PROTOCOLS FOR CULTURING *PROCHLOROCOCCUS*

Described here are the protocols we use to grow and cryopreserve *Prochlorococcus*. These protocols work with all strains of *Prochlorococcus* tested to date. We routinely grow *Prochlorococcus* in natural seawater-based (undefined) and artificial seawater-based (defined) media as well as an Instant Ocean-based medium. The natural seawater medium, PRO99, has been described in Moore et al. (2002)[1]. The artificial medium for *Prochlorococcus* is a modification of the PCR-Tu_2 recipe from Rippka et al. (2000)[2] and AMP-1 from Moore et al. (2007)[3], and is called AMP-J (Morris et al, 2011)[3]. We modified the concentrations of metals, EDTA, and bicarbonate to improve the growth of some members of our *Prochlorococcus* stock collection. Trace metals can be toxic to *Prochlorococcus*, so we designed our artificial medium to reduce the chances of metal contamination from impurities in the major salts: we only use ultrapure chemicals, and we reduced the overall metals added to the medium compared to the PRO99 medium. HEPES was replaced with TAPS because HEPES can generate hydrogen peroxide in the light (Morris and Zinser 2013). Measures we take to remove metals from our cultureware (for use with both media types) are described below. Our experience with growing *Prochlorococcus* in large volumes (1L or more) has lead us to modify the culturing procedure, and these modifications are also described. Finally, we describe our methods to cryopreserve *Prochlorococcus* for long term storage and shipment.

CONTENTS:

[1] Moore et al. (2002) Limnol. Oceanogr. 47:989-996.
[2] Rippka et al. (2000) Int. J. Syst. Evol. Microbio. 50:1833-47
[3] Moore et al. (2007) Limnol. Oceangr. Methods 5:353-362
[4] Morris et al. (2011) PLoS ONE 6: e16805
[5] Morris and Zinser (2013) J. Phycol. 49: 1223-1228

I. NATURAL SEAWATER-BASED PRO99 MEDIUM

NOTE: We observe maximal growth with open ocean seawater (i.e. Sargasso seawater), though we can routinely grow *Prochlorococcus* in some coastal seawater as well.

Table 1. Nutrient additions to filtered, autoclaved seawater

Nutrient	Manufacturer/Grade	Primary Stock (M)	Dilution Factor	Final Conc. (μM)
$NaH_2PO_4 \cdot H_2O$	(1) / ACS	0.025	1:500	50
NH_4Cl	(1) / ACS	0.50	1:625	800
$Na_2EDTA \cdot 2\ H_2O$	(2) / 99%	0.012	$1:10^4$	1.17
$FeCl_3 \cdot 6\ H_2O$	(1) / Analytic	0.012	$1:10^4$	1.18
$ZnSO_4 \cdot 7\ H_2O$	(3) / >99.5%	0.080	$1:10^7$	0.008
$CoCl_2 \cdot 6\ H_2O$	(1) / Analytic	0.050	$1:10^7$	0.005
$MnCl_2 \cdot 4\ H_2O$	(1) / Analytic	0.900	$1:10^7$	0.090
$Na_2MoO_4 \cdot 2\ H_2O$	(2) / ACS	0.030	$1:10^7$	0.003
Na_2SeO_3	(2) / ~98%	0.100	$1:10^7$	0.010
$NiCl_2 \cdot 6\ H_2O$	(1) / Analytic	0.100	$1:10^7$	0.010

Manufacturer Index: (1) Mallinckrodt, (2) Sigma, (3) Fluka

PREPARATION:

1. Prepare a glass filter funnel and flask by cleaning with acid and Milli-Q water, as described on p. 7 (clean the system after every 10 filtrations). Filter raw seawater through a 47mm 0.2μm polycarbonate filter (make sure there are no bubbles/creases).

   Autoclave seawater in a Teflon bottle (60 minutes for 2L) and allow to cool overnight

2. Prepare primary stocks of NH_4Cl, $NaH_2PO_4 \cdot H_2O$, and trace metals as follows:

   A. **0.5M NH_4Cl**
   - Weigh out 1.34g NH_4Cl using dust-free weigh paper
   - Transfer into 50 mL Teflon flask filled with about 40 mL Milli-Q water
   - Dissolve NH_4Cl by stirring
   - Adjust pH to 7.5
   - Transfer to volumetric flask and adjust volume to 50 mL with Milli-Q water
   - Filter sterilize with **pre-washed** Millipore Steriflip 0.22 μM Durapore PVDF membrane system (cat: SE179M6); pre-wash with 50 mL milli-Q water and in laminar flow hood, remove from collection tube; reset tube into filtration system
   - In a laminar flow hood, apply sterile tube cap.
   - Store sterile stock at 4°C

B. **0.025M $NaH_2PO_4 \cdot H_2O$**

- Weigh out 0.15 g $NaH_2PO_4 \cdot H_2O$ using dust-free weigh paper
- Transfer into 50 mL Falcon tube filled with about 40 mL Milli-Q water
- Dissolve $NaH_2PO_4 \cdot H_2O$ by stirring, inverting tube
- Adjust volume to 50 mL with Milli-Q water
- Filter sterilize with **pre-washed** Millipore Steriflip 0.22 µM Durapore PVDF membrane system (cat: SE179M6); pre-wash with 50 mL milli-Q water and in laminar flow hood, remove from collection tube; reset tube into filtration system
- In a laminar flow hood, apply sterile tube cap.
- Store sterile stock at 4°C

C. **Trace Metal Stocks**

i. **Primary trace metal stocks**

- Using dust-free weigh paper, weigh out:

2.30g	$ZnSO_4 \cdot 7H_2O$
1.19g	$CoCl_2 \cdot 6H_2O$
17.81g	$MnCl_2 \cdot 4H_2O$
0.726g	$Na_2MoO_4 \cdot 2H_2O$
1.73g	Na_2SeO_3
2.38g	$NiCl_2 \cdot 6H_2O$

- Transfer each trace metal into separate 100mL volumetric flasks containing about 60 mL Milli-Q water
- Dissolve contents by inverting flask several times
- Adjust volume to 100mL mark with Milli-Q water
- Store each stock in a cleaned Teflon bottle at 4°C

ii. **Trace metal working stock**

- Weigh out 0.435g $Na_2EDTA \cdot 2\ H_2O$ using dust-free weigh paper
- Transfer into 100mL volumetric flask filled with 60mL Milli-Q water
- Dissolve Na_2EDTA by inverting flask several times
- Weigh out 0.32g $FeCl_3 \cdot 6\ H_2O$ using dust-free weigh paper
- Dissolve $FeCl_3$ into same volumetric flask by inverting several times
- Individually add and dissolve 100µl of the Primary trace metal stocks
- Adjust volume to 100mL mark with Milli-Q water
- Using a polycarbonate syringe, filter through 0.2µm syringe filter into sterile container in a laminar flow hood
- Store sterile stock at 4°C

3. To make up the media, add following volumes of sterile nutrients and trace metal stock to one liter of the autoclaved seawater. It is important to *dissolve each nutrient sequentially*.

Filtered Seawater		1000.0mL
0.5M	NH_4Cl	1.6mL
0.025M	$NaH_2PO_4 \cdot H_2O$	2.0mL
Trace metal working stock		100µL

4. Store at room temperature for up to one month.

II. ARTIFICIAL SEAWATER BASED AMP-J MEDIUM

Table 2. Sea Salt and nutrient additions

Nutrient	Manufacturer/Grade	Primary Stock (M)	Dilution Factor	Final Conc.
NaCl	(2) / SigmaUltra	n/a	n/a	481 mM
$CaCl_2 \cdot 2\ H_2O$	(2) / SigmaUltra	n/a	n/a	10 mM
KCl	(2) / SigmaUltra	n/a	n/a	9 mM
$Mg\ SO_4 \cdot 7\ H_2O$	(2) / SigmaUltra	n/a	n/a	28 mM
$MgCl_2 \cdot 6\ H_2O$	(2) / SigmaUltra	n/a	n/a	27 mM
NaH_2PO_4	(2) / SigmaUltra	0.025	1:500	50 μM
$(NH_4)_2SO_4$	(2) / SigmaUltra	0.4	1:1000	400 μM
$NaHCO_3$	(2) / SigmaUltra	0.6	1:100	6 mM
TAPS	(2) / SigmaUltra	0.5	1:500	1 mM
$Na_2EDTA \cdot 2H_2O$	(2) / 99%	0.012	$1:10^5$	0.1170 μM
$FeCl_3 \cdot 6\ H_2O$	(1) / Analytic	0.012	$1:10^5$	0.1180 μM
$ZnSO_4 \cdot 7\ H_2O$	(3) / >99.5%	0.080	$1:10^8$	0.0008 μM
$CoCl_2 \cdot 6\ H_2O$	(1) / Analytic	0.050	$1:10^8$	0.0005 μM
$MnCl_2 \cdot 4\ H_2O$	(1) / Analytic	0.900	$1:10^8$	0.0090 μM
$Na_2MoO_4 \cdot 2H_2O$	(2) / ACS	0.030	$1:10^8$	0.0003 μM
Na_2SeO_3	(2) / ~98%	0.100	$1:10^8$	0.0010 μM
$NiCl_2 \cdot 6\ H_2O$	(1) / Analytic	0.100	$1:10^8$	0.0010 μM

Manufacturer Index: (1) Mallinckrodt, (2) Sigma, (3) Fluka

NOTE: To avoid contamination, dry nutrients and metals should be weighed without using spatulas.

1. Prepare nutrient stocks as following using Sigma Ultra grade chemicals:

A. **25mM NaH_2PO_4 (pH 7.5)**
- Weigh out 0.15 g $NaH_2PO_4 \cdot H_2O$ using dust-free weigh paper
- Transfer into 50 mL Falcon tube filled with about 40 mL Milli-Q water
- Dissolve $NaH_2PO_4 \cdot H_2O$ by stirring, inverting tube
- Adjust volume to 50 mL with Milli-Q water
- Filter sterilize with **pre-washed** Millipore Steriflip 0.22 μM Durapore PVDF membrane system (cat: SE179M6); pre-wash with 50 mL milli-Q water and in laminar flow hood, remove from collection tube; reset tube into filtration system
- In a laminar flow hood, apply sterile tube cap.
- Store sterile stock at 4°C

B. **0.4M $(NH_4)_2SO_4$**

- Weigh out 2.64 g $(NH_4)_2SO_4$ on dust-free weigh paper
- Transfer into 50 mL Falcon tube filled with about 40 mL Milli-Q water
- Dissolve $(NH_4)_2SO_4$ lume to 50 mL with Milli-Q water
- Filter sterilize with **pre-washed** Millipore Steriflip 0.22 µM Durapore PVDF membrane system (cat: SE179M6); pre-wash with 50 mL milli-Q water and in laminar flow hood, remove from collection tube; reset tube into filtration system
- In a laminar flow hood, apply sterile tube cap.
- Store sterile stock at 4°C

C. **0.5M TAPS (pH 8.0)**

- Weigh out 60.75 g TAPS on dust-free weigh paper
- Transfer into 1L glass beaker with stir bar and 300mL Milli-Q water
- Dissolve TAPS by stirring
- Fill remaining volume to 500mL mark with Milli-Q water
- Check pH by pipetting out small amount onto pH paper for rough estimate
- Turn stirrer on and **SLOWLY** add NaOH, check pH by pipetting out small amount onto pH paper for rough estimate periodically
- When pH is around 8.0 use a calibrated pH meter probe that has been Milli-Q water rinsed and soaked several times (add NaOH as needed to obtain pH 7.5)
- Transfer to volumetric flask or graduated cylinder and adjust volume to 500mL
- Filter, using a 500mL sterile filter system (Corning code# 430773) with .22µm nylon filter and polystyrene bottle (pre-wash the filter 2-3 times with 100mL Milli-Q water to remove residual carbon) attached to a vacuum pump (use 1 inch of Hg)
- Store sterile stock at 4°C

D. **Working trace metal stock**

Use the working trace metal stock from the PRO99 medium recipe, **but for the artificial seawater medium ten times LESS of the stock will be added to the artificial medium compared to PRO99.**

2. Prepare "Turks Island Salt Mix"[3] by adding the following:

dissolve each ingredient sequentially in 2/3 final volume of Milli-Q water

Milli-Q water	1.0L
NaCl	28.10g
Mg $SO_4 \cdot 7\ H_2O$	6.90g
$MgCl_2 \cdot 6\ H_2O$	5.49g
KCl	0.67g
$CaCl_2 \cdot 2\ H_2O$	1.47g

- Fill to appropriate volume with Milli-Q water
- Filter-sterilize with a 0.2 µm-cutoff filter (pre-rinsed to remove excess carbon)*

[3] The Merck Index (1989) Merck&Co, Inc. (Rahway, N.J.)

3. To Turks Island Salt Mix add the following sterile nutrients stocks:

	1L
$NaH_2PO_4 \cdot H_2O$	2mL
$(NH_4)_2SO_4$	1mL
TAPS	2mL
Working trace metal stock	10µl

4. Store this ASW basal medium at room temperature for up to one month.

5. Prepare 0.6M $NaHCO_3$ stock fresh on day of medium inoculation (do not store)
- Weigh out 2.52g $NaHCO_3$ on dust-free weigh paper
- Transfer into 50 mL Falcon tube filled with about 40 mL Milli-Q water
- Dissolve $NaHCO_3$ by stirring, inverting tube
- Adjust volume to 50 mL with Milli-Q water
- Filter sterilize with **pre-washed** Millipore Steriflip 0.22 µM Durapore PVDF membrane system (cat: SE179M6); pre-wash with 50 mL milli-Q water and in laminar flow hood, remove from collection tube; reset tube into filtration system
- In a laminar flow hood, apply sterile tube cap.
- Store sterile stock at 4°C

6. $NaHCO_3$ addition to basal medium:
10 mL of 0.6M $NaHCO_3$ per 1L basal medium

*** AMP-J medium is filter-sterilized; to make "AMP-A", autoclave the Turks Island Salt mix; note that this will increase the concentration of hydrogen peroxide in the medium. Note, AMP-A is not a published recipe (yet)**

III. INSTANT OCEAN MEDIUM

1. Add to 900mL Milli-Q water:

35g Instant Ocean Sea Salts*

2. Bring to 1L with Milli-Q water and stir until dissolution achieved. Autoclave. Allow to cool.

3. Add sequentially to cooled medium:

2.0 mL 0.5M TAPS, pH 8.0
1.6 mL 0.5M NH_4Cl
2.0 mL 0.025M $NaH_2PO_4\ H_2O$
100 µL Trace Metal Working solution

*Use only instant ocean sea salt mix (reef mix has Cu toxic to Pro in it)

Notes- All nutrients prepared as in "procedures for culturing Prochlorococcus" manual. Upon autoclaving white precipitate of excess bicarbonate will come out of solution giving medium a milky white appearance. When aliquotting out IO for culturing purposes agitate solution often to keep precipitate in solution. Unsterilized Instant Ocean has shown evidence of lytic activity (Prochlorococcus-infecting phage) so it is very important to sterilize the solution.

IV. AMP-J AGAR PLATES

Media preparation (1 L AMP ~50 plates)

A. Noble Agar (0.42% final)
   a. Acid washed polycarbonate bottle w/ minimum volume of 2x MQ-H_2O used (i.e. 500 ml bottle for 250ml agar)
      i. 4.2g agarose
      ii. 250 ml MQ-H_20
      iii. microwave tindalize:
         1. bring to boil in microwave – **make sure cap is loose**!
         2. agar will violently boil over, watch carefully – stop and restart microwave to get rolling boil.
         3. Carefully remove from microwave, place in hood until agar solidifies or O/N; repeat 3x.

B. Turks Island Salt Mix Basal Medium
   a. TISM – 750 ml

i.	MQ-H_20	750 ml
ii.	NaCl	21.075 g
iii.	$MgSO_4 \bullet 7H_20$	5.175 g
iv.	$MgCl_2 \bullet 6H_20$	4.118 g
v.	KCl	0.503 g
vi.	$CaCl_2 \bullet 2H_20$	1.103 g

   b. Autoclave in 1 L acid washed PC bottle
   c. Prior to last agar tindalization, prewarm Turks Island Salt Mix; place in 37C incubator or remove from autoclave and allow cool to ~37C

C. Pour AMP plates
   a. Prewarm TISM as noted in step "B. c."
   b. Add nutrients to make 1L of 1x AMP (in order shown)

i.	Stock NaH_2PO_4	2 ml
ii.	Stock $(NH_4)_2SO_4$	1 ml (or 1.6 ml NH_4Cl stock)
iii.	1 M TAPS pH 8.0	1 ml
iv.	TMWS	0.1 ml
v.	Fresh 0.6M $NaCO_3^-$	10 ml

   c. gently pour warm agar into 750 ml AMP, swirl gently, pour back and forth from 500 ml bottle to ensure homogeneity of agar.
   d. Working quickly, pour or use serological pipette to transfer ~20 ml AMP agar to petri dishes. **Note:** Use of serological pipette will make it easy to aspirate any bubbles or imperfections generated in distribution of agar and ensure consistency of agar plates.

D. Common additions or variations
   a. 0.21% agar – replace 4.2 g Noble agar for 2.1 g. Makes very soft plates.
   b. Sulfite 20mM final concentration:
      i. 1M Sulfite made fresh – 1.26 g in 10 ml. filter sterilize
      ii. 2 ml addition to AMP after bicarbonate addition

V. CULTUREWARE FOR GROWING *PROCHLOROCOCCUS*

Prochlorococcus is highly sensitive to trace metal concentrations, so special care must be given in choosing and preparing media containers and culture vessels. We have had success with the disposable and reusable cultureware, though reusable cultureware requires cleaning with acid and autoclaving (see protocol below).

A. TYPES OF CONTAINERS

Media containers

We use acid washed Nalgene polycarbonate or Teflon bottles.

Culture vessels

We use a range of vessel types. Acid washed polycarbonate or glass bottles or tubes work well, as do disposable polystyrene culture tubes: Falcon 14ml round bottom tube (Becton Dickinson #352051).

B. LIQUID TRANSFER AND MEASUREMENT DEVICES

For small volumes we use sterile disposable plastic pipettes and plastic tips. No acid washing is required. For larger volumes we use acid-washed graduated cylinders (glass or plastic) and volumetric flasks (glass).

C. PROTOCOL FOR ACID WASHING CULTUREWARE

NOTE: under no circumstances should a brush be used to clean the glassware, as this is a significant source of metal contamination. Be sure that there are no rusty objects anywhere in the vicinity of your sink or bench.

- Soak cultureware overnight in a ~2% Micro-90 (Intl. Products Corp.) detergent bath
- Rinse 6X with tap water
- Rinse 6X with deionized water
- Soak overnight in 1N HCl (trace metal free; we use Mallinckrodt #2062)
- Rinse 6X with Milli-Q water (18MΩ water)
- Fill with Milli-Q water and let sit overnight
- Autoclave cultureware filled with Milli-Q water
- Sterilely remove water prior to use

VI. GROWING *PROCHLOROCOCCUS* IN LARGE VOLUME (1-10L)

A. METHOD 1: BUFFERING WITH TAPS AND BICARBONATE:

- Acid wash a 1L or 10L Nalgene polycarbonate container. Add appropriate volume of media
- Supplement medium with TAPS pH 8.0 and sodium bicarbonate
  for 1L: 5mM TAPS, 6mM bicarbonate final concentrations
  for 10L: 10mM TAPS, 12mM bicarbonate
- Incubate with cap loosened

Note: 0.5 M TAPS and 0.6 M sodium bicarbonate stocks can be made, and should be filter-sterilized with a 0.2 μm filter. The TAPS can be stored at 4°C for several months, but the bicarbonate should be made fresh the day of inoculation.

B. METHOD 2: PUMPING STERILE AIR OR CO_2 INTO SYSTEM:

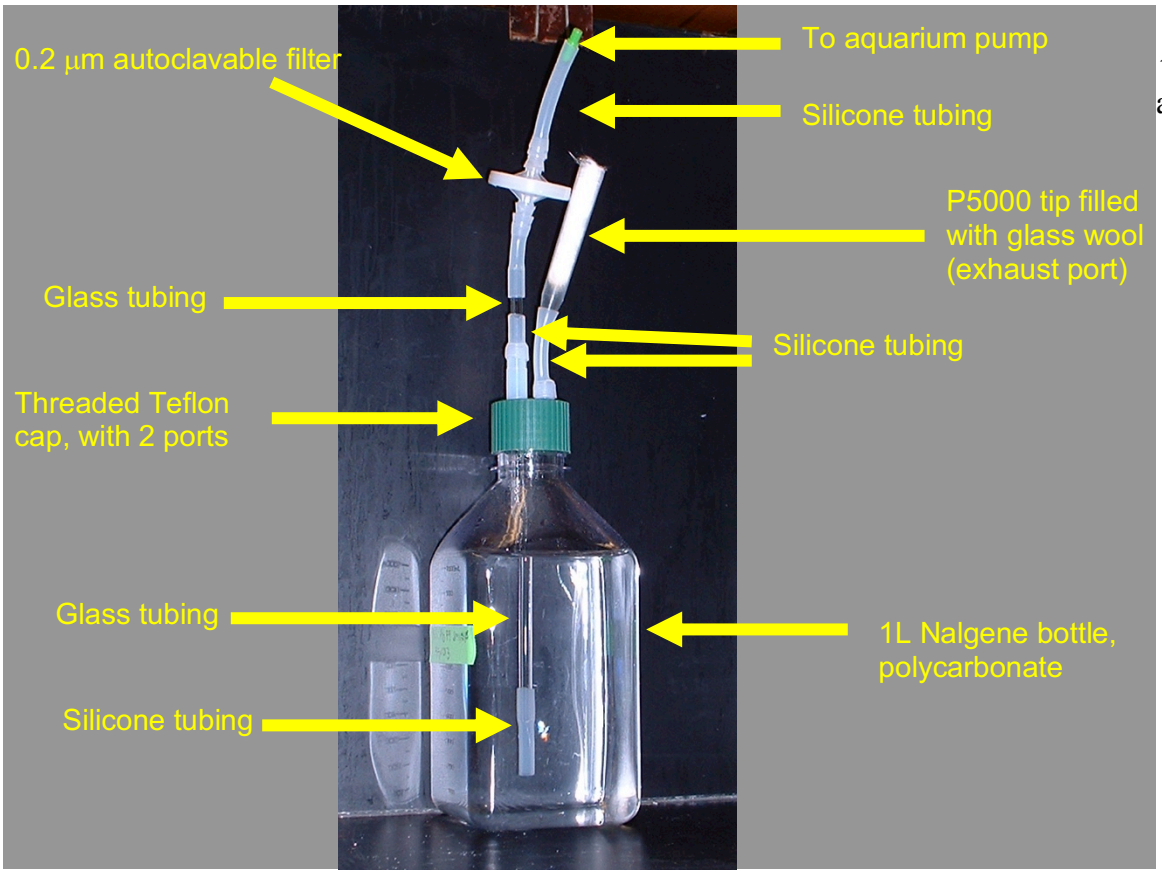
- Acid wash a 1L or 10L Nalgene polycarbonate container. Add appropriate volume of media.
- Fix top with filtered air system (see figure, next page)
- Incubate with cap tightened, low rate of airflow (1-4 bubbles/second). A higher airflow rate will cause higher evaporation and the culture may crash as a result

Note: Air source can be an aquarium pump, or compressed air, or CO_2 tank.

System components (for 1L flask):

Part	Vendor	Part number
Nalgene 50 silicone tubing (.187 ID, .312 OD)	VWR	8060-0040
6mm Pyrex glass tubing	Carolina Biol. Suppl.	WW-71-1146
Autoclavable 0.2μm filter Acro 37 TF	Pall Gelman	4464
Threaded Teflon cap for 1L flasks (1/4 inch tube diameter)	Savillex	0738-4-2

Bubbling system for *Prochlorococcus*:



tubing, air

VII. PURITY BROTH MEDIA

NOTE: both 1/10X ProAC (rich) and PLAG (minimal) media should be used to routinely test the purity of the axenic *Prochlorococcus* cultures

A. 1/10X ProAC prep

Autoclave separately:

1. 750 ml SSW, filtered with 0.2 μm PC filter
2. 250 ml H_2O + 1.7g AC Difco broth

After autoclaving:

Combine, add nutrients:

1.6 ml 0.5M NH_4Cl
2.0 ml 0.025 $NaHPO_4$
100 μl trace metal stock
0.5 ml 2000X Va vitamins (see below)

B. PLAG prep

Autoclave separately:

1. 750 ml SSW, filtered with 0.2 μm PC filter
2. 250 ml H_2O

After autoclaving:

Combine, add nutrients:

1.6 ml 0.5M NH_4Cl
2.0 ml 0.025 $NaHPO_4$
100 μl trace metal stock
0.5 ml 2000X Va vitamins (see below)
2.5 ml Pyruvate (0.05% final)
2.5 ml Lactate (0.05% final)
2.5 ml Acetate (0.05% final)
2.5 ml Glycerol (0.05% final)

Pyruvate, Lactate, Acetate, Glycerol made up as 20% stocks and filter sterilized.

Va vitamin mix (2000X stock):

To prepare stock, dissolve each compound separately, then add together and bring to 1L. Filter sterilize, store in the dark at 4°C.

Vitamin	**Final Stock Conc. (2000X)**
Thiamin-HCl	0.2g/L
Biotin	0.001 g/L
Vitamin B_{12}	0.001 g/L
Folic acid	0.002 g/L
PABA (para-aminobenzoic acid)	0.01 g/L
Nicotinic acid	0.1 g/L
Inositol	1.0 g/L
Calcium pantothenate	0.2 g/L
Pyridoxine-HCl	0.1 g/L

Reference: Waterbury and Willey. Methods in Enzymology 167: 100-105.

C. Purity assay

Routinely add 1 ml of axenic *Pro* cultures to 5-8ml 1/10 ProAC and PLAG medium.

Incubate at room temperature in dark and monitor for growth over 1+ month
(note: *Pro* will grow in PLAG in the light, so it is important to keep these tubes in the dark)

VIII. CRYOPRESERVATION PROTOCOL

A. **REUSABLE FROZEN STOCK (CONCENTRATED CELLS):**

1. **Preparation of (50X) concentrated cells**

- Grow cultures in light level approx.16 μEin/m^2/sec, continuous light, and 20-22°C temperature [if large volume (500ml+) add 6 mM sodium bicarbonate and 5mM HEPES pH 7.5].
- At mid log to early stationary phase, transfer volume of cells (50mL) into sterile centrifuge tubes/bottles.
- Spin at 8000RPM for 15 minutes at 20-22°C.
- Carefully decant liquid into waste container.
- Resuspend pellet in 1ml fresh medium (same medium used for growth).
- Withdraw concentrated cells (1mL) and transfer into 1.2mL cryogenic vial (Nalgene #5000-0012).
- Add 75 μl DMSO (7.5% final concentration) to cryogenic vial and invert several times.
- Immediately place into liquid nitrogen or into –150°C freezer.
- Note: recovery from liquid nitrogen is faster, but storage in liquid runs the risk of cross-contamination from other vials.

2. **Recovery of cells in fresh medium**

- Reduce ambient light in work area as much as possible.
- Remove cryogenic vials one at a time from liquid nitrogen or freezer and transport vials under cover to laminar flow hood as quickly as possible.
- With a sterile toothpick, immediately scrape up a small amount of the frozen cells and place into 25 mL sterile medium.
- Note: autoclaved wooden toothpicks work fine, as do ethanol-sterilized plastic toothpicks.
- Once scrape is completed return cryogenic vial to liquid nitrogen/–150°C freezer.
- Incubate cultures at 20-22°C under low continuous light (~16 μEin/m^2/sec).
- Note: **Recovery under low light is absolutely critical**.
- Monitor growth with daily fluorescence measurements. Growth can be detected within one week after thawing, but may take as long as 3-4 weeks.

B. SINGLE USE FROZEN STOCK (UNCONCENTRATED CELLS): SUITABLE FOR SHIPMENTS ON DRY ICE

1. **Preparation of unconcentrated cells**
   - Grow cultures in light level approx. 16 μEin/m^2/sec, continuous light, and 20-22°C temperature (if large volume add 6 mM sodium bicarbonate and 5mM TAPS pH 8.0).
   - At mid log to early stationary phase, transfer 1 ml cells into a 1.2mL cryogenic vial (Nalgene #5000-0012).
   - Add 75 μl DMSO (7.5% final concentration) to cryogenic vial and invert several times.
   - Immediately place into liquid nitrogen or into –150°C freezer.
   - Note: Recovery from liquid nitrogen is faster, but storage in liquid runs the risk of cross-contamination from other vials.

2. **Recovery of cells in fresh medium**
   - Reduce ambient light in work area as much as possible.
   - Remove cryogenic vials from liquid nitrogen or freezer and transfer to 37°C bath.
   - Partially thaw the cells: once half of liquid has thawed and small ice chunk remains
   - Remove vials and transport to laminar flow hood.
   - Transfer 2×500 μl cells into 2 culture tubes containing 25 mL sterile medium.
   - Incubate cultures at 20-22°C under low continuous light (~16 μEin/m^2/sec).
   - Note: **Recovery under low light is absolutely critical**.
   - Monitor growth with daily fluorescence measurements. Growth can be detected within one week after thawing, but may take as long as 3-4 weeks.

Note: Both recovery methods will work for concentrated cells, and may work for unconcentrated cells. Significantly faster recovery of unconcentrated cells occurs when the entire contents of the vial are used for inoculation.

Note: Both PRO99 and AMP-J media are suitable for preparing cells for freezing and for recovery after thawing.