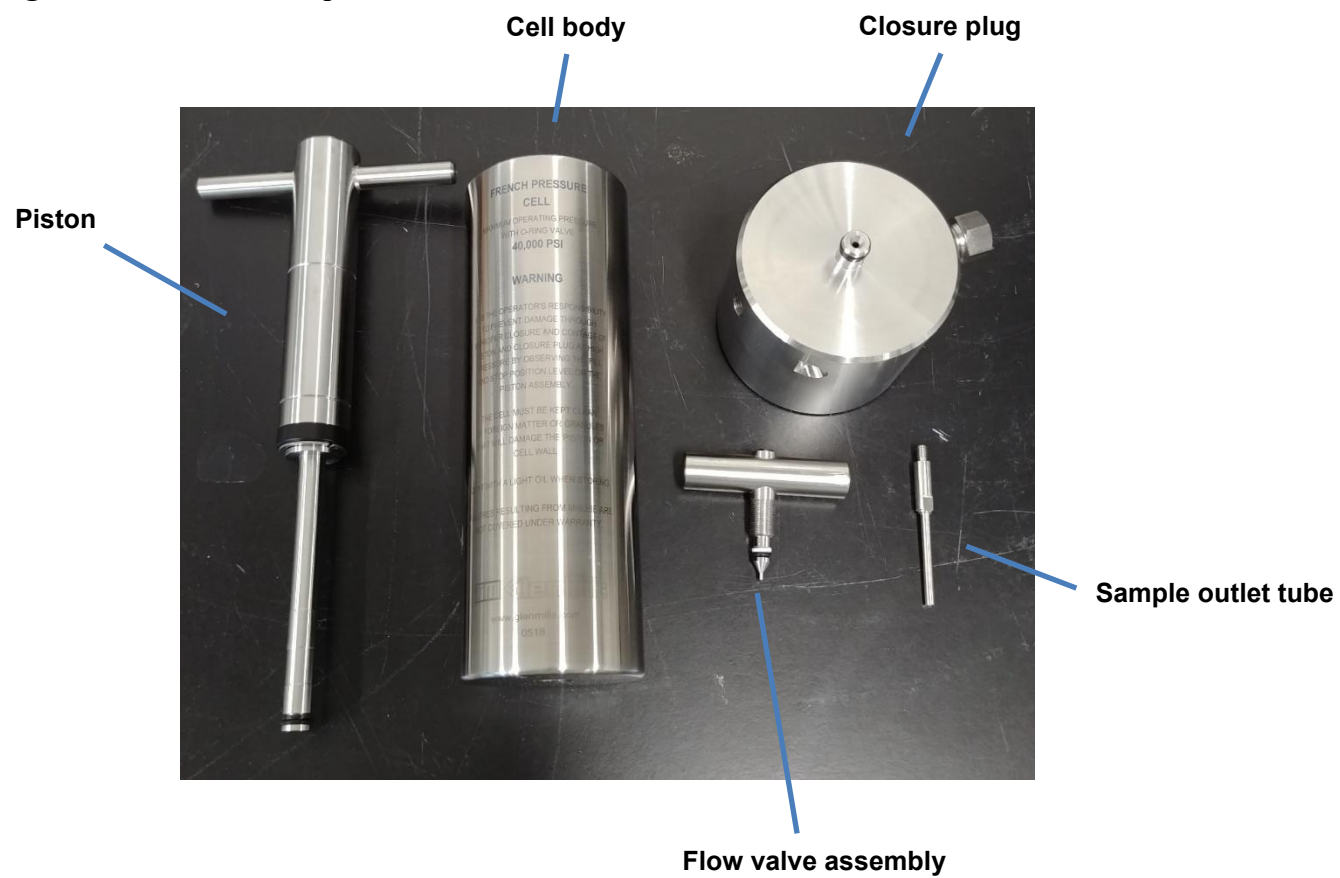
### **Notes**

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.

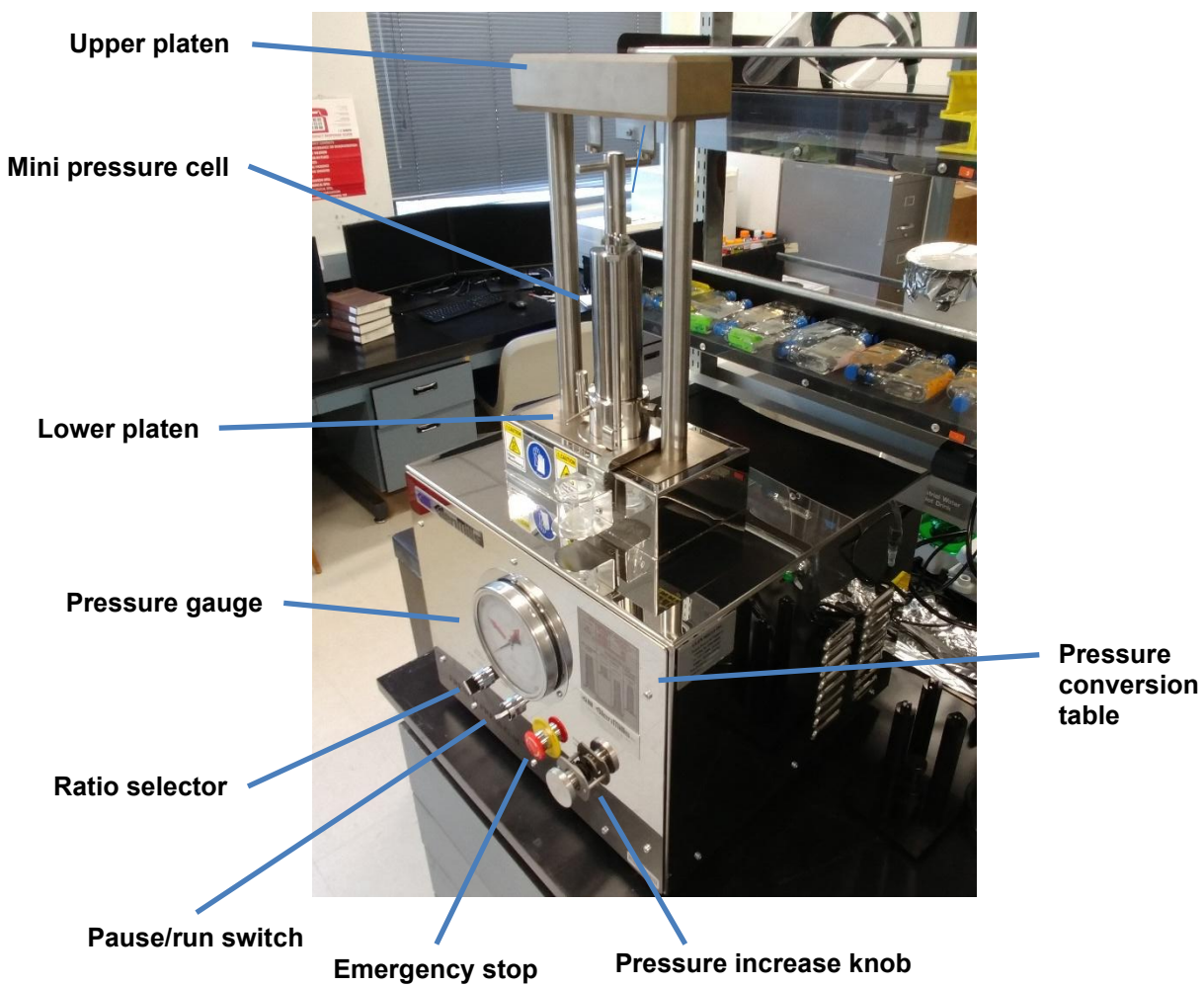
**Fig. 1.** Overview of mini pressure cell.



**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).

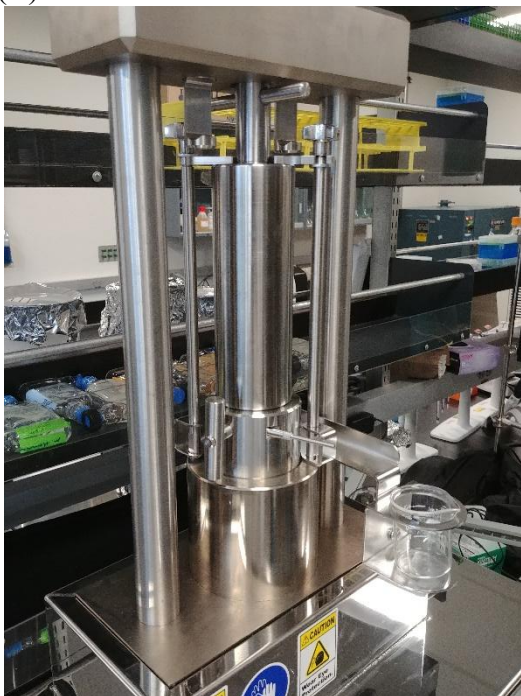
(A)



(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*.

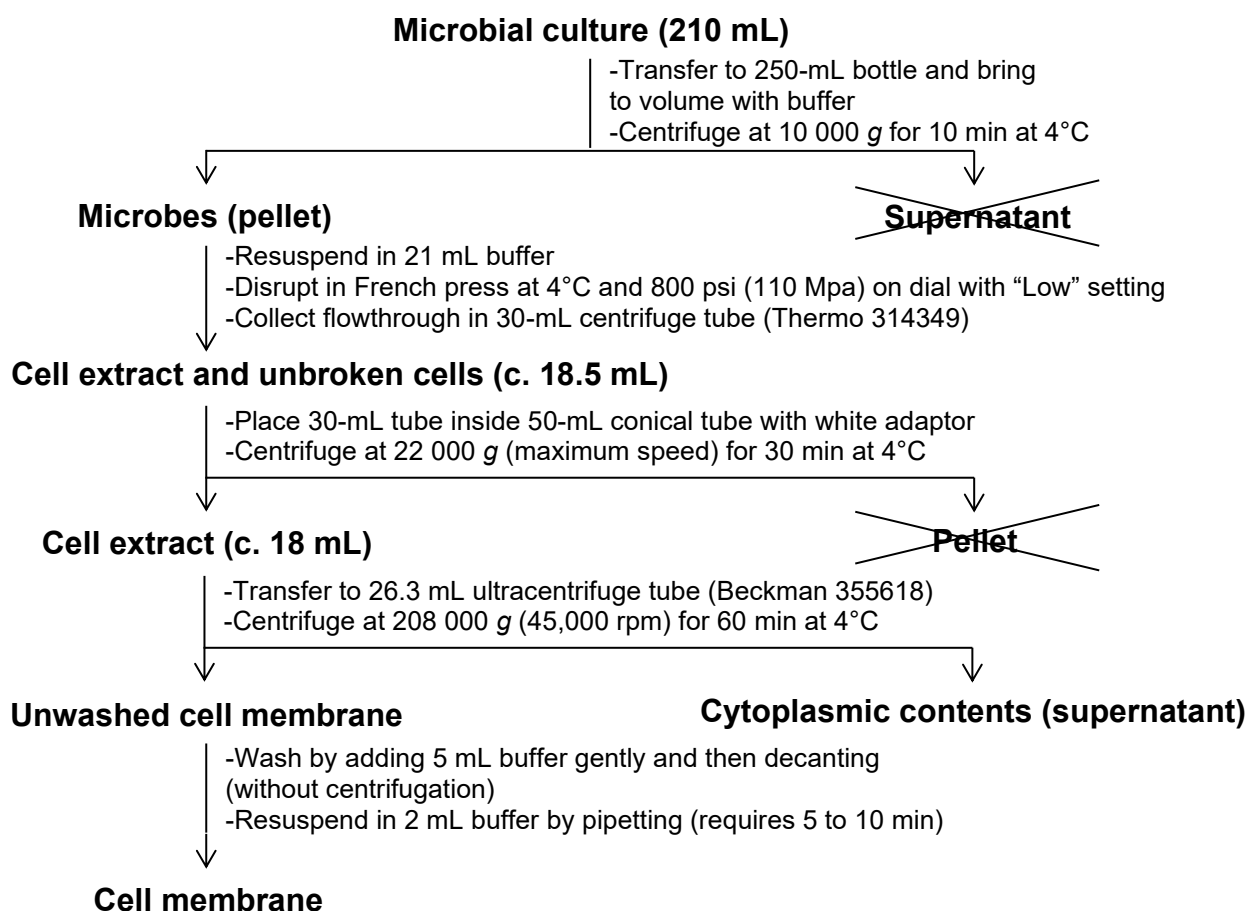
2.) Store cell membrane and cytoplasmic contents at -80°C.

### Notes

(i) For samples that need to be prepared anaerobically, flush tubes with N<sub>2</sub> 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<sub>2</sub>. Use anaerobic buffers and store cytoplasmic contents and cell membrane under N<sub>2</sub>.

(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: (68)

Dilute 4  $\mu$ L acetate kinase (available from Megazyme as special order) with 96.0  $\mu$ L 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<sub>2</sub>O. 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<sub>2</sub>O 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<sub>2</sub>O per 0.004807 g acetyl-CoA (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH<sub>2</sub>O assumes acetyl-CoA has purity of 84.2%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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: (69)

Dissolve 0.0092 g of acetyl phosphate lithium potassium salt (Sigma 01409-500MG) in 1 mL dH<sub>2</sub>O.

The volume of dH<sub>2</sub>O assumes acetyl phosphate A has purity of ~100%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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: (70)

Add 0.2136 g ADP disodium salt (Sigma A2754-1G) to 15-mL centrifuge tube and dissolve in ~4 mL dH<sub>2</sub>O. Adjust pH to a value between 7 to 9 with NaOH (e.g., 4 N). Bring to 5 mL with dH<sub>2</sub>O (using the remaining ~1 mL dH<sub>2</sub>O 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<sub>3</sub>·6H<sub>2</sub>O in ~75 mL ddH<sub>2</sub>O, then bring to 100 mL in a volumetric flask. Use AlCl<sub>3</sub>·6H<sub>2</sub>O, not anhydrous AlCl<sub>3</sub>, as the latter reacts violently with water.

### **Ammonium chloride (1 M)**

Dissolve 5.3491 g ammonium chloride ~75 mL dH<sub>2</sub>O, then bring to 100 mL in a volumetric flask.

### **APAD (50 mM)**

Reference: (71)

Weigh approximately 0.0020 g of 3-acetylpyridine adenine dinucleotide (Sigma A5251-25MG) in a small (10 mL) beaker. Add 56.2 µL dH<sub>2</sub>O per 0.002 g 3-acetylpyridine adenine dinucleotide (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH<sub>2</sub>O assumes APAD has purity of 93%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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<sub>2</sub>O.

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<sub>2</sub>O 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: (72)

Dilute 2.9  $\mu\text{L}$  citrate synthase from porcine heart (Sigma C3260-200UN) with 97.1  $\mu\text{L}$  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<sub>2</sub>O. 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<sub>2</sub>O in a volumetric flask.

### **Coenzyme A (5 mM)**

Reference: (73) 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<sub>2</sub>O per 0.003904 g coenzyme A (adjusting volume for actual mass). Dissolve by pipetting up and down.

The volume of dH<sub>2</sub>O assumes coenzyme A has purity of 98.3%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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<sub>2</sub> for a few seconds. Store at -20°C.

### **Development solution**

Reference: (74, 75)

Combine equal volumes of ferric chloride-HCl and trichloroacetic acid (30 % w/v). Store room temperature. Prepare fresh each day.

### **Dithionite (0.1 M)**

Reference: (74, 75) and instructions for DSMZ medium 346 ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium346.pdf](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<sub>2</sub>. Add 75 mL of water (anaerobic) while flushing with N<sub>2</sub> and stopper. Filter sterilize into a sterile serum bottle previously gassed with N<sub>2</sub>. 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, 21) and instructions for DSMZ medium 559 ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium559.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium559.pdf))

Weigh 1.1569 g dithiothreitol into a 160-mL culture bottle. Flush with N<sub>2</sub>. Add 75 mL of water (anaerobic) while flushing with N<sub>2</sub> and stopper. Filter sterilize into a sterile culture bottle previously gassed with N<sub>2</sub>. Long-term stability is not established.

### **DTNB (10 mM)**

Reference: (76)

Dissolve 0.0396 g of 5,5'-dithiobis(2-nitrobenzoic acid) in 10 mL of 0.1 M KPO<sub>4</sub> (pH = 7). Solution is stable for up to 6 months at 4°C and protected from light.

To prepare 0.1 M KPO<sub>4</sub> (pH = 7), see procedure below for preparing potassium phosphate buffers (pH = 5.8 to 8.0).

### **EDTA (0.5 M) (pH = 8.0)**

Reference: (77)

Add 93.05 g disodium EDTA·2H<sub>2</sub>O to 400 mL dH<sub>2</sub>O. 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<sub>2</sub>O.

### **α-ketoglutarate (100 mM)**

Reference: (78)

Dissolve 0.073 g α-ketoglutaric acid (Sigma 75890-25G) and 0.121 g Tris base to 5 mL dH<sub>2</sub>O.

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<sub>600</sub> = 1.0 to 1.2 for *P. brevis* GA33 and OD<sub>600</sub> = 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<sub>4</sub> (50 mM Tris-Cl [pH 7.6], 20 mM MgSO<sub>4</sub>, 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<sup>+</sup> oxidoreductase activity.

### **Ferric chloride-HCl**

Reference: (74)

Add 8.1105 g ferric chloride to ~25 mL ddH<sub>2</sub>O in a beaker. Add 41.3 mL concentrated (12.1 M) HCl, then bring to volume with ddH<sub>2</sub>O 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<sub>2</sub>O. 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<sub>2</sub>O. 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<sub>2</sub>O 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: (68, 79)

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<sub>2</sub>O. 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<sub>2</sub>O 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: (74)

Add 13.9 g of hydroxylamine hydrochloride to ~50 mL ddH<sub>2</sub>O 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 ddH<sub>2</sub>O 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<sub>2</sub>O. 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<sub>2</sub>O 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<sub>2</sub>O. 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<sub>2</sub>O 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<sub>2</sub> 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<sub>2</sub>. Store at 4°C. Long term stability not established.

### **L-Malate (100 mM)**

Reference: (72)

Add 1.34 g L-malic acid (Sigma 02288-10G) to 50 mL dH<sub>2</sub>O. 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: (72)

Dilute 2.1  $\mu\text{L}$  malic dehydrogenase from porcine heart (Sigma M1567-5KU) with 97.9  $\mu\text{L}$  of 2.8 M ammonium sulfate ( $\text{pH} = 6.0$ ). 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  $4^\circ\text{C}$ .

To prepare 2.8 M ammonium sulfate ( $\text{pH} = 6.0$ ), add 36.999 g ammonium sulfate to beaker and bring to  $\sim 80$  mL with  $\text{dH}_2\text{O}$ . Stir and gently heat to dissolve, add strong base (e.g., 1 M HCl) drop-by-drop to adjust to  $\text{pH}$  to 6.0, then bring to 100 mL with  $\text{dH}_2\text{O}$  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  $\text{dH}_2\text{O}$ , and dissolve by pipetting up and down. Long-term stability is not established.

### **MgCl<sub>2</sub> (1 M)**

Dissolve 10.1655 g of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  in  $\sim 40$  mL  $\text{dH}_2\text{O}$ , then bring to 50 mL in a volumetric flask.

### **MgCl<sub>2</sub> (1 M) (anaerobic)**

Add 1.6785 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  to a Balch tube. Flush tube with  $\text{N}_2$ . 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  $\text{N}_2$ . While continuing the flush, add 10 mL water (anaerobic) and dissolve. Stopper and autoclave.

### **NAD (40 mM)**

Reference: (78) 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  $\sim 4$  mL  $\text{dH}_2\text{O}$ . Check that  $\text{pH}$  has value between 2 and 6 (applying a 2- $\mu\text{L}$  aliquot to indicator paper suffices). If needed, adjust  $\text{pH}$  with 1 M Tris. Bring to 5 mL with  $\text{dH}_2\text{O}$ .

Solution is stable for 6 months at  $-80^\circ\text{C}$  and 2 weeks at  $0^\circ\text{C}$ . 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  $\text{N}_2$  for a few seconds. Store at  $-80^\circ\text{C}$ .

### **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<sub>2</sub>O 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: (79) and product information sheet for Sigma N5755-100MG

Dissolve 0.02628 g NADP disodium salt (Fisher 48-197-2100MG) in 3 mL dH<sub>2</sub>O.

The volume of dH<sub>2</sub>O assumes NADP has purity of 89.9%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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<sub>2</sub>O. 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<sub>2</sub>O in a volumetric flask.

### **Phosphoenolpyruvate (100 mM)**

Reference: (73)

Add 0.0206 g phosphoenolpyruvic acid monopotassium salt (Fisher AAB2035806) to a small (10 mL) beaker. Add 1 mL of dH<sub>2</sub>O. 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  $\mu\text{L}$  of phosphotransacetylase (3000 U/mL) with 66.7  $\mu\text{L}$  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<sub>2</sub>O 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<sub>2</sub>O in a volumetric flask.

**Potassium bicarbonate (1 M)**

Add 10.0115 g of potassium bicarbonate to a beaker. Bring to ~75 mL with dH<sub>2</sub>O, then stir to dissolve. To prevent decomposition and loss as CO<sub>2</sub>, do not heat. Bring to 100 mL with dH<sub>2</sub>O in a volumetric flask. Store in serum bottle and stopper to prevent volatilization as CO<sub>2</sub>.

**Potassium hydroxide (8 N)**

Add 44.88 g of KOH pellets to a beaker. Bring to ~75 mL with dH<sub>2</sub>O, then stir to dissolve. After cooling, bring to 100 mL with dH<sub>2</sub>O in a volumetric flask.

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

Reference: (77, 80)

Mix 1 M K<sub>2</sub>HPO<sub>4</sub> and 1 M KH<sub>2</sub>PO<sub>4</sub> in volumes indicated

pH	1 M K <sub>2</sub> HPO <sub>4</sub> (mL)	1 M KH <sub>2</sub> PO <sub>4</sub> (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

The pH in table corresponds to expected value when the buffer is diluted to concentration 0.1 M. If final concentration of buffer is much greater or less than 0.1 M, mix 1 M K<sub>2</sub>HPO<sub>4</sub> and 1 M KH<sub>2</sub>PO<sub>4</sub> in volumes indicated by ref. (80). If verifying pH with a probe, note that several minutes are required for pH probe to equilibrate.

To prepare 1 M K<sub>2</sub>HPO<sub>4</sub>, bring 87.1 g K<sub>2</sub>HPO<sub>4</sub> to 500 mL in a volumetric flask.

To prepare 1 M KH<sub>2</sub>PO<sub>4</sub>, bring 68.043 g KH<sub>2</sub>PO<sub>4</sub> to 500 mL in a volumetric flask.

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

Add 1.0713 g  $K_2HPO_4$  and 0.5239 g  $KH_2PO_4$  to a Balch tube. Flush tube with  $N_2$ . While continuing the flush, add 10 mL water (anaerobic) and dissolve. Autoclave (optional).

**Potassium phosphate buffer (pH = 7.0) (0.1 M) with dithiothreitol (2 mM) (anaerobic)**

To 75 mL water (anaerobic), add 8.52 mL of potassium phosphate buffer (pH = 7.0) (1 M) (anaerobic) and 1.70 mL of dithiothreitol (0.1 M). Add aseptically using syringes flushed with  $N_2$ ; added volumes will be approximate.

**Propionate (1 M) (pH = 7)**

Weigh 7.408 g propionic acid (a liquid; Sigma P1386-1L) in a beaker. Bring to ~60 mL with  $dH_2O$ . Add strong base (e.g., 8 N KOH) drop-by-drop to adjust to pH to 7.0, then bring to 100 mL with  $dH_2O$  in a volumetric flask.

**Propionyl-CoA (5 mM)**

Add 1061.2  $\mu L$  of ice-cold  $dH_2O$  directly to 0.005 g propionyl-CoA (Sigma P5397-5MG) in the manufacturer's vial. Dissolve by pipetting up and down.

The volume of  $dH_2O$  assumes propionyl-CoA has purity of 87.4%; see certificate of analysis for exact purity and adjust volume of  $dH_2O$  accordingly. Here, purity refers to (purity [HPLC] - water - solvent - lithium) %.

Long term stability is not established, but stability of acetyl-CoA is 6 months at  $-80^\circ C$ . Before freezing, store in aliquots (0.05 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.

**Pyruvate (100 mM)**

Reference: Product information sheet for Sigma P4562

Dissolve 0.1100 g sodium pyruvate (Fisher AC132150250) in 10 mL  $dH_2O$ . Filter sterilize. Solution is stable for 24 months at  $4^\circ C$ .

**Pyruvate (1 M) (anaerobic)**

Add 1.1004 g sodium pyruvate (Fisher AC132150250) to a Balch tube. Flush tube with  $N_2$ . While continuing the flush, add 10 mL water (anaerobic) and dissolve. Filter sterilize into sterile Balch tube previously gassed with  $N_2$ . Solution is stable for 24 months at  $4^\circ C$ .

**Pyruvate kinase (100 U/mL)**

Dilute 2.19  $\mu L$  pyruvate kinase from rabbit muscle (Sigma C3260-200UN) with 97.81  $\mu L$  of 3.2 M ammonium sulfate (pH = 6.0). The volume of pyruvate kinase assumes 4576 U/mL; see pack label for exact U/mL and adjust volumes accordingly. Solution is stable for months at  $4^\circ C$ .

To prepare 3.2 M ammonium sulfate (pH = 6.0), add 42.2848 g ammonium sulfate to beaker and bring to ~80 mL with dH<sub>2</sub>O. 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<sub>2</sub>O in a volumetric flask.

### **Sodium fluoride (100 mM)**

Add 0.4199 g NaF to a screw-cap plastic bottle. Add 100 mL ddH<sub>2</sub>O with a volumetric pipette. Dissolve. Solution will etch glass bottles and should not be stored in them.

### **Sodium vanadate (200 mM) (pH = 10.0)**

Reference: (81)

Dissolve 3.6781 g Na<sub>3</sub>VO<sub>4</sub> in ~50 mL ddH<sub>2</sub>O. 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<sub>2</sub>O. 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<sub>2</sub>O in a volumetric flask. Filter sterilize into a 160-mL culture bottle.

### **Succinyl-CoA (5 mM)**

Reference: (82)

Add 907.1 µL of ice-cold 0.01 M H<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>SO<sub>4</sub> assumes succinyl-CoA has purity of 78.7%; see certificate of analysis for exact purity and adjust volume of dH<sub>2</sub>O 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<sub>2</sub>SO<sub>4</sub>, add 275.9 µL of concentrated H<sub>2</sub>SO<sub>4</sub> to ~125 mL in a beaker. Bring up to 500 mL in a volumetric flask. The volume of H<sub>2</sub>SO<sub>4</sub> assumes assay of 96.6 % w/w; see pack label for exact assay and adjust volume of dH<sub>2</sub>O 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<sub>2</sub> for several minutes. Store at -20°C.

### **Trichloroacetic acid (30 % w/v)**

Dissolve 30.0 g trichloroacetic acid in ~30 mL ddH<sub>2</sub>O, 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O. 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<sub>2</sub>. 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<sub>2</sub> for 10 min, add 44.32 mL anaerobic dH<sub>2</sub>O to the bottle and cap it with butyryl stopper under N<sub>2</sub>. 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<sub>2</sub>O to the bottle and cap it with butyryl stopper under N<sub>2</sub>. 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<sub>12</sub> (1 mg/mL)**

Dissolve 0.01 g vitamin B<sub>12</sub> in 10 mL ddH<sub>2</sub>O. Filter through 0.22 µm membrane. Store at 4°C.

### **Water (anaerobic)**

Boil ~350 mL water under N<sub>2</sub> 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<sub>2</sub> and stopper. Autoclave (optional).

## Purification of ferredoxin from *Clostridium pasteurianum*

Adapted from ref. (83–85). 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. (77)

### 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<sub>2</sub>O.

#### **1 M Acetic acid**

Add 5.72 mL glacial acetic acid to a 100 mL volumetric flask. Bring to volume with dH<sub>2</sub>O.

#### **Ammonium sulfate (10% saturated, pH = 6.8)**

Add 5.3 g ammonium sulfate and 100 mL dH<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>HPO<sub>4</sub> and 3.85 g KH<sub>2</sub>PO<sub>4</sub> to 1 L volumetric flask. Bring to volume with dH<sub>2</sub>O. 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<sub>2</sub>O.

#### **NaCl (2 M)**

Add 11.688 g NaCl to ~75 mL dH<sub>2</sub>O in beaker and stir to dissolve. Transfer to a 100 mL volumetric flask and bring to volume with dH<sub>2</sub>O.

#### **NaCl (100 mM)**

Add 2.3376 g NaCl to 250 mL volumetric flask. Bring to volume with dH<sub>2</sub>O.

**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<sub>2</sub>O 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<sub>2</sub>O. Add 10% HCl drop-by-drop to adjust pH to 8. Transfer to a 100 mL volumetric flask and bring to volume with dH<sub>2</sub>O.

**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<sub>2</sub>O. Transfer to a 250 mL volumetric flask and bring to volume with dH<sub>2</sub>O.

**NaOH (0.5 N)**

Add 4.9996 g of NaOH to ~200 mL dH<sub>2</sub>O. Transfer to a 250 mL volumetric flask and bring to volume with dH<sub>2</sub>O.

**Water (anaerobic)**

Boil ~350 mL water under N<sub>2</sub> 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<sub>2</sub> and stopper. Autoclave.

**Preparing medium for *C. pasteurianum******C. pasteurianum* minerals A**

Add following to a 1-L volumetric flask

153.29 g K<sub>2</sub>HPO<sub>4</sub> to 1 L

Bring to volume to 1 L with dH<sub>2</sub>O and store at 4°C.

***C. pasteurianum* minerals B**

Add the following to a 1-L volumetric flask

15 g  $\text{KH}_2\text{PO}_4$   
1 g  $\text{NaCl}$   
0.9766 g  $\text{MgSO}_4$   
0.1 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$   
10 g  $\text{NH}_4\text{Cl}$

Bring to volume with  $\text{dH}_2\text{O}$  and store at  $4^\circ\text{C}$ .

***C. pasteurianum* iron sulfate**

Add 0.5 g  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  to a 1-L volumetric flask. Bring to volume with  $\text{dH}_2\text{O}$  and store at  $4^\circ\text{C}$ .

***C. pasteurianum* vitamins**

Add the following to a 1-L volumetric flask

0.05 g 4-aminobenzoic acid

0.01 g biotin (stored at  $4^\circ\text{C}$ )

Bring to volume with  $\text{dH}_2\text{O}$  and store at  $4^\circ\text{C}$ .

**Glucose (40% w/v, anaerobic)**

Add 40 g glucose to a 150-ml serum bottle. Flush the bottle under  $\text{N}_2$  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  $\text{N}_2$ , stopper the bottle. Autoclave at  $121^\circ\text{C}$  for 15 min.

***C. pasteurianum* medium (sterilized)**

Add following to a 1-L round bottom flask

230 mL  $\text{dH}_2\text{O}$

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  $\text{N}_2$  (requires about 5 min). Cool it down in ice water under  $\text{N}_2$  and bubble for 5 min. Approximately 50 mL will be lost, giving 300 mL final volume. pH should be approximately 7.1.

Bubble with  $\text{CO}_2$  to decrease pH to 6.8 (requires about 3 min).

Under  $\text{CO}_2$ , 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  $121^\circ\text{C}$  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<sub>2</sub>O to a 1-L Pyrex bottle. Stir to dissolve, then bring to 540 mL with dH<sub>2</sub>O. Wire a stopper in place, cover with aluminum foil, and autoclave at 121°C for 15 min. Pressurize the bottle with N<sub>2</sub>, cool, then remove stopper and flush with N<sub>2</sub>.

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<sub>2</sub> for 4 min, then continue bubbling with CO<sub>2</sub> for 4 min. Drain contents of separatory funnel into Pyrex bottle with glucose solution. Bubble with CO<sub>2</sub> 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<sub>2</sub>O, transfer ion exchanger to Buchner funnel lined with 2 layers of #1 Whatman filter paper.

(e) Wash ion exchanger with dH<sub>2</sub>O. Fill a 1 L graduated cylinder with dH<sub>2</sub>O, 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<sub>2</sub>O).

(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<sub>2</sub>O, 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<sub>2</sub>O).

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  $0.0015 \text{ g wet cells} \times OD_{600}^{-1} \times \text{mL culture}^{-1}$  (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  $\geq 80$  g cell pellet from  $-20^{\circ}\text{C}$ . If each tube contains 5.4 g cell pellet,  $\sim 15$  tubes will be needed.
  - 2.) Resuspend cell pellets in  $\text{dH}_2\text{O}$  at room temperature.
    - (a) To each 50-mL tube, add 1.5 mL  $\text{dH}_2\text{O}$ /g wet cells.
    - (b) Use spatula to loosen pellet from tube wall. Use some of the  $\text{dH}_2\text{O}$  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  $\mu\text{L}$  of lysozyme (50 mg/mL) and 0.1  $\mu\text{L}$  of universal nuclease (250 U/ $\mu\text{L}$ ) per mL of resuspend cells.
  - 4.) Incubate at  $37^{\circ}\text{C}$  in water bath for 40 min. Mix well with a spatula in every 5 min. The lysate will remain brown and turbid, but will no longer be sticky/viscous.
  - 5.) Cool to room temperature ( $23\text{--}25^{\circ}\text{C}$ ) by mixing on a stir plate or by placing on ice for a few minutes. Temperature can be measured with pH or conductivity probe used below.
  - 6.) Measure pH with a pH probe. Initial pH will be  $\sim 6$ .
  - 7.) Adjust pH to 6.8 using 1 M Tris-base (4 to 6 mL for 80 g cells). Adjust pH carefully, as downstream precipitation of ferredoxin will be low otherwise. If pH is too high, lower it with 1 M acetic acid.
  - 8.) Adjust conductivity.
    - (a) Prepare the conductivity probe (Fisher WD-35662-30).
    - (b) Measure conductivity of 100 mL of 100 mM NaCl at room temperature (about 10.7-11.3 mS/cm).
    - (c) Measure conductivity of the lysate at room temperature ( $\sim 4\text{--}5$  mS/cm).
    - (d) Add NaCl (2 M) to lysate until the conductivity matches that of 100 mM NaCl (15 to 19 mL for 80 g of cells).
- If conductivity is too high, dilute with  $\text{dH}_2\text{O}$ . 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 Polymixin-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  $\mu$ L of polymixin-P (10% w/v, pH = 6.8) per gram of acetone supernatant (10.25 mL for ? 205 g acetone supernatant). Add polymixin-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  $\mu$ L of DEAE-cellulose slurry per gram of original cell pellet (e.g., 9.6 mL for 80 g cell pellet). If slurry is not 0.25 g/mL, adjust the volume proportionately.

2.) Adsorb the ferredoxin onto the slurry by mixing it for 2 to 4 h. Place a stir bar into the beaker, place the beaker in a cooler with ice, and place the cooler on the stir plate.

3.) Transfer the slurry to two 50-mL tubes. Let the contents stand for 30 min (or longer) to allow the DEAE-cellulose slurry to settle. Alternatively, pellet the slurry by centrifuging it at 24,400 for 20 min at 4°C.

4.) Remove supernatant (yellow) without disturbing DEAE-cellulose pellet (deep brown). Keep both the pellet and supernatant.

5.) Wash the DEAE-cellulose pellet. Add 0.5 to 1 mL ammonium sulfate (60% and 90% saturated mix, pH = 6.8) per gram of original wet cell pellet (e.g., 40 to 80 mL per 80 g cell pellet). Centrifuge at 24,400 for 20 min at 4°C. Discard the supernatant (light yellow).

6.) Wash again by repeating #5, but keep a small amount (c. 1 mL) of supernatant. Transfer the pellet and 1 mL supernatant to a 2-mL tube.

7.) Centrifuge the DEAE-cellulose at 21,100 g 15 min at 4°C. Remove the supernatant fully by inserting a 200- $\mu$ L pipette into the DEAE-cellulose pellet and pipetting out all liquid. The DEAE-cellulose pellet should look dry.

8.) Elute the ferredoxin from DEAE-cellulose. Add a small volume (e.g., 1.5 mL) of ammonium sulfate (10% saturated, pH = 6.8) to the DEAE-cellulose pellet. Mix by vortexing (2000 to 3000 rpm for 15 s).

9.) Centrifuge the DEAE-cellulose at 21,100 g for 15 min at 4°C. Pipette off most supernatant, but do not disturb the pellet. Transfer the supernatant to a 50-mL tube and label "A1 elute". Both pellet and supernatant will be brown.

10.) The DEAE-cellulose from step #9 still contains ferredoxin, which can be re-eluted by repeating steps #8 and 9. Label the supernatant as "A1 re-elute".

11.) The DEAE-cellulose from step #10 still contains ferredoxin, which can be re-eluted a second time. Label the supernatant “A1 re-re-elute”. After this step, the DEAE-cellulose pellet should contain little ferredoxin and be whitish brown.

12.) The supernatant from #4 still contains ferredoxin, which can be adsorbed onto more DEAE-cellulose. Repeat steps #1 to 11, except use 60 µL of DEAE-cellulose slurry per gram of original cell pellet (e.g., 4.8 mL for 80 g cell pellet). The time for mixing in step #2 can be extended to overnight. Keep the supernatant and label “A2 elute”; “A2 re-elute”, “A2 re-re-elute”.

13.) The supernatant from #12 still contains ferredoxin, which can be adsorbed onto more DEAE-cellulose. Label the supernatant “A3 elute”; “A3 re-elute”, “A3 re-re-elute”. After this step, the supernatant should contain little ferredoxin.

14.) Store the purified ferredoxin at 4°C.

### **Measurement of yield and purity of ferredoxin**

1.) Measure the absorbance of the purified ferredoxin using Molecular Devices M3 plate reader. Pipette 1000 µL of each sample into a UV-cuvette. Alternatively, pipette 200 µL of each sample to a 96-well plate (UV). Measure the values of  $A_{280}$ ,  $A_{390}$ ,  $A_{430}$ , and perform a spectral scan from  $\lambda = 220$  to 750 nm. Include one sample of 10% saturated ammonium sulfate (as a blank).

2.) For each sample, determine the concentration and purity of ferredoxin. The concentration is

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

where  $A_{390}$  is the absorbance at 390 nm,  $c$  is the concentration in mM,  $\epsilon_{390}$  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  $\mu$ M (~5300 mg/L) and purity of  $A_{390}/A_{280} = 0.73$ .

6.) Dispense ~150  $\mu$ L aliquots in 500- $\mu$ L tubes. Store at -80°C. They can be stored for at least 1 year. For short-term storage, 4°C can also be used.

7.) Optional: test the activity of the ferredoxin by measuring its reduction by hydrogenase of *Clostridium pasteurianum* 5.

### **Optional: Re-purification of low-quality ferredoxin**

If the quality of ferredoxin is low, it may be salvageable by repeating some steps of the purification.

1.) Mix all bad samples together in a beaker. Calculate the volume (mL) and amount of crude ferredoxin (nmol) in them.

2.) Adjust the concentration of ammonium sulfate to 60% saturated (the concentration needed to prior to adsorption on DEAE-cellulose). Mix well for 15 min in a beaker on ice. The initial concentration of ammonium sulfate is 10% saturated. Slowly add solid ammonium sulfate to raise the concentration, minding that the volume will also be increased.

Example: Step #1 produces a 50 mL sample with 10% saturated ammonium sulfate. A total of 15.83 g ammonium sulfate needs to be added, giving a 60% saturated ammonium sulfate solution with final volume of 60 mL.

3.) Transfer the solution to 50-mL tubes and centrifuge at 24,400 g for 10 min at 4°C.

4.) Transfer the supernatant to a beaker on ice. Add 0.25 g/mL DEAE-cellulose slurry to mix with the supernatant until all ferredoxin is bound. If step #1 starts with 920 nmol crude ferredoxin, ~6 mL of 0.25 g/mL slurry can be used for the binding.

5.) Mix well on ice for 2 to 4 h.

6.) Repeat **Purification of ferredoxin with DEAE-cellulose** from step #3 onward, then repeat **Measurement of yield and purity of ferredoxin**. When purifying ferredoxin with DEAE-cellulose, it may not be necessary to perform multiple bindings, as all ferredoxin should be bound the first time.

## Assay of lactate dehydrogenase activity

Modified from ref. (67)

### 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 <sub>4</sub> (pH = 6.2) (1 M)	35	70
NADH (40 mM)	2.5	0.2
ddH <sub>2</sub> O	425	NA
Cell extract	25	NA
Pyruvate (100 mM) or ddH <sub>2</sub> O	12.5	25
Final volume	500	

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

Component	Mastermix
KPO <sub>4</sub> (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 <sub>2</sub> O	
1	Cell extract	
2	Pyruvate	ddH <sub>2</sub> O

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize (1 to 3 min) before making next addition.

After addition 2, record absorbance at short (0.5 min) intervals for 5 to 10 min.

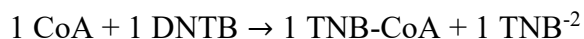
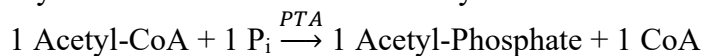
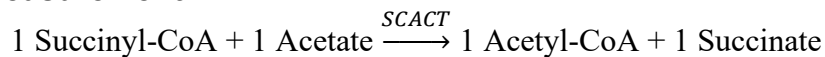
### Calculation of activity

- 1.) Denote the absorbance after addition 1 as  $A_1$ .  $A_2$  and other absorbances are defined similarly.
- 2.) Plot  $A_2$  vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.
- 3.) Calculate the reaction rate
$$\text{Rate} = (\text{slope}/6\,220)$$
where  $6\,220\text{ M}^{-1}\text{ cm}^{-1}$  is the extinction coefficient of NADH.
- 4.) Calculate the activity ( $\text{mol L}^{-1}\text{ min}^{-1}$ ) by correcting the reaction rate with control cuvettes.
$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}}$$

## Assay of succinyl-CoA:acetate CoA-transferase activity with phosphotransacetylase and DTNB

Modified from (86)

### Principle of measurement



where SCACT is succinyl-CoA:acetate CoA-transferase and PTA is phosphotransacetylase. TNB<sup>2-</sup> 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 <sub>2</sub> O	3	NA
KPO <sub>4</sub> (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 <sub>2</sub> O	30	300
Enzyme sample or ddH <sub>2</sub> O	6	NA
Succinyl-CoA (5 mM) or ddH <sub>2</sub> O	30	1.5
Final volume	100	

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

Component	Master mix
ddH <sub>2</sub> O	A
KPO <sub>4</sub> (pH = 8.0) (1 M)	A
KCl (1 M)	A
DTNB (10 mM)	A
Phosphotransacetylase (3000 U/mL)	A

Prepare the master mix in a 1.5-mL centrifuge tube or larger volume tube. Make extra (equivalent of 1/2 sample) to ensure sufficient volume.

### Measurement of activity

Add components to wells in the following order:

Addition	Well			
	1	2	3	4
1	Mastermix A			
1 Potassium acetate	ddH <sub>2</sub> O	Potassium acetate	Potassium acetate	Potassium acetate
1 Enzyme sample	Enzyme sample	ddH <sub>2</sub> O	Enzyme sample	Enzyme sample
2 SucCoA	SucCoA	SucCoA	SucCoA	ddH <sub>2</sub> O

After making addition 1, mix contents of wells and record absorbance (412 nm) on Molecular Devices M3 spectrophotometer. Take readings at 20 s intervals for 5 min.

Make addition 2, then record absorbance for 30 to 45 min. Because succinyl-CoA is unstable in solution, keep it in the freezer until shortly (5 min) before this addition. Thaw succinyl-CoA by flicking the tube and vortexing, then keep on ice until use.

### Calculation of activity

- 1.) Denote the absorbance after addition 1 as  $A_1$ .  $A_2$  is defined similarly.
- 2.) Plot  $A_2$  vs. time, find the linear part of the curve, and then calculate the slope. Do this for each well.
- 3.) Calculate the reaction rate  

$$\text{Rate} = (\text{slope}/14\ 150) / \text{path length}$$
 where  $14\ 150\ \text{M}^{-1}\ \text{cm}^{-1}$  is the extinction coefficient of  $\text{TNB}^{2-}$  (87). The path length is expressed in cm and measured according to *Measurement of path length of 96-well plates*. A typical value of path length is 0.288 cm.
- 4.) Calculate the activity ( $\text{mol L}^{-1}\ \text{min}^{-1}$ ) by correcting the reaction rate with control cuvettes.

$$\text{Activity} = \text{Rate}_{\text{well 1}} - \text{Rate}_{\text{well 3}}$$

Though the above is one way to correct the reaction rate, another way is

$$\text{Activity} = \text{Rate}_{\text{well 1}} - \text{Rate}_{\text{well 2}} - \text{Rate}_{\text{well 4}}$$

**Notes**

i) If  $A_2$  is not linear with time, then reduce the volume of enzyme sample added. If volume is not reduced, then activity will be underestimated.



## Assay of succinyl-CoA:acetate CoA-transferase activity with citrate synthase and malate dehydrogenase

Modified from ref. (88)

### Assay mixture

Prepare and add the assay mixture to ultramicrocuvettes (Brandtech 759200).

The assay mixture consists of

Component	Volume ( $\mu$ L)	Final concentration (mM or U/mL)
ddH <sub>2</sub> O	38.37	NA
Tris (1 M) (pH = 8.0)	10	100
L-malate (100 mM)	0.4	0.4
NAD (40 mM)	2.5	1
KPO <sub>4</sub> (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 <sub>4</sub> (1 M) (pH = 6.8)	B
Malate dehydrogenase (180 U/mL)	B
Citrate synthase (90 U/mL)	B

The components are added to cuvettes in the following order:

Addition	Cuvette			
	1	2	3	4
1			ddH <sub>2</sub> O	
1			Mastermix A	
1			Mastermix B	
1	Cell extract	Cell extract	Cell extract	ddH <sub>2</sub> O
2	Acetate	Acetate	ddH <sub>2</sub> O	Acetate
3	SucCoA	ddH <sub>2</sub> O	SucCoA	SucCoA

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize (1 to 3 min) before making next addition. Repeat for addition 2.

After addition 3, record absorbance at short (0.5 min) intervals for 5 to 10 min.

Because the reaction occurs quickly, combine components and record absorbance for only one cuvette at a time; it is not possible to handle all 4 cuvettes at once.

### Calculation of activity

1.) Denote the absorbance after addition 1 as  $A_1$ .  $A_2$  and other absorbances are defined similarly.

2.) Plot  $A_3$  vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.

3.) Calculate the reaction rate

$$\text{Rate} = (\text{slope}/6220)$$

where  $6220 \text{ M}^{-1} \text{ cm}^{-1}$  is the extinction coefficient of NADH.

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

$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 2}} - \text{Rate}_{\text{cuvette 3}}$$

Another plausible way to correct the reaction rate and calculate activity is

$$\text{Activity} = \text{Rate}_{\text{cuvette 1}} - \text{Rate}_{\text{cuvette 4}}$$

It is up to the experimenter to decide which measure of activity is more appropriate.

## Assay of succinyl-CoA synthetase activity with hydroxylamine

Modified from ref. (74, 89)

### 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 <sub>2</sub> O	20.8	NA
Tris (pH = 7.2) (1 M)	5	50
MgCl <sub>2</sub> (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 <sub>2</sub> (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 <sub>2</sub> O	20.8	1
Mastermix A	25.2	1
Succinate or ddH <sub>2</sub> O*	4	1
Cell extract	50	1
Development solution	28.57	2

\*One half of the pair of tubes should have succinate, and the other half should have ddH<sub>2</sub>O

Conditions for the additions are:

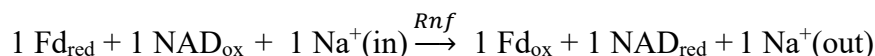
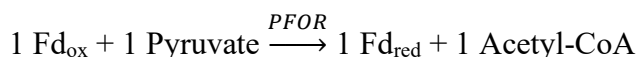
Addition	Conditions
1	Incubate at room temperature for variable time
2	Incubate at room temperature for at least 5 min, centrifuge at 18,000 g, then read $A_{540}$ of supernatant (110 $\mu$ L) in microcuvettes

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<sup>+</sup> oxidoreductase (Rnf) activity

Modified from (86)

### Principle of measurement



where Fd<sub>ox</sub> is oxidized ferredoxin, Fd<sub>red</sub> is reduced ferredoxin, NAD<sub>ox</sub> is oxidized NAD, NAD<sub>red</sub> is reduced NAD, and PFOR is pyruvate:ferredoxin oxidoreductase. NAD<sub>red</sub> 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<sub>4</sub> (50 mM Tris-Cl [pH 7.6], 20 mM MgSO<sub>4</sub>, 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 <sub>2</sub> O (anaerobic)	630	NA
Tris (pH 7.6) (1 M) (anaerobic)	50	50
MgCl <sub>2</sub> (1 M) (anaerobic)	10	10
Dithiothreitol (100 mM) (anaerobic)	40	4
NaCl (1 M) (anaerobic)	10	10
Cell membrane or dH <sub>2</sub> O (anaerobic)	100	40 <sup>a</sup>
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 <sup>b</sup>
Crude pyruvate:ferredoxin oxidoreductase (anaerobic)	100	36 <sup>a</sup>
Pyruvate (1 M) anaerobic)	10	10
NAD (200 mM) (anaerobic)	10	2
Final volume	1000	

<sup>a</sup>μg protein/mL

<sup>b</sup>U/mL

Add components to the cuvette anaerobically. Transfer small aliquots of components to 0.5 mL centrifuge tubes flushed with N<sub>2</sub>. While flushing the cuvette with N<sub>2</sub>, transfer the component to the cuvette with a micropipette. Cap the cuvette once done. Tap the cuvette to remove any bubbles.

### Measurement of activity

Add components to cuvettes in the following order:

Addition	Cuvette	
	1	2
1		dH <sub>2</sub> O
1		Tris
1		MgCl <sub>2</sub>
1		Dithiothreitol
1		NaCl
1	Cell membrane	dH <sub>2</sub> O
1		Coenzyme A
1		Ferredoxin
1		Thiamine pyrophosphate
1		Phosphotransacetylase
1	Crude pyruvate:ferredoxin oxidoreductase	
2		Pyruvate
3		NAD

Make addition 1, then incubate for 5 to 10 min. Make addition 2, then record absorbance at 340 and 430 nm with a Molecular Devices M3. Wait until absorbance at 430 nm reaches plateau (requires 5 to 10 min). Make addition 3 and continue to record absorbance.

Perform the incubations and absorbance recordings at 37°C. To configure the Molecular Devices M3 to do this, choose “Temperature ON, 37°C” with parameters “Absorbance, Kinetics, 2 wavelengths (340 and 430), time length 15 min and 10 second interval”. When absorbance is not being recorded, incubation can be done in a water bath.

Example results are shown in Fig. 1.

### Calculation of activity

- 1.) Denote the absorbance after addition 3 as A<sub>3</sub>.
- 2.) Plot A<sub>3</sub> vs. time, find the linear part of the curve, and then calculate the slope. Do this for each cuvette.
- 3.) Calculate the reaction rate  

$$\text{Rate} = (\text{slope}/6\,200)$$
 where 6 200 M<sup>-1</sup> cm<sup>-1</sup> is the extinction coefficient of NADH.

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

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

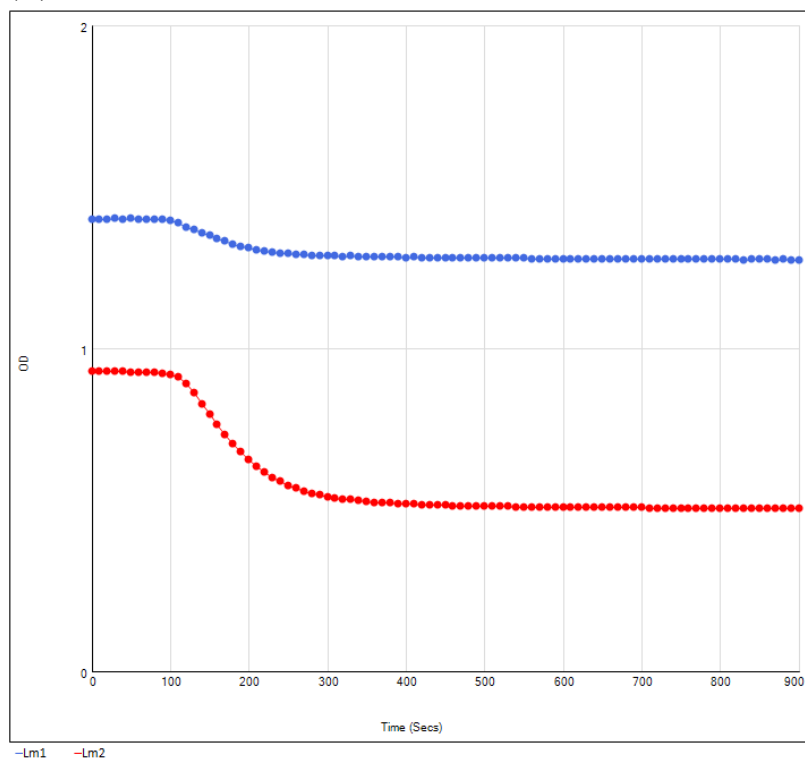
5.) Measure the protein of the membrane and express activity as  $\text{mol mg protein}^{-1} \text{min}^{-1}$ .

**Notes**

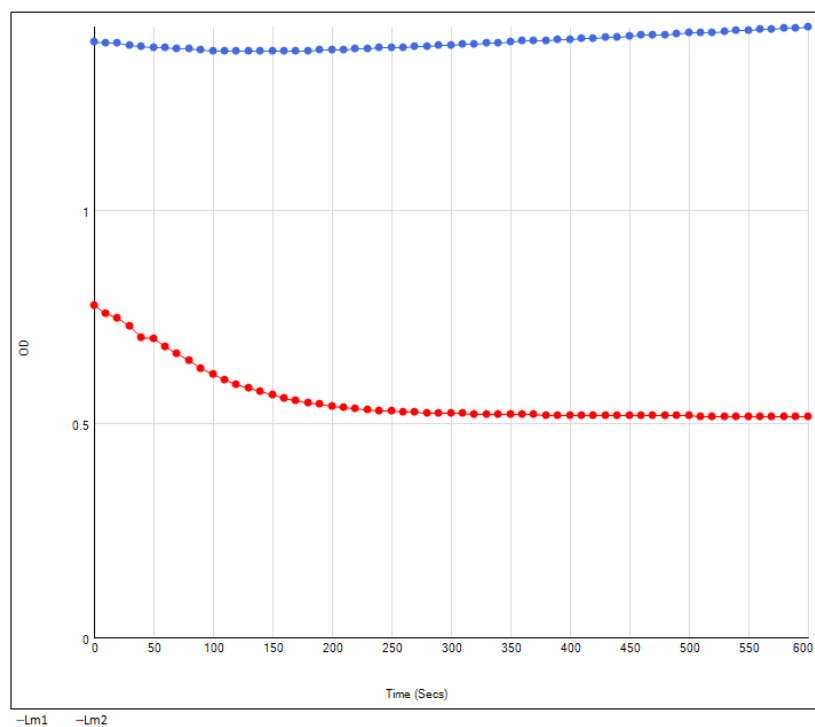
i) Low activity may be observed and be due to oxidation of NADH (by a NADH dehydrogenase). To correct for this oxidation, include a control cuvette containing NADH (in place of NAD).

**Fig. 1.** Change in absorbance over time. (A) After addition 2. (B) After addition 3. Results are for cuvette 1. Blue = absorbance at 340 nm; red = absorbance at 430 nm.

(A)



(B)





## Assay of acetate with lactate dehydrogenase, pyruvate kinase, phosphotransacetylase, and acetate kinase

Modified from (90) 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 <sub>2</sub> (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 <sub>2</sub> (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  $\mu\text{L}$  per sample. The volume of mastermix B is 6.97  $\mu\text{L}$  per sample. The volume of mastermix C is 6.67  $\mu\text{L}$  per sample. Prepare each mastermix in 2-mL centrifuge tube. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
Mastermix A	1
Mastermix B	1
Sample	1
Mastermix C	2

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least one blank (e.g., buffer).

### Calculation of analyte concentration

1.) Denote the absorbance after the first addition as  $A_1$ .  $A_2$  and other absorbances are defined similarly.

2.) Calculate analyte concentration for cuvette as

$$(A_2^* - A_1) / 6220 \times (500/435.91)$$

where  $6220 \text{ M}^{-1} \text{ cm}^{-1}$  is the extinction coefficient of NADH and  $(500/435.91)$  is the ratio of the final volume to sample volume.

$A_2^*$  is the absorbance after addition 2, extrapolated to the time of addition 2. To find it, plot  $A_2$  vs. time after addition 2. Fit a line to the linear portion of the curve. The y-intercept of the curve is  $A_2^*$ .

3.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the supernatant (done before heating in 2-mL screw-top tube).

## Assay of acetyl-CoA with citrate synthase and DTNB

Modified from ref. (67, 72)

### Preparation of samples

Prepare samples to contain up to 50  $\mu\text{M}$  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 ( $\mu\text{L}$ )	Final concentration (mM or U/mL)
KPO <sub>4</sub> (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 <sub>4</sub> (pH = 7) (1 M)	A
Oxaloacetate (10 mM)	A
DTNB (10 mM)	A

The volume of mastermix A is 150  $\mu\text{L}$  per sample. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
Mastermix A	1
Sample	1
Citrate synthase (90 U/mL)	2

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making

next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least blank (e.g., water).

### **Calculation of analyte concentration**

4.) Denote the absorbance after the first addition as  $A_1$ .  $A_2$  and other absorbances are defined similarly.

5.) Calculate analyte concentration for cuvette as

$$(A_2 - A_1)/14\,150$$

where  $14\,150\text{ M}^{-1}\text{ cm}^{-1}$  is the extinction coefficient of  $\text{TNB}^{2-}$ .

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 ref. (67, 72)

### 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 <sub>4</sub> (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 <sub>4</sub> (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  $6\,220\text{ M}^{-1}\text{ cm}^{-1}$  is the extinction coefficient of NADH and  $(500/401.25)$  is the ratio of the final volume to sample volume.

9.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the sample.

## Assay of acetyl phosphate with malate dehydrogenase, citrate synthase, and phosphotransacetylase

Modified from ref. (72, 91)

### 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 ( $\mu$ L)	Final concentration (mM or U/mL)
Triethanolamine (pH = 7.5) (1 M)	75	150
MgCl <sub>2</sub> (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 <sub>2</sub> (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  $\mu$ L per sample. Because some mastermix will stick to the tube, make extra (equivalent of 1/2 sample) to ensure sufficient volume.

The components are added to cuvettes in the following order:

Component	Addition
-----------	----------

Mastermix A	1
Sample	1
Malate dehydrogenase (180 U/mL)	2
Citrate synthase (90 U/mL)	3
Phosphotransacetylase (3000 U/mL)	4

After combining components for addition 1, mix contents of cuvette and record absorbance (340 nm) at intervals on Genesys 20 spectrophotometer. Allow absorbance to stabilize before making next addition. Repeat for remaining additions. If absorbance does not stabilize, wait until it increases (or decreases) at a constant rate.

In addition to samples, include at least blank (e.g., water).

### Calculation of analyte concentration

10.) Denote the absorbance after the first addition as  $A_1$ .  $A_2$  and other absorbances are defined similarly.

11.) Calculate analyte concentration for cuvette as

$$(A_4^* - A_3) / 6220 \times \{1 + (A_3 - A_1) / [(A_4^* - A_3) + (A_3 - A_1)]\} \times (333.57 / 401.25)$$

where  $6220 \text{ M}^{-1} \text{ cm}^{-1}$  is the extinction coefficient of NADH and  $(500/333.57)$  is the ratio of the final volume to sample volume.

$A_4^*$  is the absorbance after addition 4, extrapolated to the time of addition 4. To find it, plot  $A_4$  vs. time after addition 4. Fit a line to the linear portion of the curve. The y-intercept of the curve is  $A_4^*$ .

12.) Correct the value above by (a) subtracting the concentration for the blank and (b) multiplying by the dilution of the sample (if any).



## Measurement of path length of 96-well plates

Cuvettes have a fixed path length (usually 1 cm), but 96-well plates do not. Path length can be measured empirically using a NADH standard solution.

### NADH standard solution

Prepare the NADH standard solution

Component	Volume ( $\mu$ L)	Final concentration (mM or U/mL)
ddH <sub>2</sub> O	997.7	NA
NADH (40 mM)	2.5	0.1
Final volume	1000	

### Absorbance in cuvettes

Add NADH standard solution to cuvettes (Fisher 14-955-127).

Addition	Cuvette	
	1	2
1	ddH <sub>2</sub> O	ddH <sub>2</sub> O
2	NADH	NADH

After making addition 2, record absorbance (340 nm) using cuvette port of Molecular Devices M3 spectrophotometer

### Absorbance in well plates

Add contents to cuvettes well plates (e.g., Corning 3635).

Addition	Well	
	1	2
1	Cuvette 1	Cuvette 2

Do not add the full 1000  $\mu$ L volume; instead, add the volume required by a specific assay (often 100 to 200  $\mu$ L).

After making addition 1, record absorbance (340 nm) using plate reader of Molecular Devices M3 spectrophotometer.

### Calculation of path length

1.) Denote the absorbance of cuvettes as  $A_{\text{cuvette 1}}$  and  $A_{\text{cuvette 2}}$ .  $A_{\text{well 1}}$  and  $A_{\text{well 2}}$  are the absorbances of well plates.

2.) Calculate path length (cm) as

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

For 200  $\mu\text{L}$  in Corning 3635 plate, path length is c. 0.58 cm. For 100  $\mu\text{L}$ , 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<sub>2</sub>O, 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  $\mu\text{M}$  stock concentration of primers. Look up concentration of primers in manufacturer datasheet accompanying primers. Add 10  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$  per nmol of primer (e.g., 185  $\mu\text{L}$   $\text{H}_2\text{O}$  for tube containing 18.5 nmol) directly to primer tube. Store at  $-20^\circ\text{C}$  until ready for use.
- 3.) Prepare 10  $\mu\text{M}$  working concentration of primers. Add 90  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$  to 1.5 mL sterile centrifuge tube, then add 10  $\mu\text{L}$  aliquot of 100  $\mu\text{M}$  stock. Store at  $-20^\circ\text{C}$  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  $\text{CO}_2$  by inserting it into gassing probe (see *Making buffer and media*). Withdraw  $\sim 0.1$  mL  $\text{CO}_2$  (same volume as culture removed in next step).
  - (d) Withdraw  $\sim 0.1$  mL culture from stock tube after injecting 0.1 mL  $\text{CO}_2$ .
  - (e) Inject 1 drop ( $\sim 20$   $\mu\text{L}$ ) into sterile 1.5 mL microcentrifuge tube containing 200  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$ .
- 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  $-20^\circ\text{C}$  until ready for use, and add last. All other components can be kept on ice until ready for use.
- 6.) Aliquot 49  $\mu\text{L}$  PCR mastermix into PCR tubes. Add 1  $\mu\text{L}$  of DNA template (diluted culture prepared in #4) to each of 2 tubes. Add 1  $\mu\text{L}$  of molecular biology  $\text{H}_2\text{O}$  to another tube (negative control). Add 1  $\mu\text{L}$  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

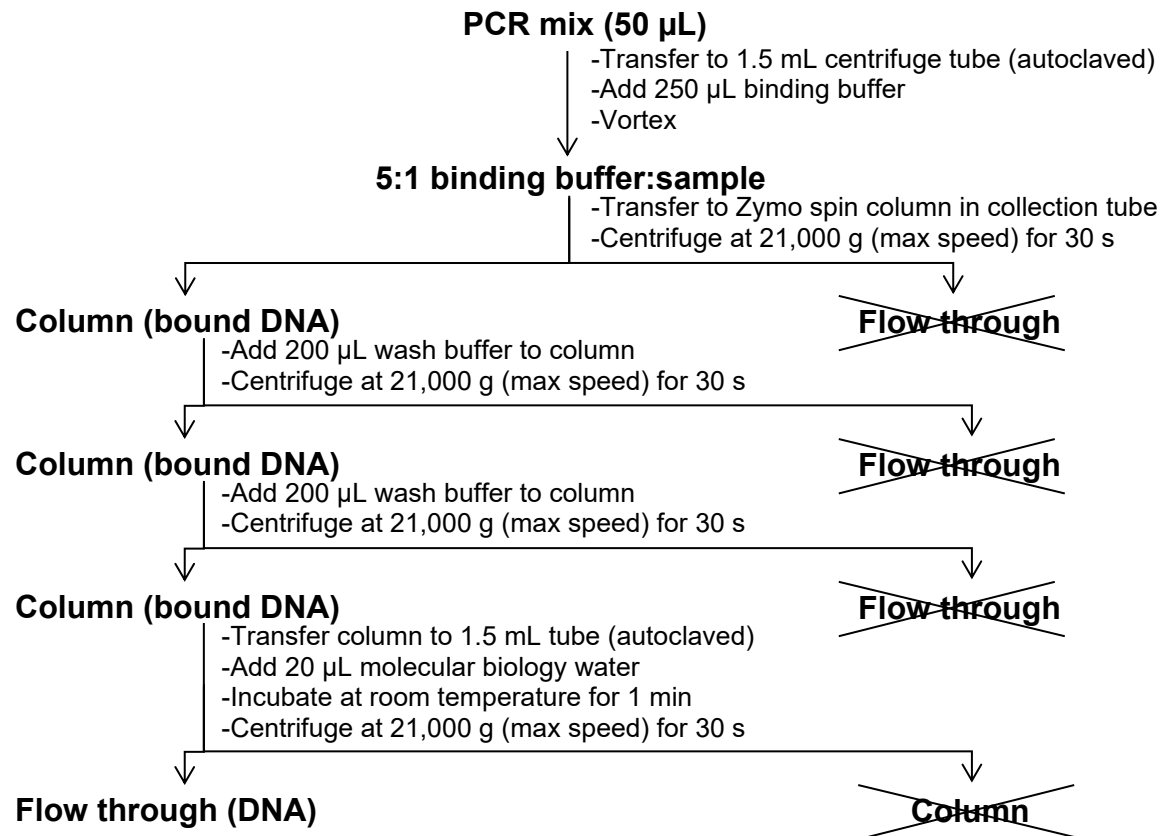
Component	μL per 50 μL reaction
5x Q5 reaction buffer (from NEB M04935)	10
10 mM dNTP (NEB N0447S)	1
Forward primer working concentration	2.5
Reverse primer working concentration	2.5
Molecular biology H <sub>2</sub> O	32.5
Q5 DNA polymerase fidelity (from NEB M04935)	0.5

**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 T<sub>m</sub> calculator (<http://tmcalculator.neb.com/#!/main>); typically 3°C above T<sub>m</sub> of lower T<sub>m</sub> 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  $\mu\text{M}$  stock concentration of primers. Look up concentration of primers in manufacturer datasheet accompanying primers. Add 10  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$  per nanogram of primer (e.g., 185  $\mu\text{L}$   $\text{H}_2\text{O}$  for tube containing 18.5 nmol) directly to primer tube. Store at  $-20^\circ\text{C}$  until ready for use.
- 3.) Prepare 10  $\mu\text{M}$  working concentration of primers. Add 90  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$  to 1.5 mL sterile centrifuge tube, then add 10  $\mu\text{L}$  aliquot of 100  $\mu\text{M}$  stock. Store at  $-20^\circ\text{C}$  until ready for use.

### PCR and sample submission

- 1.) Perform PCR according to *Colony PCR*. One 50  $\mu\text{L}$  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/ $\mu\text{L}$  is typical.
- 5.) Prepare PCR product for submission to the UC Davis DNA Sequencing Center. For each sequencing reaction, the center requires 6  $\mu\text{L}$  template at a concentration of 2 ng/ $\mu\text{L}$  per 100 bp of PCR product. Pipette enough PCR product and molecular biology  $\text{H}_2\text{O}$  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/ $\mu\text{L}$ , and it needs to be sequenced using two primers. Pipette 6  $\mu\text{L}$  of product and 6  $\mu\text{L}$  of molecular biology  $\text{H}_2\text{O}$  at bottom of tube. This will meet the volume (12  $\mu\text{L}$ ) and concentration (30 ng/ $\mu\text{L}$ ) requirements.

- 6.) Prepare the primers for submission. For each sequencing reaction, the sequencing center requires 4  $\mu\text{L}$  of primer at concentration of 3  $\mu\text{M}$ . Pipette enough primer and molecular biology  $\text{H}_2\text{O}$  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  $\mu\text{M}$ . Pipette 3.6  $\mu\text{L}$  of primer 1 and 8.4  $\mu\text{L}$  molecular biology  $\text{H}_2\text{O}$  at the bottom of one tube. Repeat for primer 2 using a second tube. This will meet the volume (12  $\mu\text{L}$ ) and concentration (3  $\mu\text{M}$ ) 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<sub>2</sub>O to 400 mL dH<sub>2</sub>O. 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<sub>2</sub>O. Bring to 500 mL final volume with dH<sub>2</sub>O.

Make 5x TBE buffer. Add to 300 mL dH<sub>2</sub>O

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 ref. (92) 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](http://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	AACTYAAAKGAATTGACGG
342r	CTGCTGCSYCCCGTAG
907r	CCGTCAATTCMTTTRAGTTT
1492r	TACGGYTACCTTGTTACGACTT

**Table 2.** PCR cycling conditions

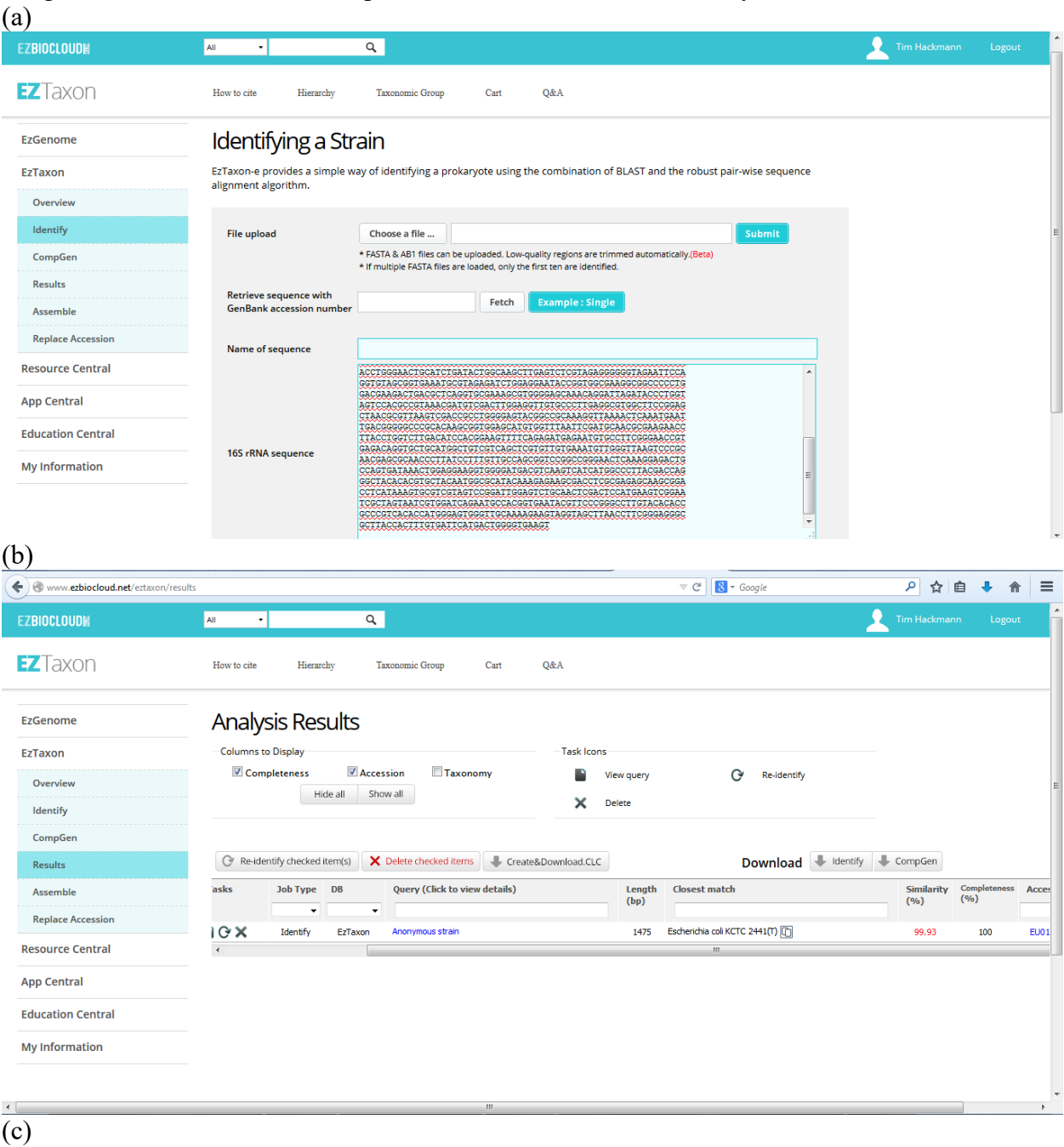
Temp (°C)	Length (min)
	98 3
35 cycles	98 0.167
	63 0.5
	72 1
	72 2
	4 ∞



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

GCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTT  
GCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGA  
TAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG  
CCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGC  
GACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGACACGGTCCAGACTC  
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT  
GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATAC  
CTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA  
TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTAAAG  
TCAGATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATCTGATACTGGCAAGCTTGAGTCTCG  
TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG  
CGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT  
AGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC  
TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAAGGTTAAAACTCAAAT  
GAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACC  
TTACCTGGTCTTGACATCCACGGAAGTTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGA  
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTGAAATGTTGGGTAAAGTCCCGCAACGAGCG  
CAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAAGTCAAAGGAGACTGCCAGTGATAAAC  
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%.



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## 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/Totant
☒ Completeness

Hide all Show all

Functions
CSV file
Excel file
FASTA(gz)
Publish
Editor file

Identification of Anonymous strain Edit
Length of sequence :1475bp View query sequence
Completeness :100%
 (1 - 1476)

Tasks	Rank	Name	Strain	Authors	Taxonomy
	1	<a href="#">Escherichia coli</a>	KCTC 2441(T)	(Migula 1895) Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia
	2	<a href="#">Escherichia fergusonii</a>	ATCC 35469(T)	Farmer et al. 1985	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia
	3	<a href="#">Shigella flexneri</a>	ATCC 29903(T)	Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Shigella
	4	<a href="#">Shigella sonnei</a>	GTC 781(T)	(Levine 1920) Weldin 1927	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Shigella
	5	<a href="#">Shigella boydii</a>	GTC 779(T)	Ewing 1949	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Shigella
	6	<a href="#">Escherichia coli</a>	O157:EC4115	(Migula 1895) Castellani and Chalmers 1919	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia
	7	<a href="#">Escherichia albertii</a>	TW07627(T)	Huys et al. 2003	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia
				(Shiga 1898) Castellani and Chalmers	

### **Example experiment: Sequencing of the mannose phosphotransferase (PTS) operon**

Modified from ref. (93)

- 1.) Perform PCR on bacterial culture using 55F and 2808R primers (**Table 1**). Use conditions in **Table 2**. See **Colony PCR**. Repeat with 2439F and 3927R primers.
- 2.) Sequence PCR products according to **Sequencing**. Use primers in **Table 2**. For the amplicon generated with 55F and 2808R, use primers 55F to 2439F and 305R to 2808R. For the amplicon generated with 2439F and 3927R, use the remaining primers.
- 3.) Generate a consensus sequence after aligning and assembling reads. See **Sequencing**. Include a reference sequence (**Fig. 1**) to aid alignment and identification of aberrant base calls.

#### **Notes**

- i) Two amplicons are generated (55F and 2808R, 2439F and 3927R) because a single amplicon that covered the entire sequence could not be easily generated.

**Table 1.** Primer sequences (from Primer3Plus with default settings)

Primer	Sequence
55F	TTTCCCGAACGTTATTTTGC
452F	TCGCACAATTTGATGCTGAT
935F	TCAAACAAGCAGCACCAAAC
1425F	CATTGAAAACTTTATAAACGAAAGGA
1936F	TTGCAGTACCAACTTCAGCAG
2439F	TGCTCTTGAACGTCACCTGG
2938F	TCCCTAAAGGAAACGTAAGTGG
3447F	CGTAACAAAGTCAGCCGTCA
305R	CCGACACCCATTCTTGTCT
787R	CACCGATGACAGTTCCTTCA
1313R	CCTGCTGAAAGCTTAATTCAA
1785R	AGGAAAAGACCTGCAACAGC
2276R	GCCATGTTTGTACCCCTTTC
2808R	TGGATGATACCACCTGAAAGG
3299R	AGCCGTTAATTCCACGGTAG
3787R	GGTTCCATCCAGCTTCACAT
3927R	CAGCAAGTTTTTCAGCAGCA

**Table 2.** PCR cycling conditions

Temp (°C)	Length (min)
35 cycles	94 2
	94 0.5
	55 0.5
	68 4
	4 ∞

**Figure 1.** Mannose PTS operon. Sequence is from ref. (94) and annotated using ref. (93)

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

TGCTGCTGAAAACTTGCTG = last reverse primer (3957R)

>2601025965 PTS system, mannose-specific IIB component  
[Streptococcus bovis JB1 : Ga0062118\_101] (+)strand

ACCTTTTCATGATTCTTTCAAAAAACATAGCGTTTTTCCCGAACGTTATT  
TTGCGAATTATACAATTTAATCGCTTTATTTTGTGGGTTTCTGAAAATTT  
TTCATGATTTCTGTTATTTTTTCTCAAAAAGCTATTGCATTTTTTGTGAA  
AACGTTTTTACAATAATATCGTTCTTAAAATTTCTTGGAACGGAAAAGAA  
TTGCCG GACTTGTCCGGAAGGA GATTAT TCTTTT TTGTTT CAACGAAAAA  
TAATCATAA GGAGG AAGAACAAGA ATGGGTGTCGGTATTATTATTGCCAG  
CCATGGTAAATTTGCTGAAGGTATTCATCAATCAGGTCTATGATCTTG  
GTGACCAAGAGAAAAGTTCAAGTTGTAACCTTTCATGCCAAGCGAAGGACCT  
GATGATTTATATGCACACTTCAACGATGCTATCGCACAATTTGATGCTGA  
TGATGAAATCCTCGTACTAGCTGACCTTTGGAGTGGTTCTCCATTTAACC  
AAGCTAGTCGCGTAATGGGTGAAAATCCAGACCGCAAGATGGCTATCATT  
ACAGGTCTTAATTTGCCAATGCTTATCCAAGCCTACACTGAGCGTATGAT  
GGACGCAAATGCAGGTGTTGAACAAGTTGTCGCAAATATCATTAAGAGT  
CTAAAGACGGTGTTAAAGCACTTCCTGAAGAACTCAACCCAGCTGAAACA  
GCTGCTGCAGCGCCTGCCGCACAAGCTGCTCCTCAAGGTGCTATCCCTGA  
AGGAAGTGTATCGGTGATGGTAAACTTAAAATCAACCTCGCTCGTATTG  
ACACACGTCTTCTTCACGGACAAGTTGCAACAACTGGACACCAGCTTCT  
AAAGCTGATCGTATCATCGTTGCATCTGACACTGTATCTAAAGATGAATT  
GCGTAAAGGCTTGATCAAACAAGCAGCACCAAACGGTGTTAAAGCAAACG  
TTGTTCCAATCAAGAAATTGATTGAAGCTTCTAAAGACCCTCGTTTTTGGT  
AACACACACGCACTTATCTTGTTTGAAACACCTCAAGAAGCTCTTGAAGC  
TATCGAAGGCGGTGTGCCAATTAAAGAACTTAACGTTGGTTCAATGGCTC  
ACTCAACAGGTAAAACAATGGTTAACAACGTATTGTCAATGGACAAAGAT  
GACGTTGCTACATTTGAAAAATTACGTGACCTTGGCGTAACATTCGATGT  
TCGTAAAGTCCCTAATGATTCTAAAAAAGACTTGTTTGATCTAATCAAAA  
AAGCAAACGTTCAATAA TTTTTGAATTAAGCTTTCAGCAGGGTGAAAAAG  
AAAGCGTTTCTATGAAGGGCTTTCACCTGTCGTGAGAATCAAACCTTCCT  
GTTTTTATCCAAAACACGTAAGCCTTGGTTTCTGGCTTACAATTGCACAT  
TGAAAACTTTTATAAACGAAAGGAAGCTAGATA ATGTCAGTTATTTCTAT  
GATTTTAGTCGTTGTAGTTGCCTTCTTCGCTGGTCTTGAAGGTATCCTTG  
ACGAATTCCAATTCCACCAACCCTAGTTGCCTGCACACTTATCGGTCTT  
GTTACTGGTAACCTTGAAGCAGGTATCATCCTTGGCGGTCTCTACAAAT  
GATCGCTCTTGGTTGGGCTAACATTGGTGCCGCTGTTGCGCCAGATGCTG  
CCCTTGCTTCTGTAGCCGCTGCTATCATCATGGTTAAAGGTGGAGACTTC  
ACTTCTAAAGGTATCGCCGTTGCAACAGCAACAGCTATCCCTCTTGCTGT  
TGCAGGTCTTTTCCTTACTATGCTTGTTTCGTAAGCTTCTGTTGCCCTTG  
TTCACGGTGCTGACGCTGCTGCTAAAGAAGGTAACATCGCTGCAGTTGAA  
CGTACTCACTTGGTAGCTCTATTCCCTTCAAGGTCTTCGTATTGCTGTTCC  
TGCTGCTCTTCTTCTTGCAAGTACCAACTTCAGCAGTACAATCTATCCTTA  
ACGCTATGCCAGACTGGTTGTCAGGTGGTATGGCTGTCGGTGGTGGTATG  
GTTGTTGCCGTAGGTTACGCTATGGTTATCAACATGATGGCTACAAGCGA  
AGTATGGCCTTTCTTCGCAATCGGTTTTTGCCGTTGCTGCTATCTCTGACC  
TTACTCTTATCGCCCTTGGTACAATTGGTGTGCTCTTGCCTTCATCTAC  
CTTAACCTTTTCAGAAAAGGTGGAAATGGTGGCGGAAGTATCTCAGGTTT  
TGGTGACCCAATCGGCGACATCTTGGAAGACTACTAG AAAGGGGTACAAA

CATGGCTGAAAACTTCAATTATCAAAATCTGATCGTCAAAAAGTTTGGT  
GGCGTTTCTACTTTCCTACAAGGTTCTTGGAACACGAACGTATGCAAAAC  
TTAGGTTGGGCATACGCTTTGATCCCTGCTATCAAAAACTTTATACATC  
TAAAGAAGACCGAGCTGCTGCTCTTGAACGTCACTTGGAATTCTTCAATA  
CTCACCACATACGTTGCTGCTCCAATCATCGGTGTAACCTTGCCCTTGAA  
GAAGAACGCGCAAATGGTGCTGAAATTGATGACACAGCTATCCAAGGGGT  
TAAAATCGGTATGATGGGACCTCTTGCTGGTGTTGGTGACCCAGTCTTCT  
GGTTTACAGTTCGTCCTATCCTTGGTGCCCTTGGTGCTTCACTTGCTATG  
GCAGGTAACATCGTTGGTCCACTTCTATTCTTCTTTGGATGGAACATCAT  
CCGTATGGCATTCTTGTGGTACACTCAAGAACTTGGTTACAAAGCTGGTT  
CTGAAATCACTAAAGACCTTTCAGGTGGTATCATCCAAAAATCACTAAA  
GGTGCTTCAATCCTTGGTATGTTTCATCTTGGCTGTCTTGGTTGAACGTTG  
GGTATCAATTAACCTTCACTGTTGATCTTCCTTCACTAACTTTCAGAAG  
GTGCCTACATCGAATTCCCTAAAGGAAACGTAACCTGGTACTGAACTTCAA  
GGTATTCTTGGTAAAGTGGCTGATGGACTTAGCCTTTCACCAGAAAAAGC  
TAACACACTTCAAGGTCAATTGAACTCATTGATTCCTGGTTTGATGGGAC  
TTGCCCTTACATTCTTTGCATGTGGTTGCTTAAGAAAAAAGTTTCTCCA  
ATCACAATCATCATCGGATTGTTTCATCGTTGGTATCATCGCTCGTTTCTT  
CGGAATCATGTAAATAAAAGAAAGGAAGGATTTCCGGTCCTTCTTTTTTTAT  
TATGTTATAATAGACTAAAAGTTTAATCAGAGGAGATTACATGGCACAAT  
CACTTAACTCTACCGTGGAATTAACGGCTACTGGTGTTTCTTACCTTGGT  
ATGGGAGGAAAAGTAGGGAAATTCCTTCTCGGTAACAAAGGTCTTGAATT  
TTATAGCGACGCTAACGTGGAAGATTATATCCAAATTCCTTGGGAAAACA  
TCGAAAAAATCGGCGCTAACGTTTCTCGTAACAAAGTCAGCCGTCACCTT  
GAAATCTTTACAGACAAAGGGAAATTTCTTTTTGCCTCAAAAGACTCAGG  
AAAAATCTTAAAAGTGGCTCGCCAGCATATCGGAAACGATAAAGTTGTTC  
GTATGCTTACGCTTGTTCAAGTCCTTATGAAAAAACTAACAGGATTTGTC  
AAAAAGAAATAAATCTAGTATAATAAATAGTACGGATAAGTAAGAGTGGA  
GCTCTTTTCAGAAAGTCTGCGGTTGCTGTGAGCAGATAGAGCGACAGGTCT  
GAAATTCTACCGTTATATCAATTAAAAATAGAAATTAAGTGCACAGGATG  
TGAAAGCTGGATGGAACCGCGCGATTGCGCTCCAGCAGTTAACATGCTGTG  
CTTTTTGTTCATCTGGGCACAGCTAACCCTATGAGCGCTAAAAAACTGG  
AGGAACTTATGTTAGACATCAAACGTATTCGTAACGATTTTCGACGAAGT  
CGCTAAAAAATTAGCTACACGTGGCGTTGCTGCTGAAAAACTTGCTGAAC  
TTAAAGAACTTGACGACAAACGTCGTGAATTGCTT

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

Modified from ref. (94)

- 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.



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Column Selector Select Page Deselected Page

Select	Domain	Status	Genome Name	Study Name	Sequencing Center	Genome Size	Gene Count
<input checked="" type="checkbox"/>	A	F	<a href="#">Methanospirillum hungatei JF-1</a>	Methanogenic Syntrophic Consortium	DOE Joint Genome Institute	3544738	3307
<input checked="" type="checkbox"/>	B	P	<a href="#">Bubrih1b10 hungatei NK4A153</a>	The Hungate 1000. A catalogue of reference genomes from the rumen microbiome.	DOE Joint Genome Institute	3372542	3164

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

JGI 242 Genomes Quick Genome Search: Go Hi Timothy Hackmann | Logout

img INTEGRATED MICROBIAL GENOMES

IMG Home Find Genomes Find Genes Find Functions Compare Genomes Analysis Cart OMICS ABC My IMG Data Marts Using

Home > Find Genomes 241 genomes retrieved

NERSC Quarterly Maintenance: All IMG systems will be down for **two days** from **Tue Feb 3 2015** to **Wed Feb 4 2015**. Sorry for the inconvenience.

### Genome Field Search

**hint:** Go to [Preferences](#) to show or hide plasmids, GFragment and viruses.  
Go to home page statistics under [IMG Genomes](#) to select individual phylogenetic domains or all genomes.

Domains(D): B=Bacteria, A=Archaea, E=Eukarya, P=Plasmids, G=GFragment, V=Viruses.  
Genome Completion(C): F=Finished, P=Permanent Draft, D=Draft.

Add Selected to Genome Cart Select All Clear All

Filter column: Domain Filter text Apply

Export Page 1 of 3 << first < prev 1 2 3 > next >> All

Column Selector Select Page Deselected Page

Function Search Search Pathways COG KEGG KEGG Pathways via KO Terms

Phosphotransferase system (PTS)

https://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=FindFunctions&page=ffoAllKeggPathways&view=ko

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

- 03 [Protein export](#) (39)
- 03 [Protein processing in endoplasmic reticulum](#) (140)
- 03 [SNARE interactions in vesicular transport](#) (34)
- 03 [Ubiquitin mediated proteolysis](#) (124)
- 03 [Sulfur relay system](#) (21)
- 03 [Proteasome](#) (48)
- 03 [RNA degradation](#) (75)
- 02 **Replication and repair**
  - 03 [DNA replication](#) (51)
  - 03 [Base excision repair](#) (41)
  - 03 [Nucleotide excision repair](#) (48)
  - 03 [Mismatch repair](#) (45)
  - 03 [Homologous recombination](#) (59)
  - 03 [Non-homologous end-joining](#) (19)
  - 03 [Fanconi anemia pathway](#) (54)
- 01 **Environmental Information Processing**
  - 02 **Membrane transport**
    - 03 [ABC transporters](#) (433)
    - 03 [Phosphotransferase system \(PTS\)](#) (79)
    - 03 [Bacterial secretion system](#) (74)
  - 02 **Signal transduction**
    - 03 [Two-component system](#) (408)
    - 03 [Ras signaling pathway](#) (161)
    - 03 [Rap1 signaling pathway](#) (151)
    - 03 [MAPK signaling pathway](#) (188)
    - 03 [MAPK signaling pathway - fly](#) (17)
    - 03 [MAPK signaling pathway - yeast](#) (41)
    - 03 [ErkB signaling pathway](#) (64)
    - 03 [Wnt signaling pathway](#) (98)
    - 03 [Notch signaling pathway](#) (28)
    - 03 [Hedgehog signaling pathway](#) (40)
    - 03 [TGF-beta signaling pathway](#) (61)
    - 03 [Hippo signaling pathway](#) (104)
    - 03 [Hippo signaling pathway - fly](#) (52)
    - 03 [VEGF signaling pathway](#) (37)
    - 03 [JAK-STAT signaling pathway](#) (123)
    - 03 [NF-kappa B signaling pathway](#) (86)

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

The screenshot shows the KEGG Pathway Details page for the Phosphotransferase system (PTS). The page has a navigation bar with tabs: "KO Terms in Pathway", "Save to My Workspace", and "View Map for Selected Genomes". The "View Map for Selected Genomes" tab is active.

Below the navigation bar, there is a section titled "KEGG Map for Selected Genomes". It includes a "Sequencing Status" dropdown menu set to "All Finished, Permanent Draft and Draft", a "Domain" dropdown menu set to "Genome Cart", and a "Selected: 242" indicator. There are also "List" and "Tree" radio buttons, and a "Show" button.

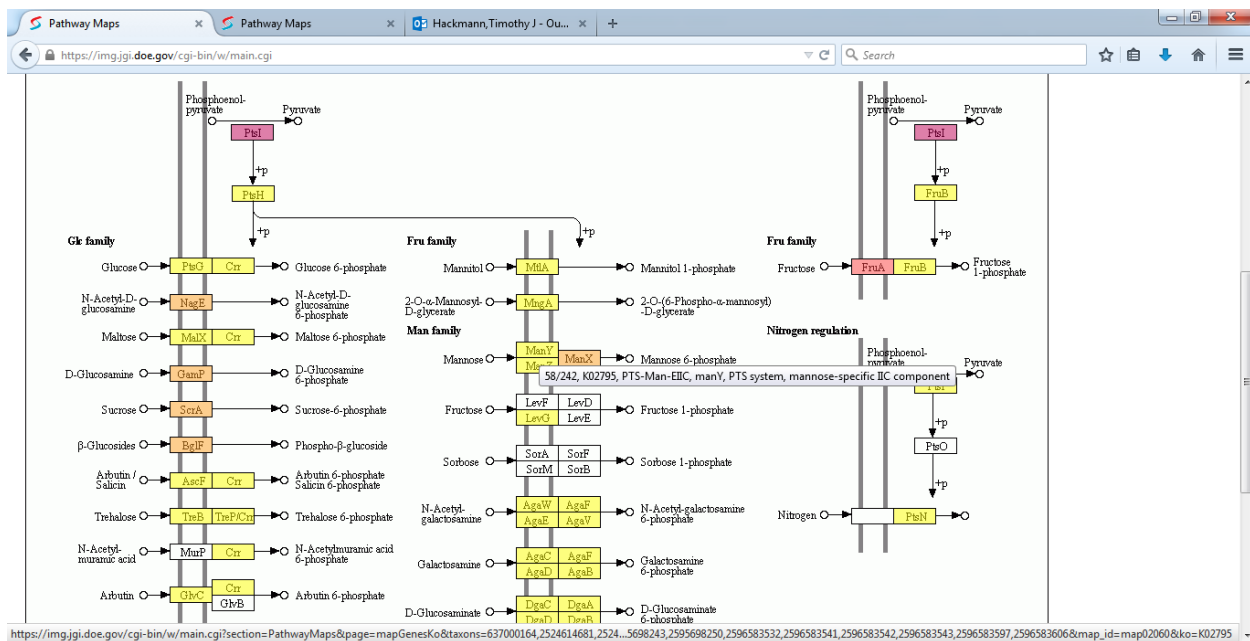
A search bar is present with the placeholder text "<enter a genome name to search>". Below the search bar is a list of 242 genomes, including *Staphylococcus epidermidis* AG42 (B) [P], *Streptococcus bovis* 2B (B) [P], *Streptococcus bovis* A645 (B) [P], *Streptococcus bovis* B315 (B) [P], *Streptococcus bovis* JB1 (B) [P], *Streptococcus bovis* SN033 (B) [P], *Streptococcus equinus* GA-1 (B) [P], *Streptococcus gallolyticus* VTM1R27 (B) [P], *Streptococcus henryi* A-4 (B) [P], *Succinivibrio dextrinosolvens* ACV-10 (B) [P], *Succinivibrio dextrinosolvens* H5 (B) [P], *Treponema bryantii* NK4A124 (B) [P], and *Treponema sp.* C6A8 (B) [P].

Below the list of genomes, there is a checkbox labeled "Find missing enzymes. (For single genome selection)".

At the bottom of the section, there are two buttons: "View Map" and "Reset".

The footer of the page includes contact information, a version number (4.510 Oct 2014), and logos for the U.S. Department of Energy and the Office of Science.

8.) Find the mannose PTS transporter in the figure. Click on the ManX, ManY, or ManZ subunit.



9.) The screen that appears shows all gene IDs for ManX, ManY, or ManZ. Click on a gene ID of interest.

Filter column: Gene ID Filter: text Apply

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

Column Selector Select Page Deselect Page

Select	Gene ID	Locus Tag	Gene Product Name	Genome	KO
<input type="checkbox"/>	<a href="#">2525402220</a>	G598DRAFT_00618	PTS system, mannose/fructose/sorbose family, IIC component	<a href="#">Selenomonas ruminantium ATCC 12561</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525403206</a>	G598DRAFT_01611	PTS system D-mannose-specific IIC component, Man family (TC 4.A.6.1.1)	<a href="#">Selenomonas ruminantium ATCC 12561</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525447621</a>	G605DRAFT_00459	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium cadaveris AGR2141</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525447876</a>	G605DRAFT_00716	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium cadaveris AGR2141</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525454837</a>	G614DRAFT_04236	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium clostridioforme AGR2157</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525455699</a>	G594DRAFT_00068	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium paraputrificum AGR2156</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525457711</a>	G594DRAFT_02100	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium paraputrificum AGR2156</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525458478</a>	G594DRAFT_02880	PTS system, fructose-specific, IIB componentPTS system, fructose subfamily, IIA componentPTS system, fructose subfamily, IIC component	<a href="#">Clostridium paraputrificum AGR2156</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525458728</a>	G594DRAFT_03133	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium paraputrificum AGR2156</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525459007</a>	G594DRAFT_03415	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Clostridium paraputrificum AGR2156</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525469216</a>	G619DRAFT_02853	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC	<a href="#">Lachnospiraceae bacterium NK4A144</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525473359</a>	G607DRAFT_00212	PTS system D-glucose-specific IIC component, Man family (TC 4.A.6.1.6)	<a href="#">Clostridium butyricum AGR2140</a>	<a href="#">K02795</a>
<input type="checkbox"/>	<a href="#">2525474413</a>	G607DRAFT_01284	Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-	<a href="#">Clostridium butyricum</a>	<a href="#">K02795</a>

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

<b>External Links</b>	
Fused Gene	No
<b>Protein Information</b>	
Amino Acid Sequence Length	303aa
COG	<a href="#">COG3716</a> - Phosphotransferase system, mannose/fructose/N-acetylglucosamine-specific component IID
KOG	
Families	- <a href="#">IPR004704</a> Phosphotransferase system, mannose/fructose/sorbose family IID component (ProSiteProfiles PS51108) - <a href="#">TIGR00828</a> PTS system, mannose/fructose/sorbose family, IID component
Transmembrane Helices	Yes
Signal Peptide	No
Statistics	<a href="#">peptide</a>
Pfam	<a href="#">pfam03613</a> - EIID-AGA
<b>Pathway Information</b>	
Enzymes	
Transport Classification	<a href="#">TC:4.A.6 - The PTS Mannose-Fructose-Sorbose (Man) Family</a>
KEGG Orthology (KO) Term	<a href="#">KO:K02798</a> PTS-Man-EIID, manZ PTS system, mannose-specific IID component
KEGG Pathway	<a href="#">Amino sugar and nucleotide sugar metabolism</a> <a href="#">Fructose and mannose metabolism</a> <a href="#">Phosphotransferase system (PTS)</a>
KEGG Orthology (KO) Modules	<a href="#">PTS system, mannose-specific IID component</a>
MetaCyc Pathway	
IMG Pathways	
IMG Clusters	
Chromosomal Cassette	Details By -- Select Protein Cluster --
<input type="button" value="Add To Gene Cart"/> <input type="button" value="Find Candidate Enzymes"/>	

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

Genes in Cart	Functions	<b>Upload &amp; Export</b>	Chromosome Map	Sequence Alignment	Gene Neighborhoods	Profile & Alignment
---------------	-----------	----------------------------	----------------	--------------------	--------------------	---------------------

### Upload Gene Cart

The Gene Cart is used for genes already in IMG. Only previously exported IMG genes can be uploaded. [To upload private data](#), you must submit it to IMG through the [submission site](#).

You may upload a Gene Cart from a tab-delimited file.  
The file should have a column header 'gene\_oid' (or 'Gene ID', or 'Gene Object ID').  
(This file can be created using the [Export Genes](#) section below with the format 'Gene information in tab delimited format to Excel')

File to upload:  
 No file selected.

### Export Genes

**hint:** Export large number of genes will be very slow.  
You will be notified for the result via email if exporting over 1000 genes.

You may select genes to export in one of the following export formats.

☐ FASTA Amino Acid format  
☒ FASTA Nucleic Acid format    -0    bp upstream    +0    bp downstream  
☐ Gene data in tab-delimited format to Excel (**Gene Cart uploadable format**)

My Email:   
 (Results will be mailed to you if selection is over 1000 genes.)

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

Unknown model, Gene, Start function primer, etc.

**Thurs Mar 17 2016** IMG will be down all day for database maintenance.  
Sorry for the inconvenience. Get updates from our [IMG Forum](#)

## Export Gene Fasta Nucleic Acid Sequence

Red = start or stop codon, Green = upstream or downstream padding.

```
>2601025967 FTS system IID component, Man family [Streptococcus bovis JB1 : Ga0062118_101] (+)strand
ATGGCTGAAAACCTCAATTATCAAAATCTGATGCTCAAAAAGTTTGGTG
GCGTTCTACTTCTCTACAGGTTCTTGGAACTACGAACTATGCAAACT
TAGGTTGGGCATACGCTTTGATCCCTGCTATCAAAAACCTTATACATCT
AAGAAGACCGAGCTGCTGCTCTTGAACGTCATTGGAATCTTCAATAC
TCACCCATACGTTGCTGCTCCAATCATCGGTGTAACCTTTGCCCTTGAAG
AAGAACGGCAATGGTGGTGAATTTGATGACACAGCTATCCAAAGGGGTT
AAATCGGTATGATGGGACCTTTGCTGGTGTGTTGGTGGACCCAGCTTCTG
GTTTACAGTTGCTGCTATCTTTGGTGCTTGGGCTCTCACTTCTATGG
CAGTAACATCGTTGGTCCACTTCTATTCTTTTGGATGGAACATCATC
CGTATGGCACTTTTGGTACACTCAAGAACCTTGGTACAAAAGCTGGTTC
TGAAATCACTAAAGACCTTTCAAGTGGTATCATCCAAAAACCTCAATAG
GTGCTTCAATCCTTGGTATGTTATCTTGGCTGCTTGGTGAACGTTGG
GTATCAATTAACCTTCACTGTTGATCTTCTTCACTAACTTTCAGAGG
TGCTTACATCGAATTCCTAAAGGAGCTAACTGGTACTGAACTTCAAG
GTATCTTGGTAAAGTGGCTGATGGACTTACGCTTACCAAAAACCT
AACACACTTCAAGGTCAATTGAACCTATTGATTCTCGTGTGATGGGACT
TGCCCTTACATTCCTTTGCATGTGGTGTGCTTAAGAAAAAGTTTCTCCAA
TCACATCATCATCGGATTGTTATCGTTGGTATCATCGCTGTTTCTTC
GGAATCATGTAA
```

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Version 4.540 Oct. 2015  
gpweb04 gemini1\_shared 5.016000 2016-03-10-14:33:48 128.227.227.195

13.) As an alternative to the above, in step #5, point to “Find Functions”, then “Function Search”, then “Pathways via KO terms”. Click. Under “Keyword”, enter the KO IDs for the genes of interest (separated by commas and spaces), then click “Go”. Continue with step #9.

This approach is faster when the KO IDs are known and many IDs need to be searched. COGs and pfam IDs can be searched analogously.

## Function Search

Function search allows you to find functions in selected genomes by keyword.

**hint:** Search term marked by \* indicates that it supports metagenomes.  
You must add your selections into **Selected Genomes**.

**Keyword:** K02795, K02796, K02797  
**Filters:** KEGG Orthology ID (list) \*

Search for functions in: ☒ Selected Genomes ☐ Selected Domain ☐ All Isolates

**Sequencing Status:** All Finished, Permanent Draft and Draft **Domain:** Genome Cart  
**List:** ☒ List ☐ Tree **Show:**  **Selected:** 72

**Search for:** <enter a genome name to search>

Butyrivibrio fibrisolvens AB2020 (B) [P]  
Butyrivibrio fibrisolvens AR40 (B) [D]  
Butyrivibrio fibrisolvens DSM 3071 (B) [D]  
Butyrivibrio fibrisolvens FE2007 (B) [P]  
Butyrivibrio fibrisolvens MD2001 (B) [P]  
Butyrivibrio fibrisolvens ND3005 (B) [P]  
Butyrivibrio fibrisolvens WTE3004 (B) [P]  
Butyrivibrio fibrisolvens YR82005 (B) [P]  
Butyrivibrio hungatei DSM 14810 (B) [D]  
Butyrivibrio hungatei NK4A153 (B) [P]  
Butyrivibrio hungatei XBD2006 (B) [D]  
Butyrivibrio proteoclasticus B316 (B) [F]

**Selected Genomes**  
72 selected  
Butyrivibrio fibrisolvens AB2020 (B) [P]  
Butyrivibrio fibrisolvens AR40 (B) [D]  
Butyrivibrio fibrisolvens DSM 3071 (B) [D]  
Butyrivibrio fibrisolvens FE2007 (B) [P]  
Butyrivibrio fibrisolvens MD2001 (B) [P]  
Butyrivibrio fibrisolvens ND3005 (B) [P]  
Butyrivibrio fibrisolvens WTE3004 (B) [P]  
Butyrivibrio fibrisolvens YR82005 (B) [P]  
Butyrivibrio hungatei DSM 14810 (B) [D]  
Butyrivibrio hungatei NK4A153 (B) [P]  
MER-FS Metagenome: Assembled

**Go** **Reset**

**hint:** All searches treat the keyword as a substring (a word or part of a word).  
The search should contain some alphanumeric characters.

## Amplification of DNA from small numbers of rumen bacteria cells

Adapted from ref. (95), Repli-g Single Cell Handbook (<https://www.qiagen.com/us/resources/download.aspx?id=38faca1c-64b0-4281-aab3-aa8324bbd181&lang=en>), instruction manual for SsoAdvanced™ Universal SYBR® Green Supermix (<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10024379pdf>), and instruction manual for CFX 96 (<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10021337pdf>).

### 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 mL centrifuge tubes (SARSTEDT 72.706.700); and 0.2 mL strip tubes (Biorad TLS 0801) and caps (Biorad TCS0803). Designate these materials for this work, and do not use for other procedures.
- 2.) Wipe down biological safety cabinet with 70% ethanol and then 10% bleach. Bleach must be made fresh on day of use.
- 3.) Similarly wipe down pipette tip boxes, pipettes, vortex mixer, and mini centrifuge.
- 4.) Place all equipment and materials in biological safety cabinet. Turn on the UV lamp of the cabinet for 60 min.

### Preparation of solutions and reagents

Do all procedures (except #4) in the biological safety cabinet.

- 1.) Prepare buffers from the Qiagen Repli-g Single Cell kit (Qiagen 150343).
  - (a) Prepare buffer DLB by adding 500 µl H<sub>2</sub>O 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  $\mu$ L of each sample (serial dilution of mixed bacteria, positive controls, negative controls) to strip tubes in duplicate.

2.) Add 1.5  $\mu$ L of buffer D2 to each sample. Mix by briefly centrifuging in a mini centrifuge.

3.) Incubate strip tubes for 10 min at 65°C (using plate thermocycler).

4.) Add 1.5  $\mu$ L stop solution to each sample. Mix by briefly centrifuging in a mini centrifuge.

5.) Transfer tubes to ice. Store for no longer than 1 h.

6.) Thaw polymerase and other component on ice. Mix by vortexing and centrifuging in a mini centrifuge.

7.) Prepare a master mix according to Table 2. Add components in order shown. Mix by briefly centrifuging in a mini centrifuge.

8.) Add 20.25  $\mu$ L mastermix to each sample. Mix by briefly centrifuging in a mini centrifuge. Transfer to ice, and use immediately for next step.

9.) Start amplification of DNA with Bio-Rad CFX96 Real-Time system.

(a) Turn the system and the computer.

(b) Open the Bio-Rad CFX Manager using the icon on the computer desktop.

(c) In the “Startup Wizard” window that appears, select the run type as “User-defined” (Fig. 1). Alternatively, “Repeat run” can be chosen if conditions are the same as the last run.

(d) In the “Run Setup” window that appears, select “Create New...” (Fig. 2).

(e) In the “Protocol Editor” window (Fig. 3), insert conditions shown in Table 3. Click “Insert Step” to insert a step, and then enter the temperature and length. Click “Add Plate Read to Step” to include a plate read at the end of each cycle. Click “Insert GOTO” in order to repeat the cycle. To hold a step forever (an infinite hold), enter zero (0.00) for the time. Under “Settings”, set the temperature of heated lid at 70°C. Click “OK” to finish editing.

(f) Go back to “Run Setup” and click “Plate” tab (Fig. 2).

- (g) Edit the plate template in the “Plate Editor” window that appears (Fig. 4). Click on a well to edit, then select the sample type (e.g., “Unknown” for samples). Under “Load”, click SYBR. Enter sample name under “Sample name”. Repeat for all remaining wells. Click “OK”.
- (h) Go back to “Run Setup” and click on “Start Run” tab (Fig. 5). Click “Open Lid”. Lid with automatically open (do not force open).
- (i) Insert strip tubes with samples. Include a second strip tube 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. (96). That reference showed that 75 min irradiation was sufficient to render DNA non-amplifiable when spiked in 15 ml liquid. The radiant exposure in that reference was  $4 \text{ mW/cm}^2$ , whereas the exposure of our multilinker only  $3.75 \text{ mW/cm}^2$ . Thus,  $75 \times 4 / 3.75 = 80$  min irradiation time is required for our multilinker.

**Table 1.** Primer sequences

Primer	Sequence
926wF	GAAACTYAAAKGAATTGRCGG
1392R	ACGGGCGGTGTGTRC

**Table 2.** Components of the Repli-g sc DNA Polymerase mastermix

Component	μL per 25.25 μL reaction
H <sub>2</sub> O sc	4.5
REGLI-g reaction buffer	14.5
50X SYBR green	0.25
REGLI-g DNA polymerase	1
Total	20.25

**Table 3.** PCR cycling conditions for Repli-g sc DNA Polymerase

	Step	Temp	Length
80 cycles	Amplification	30°C	6 min
	Plate read	30°C	NA
	Heat inactivation	65°C	3 min
	Hold	4°C	∞

**Table 4.** Components of the SsoAdvanced Universal SYBR Green mastermix

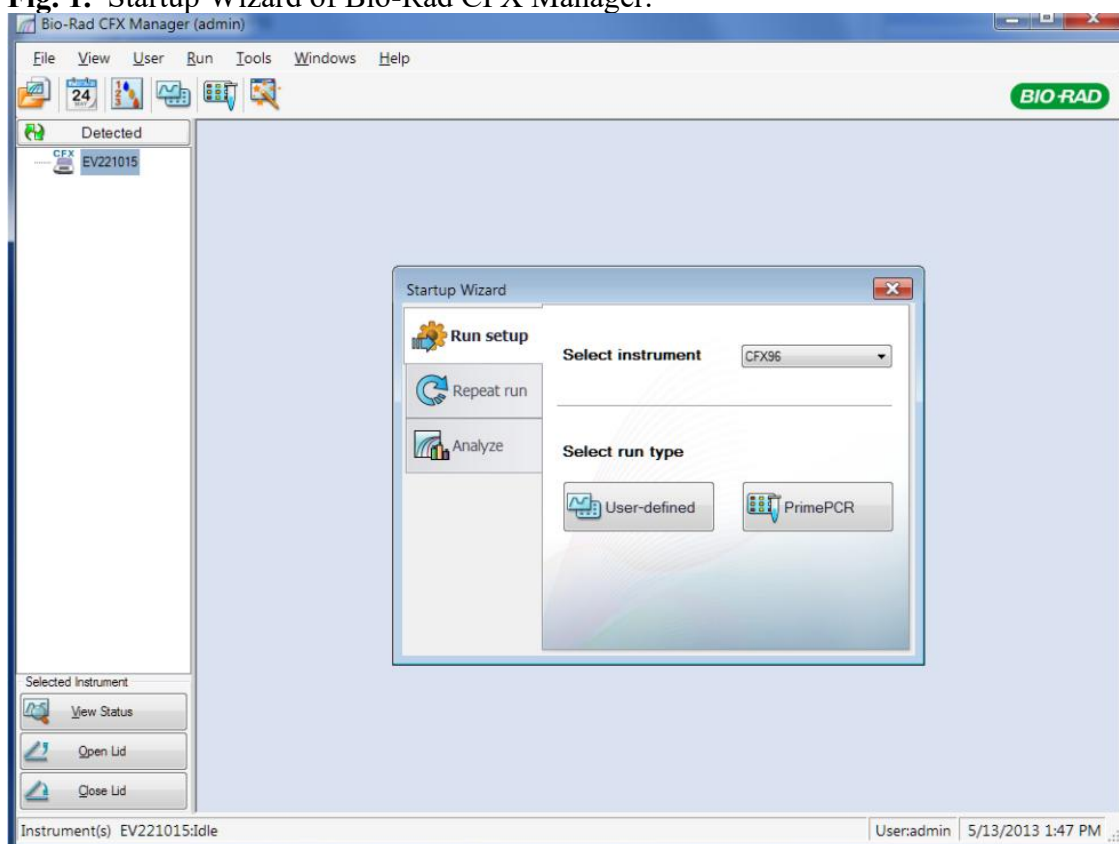
Component	μL per 10 μL reaction
SsoAdvance SYBR Green supermix 2X	5
Forward primer (926wF)	0.2
Reverse primer (1392R)	0.2
Molecular biology water	3.6
Total (does not include 1 μL amplified DNA)	9

**Table 5.** PCR cycling conditions for SsoAdvanced Universal SYBR Green Supermix

	Step	Temp	Length
40 cycles	Initial denaturation	98°C	3 min
	Denaturation	98°C	15 s
	Annealing and extension	60°C	1 min
	Plate read	60°C	NA
	Melt curve analysis	65 to 95°C <sup>a</sup>	5 s/increment
	Hold	4°C	∞

<sup>a</sup>Use 0.5°C increments

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



**Fig. 2.** Run Setup.

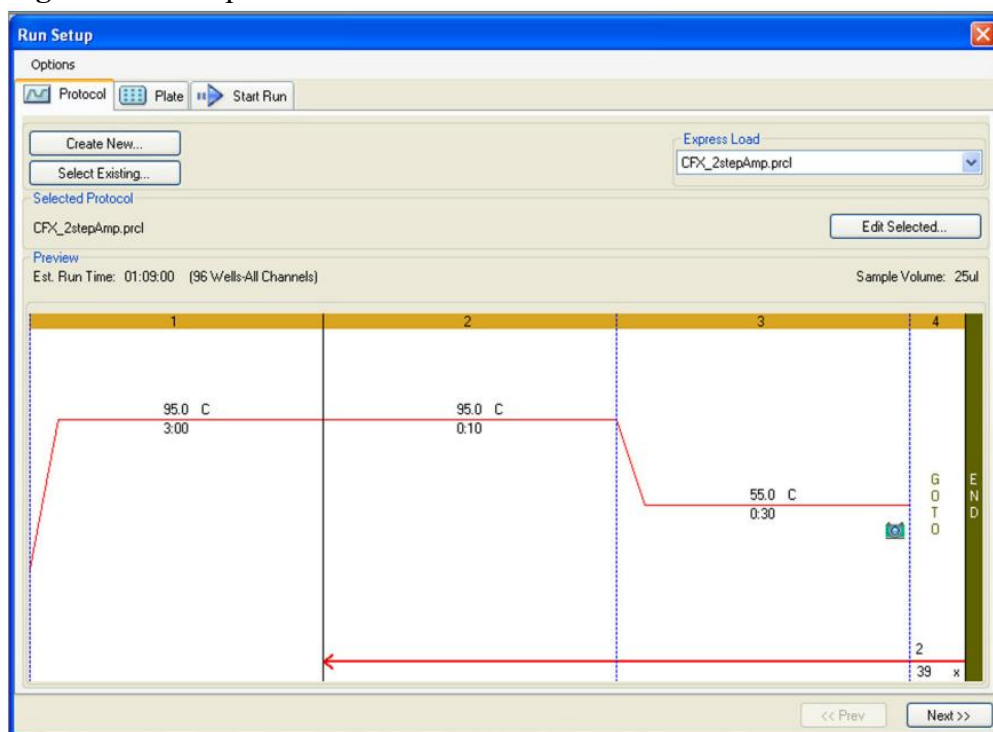


Fig. 3. Protocol Editor window

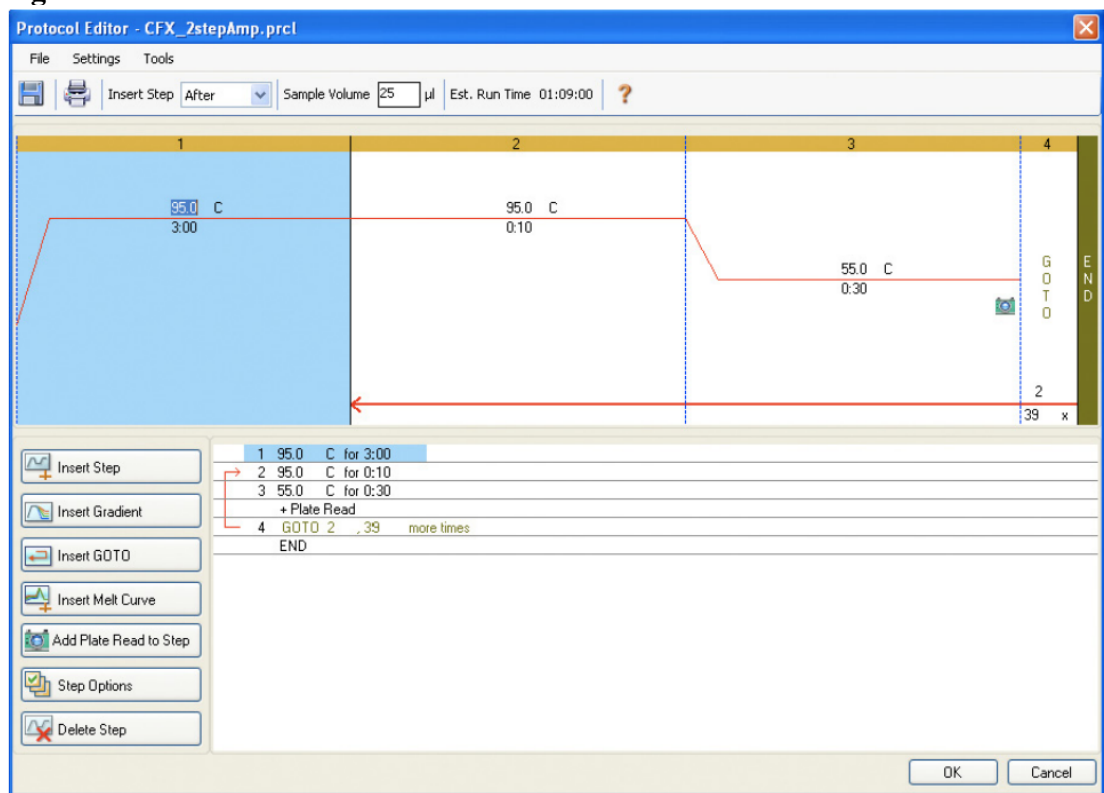
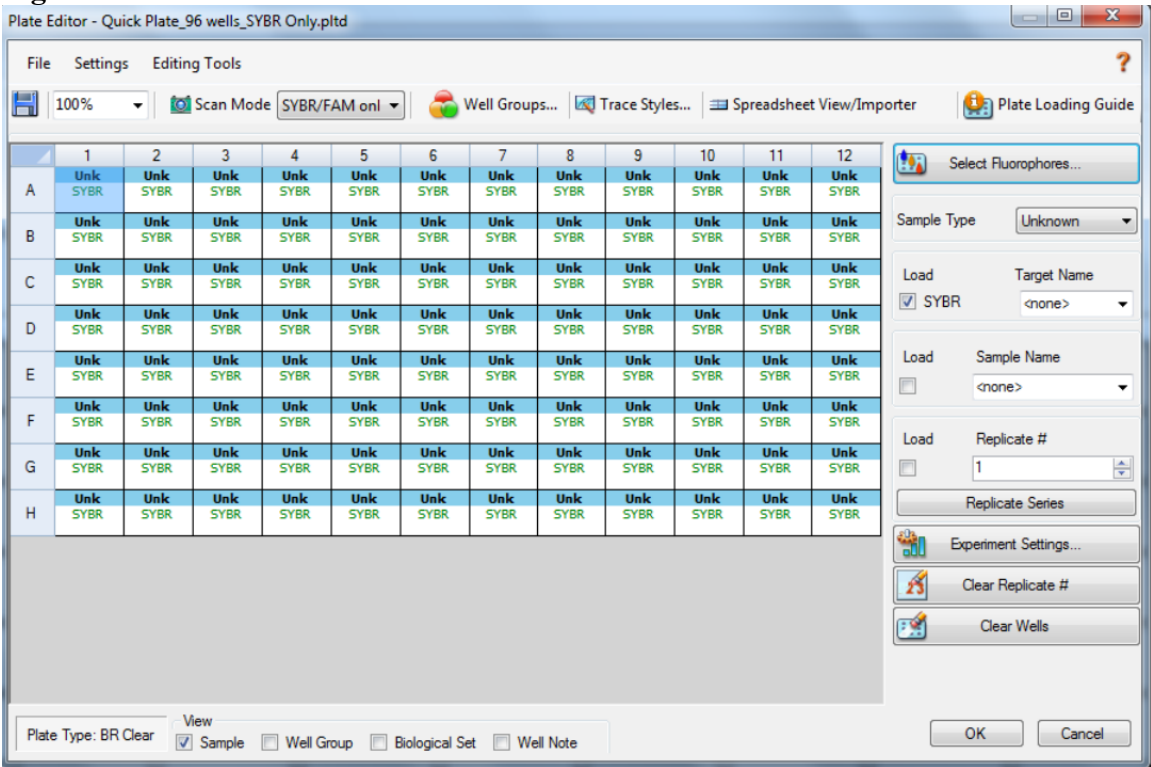
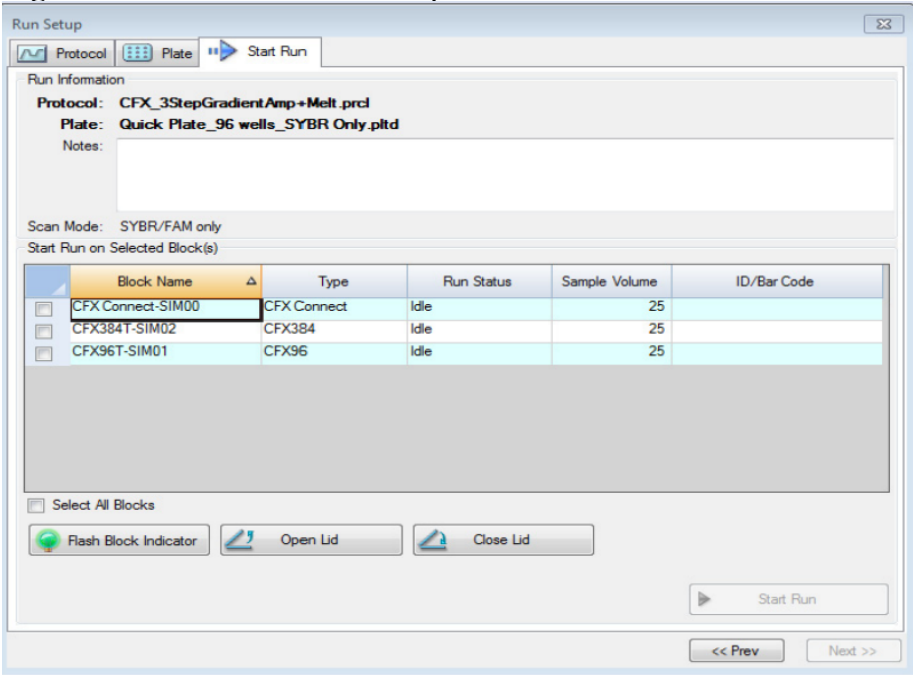


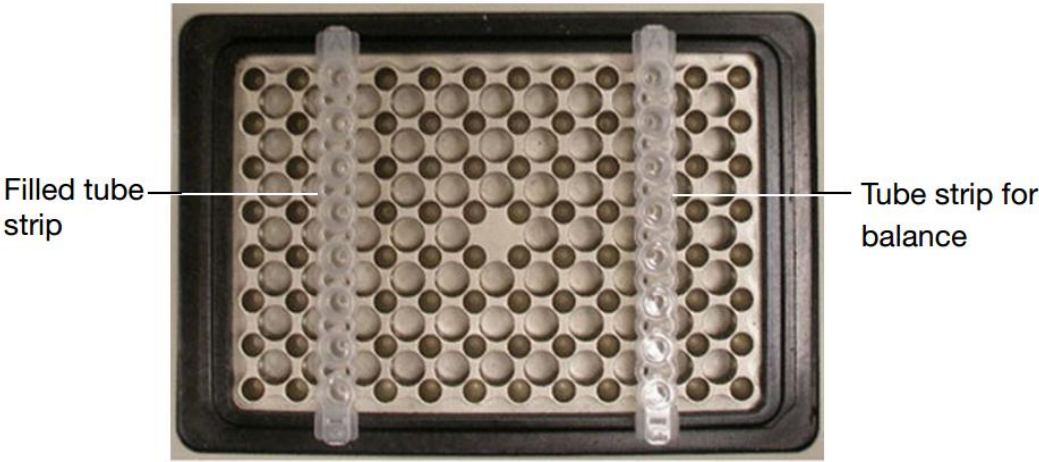
Fig. 4. Plate Editor window



**Fig. 5.** Start Run tab in Run Setup.



**Fig. 6.** Balancing of strip tubes.



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

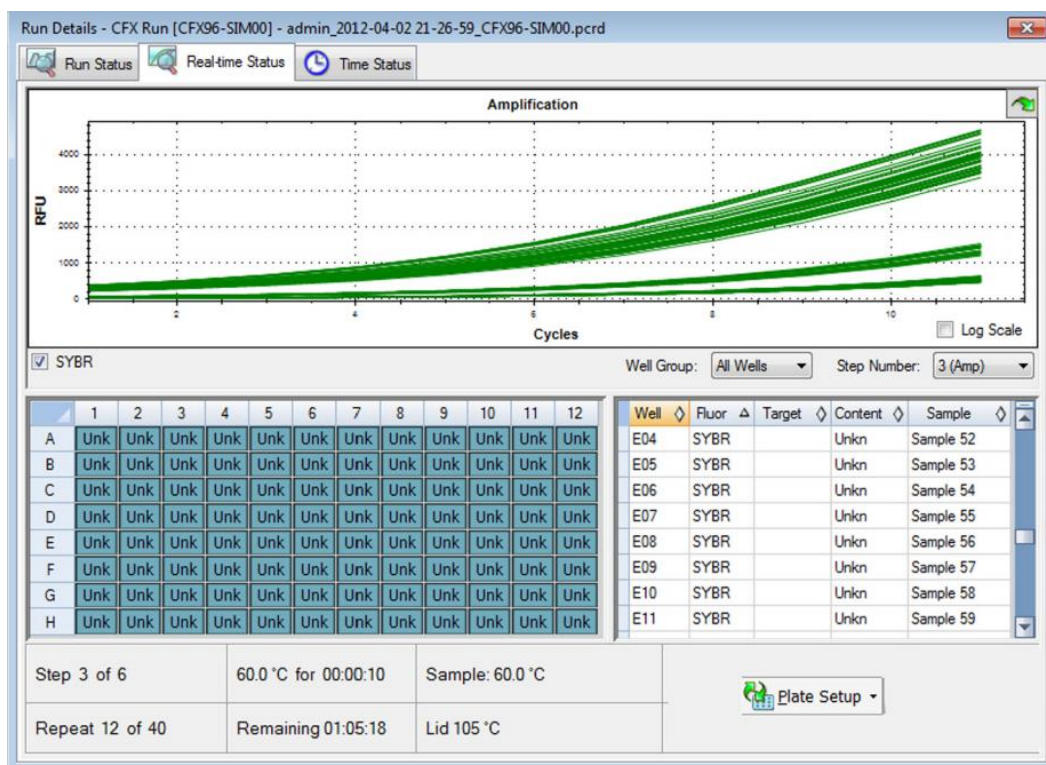
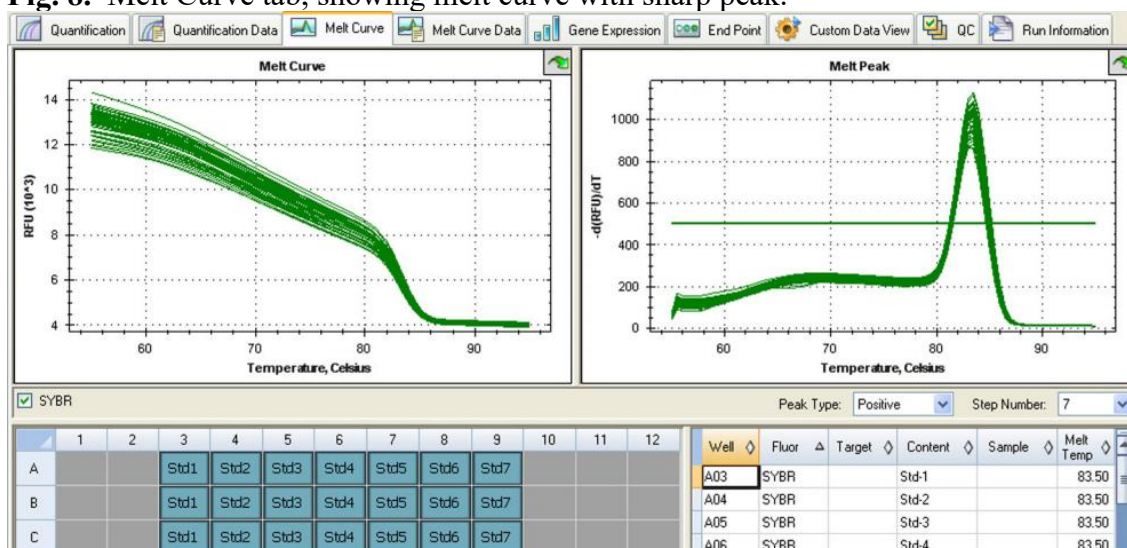
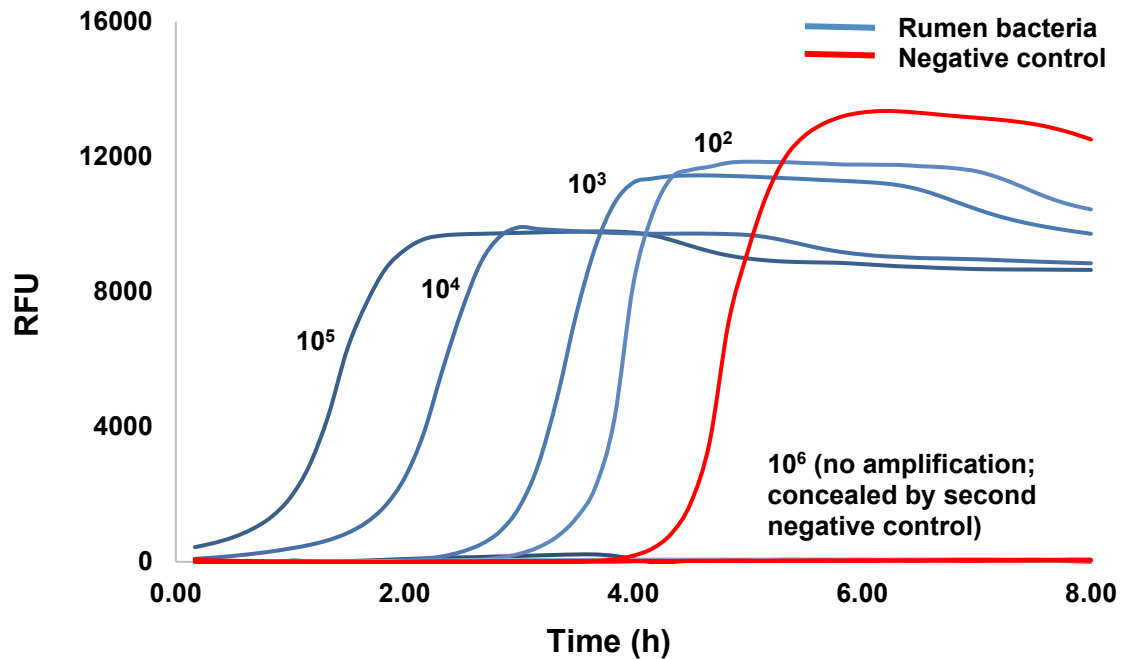


Fig. 8. Melt Curve tab, showing melt curve with sharp peak.



**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 4°C) and 0.002 g dithioerythritol (DTT) in a 2-mL tube. Dissolve in 1 mL acetone and chill at -20°C. Make immediately before use.

#### ***50 mM AMBIC***

Add 3.953 g ammonia bicarbonate (AMBIC) to a beaker. Bring to ~80 mL with LC-MS water (Fisher W6-4), then stir to dissolve. Bring to 100 mL with LC-MS water in a volumetric flask.

#### ***6 M urea in AMBIC***

Add 3.6036 g urea to 15 mL tube. Add ~6 mL of 50 mM AMBIC and dissolve. Bring to 10 mL with more AMBIC.

#### ***200 mM DTT in AMBIC***

Add 0.03 g dithioerythritol (DTT) to 2-mL tube. Dissolve in 1 mL of 50 mM AMBIC. Store at 4°C.

#### ***194.6 mM IAM in AMBIC***

Add 0.036 g of iodoacetamide (IAM) 2-mL tube. Dissolve in 1 mL of 50 mM AMBIC. Cover the tube with aluminum film and store at 4°C in the dark.

#### ***Trypsin/Lys-C***

Order trypsin/Lys-C (Promega V5073). Thaw resuspension buffer (50 mM acetic acid) on ice. Add 20 µL of the resuspension buffer to one vial of trypsin/Lys-C. Transfer contents to a 0.5 mL tube. Wash vial with 20 µL resuspension buffer, then transfer wash to 0.5 mL tube. Final volume will be approximately 40 µL, giving 0.5 µg/µL enzyme. Dispense 10 µL aliquots into PCR tubes and store at -80°C.

#### ***4% (v/v) TFA***

Add 20 mL of LC-MS water (Fisher W6-4) to a 25-mL volumetric flask. Break open a 1-mL vial of trifluoroacetic acid (Fisher A116-1 AMP) and add to water. Rinse vial and bring solution to 25 mL with LC-MS water. Cover the bottle with aluminum film and store in the dark at room temperature.

#### ***Equilibration solution (0.1% [v/v] TFA in water)***

Add 975 µL of LC-MS water (Fisher W6-4) and 25 µL of 4% (v/v) TFA to a 2-mL tube.

#### ***Rinse solution (0.1% [v/v] TFA in 5% [v/v] acetonitrile)***

Add 925 µL of LC-MS water (Fisher W6-4), 50 µL acetonitrile (Fisher A955-4), and 25 µL of 4% (v/v) TFA to a 2-mL tube.

#### ***10% (v/v) acetic acid***



Add 900  $\mu\text{L}$  of LC-MS water (Fisher W6-4) and 100  $\mu\text{L}$  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  $\mu\text{L}$  of LC-MS water (Fisher W6-4), 600  $\mu\text{L}$  acetonitrile (Fisher A955-4), and 10  $\mu\text{L}$  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  $\mu\text{g}$  of protein sample into a 2-mL tube. The volume of protein sample should be 250  $\mu\text{L}$  if the concentration of protein is to 0.8 g/L (800  $\mu\text{g}/\mu\text{L}$ ).

3) The volume of protein sample can be adjusted to make total protein to be 200  $\mu\text{g}$  if concentration of protein is lower or higher than 0.8 g/L. For example, the volume of protein sample should be 200  $\mu\text{L}$  if the concentration of protein is 1.0 g/L; the volume of protein sample should be 400  $\mu\text{L}$  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  $\mu\text{L}$  of protein sample, add 1000  $\mu\text{L}$  of chilled 15% (w/v) TCA and 0.2% (w/v) DTT in acetone. Mix by inverting tube.

2) Incubate 20 min at  $-80^{\circ}\text{C}$ .

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

4) Continue incubation overnight at  $-20^{\circ}\text{C}$ .

5) Centrifuge at 21,100 g for 20 min at  $4^{\circ}\text{C}$ . 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  $-80^{\circ}\text{C}$  or continue to next step.

**Digesting with trypsin/Lys-C**

1) Resuspend pellet in 10  $\mu\text{L}$  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  $\mu$ L. If the volume is not 100  $\mu$ L, adjust the volumes of the DTT, IAM, and AMBIC in the following steps accordingly.
- 5) Add 2.5  $\mu$ L of 200 mM DTT in AMBIC to the 100  $\mu$ L of dissolved sample in the 1.5 mL tube. Incubate at 37°C for 30 min.
- 6) Add 7.7  $\mu$ L of 194.6 mM IAM in AMBIC. Incubate at room temperature in dark for 30 min.
- 7) Quench IAM by adding 20  $\mu$ L of 200 mM DTT in AMBIC. Incubate at room temperature for 10 min.
- 8) Add trypsin/Lys-C in a 1:25 (enzyme:protein, w/w) ratio and incubate at 37°C for 4 h. The amount of sample protein is assumed to be equal to protein used for TCA/acetone precipitation (i.e. 200  $\mu$ g).
- 9) Activate trypsin by adding 550  $\mu$ L of 50 mM AMBIC. Use pH paper to verify pH > 7.
- 10) Incubate at 37°C for 14 to 16 h (or overnight).
- 11) Terminate digestion by adding 4% (w/v) TFA to a final concentration of 1% TFA. Use pH paper to verify pH < 4.
- 12) Centrifuge at 16,000 g for 10 min at room temperature.
- 13) Transfer supernatant to new 1.5 mL tube (Sarstedt 72.706.700). If amount of protein in TCA/acetone precipitation step is high, split supernatant into multiple tubes so that each has about 50  $\mu$ g.

#### **Desalting with ZipTips**

- 1) Attach a Millipore C18 ZipTips (ZTC18S096) to a 10  $\mu$ L-pipette. Make sure that tip is well-seated. Set pipette to 10  $\mu$ L.
- 2) Wet tip by aspirating 10  $\mu$ L of LC-MS acetonitrile (Fisher A955-4), and then discard solvent. Repeat once. Here and throughout, do not introduce air into the tip (or recovery of peptides will be lower).
- 3) Equilibrate tip by aspirating 10  $\mu$ L of equilibration solution, then discard solvent. Repeat once. After discarding solvent the second time, immediately proceed with steps below (do not let tip dry).

- 4) Aspirate up to 10  $\mu\text{L}$  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  $\mu\text{L}$  and repeat binding for 10 cycles.
- 5) Rinse the tip by slowly aspirating 10  $\mu\text{L}$  of rinse solution and then discarding solvent. Repeat once.
- 6) Elute the sample with 100  $\mu\text{L}$  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  $\mu\text{L}$  aliquot to measure peptides concentration. Use the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo 23290) according to instructions. Store sample at  $-80^{\circ}\text{C}$ .

### Drying samples

- 1) Prepare the SpeedVac system (Savant RT-400A condensation trap and SpeedVac concentrator)
  - (A) Check the level of coolant in condensation trap. Lift up white foam disc and see if level of liquid is near top of glass insert. If not, add coolant (100% ethanol). Trap should contain 800 mL coolant in total.
  - (B) Check vacuum tubing is properly connected between condensation trap and concentrator. Make connections as shown below (lines indicate direction of vacuum)

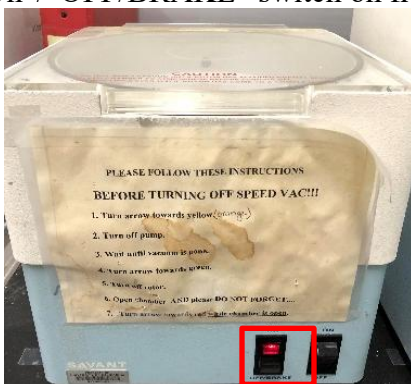


- (C) Turn on trap and wait for 1 hour to allow the temperature to decrease to about  $-50^{\circ}\text{C}$ .
- 2) Load samples in SpeedVac concentrator.
  - (A) Take out samples from  $-80^{\circ}\text{C}$  and thaw at room temperature. Samples should be in 1.5-ml tubes.

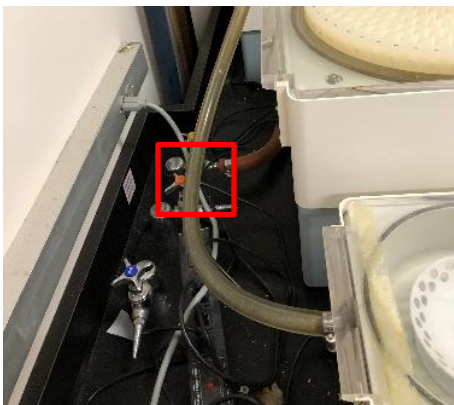
- (B) Place samples in rotor of the concentrator. Arrange the tubes so the rotor is balanced (just as with a centrifuge rotor). If needed, use 1.5-mL tubes filled with solvent to balance.
- (C) Locate blue valve on vacuum tubing, then turn so that vacuum can be applied to trap.



- (D)
- (E) Turn on concentrator using "On"/"OFF/BRAKE" switch on front.



- (F)
- (G) Turn on house vacuum (yellow, labelled "LV"). Lid of concentrator should seal shut. If it does not, check tubing and connections.



- (H)
- (I) Solvent will begin to evaporate. Monitor level of solvent frequently. Continue evaporation until solvent is about to disappear.

(J) Remove samples from rotor. Locate blue valve on vacuum tubing, then turn to release vacuum. Turn off rotor. Open lid and remove samples.

(K) Turn off house vacuum and turn off trap.

(L) Empty glass insert of solvent. Wait until trap to warm up (several hours or overnight), disconnect vacuum tubing from glass insert, then lift insert and white foam out of trap. Pour out contents. Handle glass insert with care because it is fragile and expensive. Handle glass insert with care because it is fragile and expensive.



(M)

(N) Place insert back into trap for next use.

3) Resuspend dried sample in about 40  $\mu\text{L}$  of 0.1% TFA.

4) For measuring peptides, remove a 3  $\mu\text{L}$  aliquot and mix with 9  $\mu\text{L}$  of 0.1% TFA in a 1.5 mL tube. Store the remaining sample at  $-80^{\circ}\text{C}$  for up to 3 months.

5) Measure peptides using the Pierce Quantitative Fluorometric Peptide Assay kit (Thermo 23290) according to instructions. For cell extract, a typical peptides concentration is 220 to 270  $\text{ng}/\mu\text{L}$ , about 35 to 37  $\mu\text{L}$ , giving a yield of 8 to 9  $\mu\text{g}$  from 200  $\mu\text{g}$  protein in 250  $\mu\text{L}$  original extract if desalt with two 10- $\mu\text{L}$  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  $\mu\text{g}$  total peptide (for recombinant proteins), or 2.5 to 5  $\mu\text{g}$  (for cell extract) per injection.

### **Identifying proteins from LC-MS/MS files**

1.) Install following software

- MSConvert
- X!Tandem pipeline

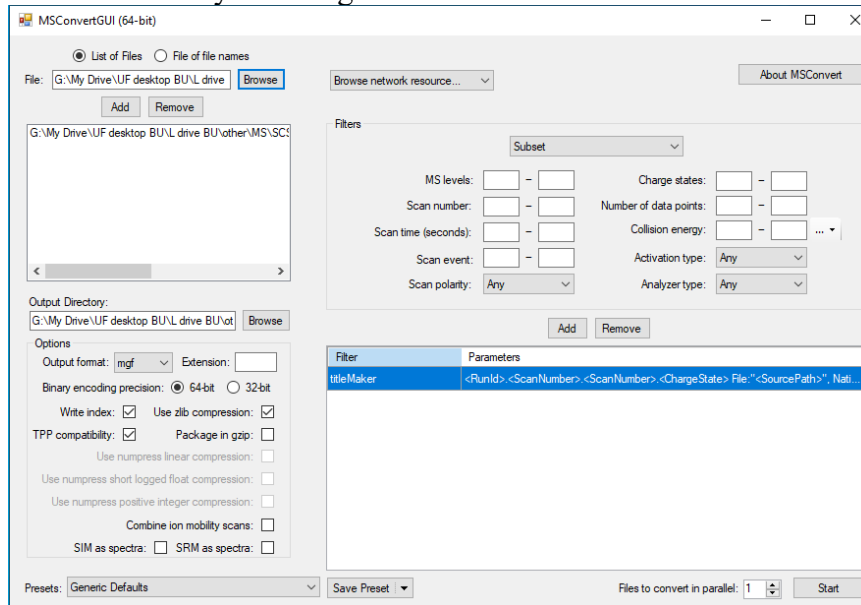
- Skyline (optional)

2.) Obtain the Thermo RAW file from UC Davis Proteomics core. File will be sent to email in the form of a Bioshare link. This file contains the chromatograms from the LC-MS/MS instrument.

3.) Convert Thermo RAW file (\*.RAW) to Mascot Generic format (\*.mgf).

(A) Open MSConvert.

(B) Select \*.RAW file by choosing “Browse”



Click “Add.” Click “Start.”

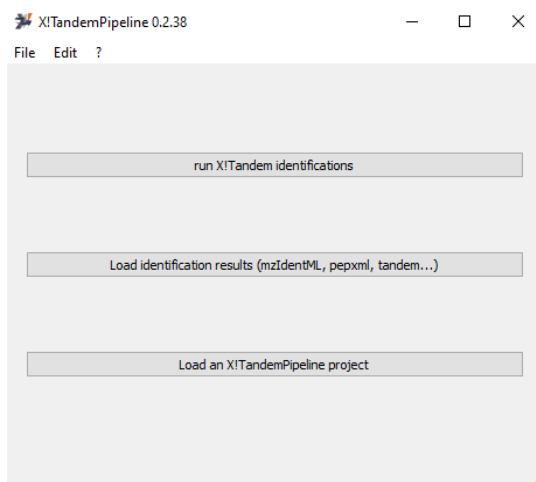
4.) Obtain a \*.faa or \*.fasta file containing the sequence of the protein. The file should contain sequences of possible contaminating proteins, also. For cell extracts, it would include all proteins predicted in the genome. For *Cutibacterium granulosum* VPI 0507, protein sequences can be downloaded from

[https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=TaxonDetail&page=taxonDetail&taxon\\_oid=2814123075](https://img.jgi.doe.gov/cgi-bin/mer/main.cgi?section=TaxonDetail&page=taxonDetail&taxon_oid=2814123075)

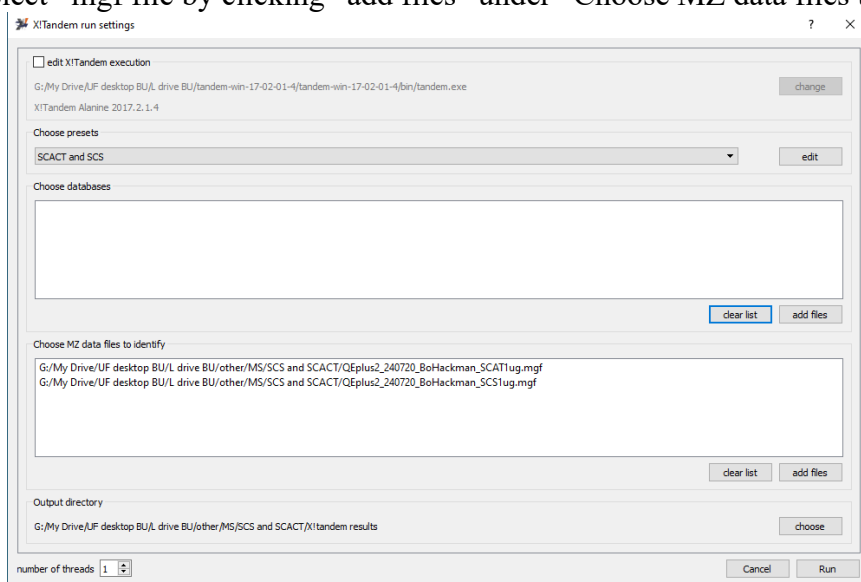
5.) Identify proteins in \*.mgf file.

(A) Open X!Tandem pipeline (xtcpp.exe).

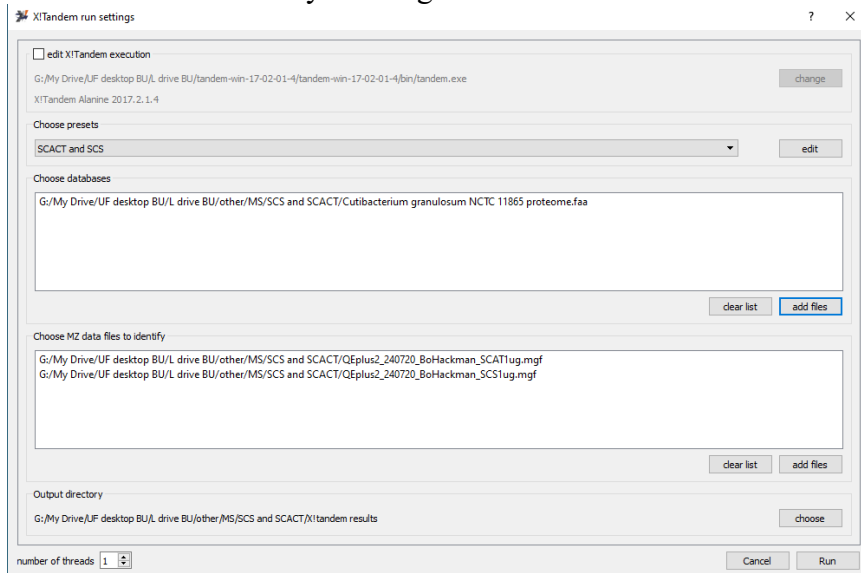
(B) Click “run X!Tandem identifications”



(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).

The screenshot shows the 'X!Tandem preset editor' window with the 'Protein' tab selected. The 'Define protein cleavage site' is set to '[KR][P]'. Other parameters include 'Use semi-enzymatic cleavage rules' (no), 'Modify the residue masses of one or all amino acids' (0.0), and 'Protein N-terminus common modifications' (yes). The 'Load' and 'Save' buttons are at the top right.

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

The screenshot shows the 'X!Tandem preset editor' window with the 'Spectrum' tab selected. The 'Fragment ion mass tolerance (monoisotopic)' is set to 0.1. Other parameters include 'Parent on MH+ mass tolerance lower window' (10), 'Anticipate carbon isotope parent on assignments errors' (yes), and 'Use chemical average or monoisotopic mass for fragment ions' (monoisotopic). The 'Load' and 'Save' buttons are at the top right.

- 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”.



**Load identification results**

mode  
☒ combine ☐ individual

Choose result files  
 G:/My Drive/UF desktop BU/L drive BU/other/MS/SCS and SCACT/Xtandem results/QEplus2\_240720\_BoHackman\_SCS1ug.xml

clear list add files

Contaminants  
☒ contaminant file ☐ contaminant regular expression

clear list add files

Cancel OK

Click OK.

(B) Inspect all entries in output. For more details, click on entry.

Protein list

export show only

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

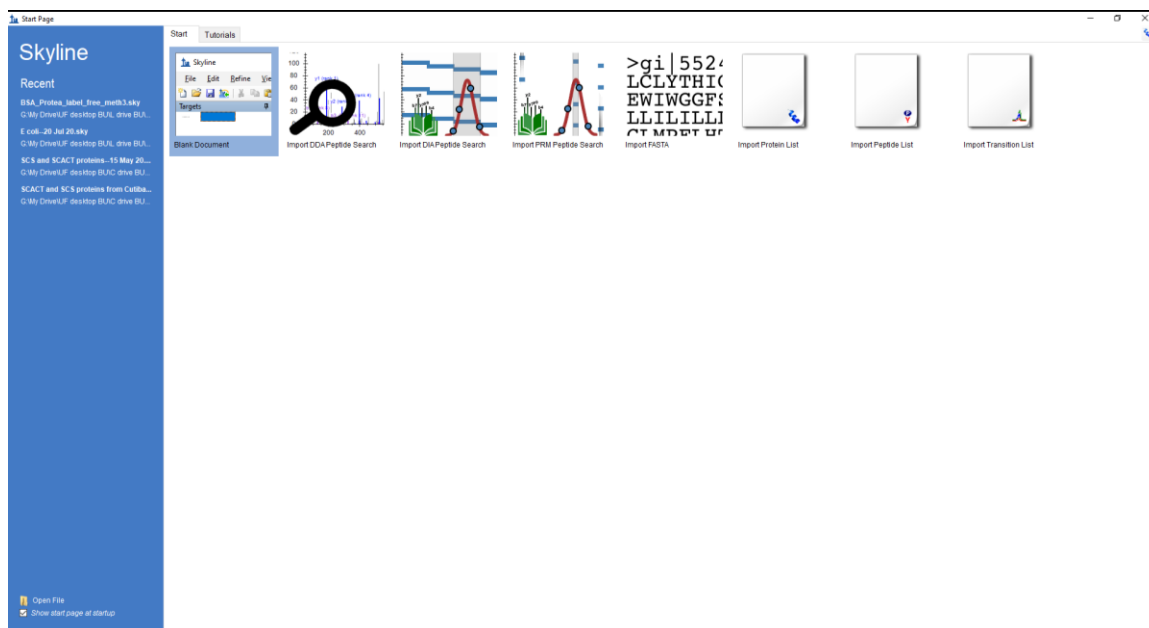
search accession

proteins all:64 valid:6 valid&checked:6 grouped:6 displayed:6

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

(A) Open Skyline.

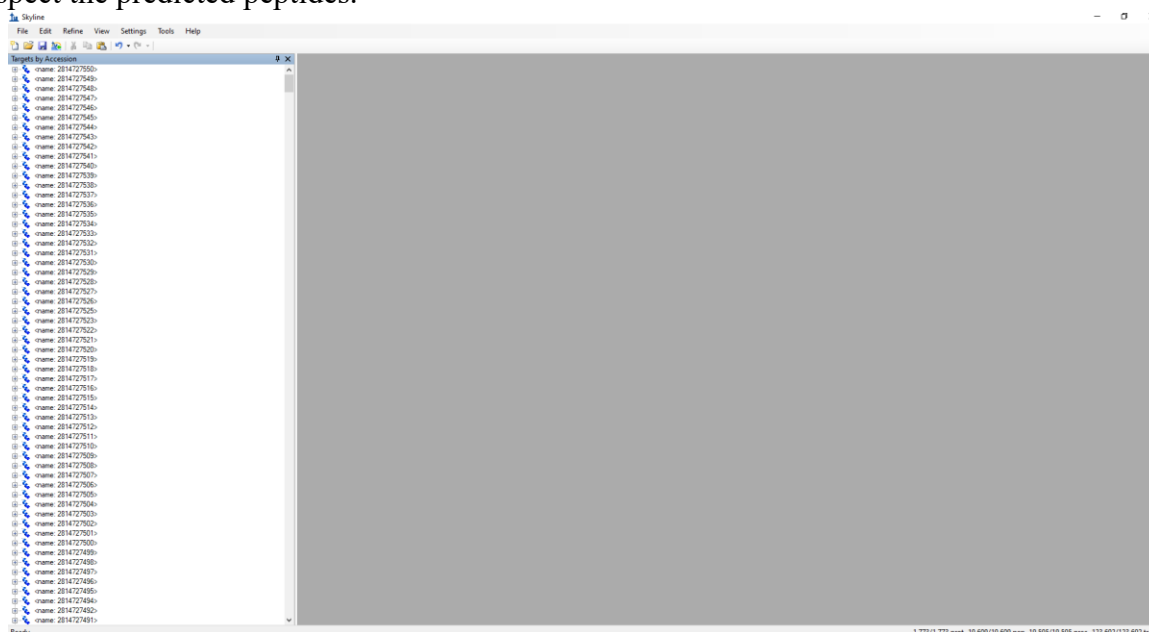
(B) Click “Blank Document”



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

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

(E) Inspect the predicted peptides.



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

Transition Settings

Prediction Filter Library Instrument Full-Scan

MS1 filtering

Isotope peaks included: Count Precursor mass analyzer: Orbitrap

Peaks: 3 Resolving power: 60,000 At: 400  $m/z$

Isotope labeling enrichment: Default

MS/MS filtering

Acquisition method: None Product mass analyzer:

Isolation scheme: Resolution:  $m/z$

☐ Use high-selectivity extraction

Retention time filtering

☒ Use only scans within 5 minutes of MS/MS IDs

☐ Use only scans within 5 minutes of predicted RT

☐ Include all matching scans

OK Cancel

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

Import Results

☒ Add single-injection replicates in files

Optimizing: None

☐ Add multi-injection replicates in directories

☐ Add one new replicate

Name:

☐ Add files to an existing replicate

Name:

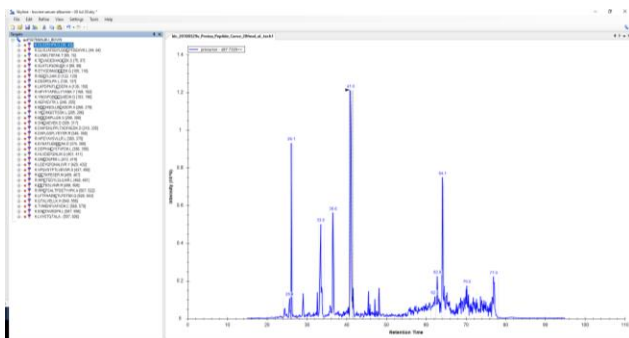
Files to import simultaneously: Many

☒ Show chromatograms during import

☒ Retry after import failure

OK Cancel

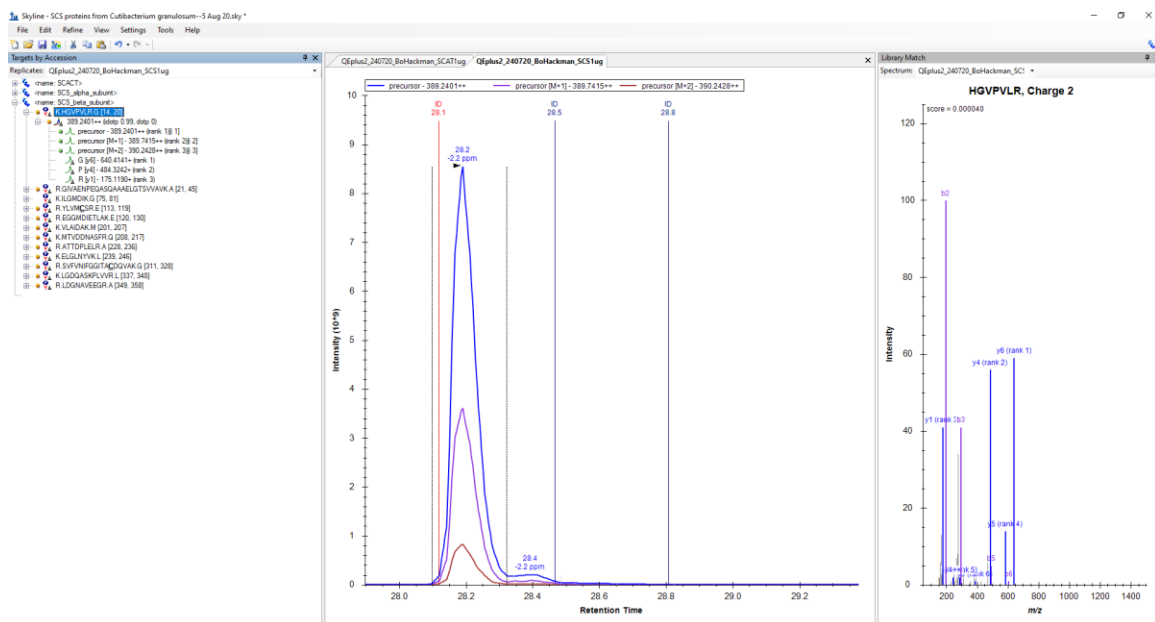
(H) Inspect the chromatograms.



(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”.

The image shows a dialog box titled "Import Peptide Search". It has a close button (X) in the top right corner. The "Spectral Library" section has two radio buttons: "Build" (selected) and "Use existing". Below this is a "Cut-off score:" field with the value "0.95". The "Search files:" section has a list box containing "QEplus2\_240720\_BoHackman\_SCS1ug.xtan.xml" and two buttons: "Add Files..." and "Remove Files". The "iRT standard peptides:" section has a dropdown menu set to "None" and two checkboxes: "Include ambiguous matches" (unchecked) and "Filter for document peptides" (unchecked). The "Workflow" section has three radio buttons: "DDA with MS1 filtering" (unchecked), "DIA" (unchecked), and "PRM" (selected). At the bottom are three buttons: "Finish", "Next >" (highlighted with a blue border), and "Cancel".

(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 (97) and  $5 \cdot 10^{-6}$  J/s detection limit]. For an experiment with  $10^{10}$  cells, this corresponds to only  $\sim 1500$  glucose molecules/cell/s. This manual describes the installation, calibration, and use of one calorimeter.

The manual culminates in describing an experiment to determine metabolic response of rumen microbes to pulse dose of glucose (20 mM). It combines many of the procedures listed earlier in the manual.

## Heat production of microbial cells by calorimetry

Adapted from Thermal Hazard Technology  $\mu$ RC manual and ref. (98, 99)

### Use

- 1.) Turn on calorimeter (Thermal Hazard Technology  $\mu$ RC) using switch on back (**Fig. 1**) if calorimeter is installed and calibrated. If not already, install and calibrate first, following instructions below.
  - 2.) Load uRC.exe.
  - 3.) Apply experiment settings in “Experiment” tab of urc.exe. **Fig. 2** shows settings for typical experiments. Click “Update” button.
  - 4.) Prepare sample and reference vials (**Fig. 3**). Place stirbars into two vials. Pipette 1 mL of sample (e.g., rumen microbes) into one vial (sample vial). Flush sample with CO<sub>2</sub> while screwing on cap with rubber septum. Pipette 1 mL of dH<sub>2</sub>O 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  $\mu\text{L}$  of this solution into 1 mL 0.01 NaOH. To correct for heat of diluting HCl, run a second experiment and inject 15  $\mu\text{L}$  0.1 M HCl into 1 mL  $\text{dH}_2\text{O}$ . For each injection, determine heat released (J) by integration. Subtract heat released from second injection (HCl into  $\text{dH}_2\text{O}$ ) from heat released by first injection (HCl into NaOH).

Expected heat production at 39°C is 79.05 mJ (-52.70 kJ/mol HCl). Calculation of this expected heat production assumes infinitely dilute HCl and NaOH, for which heat released is -55.84 kJ/mol at 25°C (100). To correct to 39°C, the calculation also assumes  $\Delta_r C_p^\circ = 0.22 \text{ kJ}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$  (100).

## 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  $\text{dH}_2\text{O}$  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.

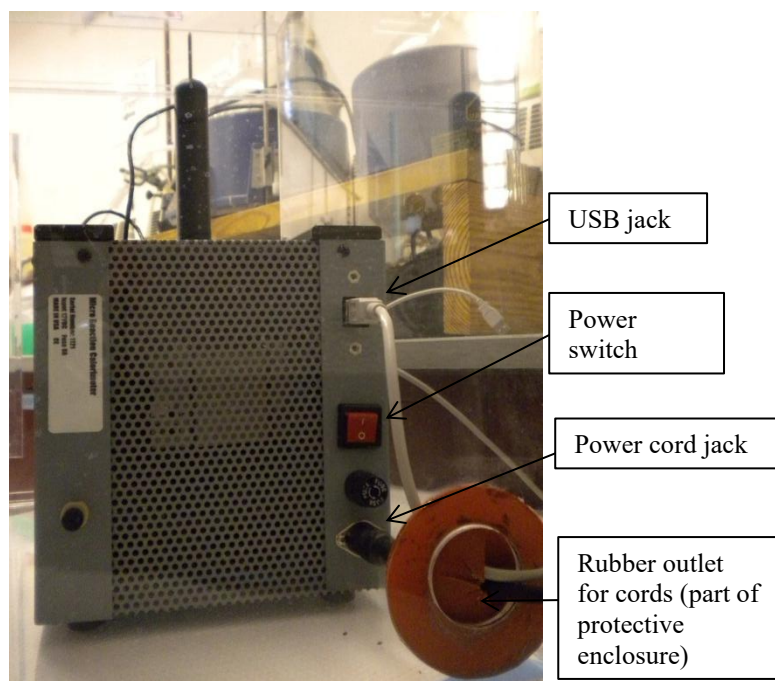
(iii) Handle all parts using gloves and, when possible, forceps to avoid transferring heat and oil from hands. Wipe calorimetry vials, metal stoppers, and syringe needle with Kimwipe before insertion to remove residual moisture.

(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  $\mu$ L) 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  $\mu$ L), syringe size (250  $\mu$ L), 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.

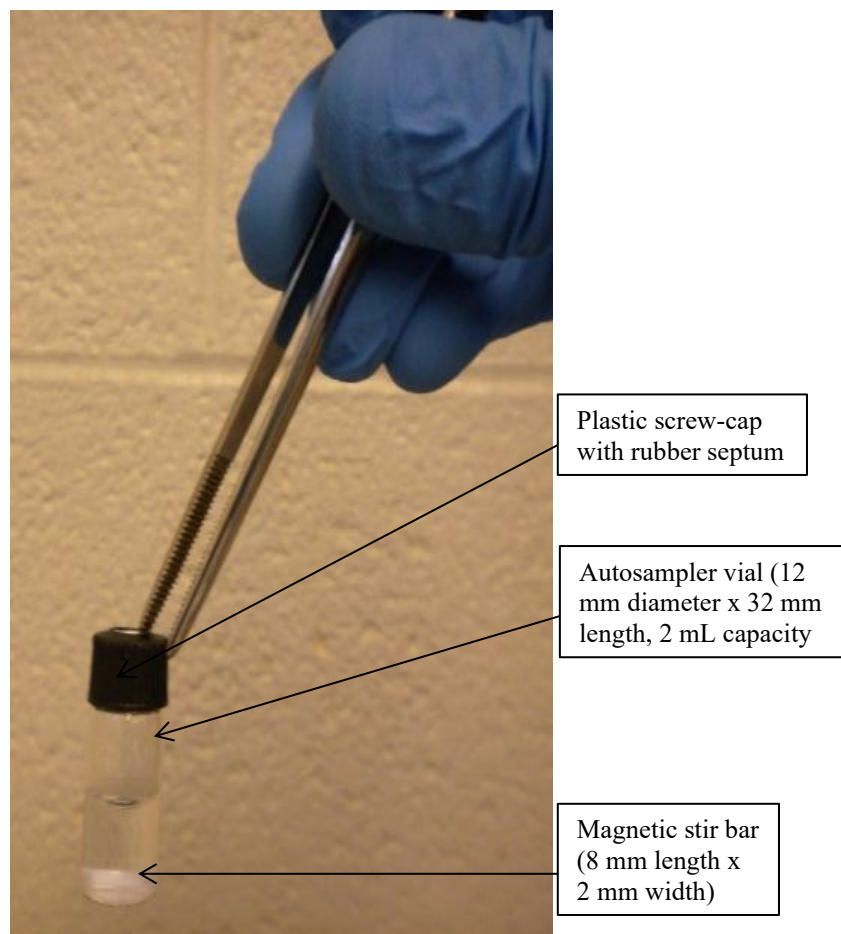
(A)

The screenshot shows the 'Titration' experiment settings. On the left, under 'Experiment Type', 'Titration' is selected with a radio button. The 'Settings' panel on the right includes: 'Stirrer ON/OFF' set to 'ON' (highlighted in green), 'Stir Speed (rpm)' at 60, 'Syringe Speed (ul/sec)' at 1, 'Syringe Size (ul)' at 250 (via a dropdown), 'Temperature (C)' at 39, and 'Sample Rate (sec)' at 1. Below this, the 'Titration' section contains: 'Number of Injections' at 1, 'Injection Size (ul)' at 20, 'Injection Interval (sec)' at 25000, and 'Lead Time (sec)' at 1200. The 'Experiment' panel on the right shows 'Method Name' as 'mixed rumen microbes + 20 mM glucose', 'Operator' as an empty field, 'Titrant' as '20 uL, 1 M glucose', 'Sample' as 'mixed rumen microbes', and a large empty 'Comments' box. At the bottom right, the 'Syringe' section has 'Move (0-100%)' as an empty field, 'Move UP' selected with a radio button, 'Move DOWN' as an unselected option, and a 'Move' button. 'Cancel' and 'Update' buttons are at the bottom center.

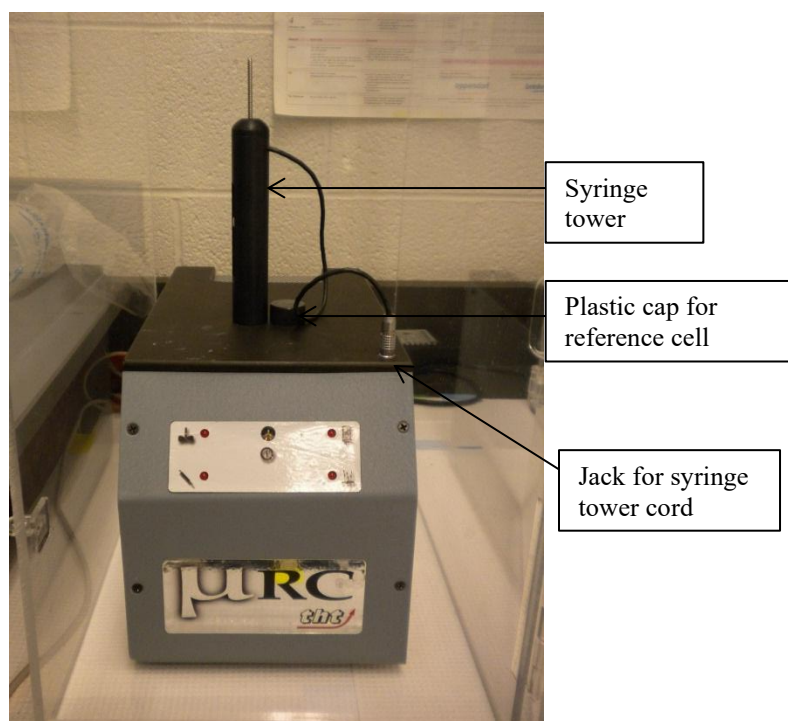
(B)

The screenshot shows the 'Collection' experiment settings. Under 'Experiment Type', 'Collection' is selected with a radio button. The 'Settings' panel on the right is identical to screenshot (A). The 'Collect' section on the left contains 'Experiment Duration (sec)' set to 75000. The 'Experiment' panel on the right is identical to screenshot (A). The 'Syringe' section at the bottom right is identical to screenshot (A). 'Cancel' and 'Update' buttons are at the bottom center.

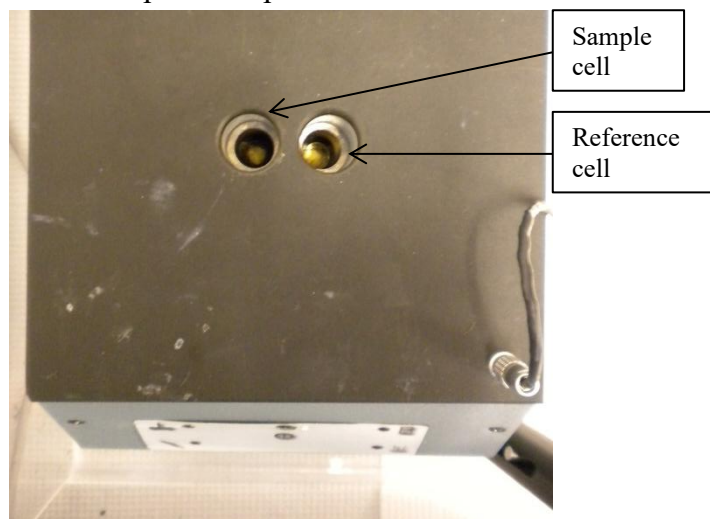
**Fig. 3.** Reference vial fully prepared. Grasped by long forceps, it is ready to be lowered into reference cell.



**Fig. 4.** Front view of calorimeter.



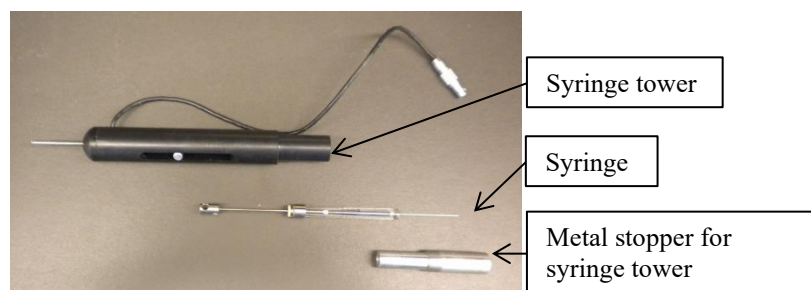
**Fig. 5.** Top view of calorimeter, with sample and reference cells exposed by removing syringe tower and plastic cap for reference cell.



**Fig. 6.** Lowering metal stopper into reference vessel.



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



(B)



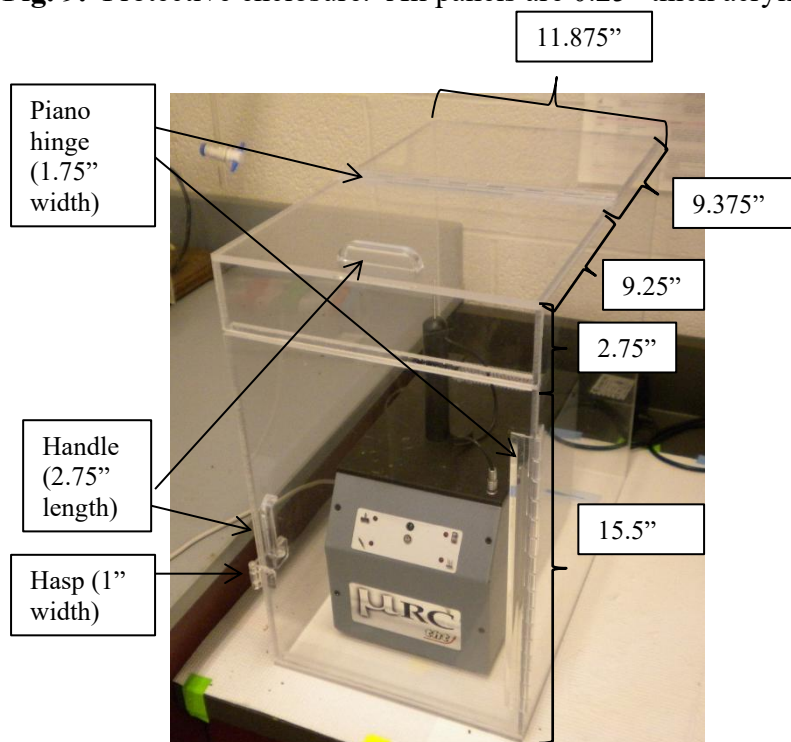
(C)



**Fig. 8.** Typical settings for calibration

Experiment Type		Settings		Experiment									
<input type="radio"/> Collection		Stirrer ON/OFF <span style="background-color: green; color: white;">ON</span>		Method Name: <input type="text" value="electrical calibration"/>									
<input checked="" type="radio"/> Calibration		Stir Speed (rpm) <input type="text" value="200"/>		Operator: <input type="text"/>									
<input type="radio"/> Titration		Syringe Speed (ul/sec) <input type="text" value="1"/>		Titrant: <input type="text"/>									
<input type="radio"/> Scan		Syringe Size (ul) <input type="text" value="250"/>		Sample: <input type="text" value="water-water blank"/>									
<input type="radio"/> cp Measurement		Temperature (C) <input type="text" value="39"/>		Comments: <div style="border: 1px solid black; height: 100px;"></div>									
		Sample Rate (sec) <input type="text" value="1"/>											
<div>Calibration</div> <table><tr><td>Number of pulses</td><td><input type="text" value="5"/></td></tr><tr><td>Pulse Size (mJ)</td><td><input type="text" value="50"/></td></tr><tr><td>Pulse Interval (sec)</td><td><input type="text" value="300"/></td></tr><tr><td>Lead Time (sec)</td><td><input type="text" value="20"/></td></tr></table>						Number of pulses	<input type="text" value="5"/>	Pulse Size (mJ)	<input type="text" value="50"/>	Pulse Interval (sec)	<input type="text" value="300"/>	Lead Time (sec)	<input type="text" value="20"/>
Number of pulses	<input type="text" value="5"/>												
Pulse Size (mJ)	<input type="text" value="50"/>												
Pulse Interval (sec)	<input type="text" value="300"/>												
Lead Time (sec)	<input type="text" value="20"/>												

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





## Example experiment: Measuring response of rumen microbes to 20 mM glucose

Results from this experiment and a similar one with 5 mM glucose are shown in ref. (97)

### 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  $\mu$ L GC syringe (with dH<sub>2</sub>O). Run at 39°C overnight.
- 3.) Pre-warm centrifuge rotor (if preparing mixed rumen microbes or bacteria), Simplex buffer, and separatory funnel to 39°C in incubator.
- 4.) Pack supplies for collecting rumen fluid according to *Collecting rumen fluid*.
- 5.) Prepare 1 M sterile glucose solution. Sterilize in Hungate tubes by autoclaving and store at -20°C (for further protection against microbial degradation). Solution is stable indefinitely.

### Day 2

- 1.) Collect fluid in the morning (e.g., 8 a.m.). Begin flocculating rumen fluid to prepare sample.
- 2.) While flocculating, fill GC syringe with 1 M glucose and re-insert calorimeter. Withdraw 1 mL, 1 M glucose from Hungate tube using 1 mL syringe according to *Making and dispensing media of Feeding and transferring protozoa in culture tubes*. Fill 250  $\mu$ L GC syringe with an aliquot of this glucose, and store an aliquot at -20°C for analysis.
- 3.) Prepare sample (mixed rumen microbes, protozoa, or bacteria) by following earlier procedures. Transfer 20 mL of sample to 60 mL culture bottle using glass graduated pipette. Gas and cap with rubber bung.
- 3.) Remove 1 mL sample and place in autosampler vial for measurement of heat production with microcalorimeter. Here and throughout, mix contents of culture bottle well by swirling and pipetting contents vigorously before sampling.
- 4.) Allow heat production of calorimeter to equilibrate. Start titration experiment. Inject 20  $\mu$ L, 1 M glucose (giving 20 mM final concentration) with 1200 s lead time.
- 5.) Near start of titration experiment (-1200 s relative to glucose injection), remove 1 mL from culture bottle sample and place in P1. Place on ice and record time. Repeat again at c. -600 s and -60 s with P2 and P3.
- 6.) Wait for calorimeter to inject glucose. At same time, inject 320  $\mu$ L 1 M glucose into culture bottle (now containing 16 mL culture). Collect sample and place in P4.

- 7.) Centrifuge P1 to P4 at 4°C at 10 000 g for 10 min (use cold room centrifuge in ZT's lab and keep on ice during transport). Although P1 to P3 were collected long before, do not attempt to centrifuge them before this point; sampling, monitoring the calorimeter, and making the glucose injection are more important.
- 8.) Return to main lab. At ~600 s after P4 was collected, collect another sample and place in P5.
- 9.) Remove supernatant from P1 to P4 and place in S1 to S4. Resuspend P1 to P4 in 1 mL 0.9% NaCl. Centrifuge P1 to P5 together at 4°C at 10 000 g for 10 min.
- 10.) Return to main lab. At ~600 s after P5 was collected, collect another sample and place in P6.
- 11.) Remove supernatant from P1 to P4 and compile with supernatant already in S1 to S4. Place P1 to P4 and S1 to S4 on ice and prepare for transport. Remove supernatant from P5, place in S5, and resuspend P5 in 1 mL 0.9% NaCl. Centrifuge P4 to P5 together at 4°C at 10 000 g for 10 min.
- 12.) Return to main lab and place P1 to P4 and S1 to S4 in -20°C as soon as possible. Repeat collecting samples at 600 to 3600 s intervals and centrifuging until P12 and S12 are complete.
- 13.) Prepare VFA samples from S1 to S12 according to ***Determination of volatile fatty acid (VFA) content of culture supernatant***. Use V1 to V12 to hold VFA samples. Thaw S1 to S12 if needed. Store at -20°C.
- 14.) Measure background heat production according to ***Heat production of microbial cells***. For precise work, measure background heat production prior to running experiment (overnight between Day 1 and 2).

### Day 3 and beyond

Perform chemical analysis according to procedures listed earlier.

- 1.) Analyze P1 to P12 for total hexoses according to ***Total hexoses of microbial cells using the anthrone reaction***. Dilute the resuspended cell pellet 1→8 by adding 200 µL pellet to 1400 µL dH<sub>2</sub>O in a separate 2-mL boil proof tube.

Analyze P1 to P12 for protein according to ***Protein content of microbial cells***. Expected protein is 3000 to 5000 µg.

If both analyses cannot be done of the same day, give priority to total hexoses. Multiple freeze-thaw cycles lower content of hexoses, but they do not affect protein.

- 2.) Analyze S1 to S12 and 1 M glucose for glucose according to ***Free glucose content of culture supernatant***. For glucose, add 91  $\mu\text{L}$  *N*-ethylmaleimide to 200  $\mu\text{L}$  supernatant, then add 582  $\mu\text{L}$   $\text{dH}_2\text{O}$ . For 1 M glucose, add 20  $\mu\text{L}$  1 M glucose to 10 mL  $\text{dH}_2\text{O}$ .
- 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<sub>2</sub>O. 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<sub>2</sub>O. 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<sub>2</sub>) into 30 mL dH<sub>2</sub>O (instead of 300 mL) and omitting CaCl<sub>2</sub>. 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<sub>600</sub>) over time.

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

Simplex buffer under CO<sub>2</sub>. 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<sub>2</sub>.

### **Incubation in 2-NBDG and radiolabeled sugars**

1.) Prepare aliquots of culture and pre-incubate. Under CO<sub>2</sub>, 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 ( $\leq 7$ ) 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  $\mu$ L of 2-NBDG or radiolabeled sugar stock. Lift tube slightly from incubator, add and mix stock by pipetting up and down, then place tube back into incubator. Record (mentally) the second when 2-NBDG is added; a stopwatch counting upwards is helpful.

4.) At end of incubation, stop reaction by quickly dispensing 2-mL stop solution with bottle top dispenser. To avoid contact between tube and warm incubator, lift the tube up slightly before adding stop solution. With practice, incubation can be timed to nearest second, and total incubation times as short as 2 s can be achieved.

5.) Using transfer pipette, quickly transfer contents of tube to filter holder with pre-wetted Nuclepore filter (shiny side up; 0.4  $\mu$ m, 25 mm diameter; Whatman 110607). To draw up contents all at once, squeeze bulb firmly and completely, then release while holding tip at bottom of tube. Add contents directly on top of filter, avoiding splashing the walls of the holder.

6.) Add 2-mL additional stop solution to tube and transfer to filter holder with same pipette. This step washes the tube, pipette, filter tower, and cells on filter. With practice, transferring contents of tube to filter holder and washing can be done in <10 s.

7.) Wait for liquid to drain from filter, then immediately disassemble holder, remove vacuum tubing, and remove filter with forceps.

8.) For 2-NBDG samples, transfer the filter to bottle containing 2 mL NMP. Try to drop filter directly in NMP, avoiding contact with walls of bottle. Swirl contents of bottle vigorously to dissolve filter. Mix with transfer pipette to complete dissolution. If filter adheres to bottle walls at any point, wash walls thoroughly with pipette. Transfer ~1 mL with transfer pipette to cuvette. Seal with cap, and read fluorescence.

9.) For radiolabeled sugar samples, transfer filter to scintillation vial, add 5 mL scintillation cocktail, shake the vial vigorously and determine DPM following *Liquid scintillation counting*.

10.) Repeat experiment for full range of concentrations and incubation lengths. Include an experiment with incubation length of 0 s, in which stop solution is added before 2-NBDG or radiolabeled sugar stocks. With practice and 2 people splitting duties, each experiment will take 3 min.

11.) Harvest 1-mL aliquots of bacteria for protein analysis (see *Protein content of microbial cells*).

## Notes

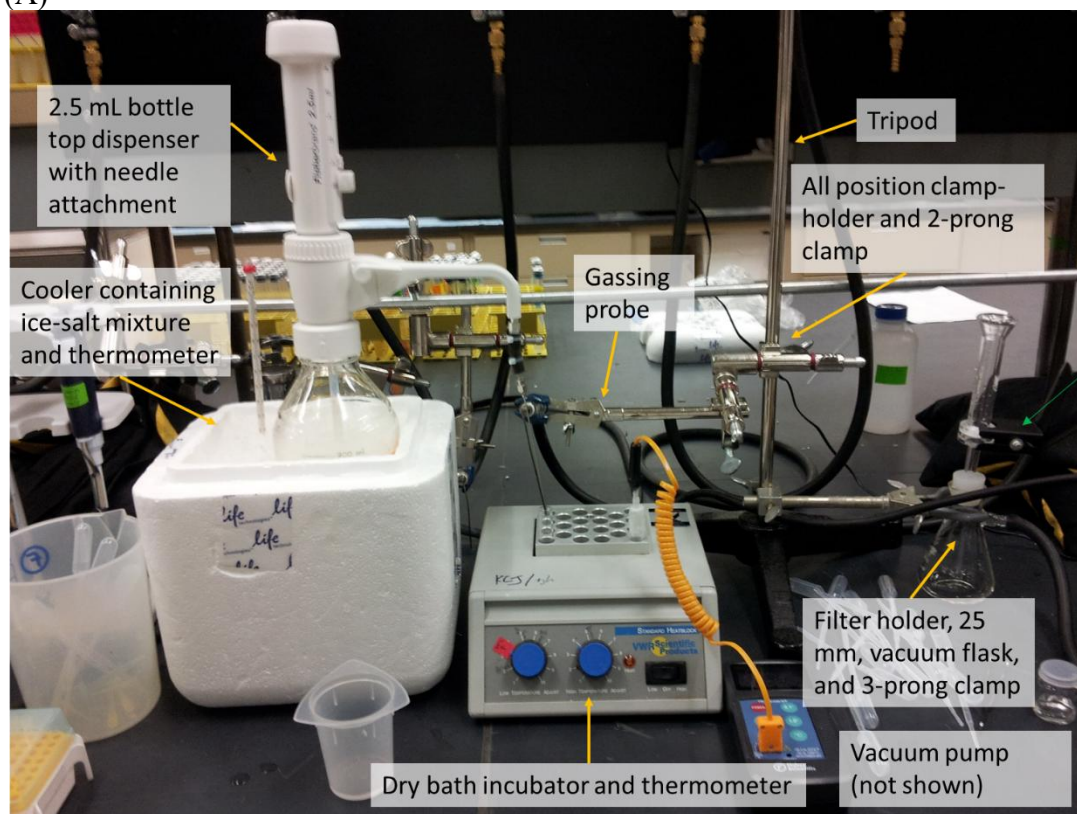
(i) For #3 of *Inoculation of cultures*, cell protein can be roughly estimated by measuring optical OD<sub>600</sub> and referring to past experiments that have measured both OD<sub>600</sub> and protein. This rough estimate facilitates adjustment of protein to 0.4 mg/L.

(ii) For some bacteria, 0.4 mg protein/mL is too concentrated and will lead to unacceptably long filtration times in step #7 of *Incubation of 2-NBDG and radiolabeled sugars*. For these bacteria, determine with a preliminary experiment the maximum concentration that will lead to filtration in <15 s.

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

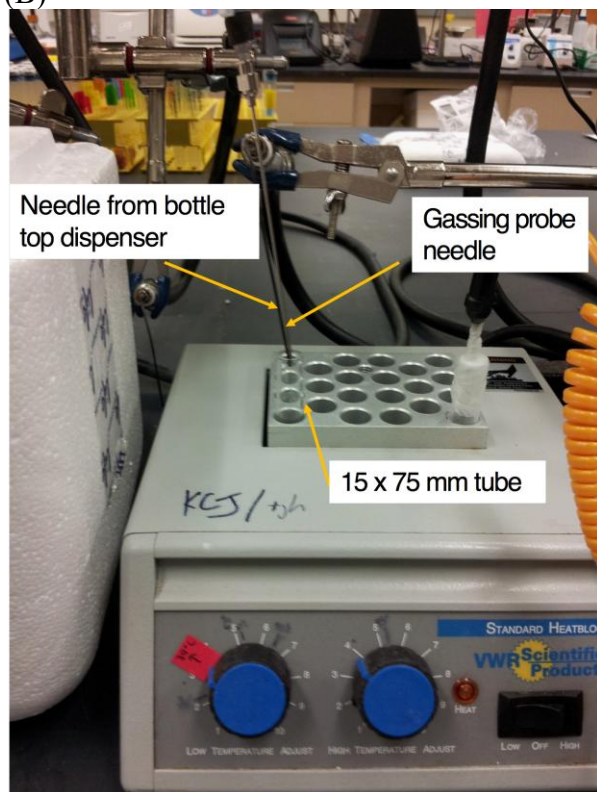
**Fig. 1.** Apparatus for incubating tubes and stopping reaction. For radiolabeled sugar samples, items used should be those designated for radioactive use. (A) Overview. (B) Close up of tube with gassing probe and bottle-top dispenser in lip. (C) Bottle top dispenser with needle attachment.

(A)

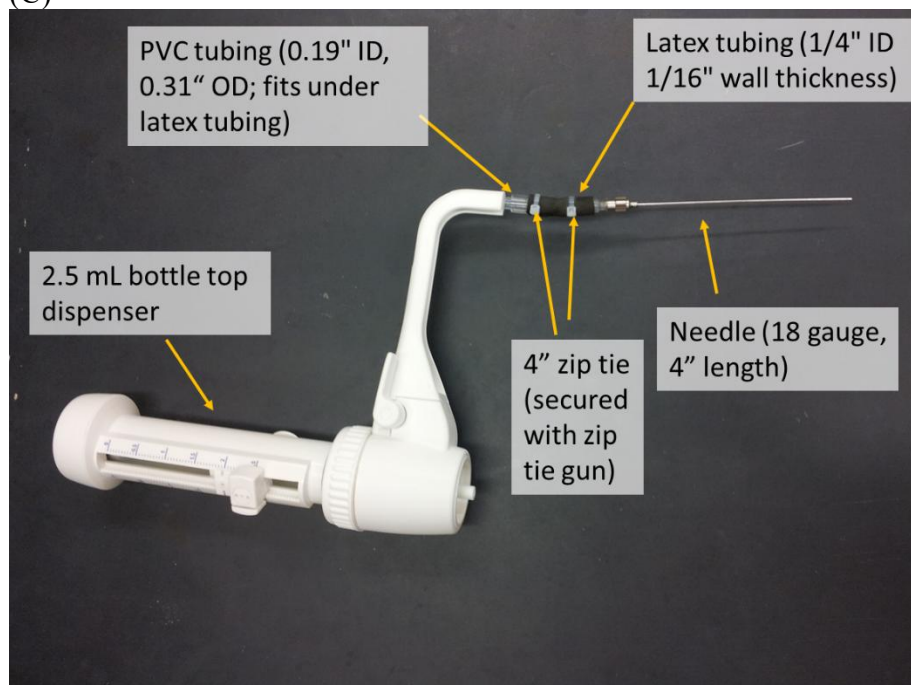




(B)



(C)



## Stocks for radiolabeled sugar transport experiments

1.) Prepare 10 mM glucose. Add 0.18016 g glucose to 100 mL volumetric flask and bring to volume with ddH<sub>2</sub>O. Sterilize by filtering through 0.2 µm membrane into autoclaved Balch tube and store at -20°C.

Prepare 10 mM mannose. Repeat procedure for 10 mM glucose but use 0.18016 g mannose.

Prepare 10 mM 2-deoxyglucose. Repeat procedure for 10 mM glucose but use 0.16416 g 2-deoxyglucose.

Prepare sterile water. Filter-sterilize or autoclave water in Balch tube.

2.) Set up lab for radioactive use. Follow *Safe use of radioactive materials*.

3.) Prepare stocks following Tables 1 to 3.

(a) Add ddH<sub>2</sub>O and 10 mM sugar to 0.5 mL sterile microcentrifuge tube in volumes indicated in tables. Do not yet add radiolabeled sugar. Here and throughout, work under flame and otherwise maintain aseptic conditions.

(b) Move radioactive materials from hood to bench and finalize bench for radioactive use.

(c) Take stock vial of a radiolabeled sugars out of refrigerator.

(d) Withdraw an aliquot of radiolabeled sugar from the vial (volume sufficient for preparing stocks). Sterile vial septum with 70% ethanol and Kimwipe (attempting to flame will melt plastic of vial). Use 1-mL syringe with 22-gauge needle to retrieve sugar. Place aliquot in sterile 0.5 mL, screw-cap centrifuge tube.

(e) Place stock vial back into refrigerator.

(f) With pipette labelled for radioactive use, add radiolabeled sugar to 0.5 mL centrifuge tube in volume indicated. Mix well. Keep at -20°C for storage.

4.) Verify stocks were prepared correctly by determining radioactivity. Pipette 10 µL of each stock and undiluted radiolabeled sugar into scintillation vial containing 5 mL scintillation cocktail. Count with Beckman LS 6000 IC β-counter using “Auto DPM” card. Compare actual DPM with expected.

**Table 1.** <sup>14</sup>C-glucose stocks (presuming 0.1 mCi/mL and 289 mCi/mmol <sup>14</sup>C-glucose)

Total glucose	Activity	Sterile ddH <sub>2</sub> O	10 mM glucose	<sup>14</sup> 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.**  $^{14}\text{C}$ -deoxyglucose stocks (presuming 0.1 mCi/mL and 274.6 mCi/mmol  $^{14}\text{C}$ -deoxyglucose)

Total deoxyglucose	Activity	Sterile ddH <sub>2</sub> O	10 mM deoxyglucose	$^{14}\text{C}$ -deoxyglucose
$\mu\text{M}$	$\mu\text{Ci}/5\ \mu\text{L}$	$\mu\text{L}$	$\mu\text{L}$	$\mu\text{L}$
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.**  $^3\text{H}$ -mannose stocks (presuming 1 mCi/mL and 19.3 Ci/mol  $^3\text{H}$ -mannose)

Total mannose	Activity	Sterile ddH <sub>2</sub> O	10 mM mannose	$^3\text{H}$ -mannose
$\mu\text{M}$	$\mu\text{Ci}/5\ \mu\text{L}$	$\mu\text{L}$	$\mu\text{L}$	$\mu\text{L}$
5000	1	75.3	124.7	50
2500	0.80	147.7	62.3	40
500	0.40	217.6	12.4	20
250	0.32	227.8	6.17	16
125	0.32	231.0	3.04	16
50	0.16	240.8	1.21	8

## Stocks for 2-NBDG transport experiments

1.) Prepare 5 mM 2-NBDG by adding 584  $\mu$ L ddH<sub>2</sub>O 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 ( $\mu$ M)	Source of 2-NBDG	Volume of source ( $\mu$ L)	Volume of dH <sub>2</sub> O ( $\mu$ L)
5000	5 mM solution	100	0
500	5 mM solution	40	360
50	500 $\mu$ M stock	40	360
12.5	500 $\mu$ M stock	10	390
5	50 $\mu$ M stock	40	360
2.5	50 $\mu$ M 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 ( $\mu$ M)	Source of 2-NBDG	Volume of source ( $\mu$ L)	Volume of NMP (mL)
10	500 $\mu$ M stock	40	2
1	50 $\mu$ M stock	40	2
0.25	12.5 $\mu$ M stock	40	2
0.1	5 $\mu$ M stock	40	2
0.05	2.5 $\mu$ M stock	40	2
0	ddH <sub>2</sub> O	40	2

1-methyl-2- pyrrolidone (NMP) should be spectroscopy grade. Store on ice and use fresh.

4.) Read fluorescence of standards. With VersaFluor fluorometer (480/20 nm excitation, 520/10 nm emission), record fluorescence of empty, chemically-resistant cuvettes (Brandtech #759150). Pipette 1 mL standard into cuvettes. Record fluorescence and subtract value of empty cuvette.

## Safe use of radioactive materials

- 1.) Put on 2 layers of gloves before handling radioisotopes or any items designated for radioactive use. Change gloves frequently and when contamination is suspected.
- 2.) Tape down bench paper (absorptive side up) on area where radioisotopes will be handled. Clear area of materials not designated for radioactive use.
- 3.) Retrieve items designated for radioactive use from the radioactive cabinet. Items in the radioactive cabinet include pipettes, tips, filter holders, beakers, forceps, vacuum flasks, pens, gassing probe, heat block, water bottle, and polystyrene cooler. If an item is not in cabinet, take one from general stock and label with yellow radioactive tape to designate it for radioactive use. Disposable items do not have to be labeled if they will be discarded at the end of the experiment.
- 4.) Retrieve radioisotopes from refrigerator or freezer designated for radioactive use. Keep the refrigerator and freezer locked when not in use.
- 5.) Conduct experiment on top of bench paper and with items designated for radioactive use. Do not touch your person or items not designated for radioactive use. If non-radioactive items must be handled, change gloves or ask labmate for assistance.
- 6.) If samples in microcentrifuge tubes need to be kept on ice, fill a 1-L plastic beaker with ice, place rack in beaker, and place microcentrifuge tubes in rack. Discard ice down drain only if swipe test shows it is not contaminated.
- 7.) Record notes using pen designated for radioactive use and disposable paper (not a lab book). Transfer notes to lab book after experiment.
- 8.) After completing experiment, decontaminate non-disposable radioactive items with NoCount, ddH<sub>2</sub>O, and paper towels. Collect contaminated liquids in liquid waste container. Discard disposable, incinerable materials in polypropylene barrel for Type A waste; discard disposable, non-incinerable materials in Type B waste; and discard liquid scintillation vials (full, not poured out) in designated waste.
- 8.) Follow *Swipe assay* for doing a swipe test.

## Liquid scintillation counting

Adapted from Beckman LS6000 Series manual

- 1.) Place sample (e.g., membrane filter or liquid) to be counted in 7 mL scintillation vial (Fisher 03-337-26).
- 2.) Add 5 mL Scintiverse BD scintillation cocktail (Fisher SX18-250) with designated bottle-top dispenser.
- 3.) Mix sample by vortexing or shaking vial.
- 4.) Load vials in a blue scintillation rack. Insert “AutoDPM” card into rack.
- 5.) Load racks in Beckman LS 6000 IC  $\beta$ -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  $^3\text{H}$  or  $^{14}\text{C}$ , 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 (Perkin Elmer 6008400).
- (v) Corrections for quenching are done automatically by using a  $^{137}\text{Cs}$  source.

## Swipe assays

After ref. (101) 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<sup>2</sup> 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 <sup>14</sup>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  $\beta$ -counter. Because of low CPM, efficiencies are not reliable (and sometimes cannot even be calculated).

**Fig. 1. Survey map.**

# **RADIATION/CONTAMINATION SURVEY**

PRINCIPAL INVESTIGATOR: HACKMANN, TIMOTHY

SURVEYOR: TAO, JUNYI

ROOM NO: 201

RADIONUCLIDES USED: 3H, 14C

SURVEY NO: 1

SURVEY TYPE: WEEKLY ☐ SPECIAL ☒

DATE: 6/22/ 15

	Yes	No	N/A
Caution Signs Posted	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Waste Facilities Posted & Neat	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Inventory up to Date	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Work Areas Clean	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Survey Meter Calibrated	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>
Survey Meter Operation Verified with Dedicated Check Source	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>

Survey Meter Model & Serial #

NA

Work Area NA mR/hr

Waste Facilities NA mR/hr

Storage Areas NA mR/hr

## **CONTAMINATION SURVEY**

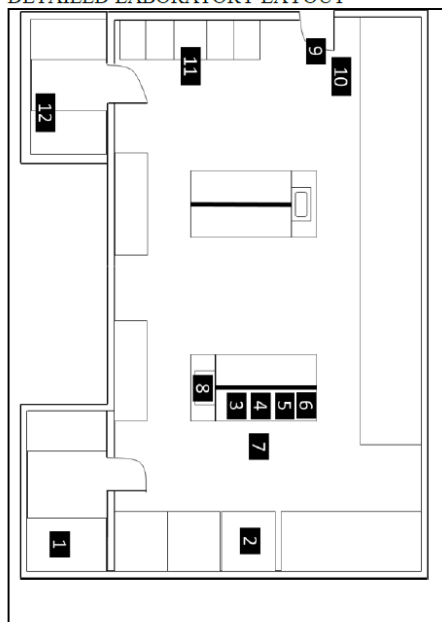
### **RESULTS**

Counting Instrument Model & Serial #

Beckman LS 6000 IC

Detection Efficiency 60% for 3H and 96% for 14C

#### **DETAILED LABORATORY LAYOUT**



#	Swipe Identification	Net Cpm	Net dpm*	Post Decon dpm
1	Outside LSC	7	11.05	
2	Lip and base of hood	3	4.07	
3	Metal tripod	11	16.78	
4	Bottle top dispenser	18	27.52	
5	Dry block	5	6.47	
6	Bench	4	5.74	
7	Floor by bench	-0.5	-1.76	
8	Sink	11.5	16.68	
9	Door knob	7.5	9.71	
10	Floor by door	6.5	8.97	
11	Refrigerator door	3	5.00	
12	Freezer door	0	0.93	

\*Areas of greater than 100 dpm/swipe must be resurveyed and documented.

DPM = Gross CPM minus background divided by the efficiency of the counting instrument for the particular radioisotope

RADIATION READINGS:



**Table 1.** Settings for swipe survey for Beckman LS 6000 IC beta counter

<b>Menu/submenu</b>	<b>Option</b>	<b>Setting</b>
Review/Edit	Counting time	2 min
	Scintillator	Liquid
	Isotope 1	<sup>14</sup> C
	Isotope 2	<sup>3</sup> H
	Isotope 3	NONE
Data calculation	Calculation mode	CPM
	Count sample	1
	Replicates	1
	Count sample set	1
	Factor for <sup>14</sup> C	1.000000
	Factor for <sup>3</sup> H	1.000000
Background/blank	Count blanks	No
	Background <sup>14</sup> C	0.00
	Background <sup>3</sup> H	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 ref. (34, 35, 102)

### Reagents

- 1.) Prepare 20 mM NADH. Weigh out 0.025 g NADH (Sigma 43420-100MG) in a sterile 2-mL centrifuge tube and add 1762  $\mu$ L molecular-biology ddH<sub>2</sub>O. Before storing at -20°C, make aliquots (17, 0.1 mL volumes in sterile 0.5 mL centrifuge tubes) to avoid repeated freeze-thawing of the entire solution during routine use.
- 2.) Prepare 0.5 M phosphoenolpyruvate (PEP) by adding 970.2  $\mu$ L molecular-biology ddH<sub>2</sub>O to 0.1 g PEP monopotassium salt. Do this by adding 500  $\mu$ L molecular-biology ddH<sub>2</sub>O 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<sub>2</sub>O to manufacturer vial, and with first pipette tip, transfer back-and-forth between vial and centrifuge tube (to wash and mix). Final solution will be clear. Store at -20°C in 0.2 mL aliquots.
- 3.) Order L-lactate dehydrogenase (LDH; Roche 1012802300) and store at 4°C.
- 4.) Prepare 1:9 toluene-ethanol. Add 1 mL toluene to 9 mL ethanol and mix.
- 5.) Prepare phosphate buffer (see *Buffer and media recipes* and *Preparing small volumes of buffer/media*). Prepare phosphate buffer + 2 mM DTT by adding 0.18 mL 0.1 M DTT to 9-mL aliquots of buffer. Add DTT after autoclaving. Make sure to prepare buffer without DTT, also (see below).
- 6.) Prepare 5 mM glucose.
- 7.) Prepare 5 mM 2-NBDG. Add 584  $\mu$ L ddH<sub>2</sub>O 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<sub>600</sub> over time.
- 2.) Harvest bacteria when culture reaches late log phase. For *Streptococcus bovis* JB1, this corresponds to OD<sub>600</sub> = 1 and requires 3 to 4.5 h from inoculation. Place tubes on ice, transfer to 10-mL screw-cap centrifuge tube under N<sub>2</sub>. Centrifuge at 4°C, and wash twice with ice-cold phosphate buffer + 2 mM DTT under N<sub>2</sub>. For *S. bovis*, 10 000 g for 5 min is sufficient for each centrifugation.
- 3.) Resuspend pellet in 3-mL phosphate buffer + 2 mM DTT (or that volume which achieves 0.1 to 1 mg protein/mL). Transfer to 12x75 mm culture tube. Keep on ice and under N<sub>2</sub>.

### 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<sub>2</sub>, 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<sub>2</sub>
  - 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<sub>2</sub>, stopper, and pre-incubate in 39°C air incubator for 10 min.

- 2.) Initiate the reaction. Add 20 µL 5 mM glucose to one cuvette, 20 µL 5 mM 2-NBDG a second cuvette, and 20 µL phosphate buffer (without DTT) to a third cuvette. Immediately mix, flush with N<sub>2</sub>, 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<sup>-1</sup> cm<sup>-1</sup> is the extinction coefficient of NADH and 10<sup>-3</sup> 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<sup>-1</sup> min<sup>-1</sup>. Repeat all experiments in triplicate.

### Notes

- (i) Most PTS assays include higher concentrations of sugar [e.g., 5 mM; (34, 35, 102)]. The concentration in the present experiment is limited to 100 µM because of the high extinction coefficient of 2-NBDG (7100 M<sup>-1</sup> cm<sup>-1</sup>).

## **SECTION VIII: MASS SPECTROMETRY**

Mass spectrometry can identify and quantify analytes important in microbiology, and it is especially useful for analysis of fatty acids from microbes. This procedure explains how to a single-quadrupole mass spectrometer (Waters Radian) for this purpose.

## Operation of Waters Radian Mass spectrometer

### Turning on

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



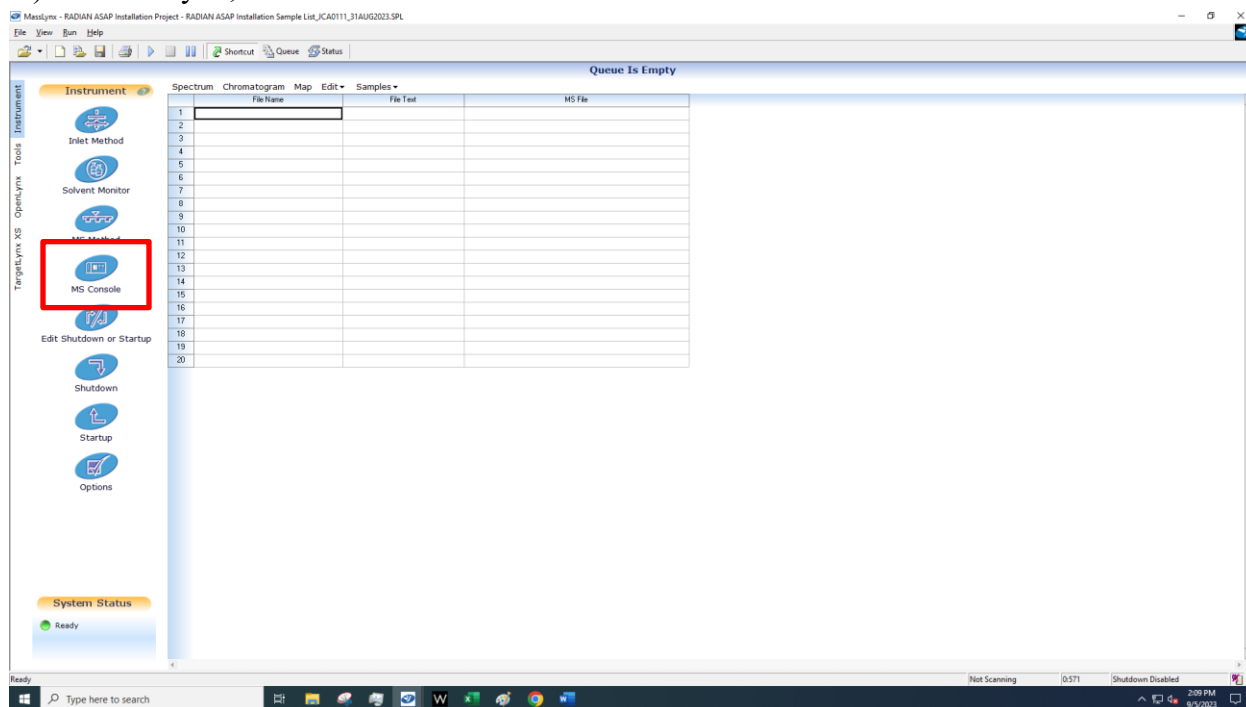
2.) Turn on instrument by pressing button on front.



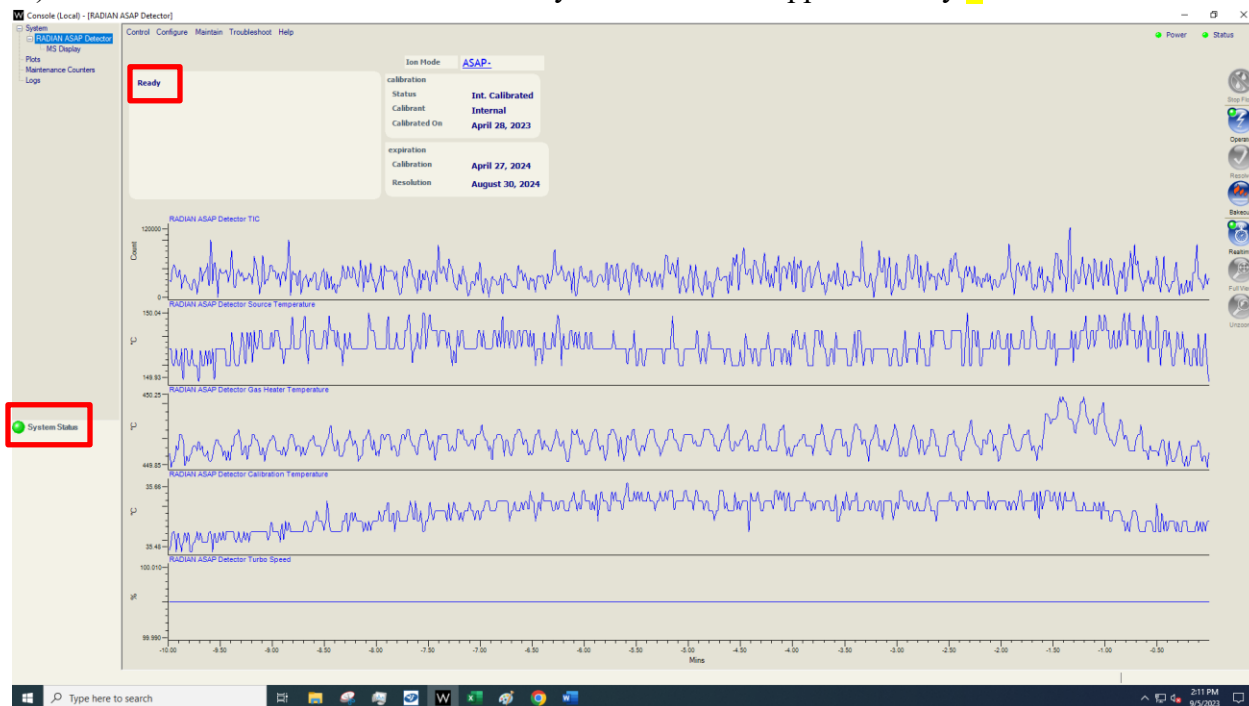
The vacuum pump, which is connected to the instrument with a relay box, will start automatically.

3.) After 5 minutes (time needed for instrument to boot), start MassLynx software on computer. Instrument will be placed in standby mode. Either pause at this step or continue below.

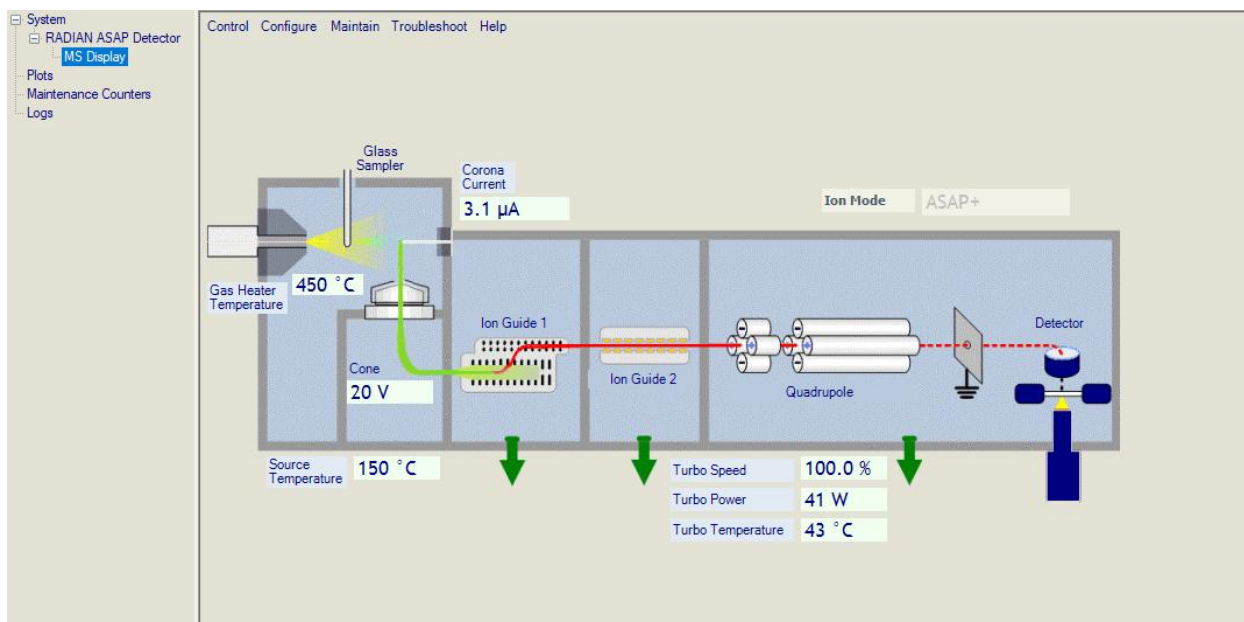
4.) In MassLynx, click on MS Console.



5.) Wait until instrument is shown as ready. This can take approximately 5 min.



6.) Optional: Check instrument status in System -> MS Display. Normal values are shown below.



Normal turbo power is 35 to 65 W, and normal turbo speed is 100%. Power above normal indicates an overload, which may be fixed by ballasting the backing pump. Speed below normal indicates a severe failure.

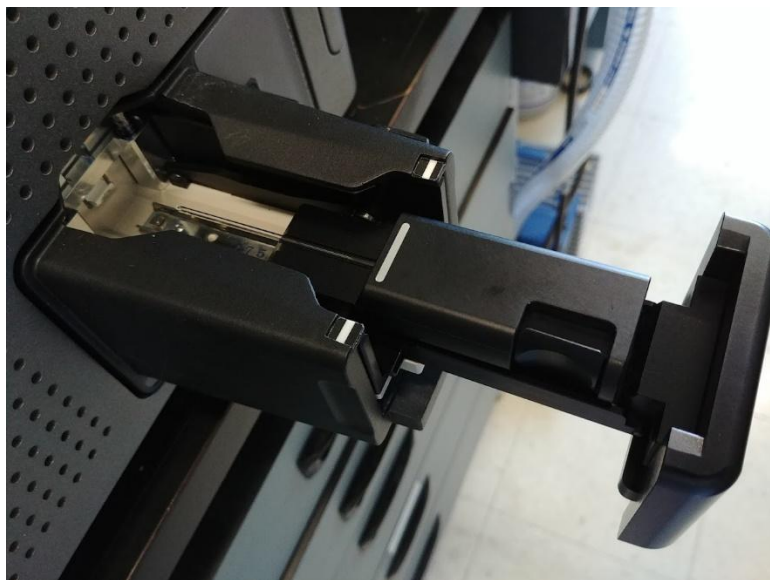
## Running samples

1.) Prepare capillary and capillary holder.

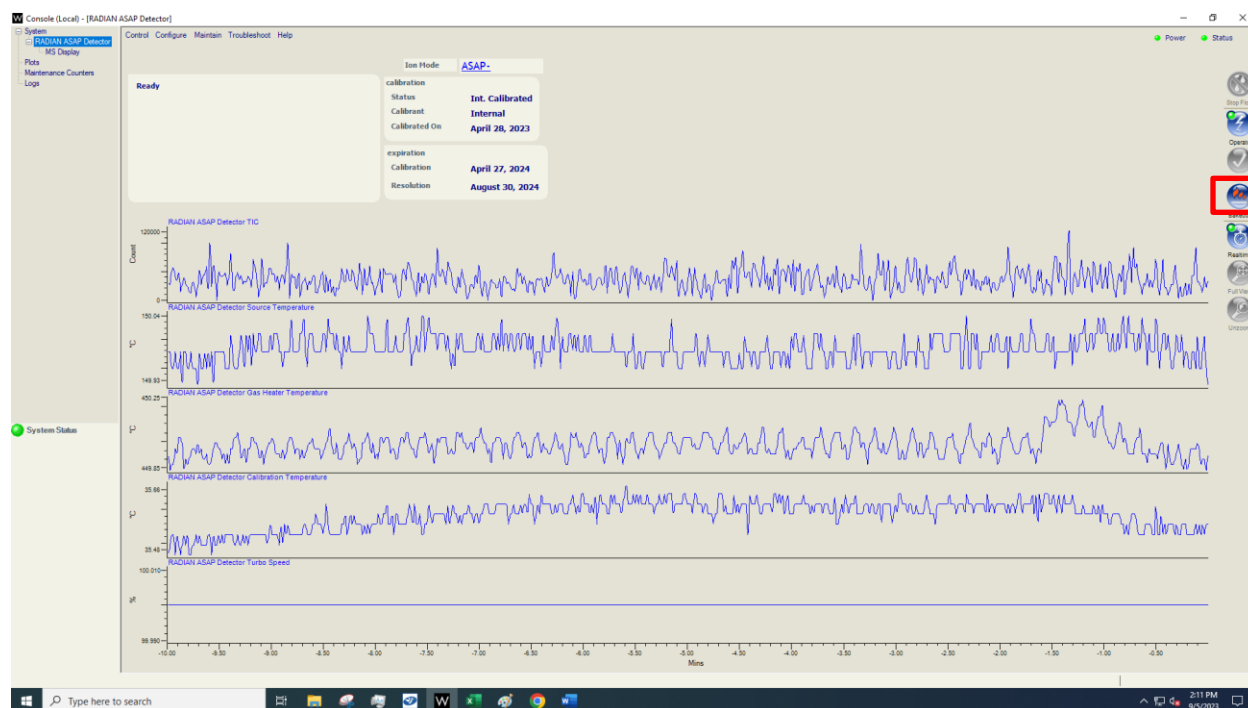
(a) Insert capillary into holder



(b) Insert capillary into source enclosure of instrument. Place holder in sample loader on instrument, making sure they are aligned at white marks and holder is fully seated.



(C) Start cleaning the capillary by pressing Bakeout button in MS Console of MassLynx.

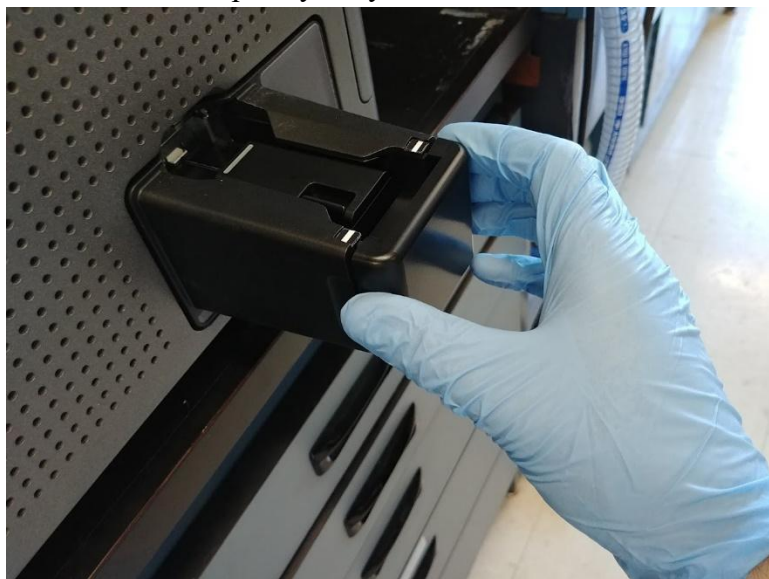


Push sample loader forward





Make sure that capillary fully enters source enclosure.

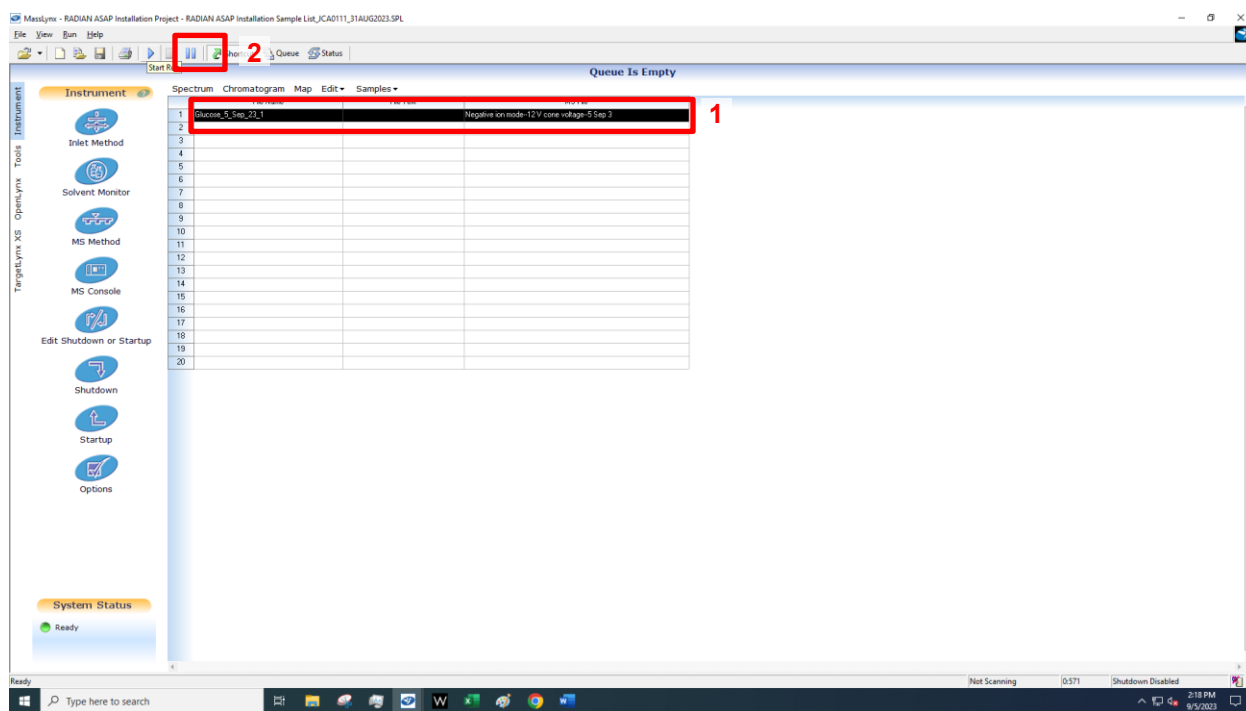


Allow to cleaning to complete (takes ~1 min).

(f) Pull out sample loader and allow capillary to cool.

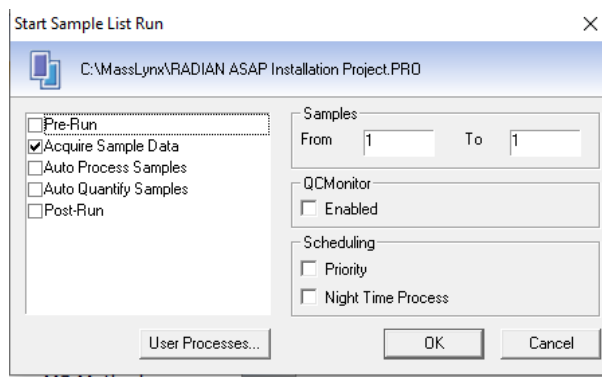


2.) In main window of MassLynx, click on and highlight row for sample to run, then press blue play button.



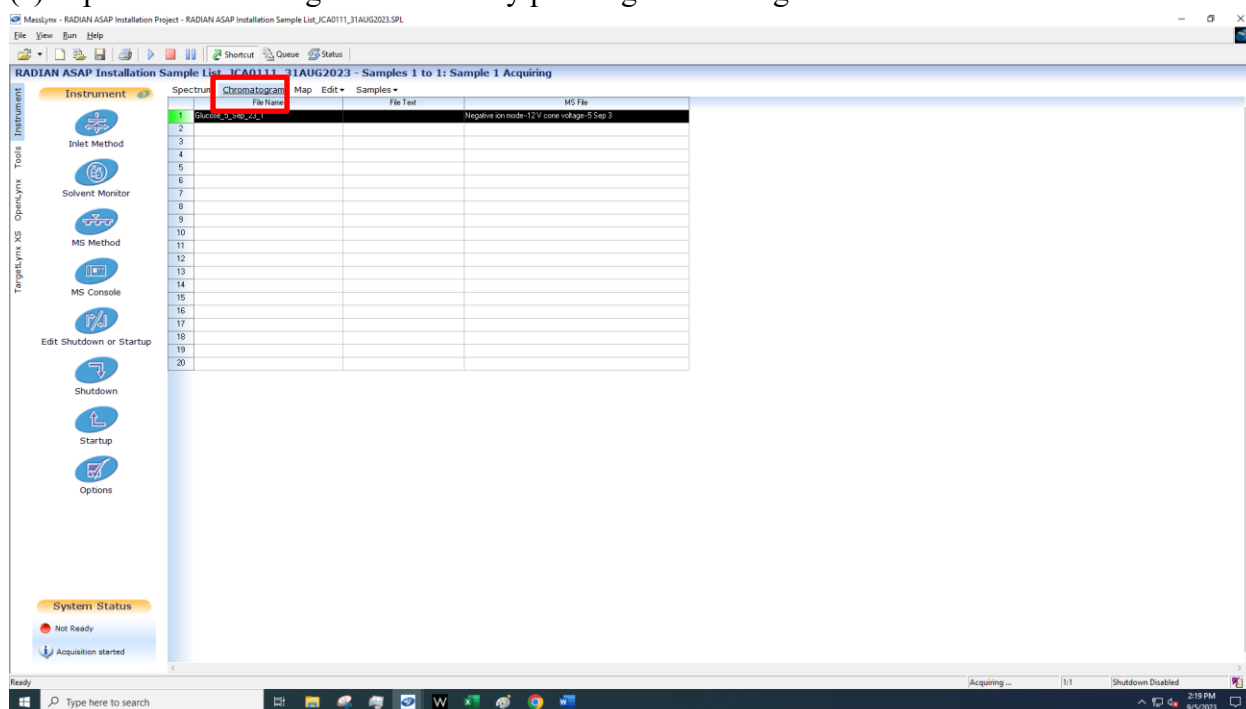
To set up a sample in the software, see *Setting up a project, sample list, and analysis method for Radian mass spectrometer*.

3.) In window that appears, verify that Acquire Sample Data is checked and press OK.

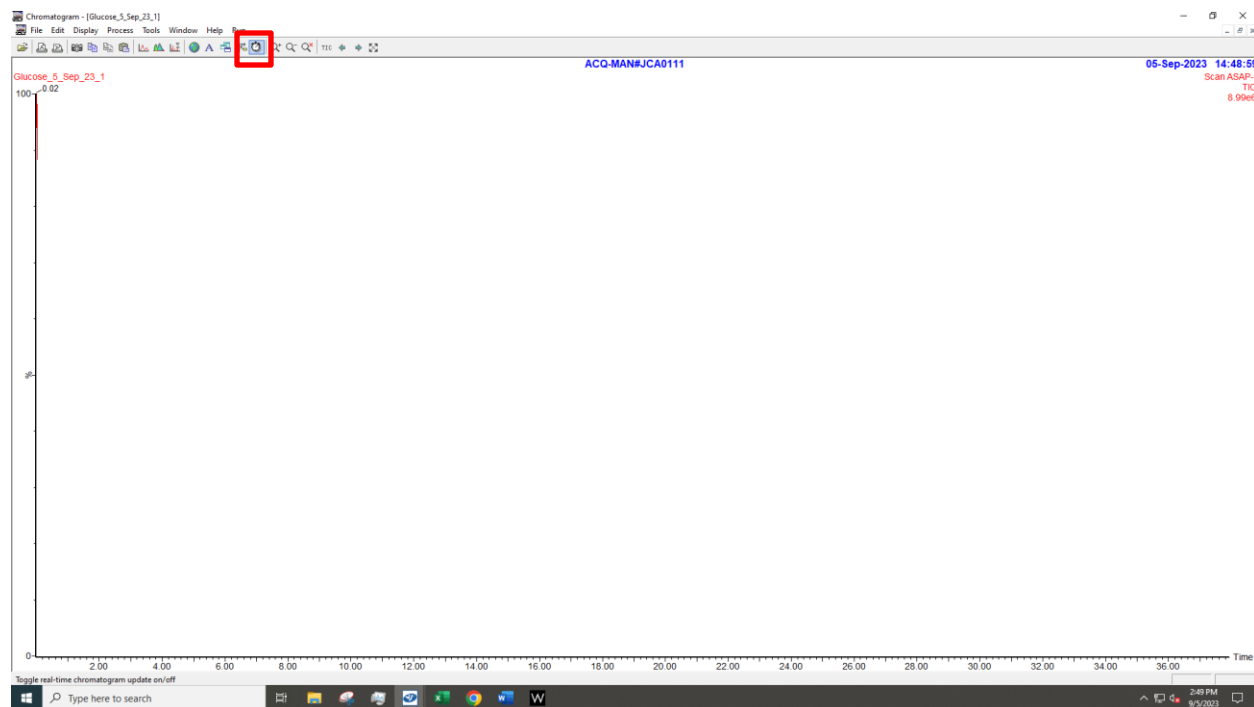


4.) Set up the chromatogram to see results in real time.

(a) Open the chromatogram window by pressing Chromatogram.

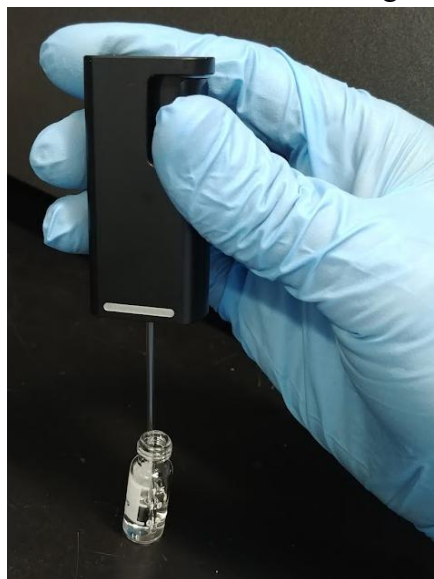


(b) Turn on real-time display of data by clicking the time icon.



5.) Start acquiring background signal. If it is not in it already, place capillary holder into the sample loader (or just press down the spring-loaded platform of the sample loader by hand).

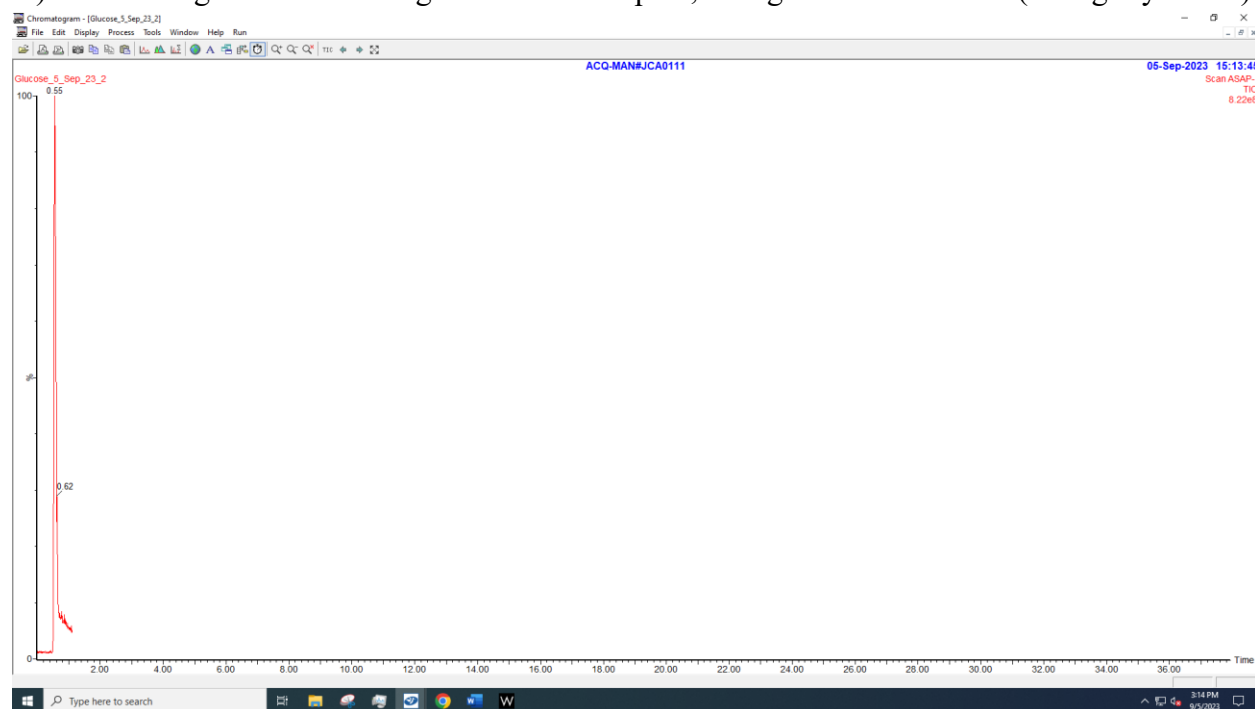
6.) Apply sample to capillary. Open GC vial with sample, then dip end of capillary in sample. Swirl to mix and ensure enough sample is applied.



For guidance in preparing samples, see *Preparing samples for Radian mass spectrometer*.

7.) Insert capillary into source enclosure of instrument (see step 1).

8.) Monitor signal on chromatogram. It should spike, then go back to baseline (or slightly above).

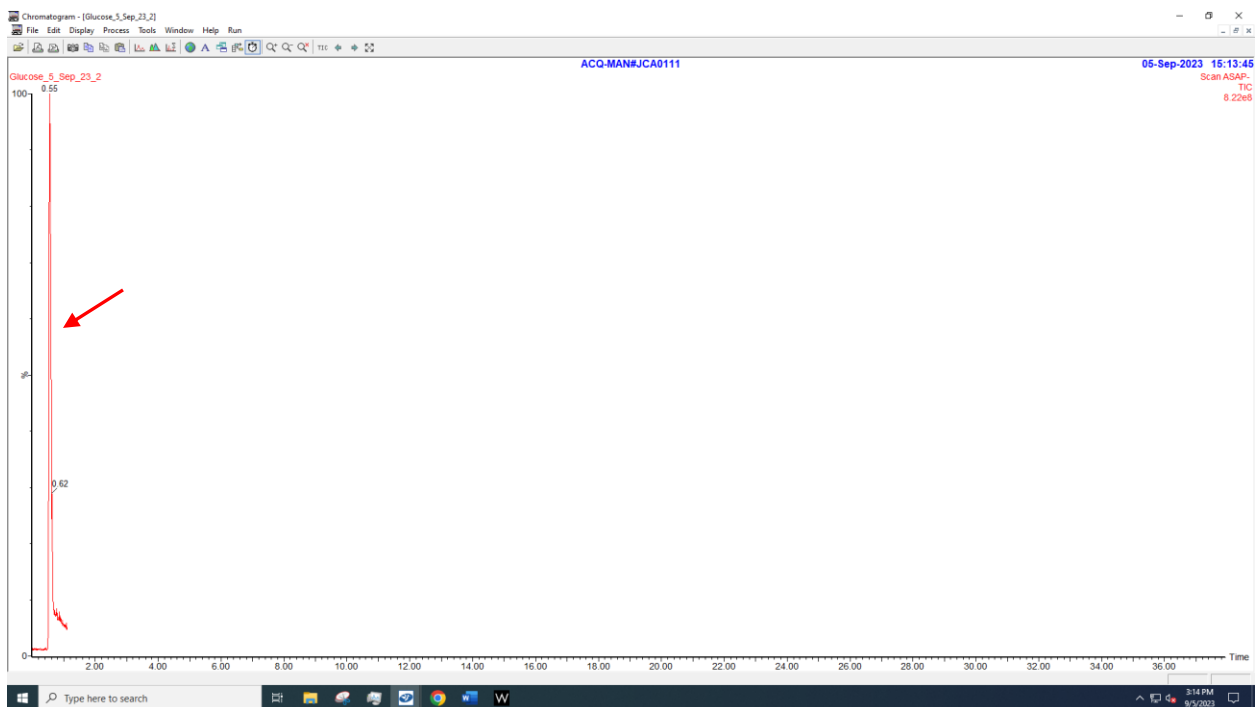


The maximum signal (TIC) is shown in the right hand corner.

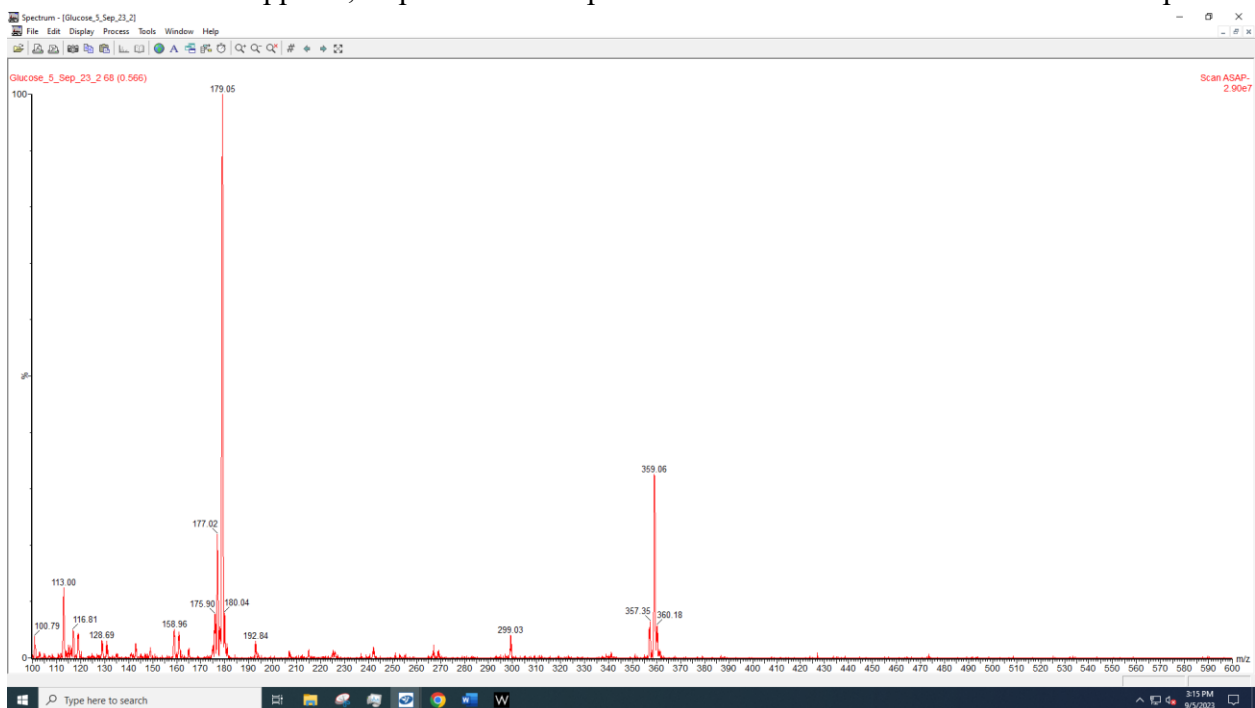
9.) Once signal returns to baseline, remove capillary and holder from instrument. Allow to cool for ~30 s.

10.) View mass spectrum.

(a) Right click on the main chromatogram near a peak.



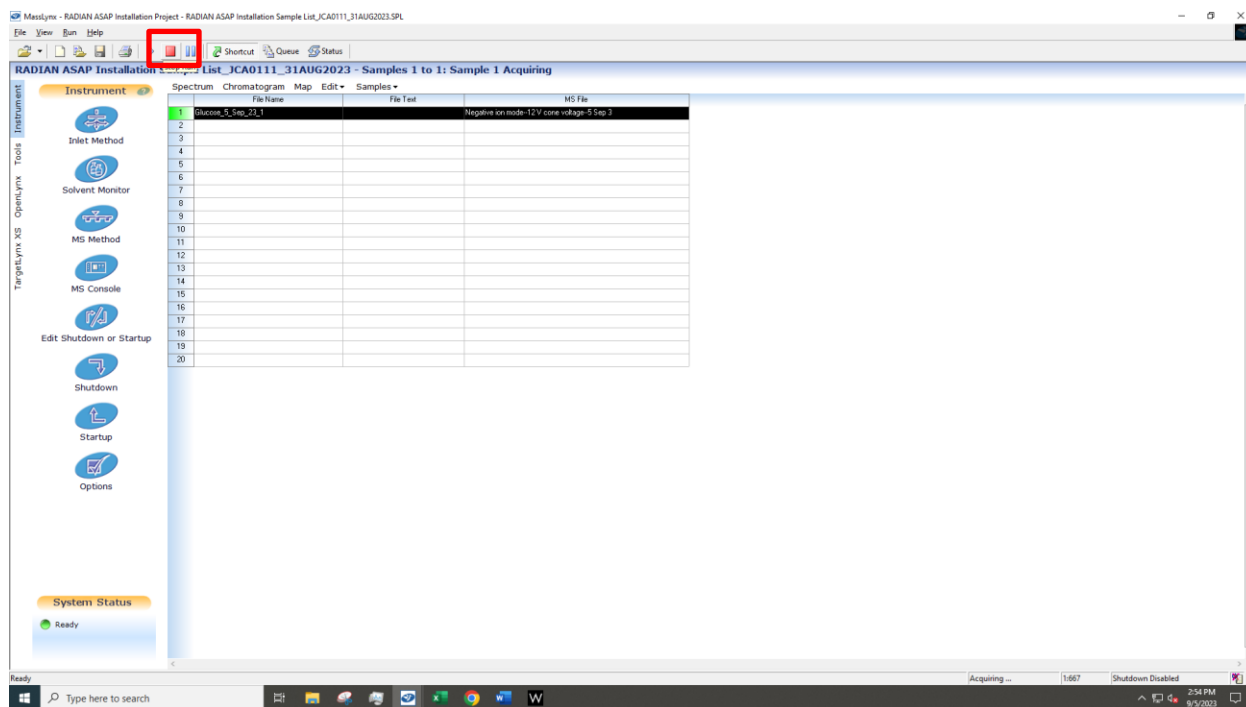
In the window that appears, inspect the mass spectrum and determine if  $m/z$  values are as expected.



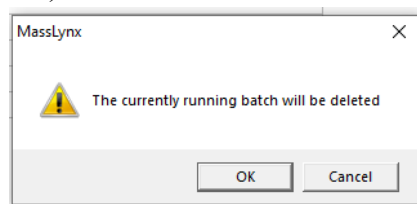
To obtain a more processed mass spectrum, see *Processing and exporting mass spectrum for the Radian mass spectrometer*.

11.) Repeat #6 to 10 at least once (to obtain a minimum of two peaks).

12.) When done collecting data, click red stop icon.

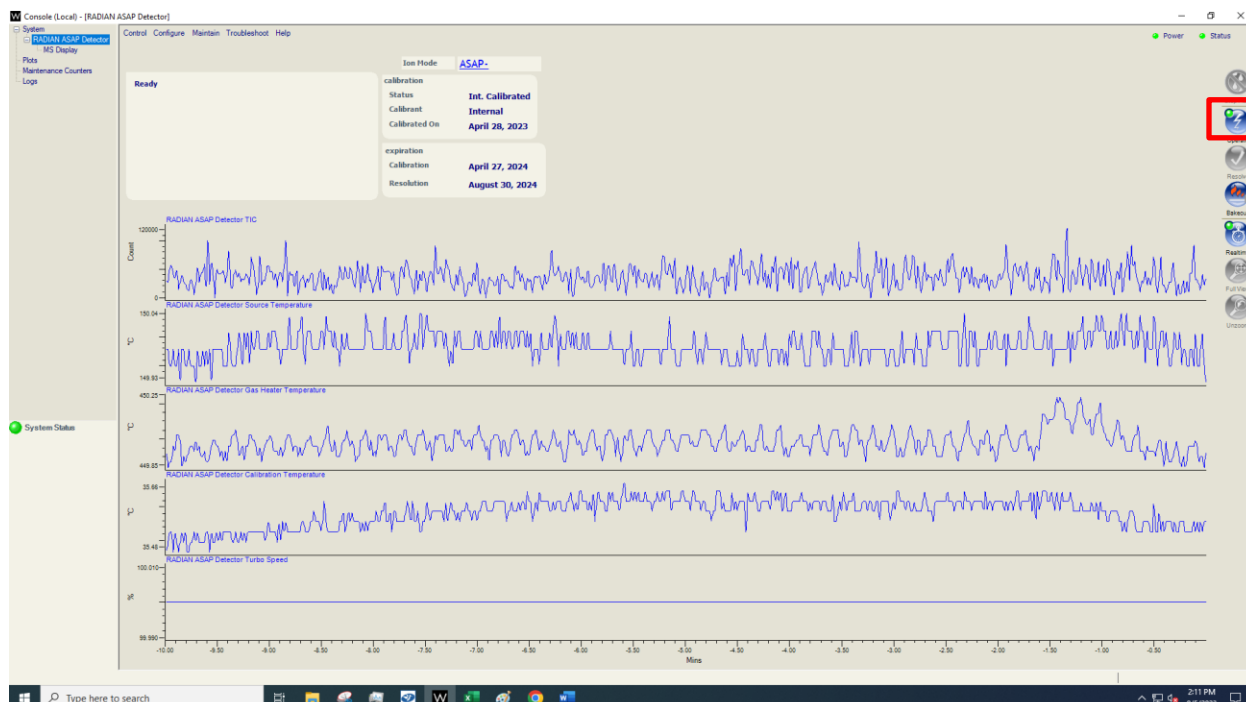


13.) Press OK when new window appears (data will not be discarded).



### Placing into standby and shutting down

1.) To place in standby, click on MS Console, and click Operate button.



Button will switch from green to red.

- 2.) Keep in standby mode if using within short period (~1 wk). In standby mode, gas is turned off, but vacuum is kept on. It is now safe to turn off compressed gas (nitrogen).
- 3.) To shut down entirely, press power button on front of instrument.

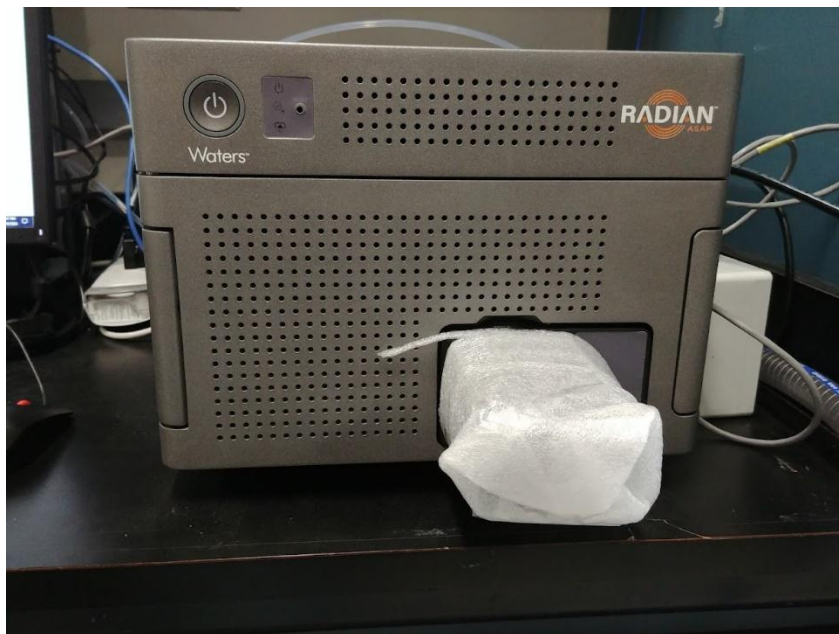


Avoid turning off without first placing into standby mode.



The vacuum pump, which is connected to to the instrument with a relay box, will stop automatically.

4.) Place cover over sample loader.



## Preparing samples for the Radian mass spectrometer

This explains how to prepare a sample using pure material (e.g., glucose). For biological samples, preparation will differ and individual protocols need to be consulted.

- 1.) Weigh 0.01 g sample in 2-mL vials (Fisher 13-622-186). For liquids, pipette 10  $\mu$ L.
- 2.) Create a 10 mg/mL solution. Add 990  $\mu$ L solvent (e.g., methanol) to the vial and shake to dissolve.

Use only compatible solvents, and use MS-grade reagents. See Fig. 1 for compatible solvents. If a buffer is needed, refer to Fig. 2.

- 3.) Create a 1 mg/mL solution. Add 100  $\mu$ L of 1 mg/mL solution to 900  $\mu$ L solvent in a new vial.
- 4.) Create a 0.1 mg/mL solution. Add 100  $\mu$ L of 0.1 mg/mL solution to 900  $\mu$ L solvent in a new vial.
- 5.) Analyze the most dilute (0.1 mg/mL) sample first. Determine if signal is adequate (maximum TIC between  $10^8$  to  $10^9$ , or well above baseline signal of  $10^6$  to  $10^7$ ). If signal is not adequate, use next most concentrated solution.

Avoid using concentrated solutions outright. They will dirty instrument components faster, and they may also saturate the detector.

**Fig. 1.** Compatible solvents for samples

**Solvents used to prepare samples:**

- Water
- Methanol
- Acetonitrile
- Ethanol
- Isopropanol
- Propanol
- Acetone
- Toluene
- Methyl tert-butyl ether (MTBE)
- Hexane
- Dichloromethane
- Dimethylformamide (DMF)
- Dimethyl sulfoxide (DMSO)
- Heptane
- Tetrahydrofuran (THF)

**Fig. 2.** Compatible and incompatible buffers for samples.

Mobile Phase Chemical Name	Formula	Concentration	Volume or Mass (per 1 L)	Preparation Procedure Number*	pH Adjustment Acid/Base	MS Compatible?
Acetic Acid	CH <sub>3</sub> COOH	0.1%	1.0 mL	1	–	Yes
Ammonium Hydroxide	NH <sub>4</sub> OH	0.1%	1.0 mL	1	–	Yes
Ammonium Hydroxide	NH <sub>4</sub> OH	0.2%	2.0 mL	1	–	Yes
Ammonium Hydroxide	NH <sub>4</sub> OH	1.0%	10.0 mL	1	–	Yes
Ammonium Hydroxide	NH <sub>4</sub> OH	100 mM	6.9 mL	1	–	Yes
Formic Acid	HCOOH	0.05%	0.5 mL	1	–	Yes
Formic Acid	HCOOH	0.1%	1.0 mL	1	–	Yes
Formic Acid	HCOOH	0.2%	2.0 mL	1	–	Yes
Formic Acid	HCOOH	0.5%	5.0 mL	1	–	Yes
Formic Acid	HCOOH	50 mM	2.1 mL	1	–	Yes
Formic Acid	HCOOH	100 mM	4.2 mL	1	–	Yes
Phosphoric Acid	H <sub>3</sub> PO <sub>4</sub>	0.1%	1.0 mL	1	–	No
Trifluoroacetic acid (TFA)	CF <sub>3</sub> COOH	10 mM	0.8 mL	1	–	Yes <sup>3</sup>
Trifluoroacetic acid (TFA)	CF <sub>3</sub> COOH	0.1%	1.0 mL	1	–	Yes <sup>3</sup>
Acetic Acid <sup>2</sup> Triethylamine (TEA) <sup>2</sup> EDTA	CH <sub>3</sub> COOH (CH <sub>3</sub> CH <sub>2</sub> ) <sub>3</sub> N C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> Na <sub>2</sub> O <sub>8</sub> ·2H <sub>2</sub> O	50 mM CH <sub>3</sub> COOH 50 mM TEA 2 mM EDTA	2.8 mL 6.9 mL 0.75 g	2	CH <sub>3</sub> COOH or (CH <sub>3</sub> CH <sub>2</sub> ) <sub>3</sub> N	No
Acetic Acid <sup>2</sup> Triethylamine (TEA) <sup>2</sup>	CH <sub>3</sub> COOH (CH <sub>3</sub> CH <sub>2</sub> ) <sub>3</sub> N	100 mM CH <sub>3</sub> COOH 100 mM TEA	5.6 mL 13.9 mL	3	CH <sub>3</sub> COOH or (CH <sub>3</sub> CH <sub>2</sub> ) <sub>3</sub> N	Yes
Ammonium Bicarbonate	NH <sub>4</sub> HCO <sub>3</sub>	5 mM	0.40 g	2	HCOOH or NH <sub>4</sub> OH	Yes
Ammonium Bicarbonate	NH <sub>4</sub> HCO <sub>3</sub>	10 mM	0.79 g	2	HCOOH or NH <sub>4</sub> OH	Yes
Ammonium Bicarbonate	NH <sub>4</sub> HCO <sub>3</sub>	20 mM	1.58 g	2	HCOOH or NH <sub>4</sub> OH	Yes
Ammonium Bicarbonate	NH <sub>4</sub> HCO <sub>3</sub>	100 mM	7.91 g	2	HCOOH or NH <sub>4</sub> OH	Yes
Ammonium Formate	NH <sub>4</sub> COOH	10 mM	0.63 g	2	HCOOH	Yes
Ammonium Formate	NH <sub>4</sub> COOH	15 mM	0.95 g	2	HCOOH	Yes
Ammonium Formate	NH <sub>4</sub> COOH	100 mM	6.31 g	2	HCOOH	Yes
Ammonium Acetate	CH <sub>3</sub> COONH <sub>4</sub>	10 mM	0.77 g	2	CH <sub>3</sub> COOH	Yes
Ammonium Acetate	CH <sub>3</sub> COONH <sub>4</sub>	20 mM	1.54 g	2	CH <sub>3</sub> COOH	Yes
Ammonium Acetate	CH <sub>3</sub> COONH <sub>4</sub>	100 mM	7.71 g	2	CH <sub>3</sub> COOH	Yes
Hexafluoroisopropanol (HFIP)	C <sub>2</sub> F <sub>5</sub> CHOH	0.4 M HFIP	41.5 mL	4	TEA	Yes
Triethylamine (TEA)	(CH <sub>3</sub> CH <sub>2</sub> ) <sub>3</sub> N	16.3 mM TEA	2.3 mL			
Sodium Phosphate, Dibasic	Na <sub>2</sub> HPO <sub>4</sub>	20 mM	2.84 g	2	H <sub>3</sub> PO <sub>4</sub>	No

(1) – See specified preparation procedure on next page

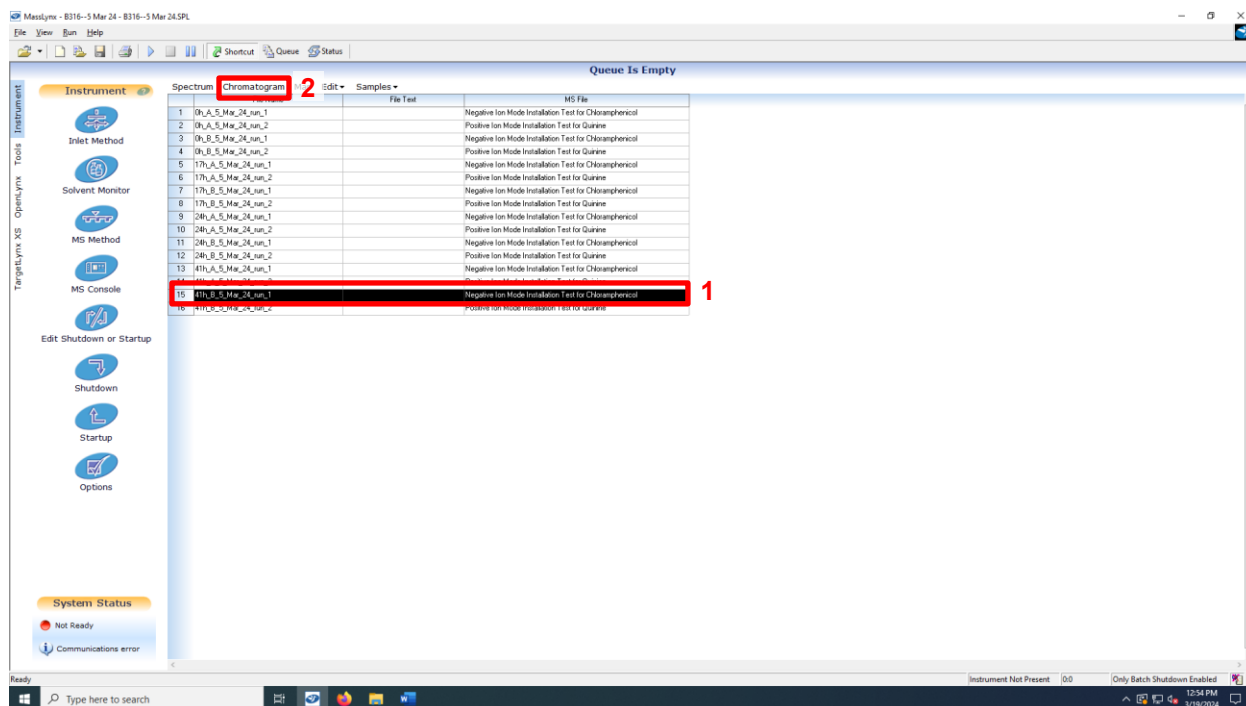
(2) – Triethylammonium Acetate (Acetic Acid + Triethylamine → Triethylammonium Acetate)

(3) – Can suppress MS signal at higher concentrations

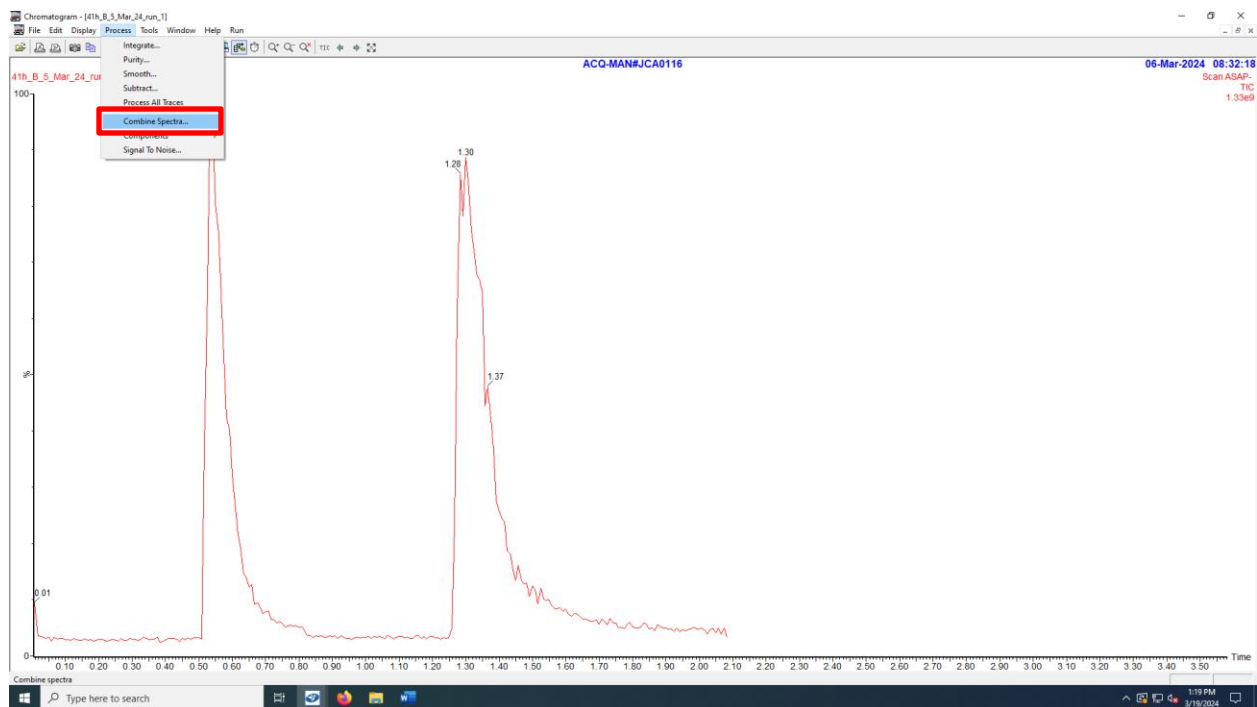
## Processing and exporting mass spectrum for the Radian mass spectrometer

### Processing the mass spectrum.

- 1.) In main window of MassLynx, click on and highlight row for sample to analyze, then click on Chromatogram.



- 2.) In window that appears, click on Process -> Combine Spectra.

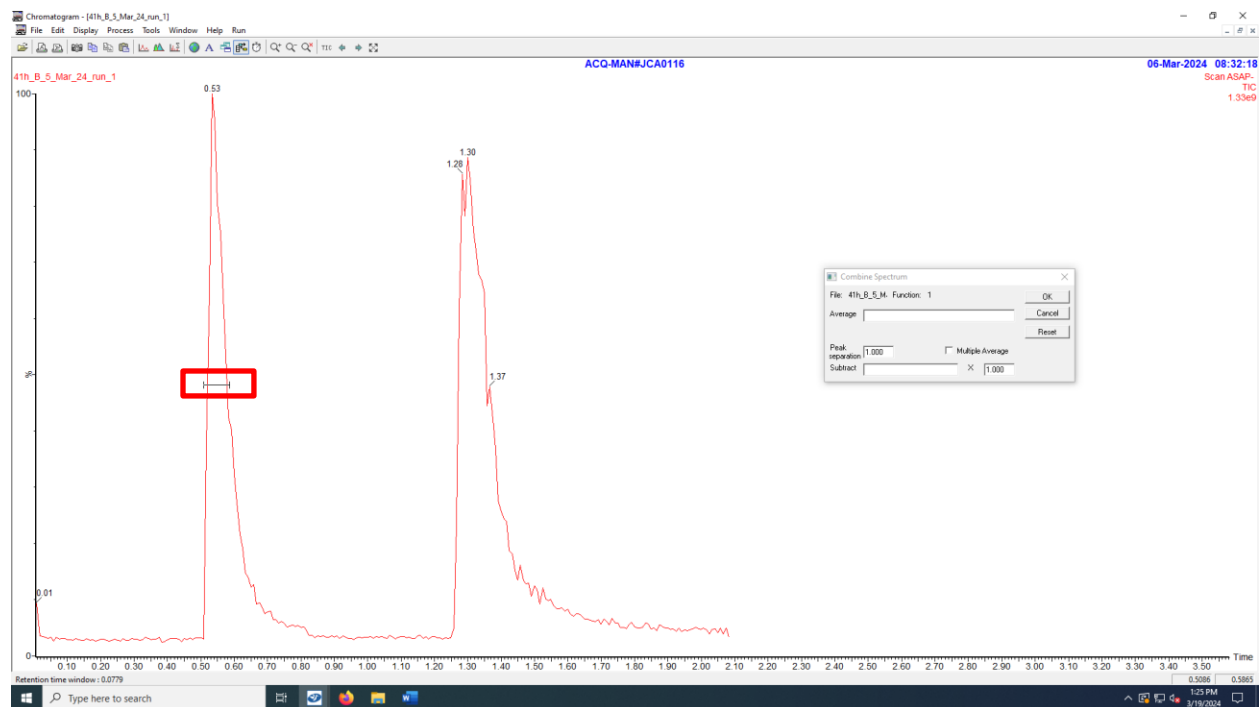


A popup will appear.

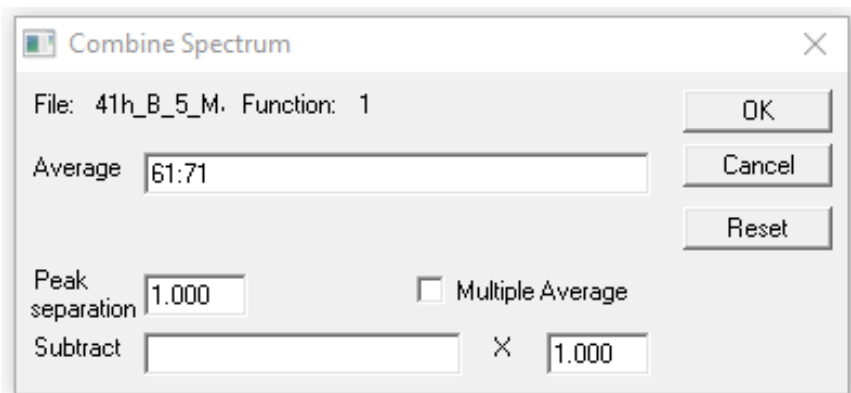
The figure shows a 'Combine Spectrum' dialog box. It has a title bar with a close button. The main area contains the following fields and controls:

- File:** 41h\_B\_5\_M. **Function:** 1
- Average:** A text input field.
- Peak separation:** A text input field containing the value '1.000'.
- Subtract:** A text input field followed by a multiplication symbol '×' and another text input field containing the value '1.000'.
- Multiple Average:** A checkbox that is currently unchecked.
- Buttons:** 'OK', 'Cancel', and 'Reset' buttons are located on the right side of the dialog.

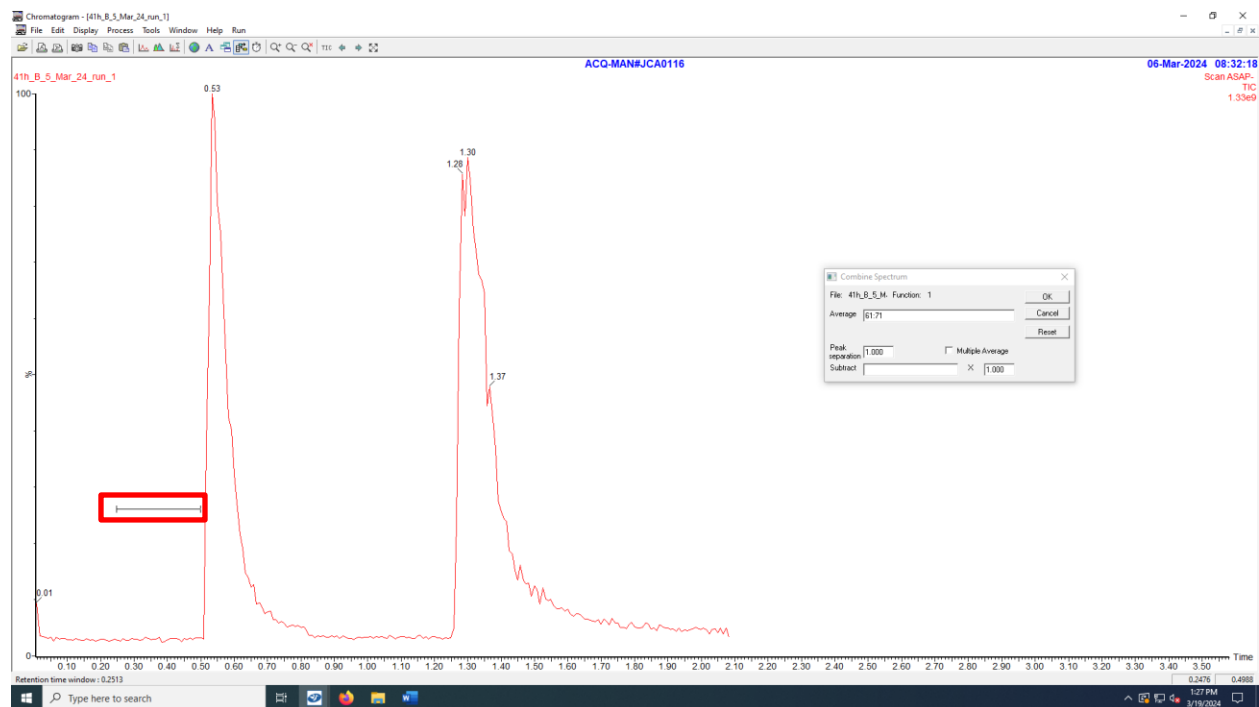
3.) Choose the signal to average. On the chromatogram, right click the main chromatogram, and drag the cursor to select a range of time corresponding sample peak.



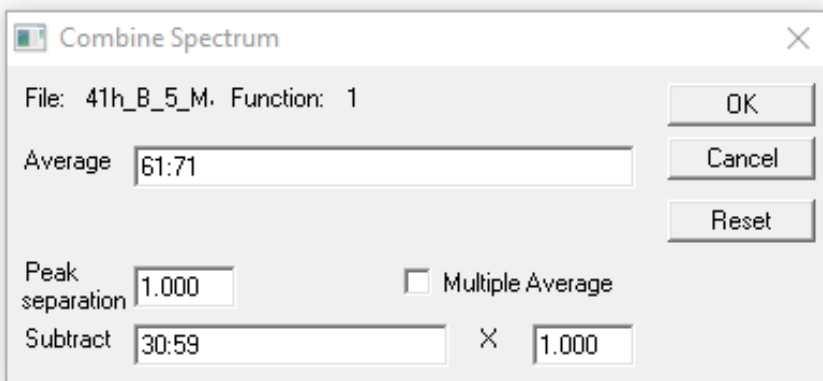
The popup will update with the range of time (scan number).



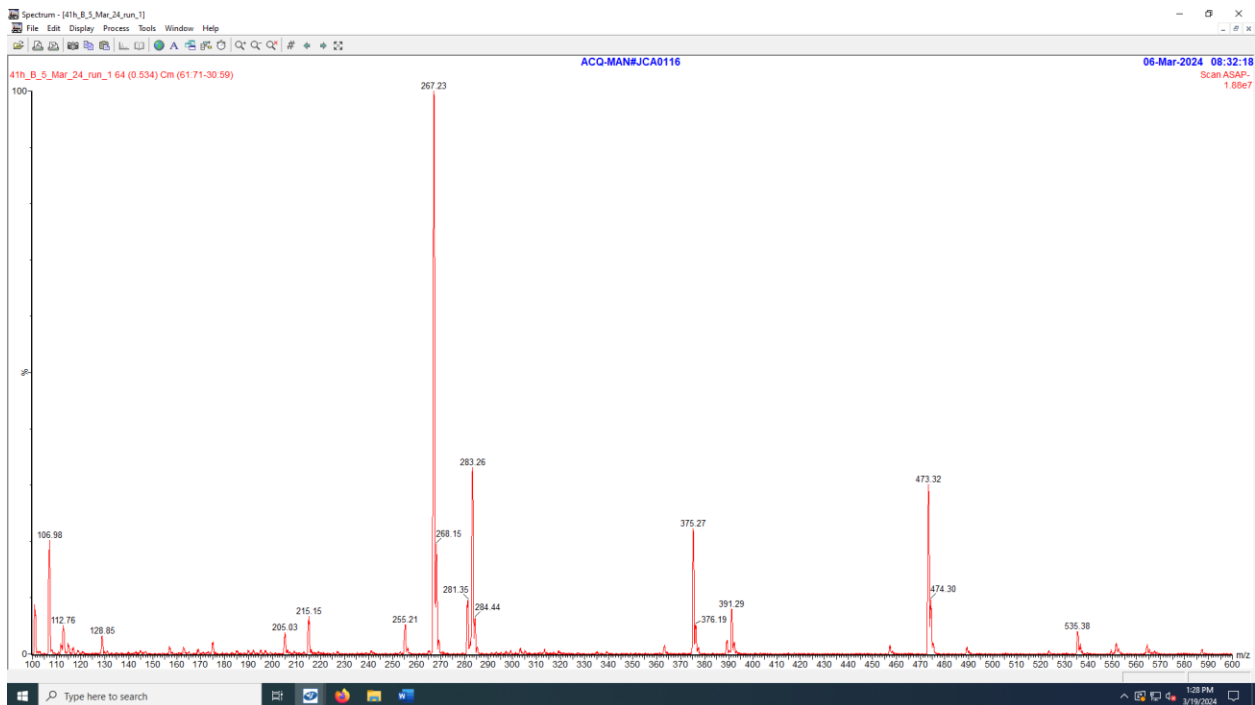
4.) Choose the background to subtract. On the chromatogram, right click the main chromatogram, and drag the cursor over a range of time corresponding to background (before injecting sample).



5.) In the popup, click OK.

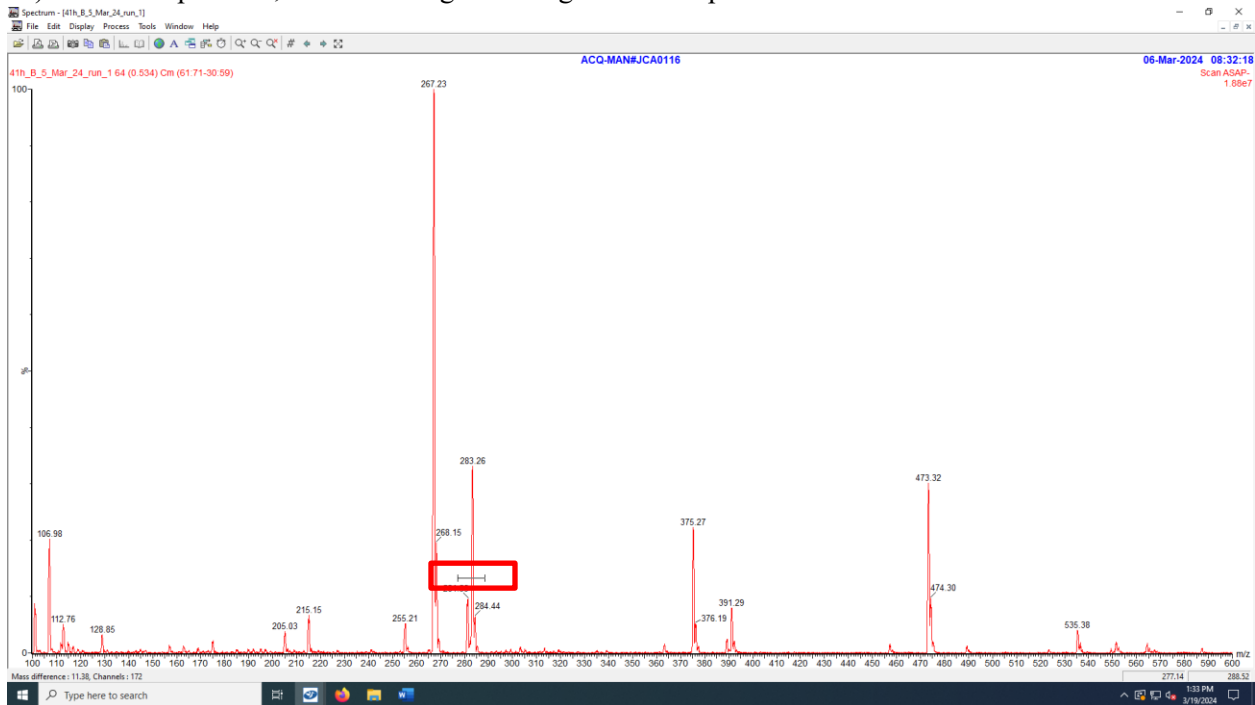


6.) In the window that appears, inspect the processed mass spectrum. Mass spectrum should resemble #10 of *Running samples*, but noise should be reduced.



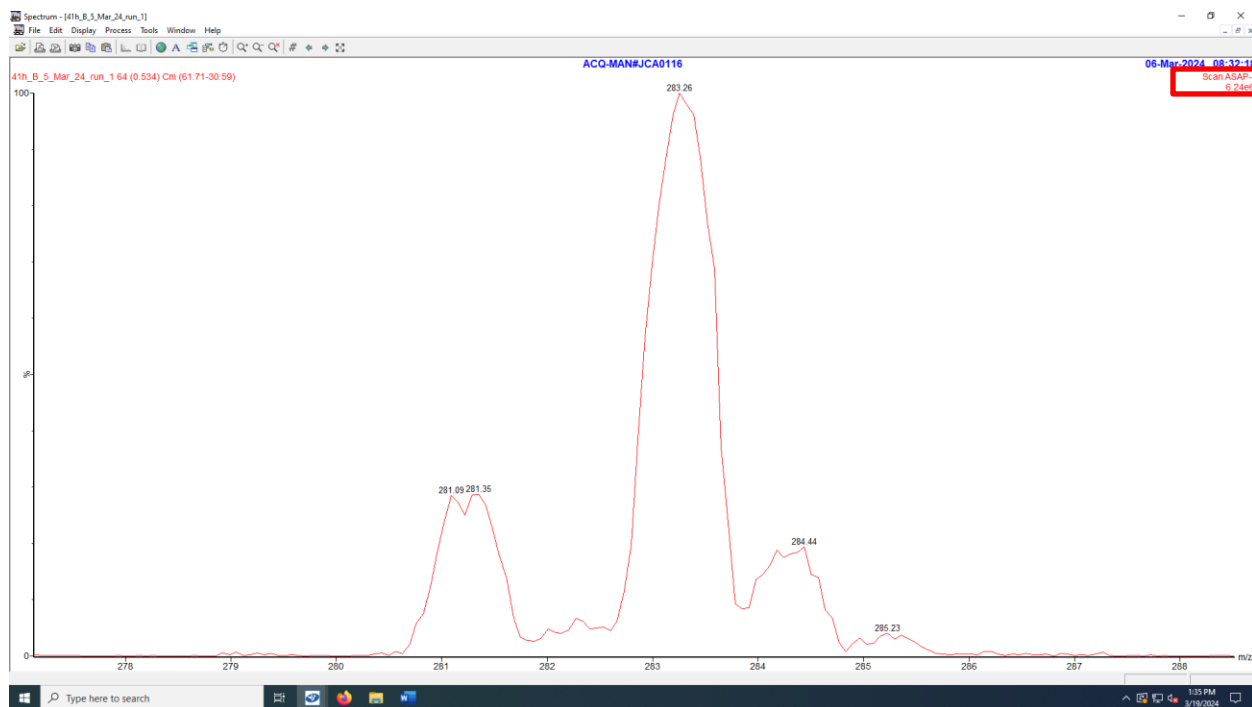
**Measuring signal (ion count) for peaks in mass spectrum.**

1.) In mass spectrum, click and drag over range of  $m/z$  of peak.



2.) Write down the signal in the upper right corner.

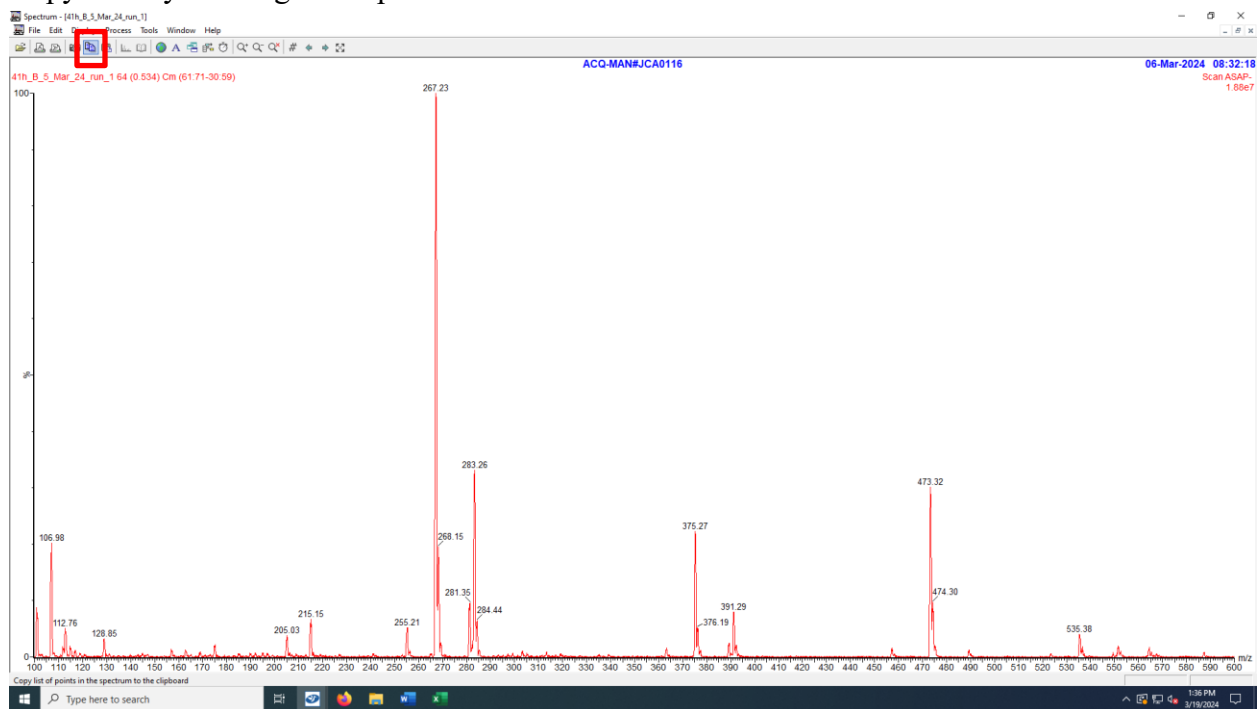




For large numbers of peaks, export data instead.

## Exporting data

Copy data by clicking the clipboard icon.



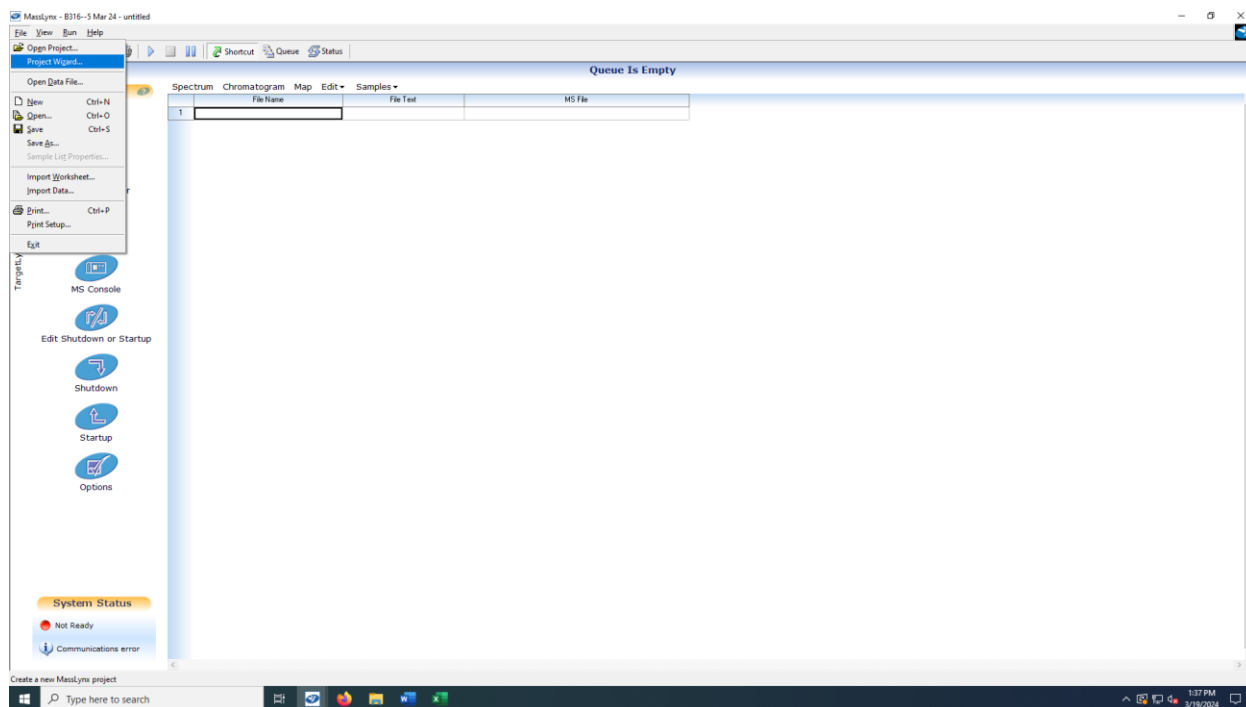
This data can be pasted into Excel or another program.



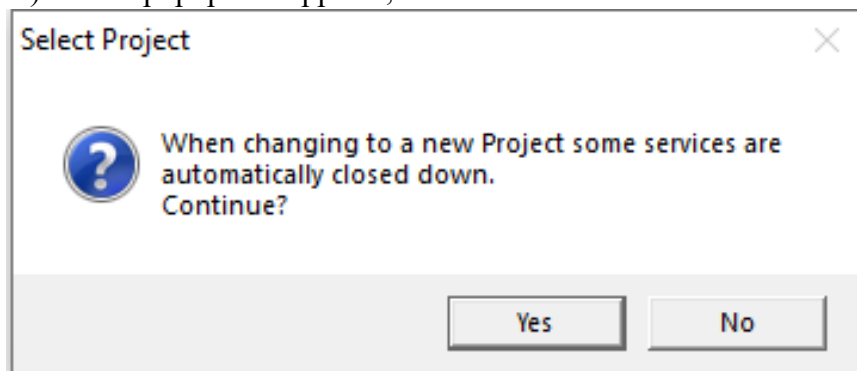
## Setting up a project, sample list, and analysis method for Radian mass spectrometer

### Setting up a project

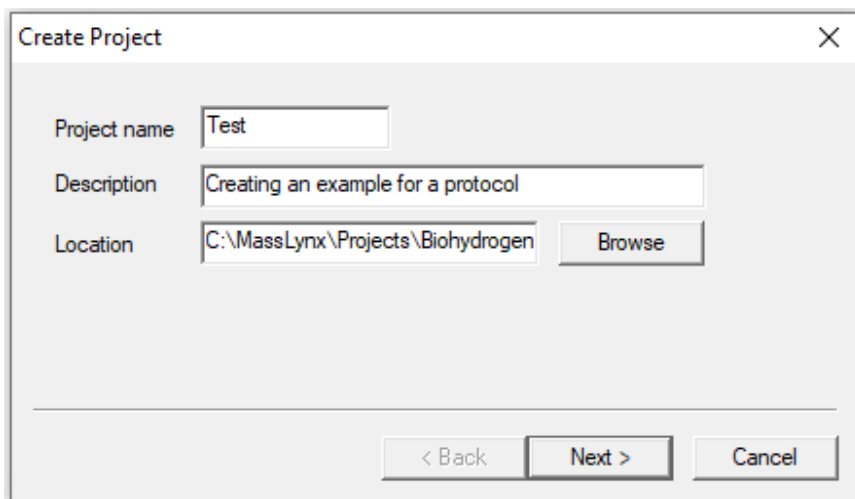
1.) In the main window of MassLynx, go to File -> Project Wizard.



2.) In the popup that appears, click OK



3.) In the popup that appears, enter project name, project description, and location.

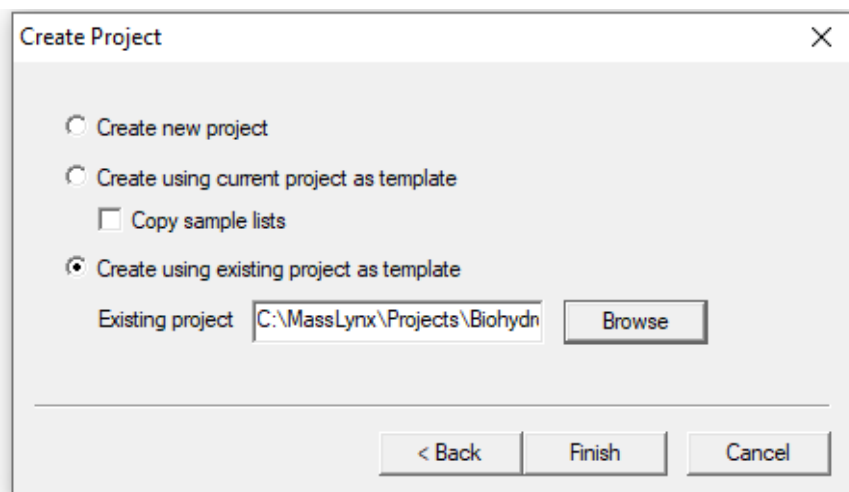


The 'Create Project' dialog box contains the following fields and buttons:

- Project name:** Text box containing 'Test'.
- Description:** Text box containing 'Creating an example for a protocol'.
- Location:** Text box containing 'C:\MassLynx\Projects\Biohydrogen' and a 'Browse' button.
- Navigation:** '< Back' button (disabled), 'Next >' button (active), and 'Cancel' button.

Click Next.

4.) After clicking next, choose an option for creating a project. The last option is simplest if the project is similar to one run in the past.

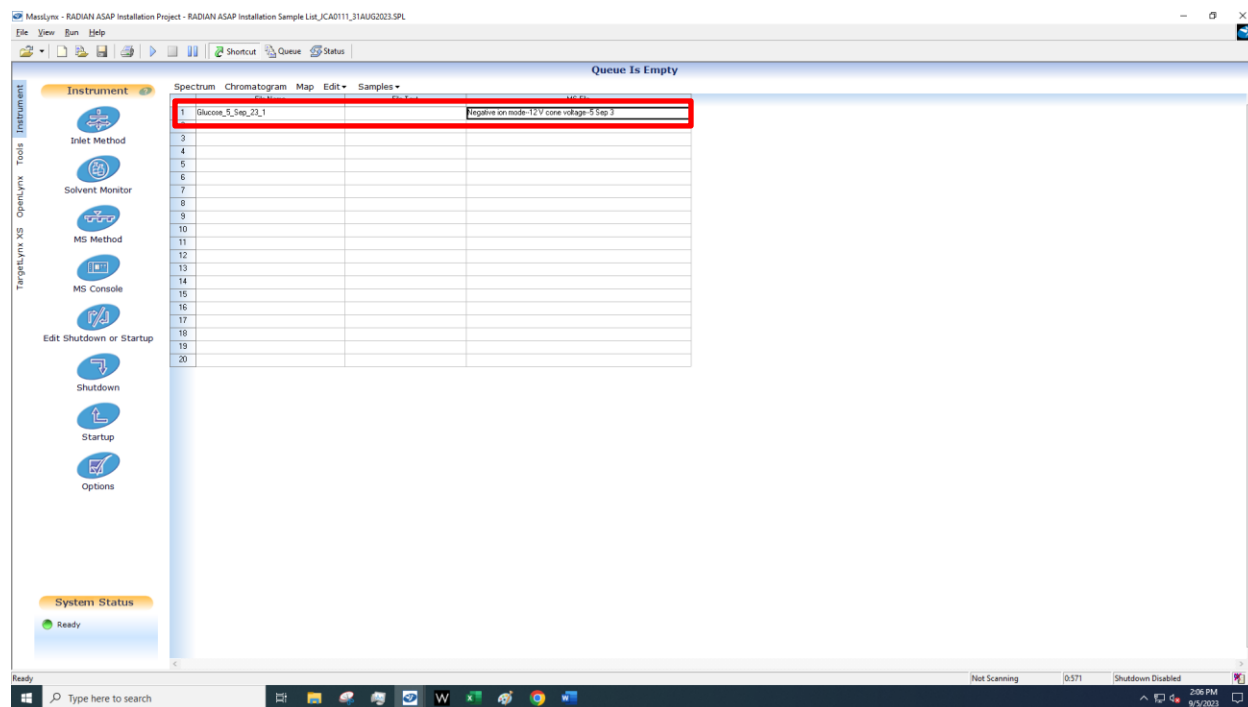


The 'Create Project' dialog box shows the following options and fields:

- Options:**
  - ☐ Create new project
  - ☐ Create using current project as template
    - ☐ Copy sample lists
  - ☒ Create using existing project as template
- Existing project:** Text box containing 'C:\MassLynx\Projects\Biohyd' and a 'Browse' button.
- Navigation:** '< Back' button (disabled), 'Finish' button (active), and 'Cancel' button.

### Setting up sample list

In the main window of MassLynx, click on a row in the sample list, then insert sample information as shown.

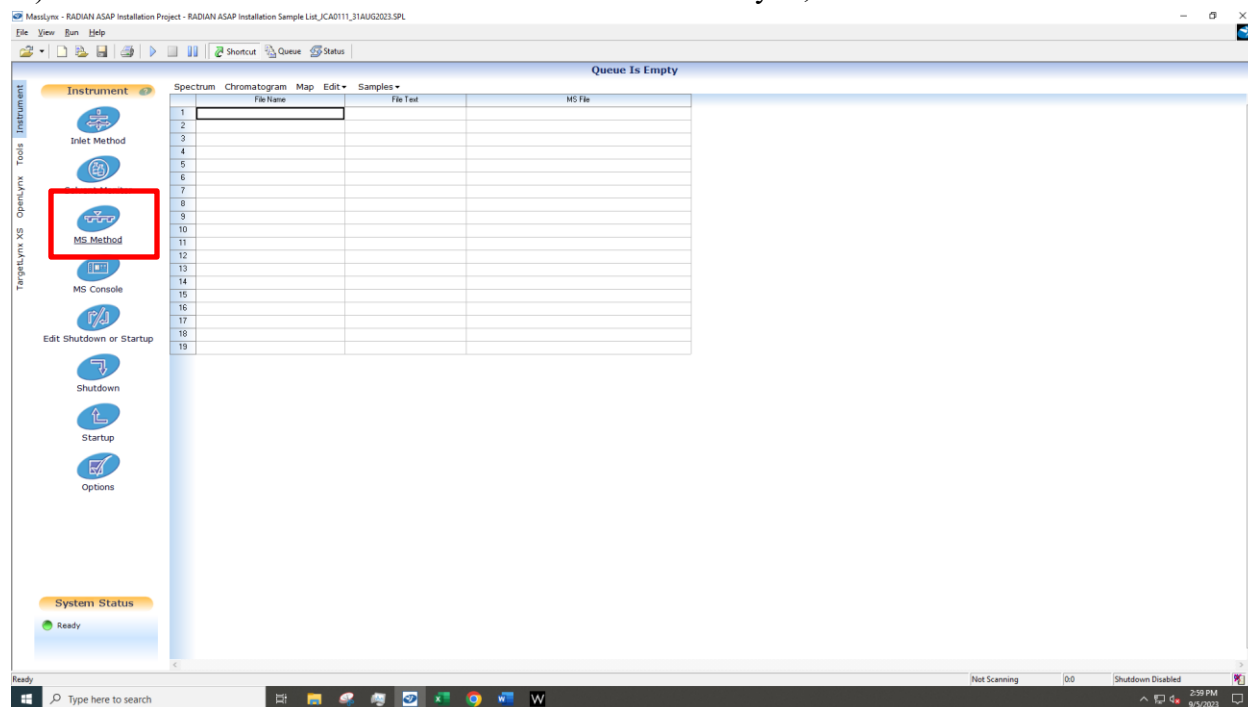


File name should be unique (e.g., sampleType\_date\_number), or data of previous samples be overwritten.

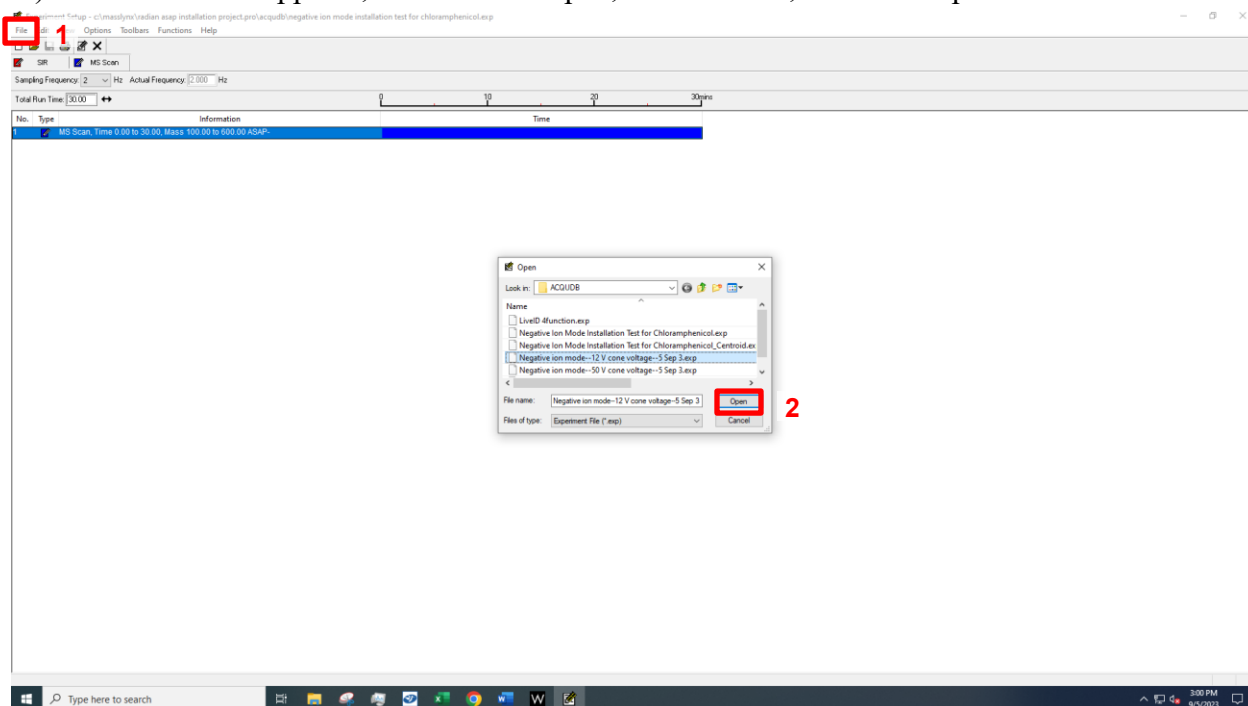
The MS file should point to the appropriate method of analysis. To set up a method, see ***Setting up a method***.

## Setting up a method

1.) In the main window of MassLynx, click on MS Method

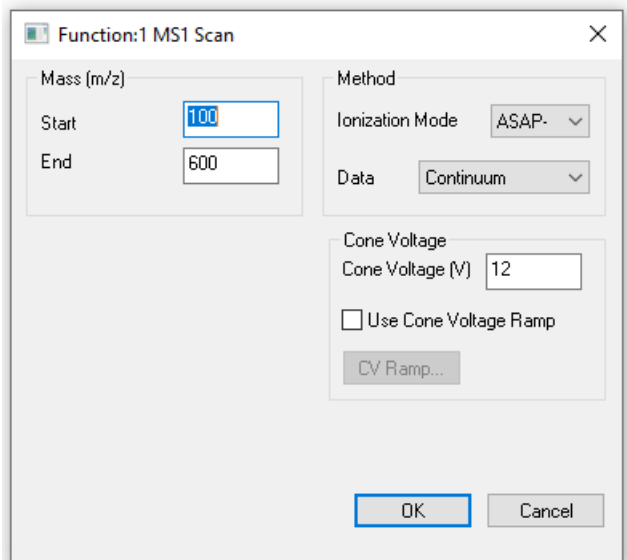


2.) In window that appears, click File -> Open, choose a file, and click Open.



The best file to open will be for a method similar to the one being created. If no existing method is similar, open any file.

3.) Review the settings of the opened file. To see all settings, double click on the row  
A new window will be opened. Typical settings (for negative ionization mode) are shown below.

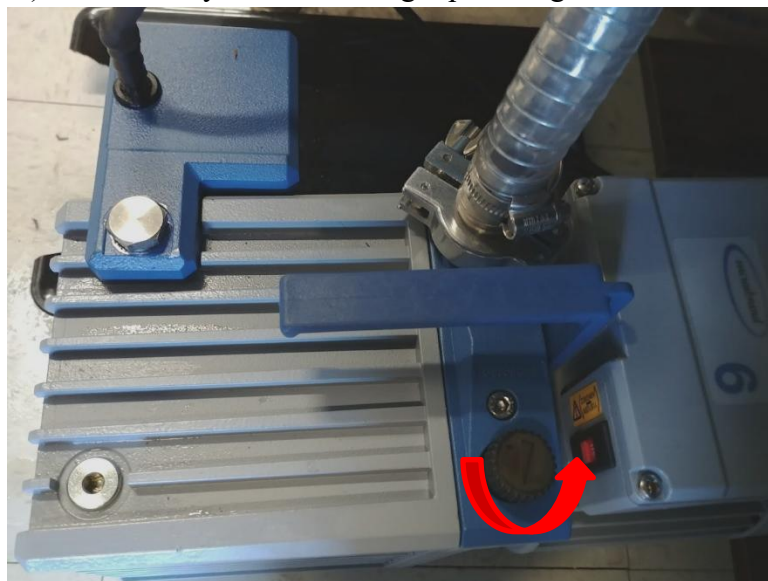


## Maintenance of Radian mass spectrometer

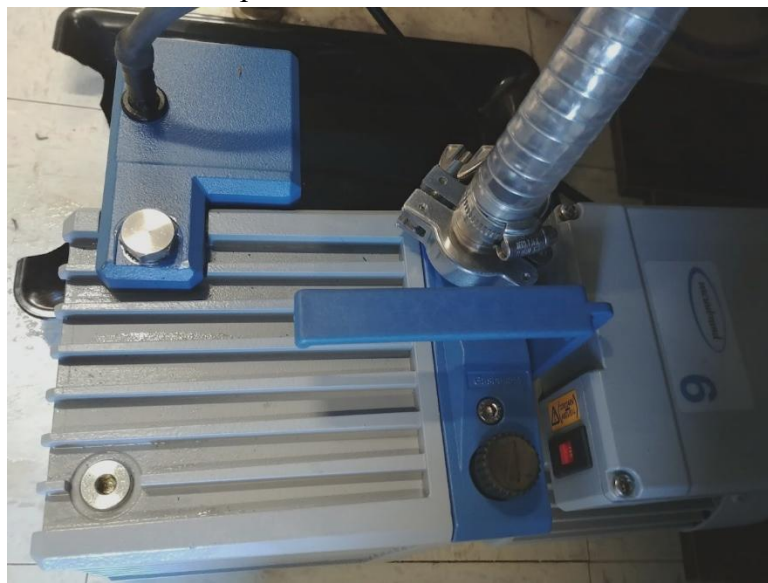
### Gas ballasting the backing pump

This maintenance needs to be performed approximately every week. Failure to do so will overload the turbo pump (indicated by abnormally high power usage of the pump in MS Console).

1.) While the system is running, open the gas ballast valve



The arrow should point towards the handle



2.) Wait 3 min.

3.) Close the valve.

### Checking oil of backing pump

This maintenance needs to be performed approximately every week.

Check oil using gauge on front. Verify that the level of oil is between max and min.



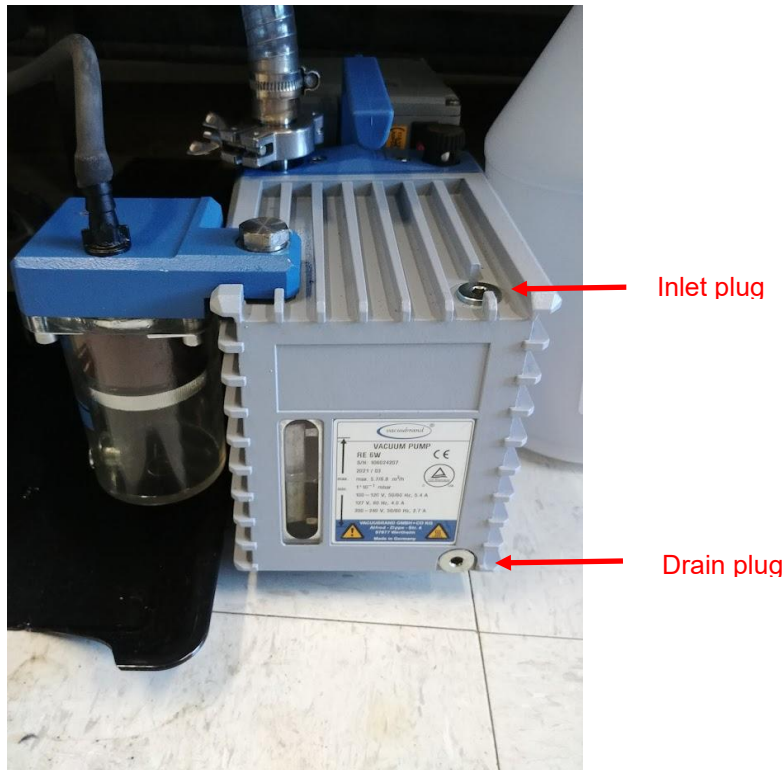


Also verify that that color of oil is clear. Bubbles are normal, but dark color is not.

### **Changing oil of backing pump**

This maintenance needs to be performed once or twice a year.

- 1.) Turn off the system, including pump.
- 2.) Wait 5 min for instrument to vent.
- 3.) Place pump on tray for catching oil.
- 4.) Remove the drain plug (requires size 6 allen wrench).

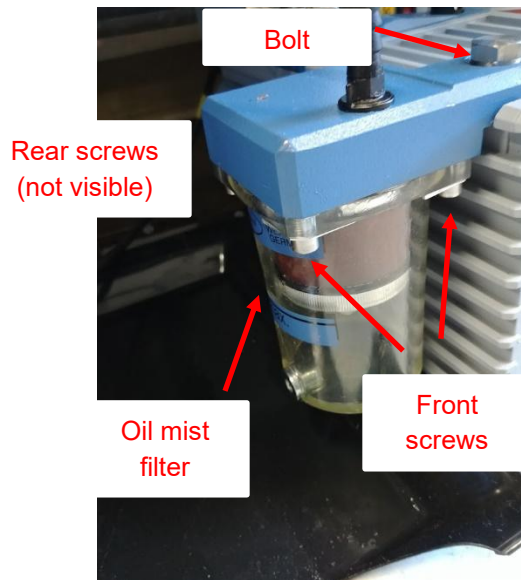


- 5.) Tilt the pump to drain of all oil.
- 6.) Reinstall the drain plug.
- 7.) Remove the inlet plug (requires size 6 allen wrench).
- 8.) Add oil. The original vacuum pump oil (Vacuubrand 700009679) can be used, but most oils from a hardware store will also work. Do not mix different types/weights of oil.
- 8.) Turn system back on. Though the manual advises it, it is not necessary to flush the pump with 50 mL of oil.

### **Replacing oil mist filter**

This maintenance needs to be performed once a year. Despite what is reported in the manual, this can be done by the user.

- 1.) Turn off the system, including pump.
- 2.) Remove the bolt attaching the mist filter assembly to the pump.



- 2.) Turn assembly upside down.
- 3.) Remove the front and rear screws attaching the housing to the assembly.
- 4.) Unscrew the oil mist filter (made of brown cork).
- 5.) Replace the filter with a new one (part available from Waters).

#### **Cleaning the instrument air filter**

This maintenance needs to be performed once a year.

- 1.) Open the panel on top of the instrument.
- 2.) Unscrew the Allen keys.
- 3.) Remove the metal frame.
- 4.) Remove the filter and wash.
- 5.) Reinstall the filter.

#### **Cleaning source enclosure and other parts**

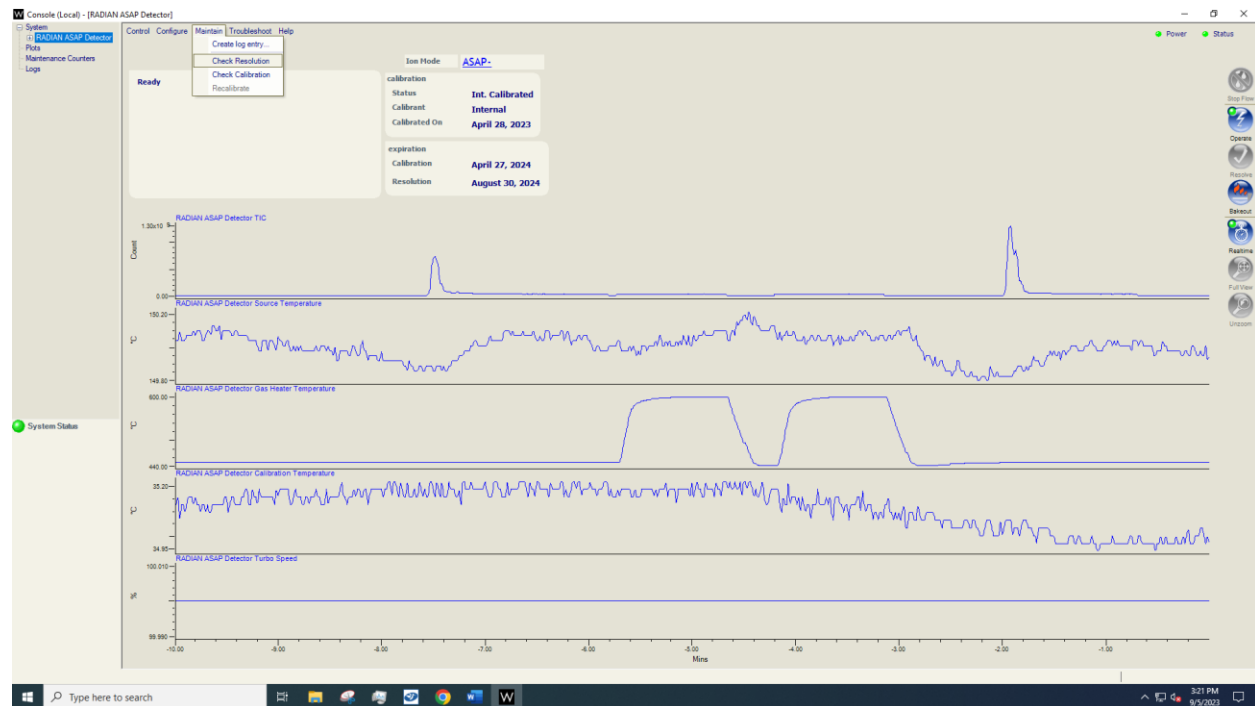
This maintenance should be done as needed (if instrument sensitivity drops). Follow the manual for disassembly and cleaning.

## Other details of using the Radian mass spectrometer

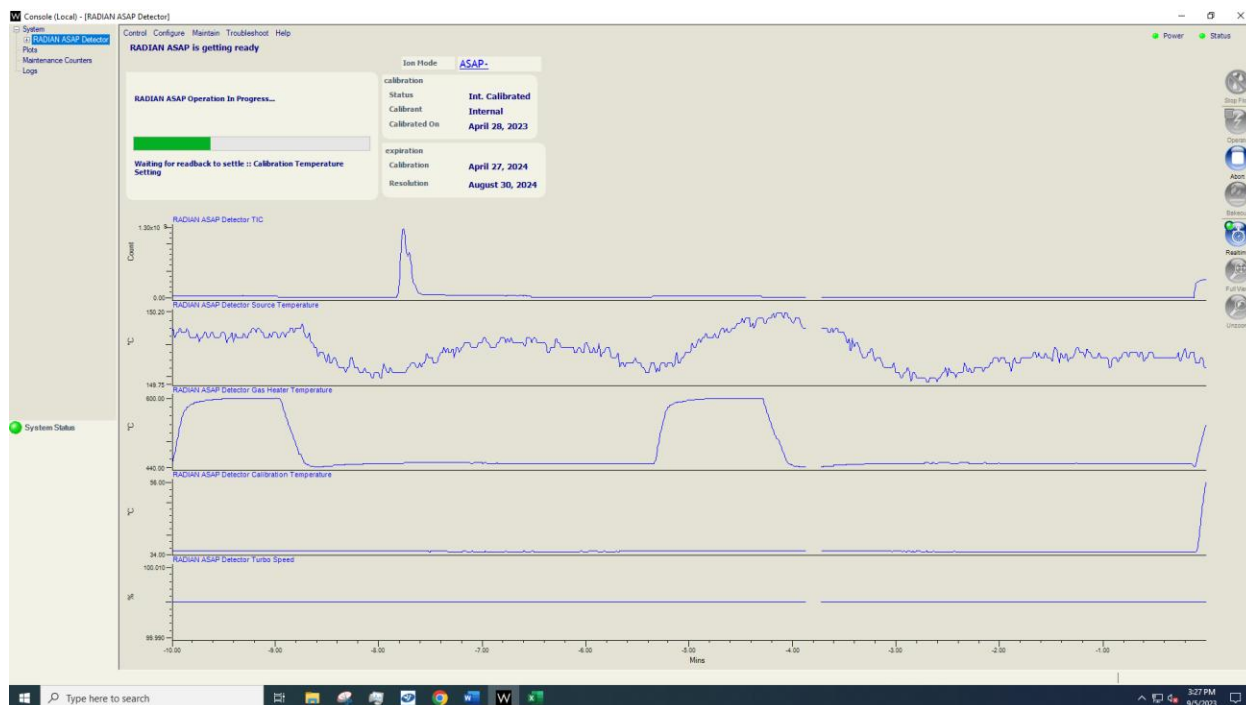
### Checking resolution and calibration

Resolution and calibration can be checked if desired.

To check resolution, Go to Maintain -> Check resolution



Allow to complete.



System passes if no warning is displayed at the end.

To check calibration, do the same, but go to Maintain -> Check calibration.

### Resetting the embedded PC (EPC)

Occasionally the embedded (EPC) needs to be reset, such as when MassLynx reports the instrument is not found.

To reset, using a long object (4" gassing probe needle works) and insert in hole on front of instrument.

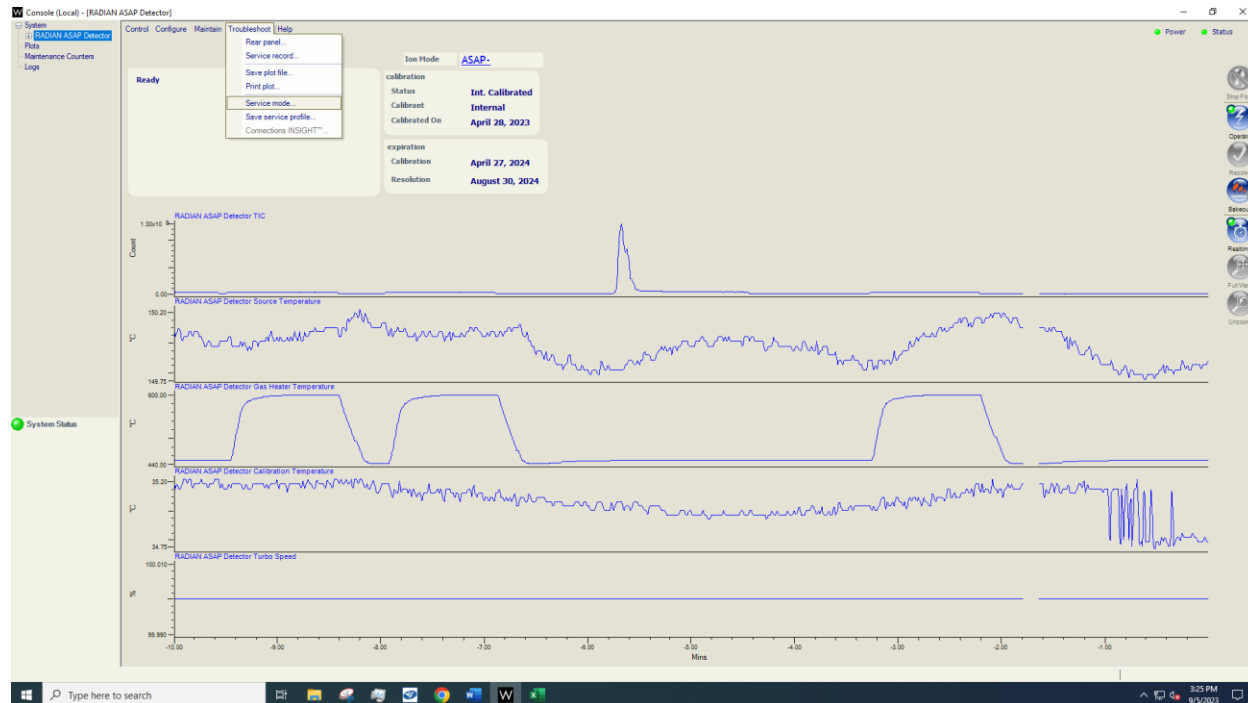


The clicking sound indicates it is reset.

## Service mode

Advanced functions can be accessed under Service Mode. Enter this mode only if understanding the risks; irreparable damage can be done to the system.

To enter service mode, go to MS console → Troubleshoot



The password is 1147.

## Tuning

The instrument can be tuned under service mode, though this should be done only if understanding the risks. Settings for tuning are shown below, and they cannot be restored automatically if changed.

RADIAN ASAP Detector

File

Ion Mode

Gas

Ramps

Setup

Acquire

Help

ASAP+

Lens Controls

Engineers

Diagnostics

Statuses

PLD Version

Version

G

Power Supply

+1.2 Volts

1.19

+2.5 Volts

2.49

+3.3 Volts

3.30

+5.0 Volts

4.99

-12.0 Volts

-12.2

+12.0 Volts

12.0

Test Point

66.0

Positive HT

429.0

Negative HT

-429.7

ETD

0.00

DC +ve

95.2

DC -ve

-93.8

Corona Voltage (kV)

3.05

Corona Current (uA)

3.05

Lens Readbacks

Sample Cone Voltage

23.6

Source Offset

106.7

StepWave Head Gradient

62.3

StepWave Offset

41.9

StepWave Body Gradient

76.3

Ion Guide 1 Offset

26.9

Ion Guide 2 Gradient

4.1

Ion Guide 2 Offset

1.0

Prefilter

-9.9

StepWave Readbacks

StepWave RF Current

52.66

StepWave RF Amplitude

152.23

Sensor Readbacks

Nitrogen Sensor (bar)

6.72

Source

PwM (%)

23

Temp (°C)

143.9

Uncorrected Temp (°C)

86.0

Heater Current (A)

0.00

Probe

PwM (%)

16

Temp (°C)

450.0

Uncorrected Temp (°C)

422.8

Heater Current (A)

0.03

Calibration

PwM (%)

0

Temp (°C)

33.3

Uncorrected Temp (°C)

2.7

Heater Current (A)

0.04

Vacuum

Turbo Speed (%)

100.0

Set Rotation Speed (rpm)

90000

Rotation Speed (Hz)

1500

Vacuum Pump Power

41

Pleffier Error Code

0000

Pressure Sensor (mbar/V)

1.5

Detector

PMT Voltage (V)

533

Dynode Voltage (V)

-8070

Phosphor Voltage (V)

9000

Instrument Temperatures

Ambient (°C)

48

Pump Bottom (°C)

43

Bearing (°C)

46

Motor (°C)

60

Quad (°C)

49.5

Mass

Span

Gain

1

1147

3

5

2

479

5

4

3

761

5

25

4

1159

5

43

1147.0

479.0

2.65e+4

1.13e5

**RADIAN ASAP Detector**

File Ion Mode Gas Ramps Setup Acquire Help

ASAP+ Lens Controls Engineers Diagnostics Statuses

**Voltages**  
 Mode ☒ Current ☐ Voltage  
 Corona (μA)     
 Cone (V)

**Temperatures**  
 Source Temp (°C)    
 Gas Heater Temp (°C)

**Detector**  
 Gain

**Internal Calibrant**  
 Calibrant Heater ☒ Disabled ☐ Enabled  
 Calibrant Temp (°C)   
 High Voltage Supply   
 Calibrant Valve

	Mass	Span	Gain
<input checked="" type="checkbox"/> 1	1147	3	5
<input checked="" type="checkbox"/> 2	479	5	4
<input checked="" type="checkbox"/> 3	761	5	25
<input checked="" type="checkbox"/> 4	1159	5	43

	1147.0	479.0
	1.72e4	1.22e5

**RADIAN ASAP Detector**

File Ion Mode Gas Ramps Setup Acquire Help

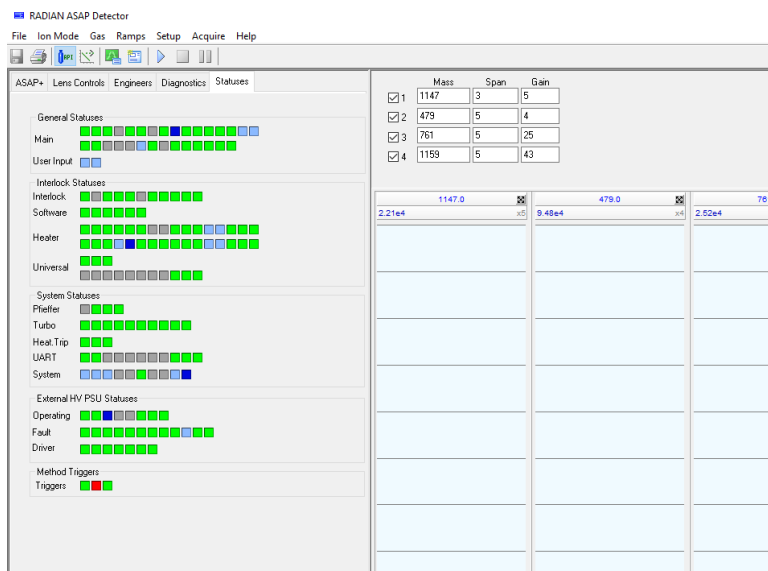
ASAP+ Lens Controls Engineers Diagnostics Statuses

**Controls**  
 Source Offset    
 StepWave Body Gradient    
 StepWave Offset    
 StepWave Head Gradient    
 Ion Guide 1 Offset    
 Ion Guide 2 Gradient    
 Ion Guide 2 Offset    
 Prefilter

	Mass	Span	Gain
<input checked="" type="checkbox"/> 1	1147	3	5
<input checked="" type="checkbox"/> 2	479	5	4
<input checked="" type="checkbox"/> 3	761	5	25
<input checked="" type="checkbox"/> 4	1159	5	43

	1147.0	479.0	7
	1.91e4	1.02e5	3.50e4





## **Selection of compressed gas and regulators for Radian mass spectrometer**

Compressed nitrogen must be >95% purity, dry, and oil free. The gas used routinely used is Airgas NI UHP300 (Ultra High Purity 5.0 Nitrogen).

The regulator must be dual stage and be capable of delivering at least 100 psi. The regulator routinely used is Airgas Y12N245E580-AG.

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