LABORATORY MANUAL

Semrau's Lab

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Table of Contents

1. Media Preparation

1a. Nitrate Mineral Salt (NMS) media preparation (Whittenbury et al.,1970)

- Add following ingredients in 700mL of water and dissolve before make-up to 1L.

Ingredient	Amount (per liter)
$MgSO_4 \cdot 7H_2O$	1.0 g
KNO_3	1.0 g
$CaCl_2 \cdot H_2O$	0.2 g
3.8% (w/v) solution Fe-EDTA	0.1 ml
0.1% (w/v) $NaMo \cdot 4H_2O$	0.5 ml
Trace element solution (recipe below)	1.0 ml

NOTE: The Fe-EDTA and $NaMO_4 \cdot 4(H_2O)_4$ solutions are typically made at 1X and stored at 4°C; Fe-EDTA solution not to be exposed to light to prevent photo-degradation.

- If you want to prepare NMS-agar plates, add 18g Agar for 1 L of NMS media (add magnetic stir bar, may help to stir the media while adding below components)
- Autoclave the medium and then add following ingredients.

Ingredient	Amount (per liter)
Phosphate stock solution (1X) (recipe below)	10mL
Vitamin Stock solution (10X) (recipe below)	1mL

- Copper can be added as required. $CuCl_2$ (10 mM) is usually prepared, filter sterilized and stored for use.
- NMS-liquid media (NMS-liquid) use 1μM-final Cu concentration. For 1 μM final Cu-concentration 10 μL of 10 mM $CuCl_2$ is mixed with 100 mL of NMS in culture flask (DF=10,000x).
- NMS-solid media (NMS-agar) contain 10 μM-final Cu concentration. For 10 μM final Cu-concentration 100 μL of 10 mM $CuCl_2$ is mixed with 100 mL of NMS in culture flask (DF=1,000x).

Stock Trace element solution (1x)

- Add below components to 700 mL water and dissolve. Then add additional water to 1 L.

Ingredient	**Amount (per liter)**
$FeSO_4 \cdot 7H_2O$	500 mg
$ZnSO_4 \cdot 7H_2O$	400 mg
$MnCl_2 \cdot 7H_2O$	20 mg
$CoCl_2 \cdot 6H_2O$	50 mg
$NiCl_2 \cdot 6H_2O$	10 mg
H_3BO_3 (boric acid)	15 mg
EDTA	250 mg

NOTE: This solution need not to be sterilized as it is added to the base components of NMS medium prior to autoclaving. Stock solutions of 1X or 10X can be prepared and stored at 4°C

Stock Phosphate solution (1x)

- Add above components to 700 mL water and dissolve. Then add additional water to 1 L. Autoclave and store at room temperature.

Ingredient	**Amount (per liter)**
KH_2PO_4	26 g
$Na_2HPO_4 \cdot 7(H_2O)$ (or Na_2HPO_4)	62 g (or 33g)

NOTE: pH should be 6.8

Stock Vitamin solution (10x)

- Add above components to 700 mls water and dissolve. Then add additional water to 1 liter and filter sterilize using a 0.2 µm filter.

Ingredient	**Amount (per liter)**
Biotin	20 mg
Folic acid	20 mg
Thiamine HCl	50 mg
Ca pantothenate	50 mg
Vitamin B12	1 mg
Riboflavin	50 mg
Nicotiamide	50 mg

NOTE: It is easier to make 10X stock solutions and then dilute to1X prior to use. Store at 4°C. It is important that the vitamin solution not to be exposed to light to prevent photo-degradation

1b. NMS-Antibiotic Plates/media preparation

- Some of the OB3b-mutants required specific antibiotics to retain the plasmids. Hence, it's necessary to maintain the cultures in appropriate plates or enriched in NMS-liquid supplied with antibiotics.
- Follow the NMS-liquid or agar media preparation protocol as detailed above (i.e., 1a). Add antibiotics when the media cool down to < 60°C.
- Prepare the antibiotic solutions, filter sterilize and store at appropriate temperature.
- Add to NMS-liquid or agar as appropriate (check below table for reference)

Ingredient (Storage requirements)	**Stock Concentration (mg/mL)**	**Amount (µL) of stock solution for 1L of NMS**	**Final concentration in NMS (µg/mL)**
Gentamicin **(15-30°C)**	50	50	2.5
Ampicillin **(-20°C)**	100	100	100
Kanamycin **(4°C)**	50	200	10
Spectinomycin **(-20°C)**	100	200	20

NOTE: The antibiotics stocks cannot be stored for more than one year.

2. Culture preparation, Harvest and maintenance

2a. Methanotroph culture stock maintenance (Freeze stock at -80°C)

- 15% Glycerol stock can be prepared for long-term storage. Glycerol is inhibitory to few methanotrophs. A protocol that is successful for most strains is to freeze in 1%, TT and 5% DMSO or only 10% DMSO (0.9 ml of a culture with OD600 0.5-1 plus 100 µl DMSO, stored at -80C).
  (For more details please read this article "Survival or Revival: Long-Term Preservation Induces a Reversible Viable but Non-Culturalable State in Methane-Oxidizing Bacteria" by Hofeman et al., 2012 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034196)
- It is advisable to check the cultures for freezer stocks carefully for purity and discard any that are suspect.
- Before use, ensure that the culture is same as written on the lid by PCR amplification or sequencing of suspect short region (if required) using specific primer sets.

2b. Methanotroph culture stock maintenance (NMS-Agar plates)

- For routine use and culture maintenance, streak and transfer the cultures on NMS-agar plates once in every 25-30 days (10µM-final Cu concentration and if required appropriate antibiotics).
- Incubate under pure-CH_4 conditions and at 30°C (optimum for most methanotrophs) at gas tight chamber. Some impurities inhibit the growth of methanotrophs (e.g. natural gas cannot be used as methane source).
- On plates, it is common for contaminants to grow under methanotroph colonies, and only after the methanotroph cells have lysed (often after weeks of incubation) is it possible to see thin films of contaminants growing out from the colonies.
- It is recommended that cultures routinely be checked under the microscope for low levels of contamination (at the few % level), and routinely streaked onto a minimal medium with methanol and also onto a rich medium, such as nutrient broth, to test for the presence of contaminants.
- LB is not recommended as a test medium, since the higher salt may inhibit heterotrophic contaminants.

2c. Methanotroph culture preparation from stock/NMS-agar for experiment (NMS-liquid)

- As a standard procedure, the culture from freeze stocks (~1 mL) will be added in 30 mL of NMS medium and sealed with rubber septa and purged with CH_4. The final OD600 ~ 0.07-0.08 on time 0.
- If it's from NMS-agar, scoop a loop full of culture and inoculate in 30 mL NMS-liquid.
- For purging, use gas manifold. Start the compressor and open the valves at gas cylinder. Connect the gas manifold tube with the flask outlet tube, turn the red-valve anti-clockwise and create negative pressure of ~15psi. Then turn the same valve on clockwise to feed the CH_4 until the gas pressure reaches 0 psi (Note: make sure that blue vale at the gas manifold is on for CH_4).
- Measure OD600 at point 0 and every 24 h until the culture reaches its OD600~0.6-0.7.

- Once culture is up, streak on NMS-agar plates and check for any contamination using Nutrient Agar plates. (Note: LB-agar usually not allow many heterotrophs to grow).
- Also, do PCR using specific primers to confirm the strain before use for any experiment. Follow colony PCR steps (see section:??) or liquid DNA extraction step followed by PCR (see section:??)
- For any subsequent experiments using this stock culture, plan the volume of culture required well before and inoculate in appropriate volume 24-48 h before starting any experiment (to harvest the cells at early-mid stationary phase).
- Add $CuCl_2$ to get 1μM –final Cu concentrations, if required. Some methanotrophs may not require Cu. (Note: can grow those using Cu-free NMS).
- If needed add specific antibiotic to grow the mutant strains (Check the table 1 in Appendix).
- To start with the OD600~0.05-0.1 should be ideal for any experiment. Measure OD600 intermittently and record the growth of culture.
- Harvest the cells when OD600~0.65-0.7 (at early stationary phase) that may reach between 24 and 48h incubations at 30°C and 220 rpm.
- Cell harvesting: Centrifuge the cultures @6500 rpm, 10 min; use 50 mL sterile falcon tubes and wash the pellets with fresh Cu-free NMS. Re-suspend the pellet and use it as inoculum for any experiment.
- To maintain the sterility, always work close to the flame.

3. Shake flask experiments

3a. Cell culture preparation

- Follow the steps detailed in section 2c. Prepare 24-48h culture to use it for any experiment. Harvest the cells once it reaches its early stationary phase (OD600~0.6-0.7) and re-suspend in 30-50 mL of Cu-free NMS and use as inoculum.
- Any experiment, make sure that the initial OD600 is measures as 0.05-0.1.

3b. Experiment set-up and monitoring.

- Prepare 30 or 50 mL final volume in 250 mL side arm flask for any experiment (Note: as a rule of thumb always have 1/4 of flask volume free head space). In which 27 or 45 mL will be the fresh NMS and 3 or 5 mL will be the inoculum (~10%).
- Always use triplicate flasks for each conditions and work close to flame to avoid any contamination.
- If working with Cu-free tests or with any other metals, after washing with soap solution, soak the flasks in acid bath overnight, rinse it with tap water followed by distilled water and sterilize it.
- Use fresh NMS every time. Also use the same NMS as Blank for any subsequent analysis from the same experiment (e.g. ICP-MS; Naphthalene assay, etc.).
- Ensure the required final Cu concentration and antibiotic requirements for the test strain and maintain throughout the experiment.
- If you plan to use Cu-preloaded Methanobactin (Cu-MB complex) instead of free Cu, prepare your Cu-MB incubations well before the start of your experiment (i.e., Cu-MB complex required 1 h incubation at 30^{o}C and 220 rpm)
- Incubate the flasks at 30^{o}C and 220 rpm and monitor the growth by OD600 measurement.
- Monitor the OD600 for every 6-8 h initial incubation period and once the OD600~0.35 or above, every 2-4h until it reaches its early stationary phase.
- Harvest the cells for different tests when the culture reaches its mid or late-exponential phase (or at early stationary phase).

3c. Harvesting and sample preparation for analysis

- Immediately after opening the experimental flask (do one flask at a time), set samples aside for following analysis;

    (i) RNA extraction: collect 10 mL of samples in a 50 mL falcon tube that contains stop solution. Set aside until you complete the naphthalene assay and sample preparation for metal extraction. However, finish first day extraction as quick as possible and cannot be stored for long (see section: ??).

(ii) Naphthalene assay: take 1.6 mL out and incubate the tubes at 30°C and 220 rpm for 1 h (see section: ???).

(iii) DNA extraction: collect 10 mL of samples, centrifuge at 6500 rpm for 10 min and store the biomass at -20°C.

(iv) Metal extraction: Collect 10-20 mL of samples in a 50 mL falcon tube. Centrifuge at 6500 rpm for 10 min and collect the supernatant in a new-labelled 15 mL flacon tube. Add equal amount of Cu-free NMS to wash the pellet (i.e. re-suspend the pellet and centrifuge again). Collect the wash in a different 15 mL labelled falcon tube. Add 1 mL of Cu-free NMS to the pellet and re-suspend and transfer it into 2 mL screw cap vial. Store all samples at -20°C until use (see section: ??).

4. DNA and RNA extractions and Gel documentation

4a. DNA extraction from cultures grown in NMS agar plates

- Prepare a small PCR tubes in a rack and labelled them properly, if you have more than one culture for DNA extraction.
- Add 5 µL of 0.1 M NaOH in each tubes.
- Pick a sterile micropipette tip or sterile toothpick and gently scoop scrap a culture from agar plates.
- Dissolve and mix the culture in NaOH. Incubate in thermocycler for DNA extraction.
- Choose the program DNA extraction in thermocycler, which 95°C for 10 minutes followed by 4°C cooling (forever). Also make sure that the volume of reaction mixture is selected as 5 µL in thermocycler.
- Use the extract as template for PCR reactions.

4b. DNA extraction from E.coli TOP 10 Transformant

- Prepare a PCR tubes with proper labelling and add 5 µL of 0.1 M NaOH in each tubes.
- Pick a single colony (which is positive transformation) and dissolve in 15 µL DNase- free water.
- Take 5 µL culture dissolved in DNase-free water and mix with 5 µL 0.1 M NaOH and incubate under same conditions as in 4a.
- Use the extract as template for PCR reactions.
- Store the remaining 10 µL culture for other use.

4c. DNA extraction from liquid NMS cultures

- Prepare a PCR tubes with proper labelling and add 5 µL of 0.1 M NaOH in each tubes.
- Take 5 µL culture directly from the side arm flask and mix in PCR tube that contain NaOH. Follow the DNA extraction as detailed in 4a.
- Use the extract as template for PCR reactions.

4d. RNA extraction from liquid NMS cultures

- Need two days to complete the extraction.
- Try to finish first day extraction as soon as the sample is collected and followed by extraction incubate at -80°C overnight.
- Complete the second day extractions as soon as possible or before two weeks from the date of overnight incubate at -80°C.

(i) Key points to remember:

- Prepare the 50 mL falcon tubes before sample collection and properly labelled.
- Add 1 mL of stop solution in collection tubes and keep it ready.
- Prepare stop solution fresh and store in 4°C before use (don't use old stock)
- Make sure that all tubes are chilled and placed in ice all the time.
- Add samples one by one keep it aside. Finish your Napthalene assay and sample preparation for metal analysis (which are sensitive parameters too).
- Also, make sure that the other reagent required for the RNA extraction is chilled and readily available to use.

(ii) Reagents preparation:

1. Stop solution - 5% equilibrated phenol in ethanol: take 5 ml of Water Saturated Phenol (Ambion, cat# AM9710) add 45 ml of ethanol (200 proof). Store at 4°C. Use within 1-2 weeks.

2. CTAB solution: dissolve 14g of NaCl in 80 ml H_2O, add 10g of CTAB (Cetyltrimethylammonium Bromide, also known as Hexa-decyl-trimethyl-ammonium Bromide) and adjust volume to 100 ml. Add 100µl DEPC (Diethylpyrocarbonate), incubate overnight and autoclave. Store at Room Temperature

3. Phosphate buffer: To prepare 0.2M sodium phosphate buffer (pH 7.6) dissolve 15.5 g $Na_2HPO_4x2H_2O$ and 2 g of $NaH_2PO_4xH_2O$ in 800 ml of ddH_2O. Adjust volume to 1L, add 1 ml DEPC, and incubate overnight and autoclave. Store at Room Temperature

(iii) Day-I extraction:

- Prepare 2 ml screw-cap tubes (1 tube for one cell culture sample, or 10-14 tubes for one sediment sample)
- Add 0.5 g of 0.1 mm zirconia-silica beads (Biospec products)
- Add 35 µL of SDS 20% and 35 µL of Lauryl Sarkosine 20%
- Add 750 µL of chilled phenol:chloroform:isoamylic alcohol (25:24:1) (this can be added after adding samples).
- Collect biomass pellets from 10 mL culture samples by centrifugation at 5000 rpm for 10-20 min at 4°C. Use 50 mL falcon tube and appropriate rotors.
- Discard the supernatant and add 0.75 ml (for cell culture sample pellet) or 4-5 ml (for sediment sample pellet) of extraction buffer (10% CTAB in 2.4 M NaCl and 0.2 M phosphate buffer pH 7.6, 1:3*)

- mix well to resuspend the pellets into buffer and transfer 750 -800 µL of the mixture to 2 ml screw-cap tubes that is prepared above (should be 1 tube for cell culture sample pellet and 1014 tubes for sediment sample)
- Homogenise in a mini-beater (Biospec products) for 1 min (Note: set 6 for 60 sec)
- Centrifuged at 14,000rpm for 5 min at 4°C
- Take the upper aqueous phase, transfer into a clean 2 ml tube
- Add 750 µL of chilled chloroform:isoamylic alcohol (24:1) (Note: store it in 4°C)
- Centrifuge at 14,000rpm for 5 min at 4°C.
- Prepare 1.5 ml tubes for the next steps
- Transfer the upper aqueous phase (~700 µL) into new 1.5 ml tube and add
    - 0.5 M MgCl2(final concentration 2.5 mM) ~ 3.5 µL (for 700 µL of sample)
    - 0.1 volume of 3 M sodium acetate ~ 70 µL (for 700 µL of sample)
    - 0.7 volume of iso-propanol ~ 490 µL (for 700 µL of sample)
- Incubate the tubes overnight at -80°C.

(iv) Day-II extraction:

- Centrifuge the tubes at 14,000 RPM (18,000 g) for 45 min at 4°C,
- Remove supernatant and add 500 µl of 75% ethanol
- Centrifuge at 18,000 g for 5 min at 4°C,
- Remove supernatant and centrifuge at 18,000 g for 5 min at 4°C
- Remove any liquid residue from the tubes by pipetting and dry samples for 15 min at RT
- Follow the subsequent steps from Qiagen extraction kit to complete the extraction
- Store the samples at -20°C for RT-qPCR.

PCR Mixture preparation

	15 μL rxn	**50 μL rxn**
DNase-free H_2O	~ 6 μL	~20 μL
Forward primer (10 μM)	0.75 μL	2.5 μL
Reverse primer (10 μM)	0.75 μL	2.5 μL
DNA template	0.3 μL	0.3 μL
Polymerase mix	7.5 μL	25 μL

Note: For 15 μL, add <0.3 μL NaOH mix as DNA template (adding more NaOH may interfere with PCR reaction).

Notes: * For transformed *E. Coli*; Can save intact cells left over for growth in liquid, then plasmid extraction