SOP for AMMBI Pilot Project

Samples (2 L) collected at various depths and filtered each month from the 3 sites, Maria Island (MAI), Port Hacking Bay (PHA) and North Stradbroke Island (NSI). Sterivex filters used; at the end, filters are dried by pushing some air through them. Filters are then stored at -80 °C from collection and transported to CMAR Hobart on dry ice. Upon arrival filters should be logged into the spreadsheet:

..\SampleDNA-Logs\AMMBI microbial samples.xlsx

And stored at -80 °C, bottom shelf, in plastic box provided.

DNA Extraction Protocol for Sterivex Samples

(as modified by Sharon Appleyard)

Materials

Lysis buffer

200mM $NaH_2PO_4 2H_2O$ (monobasic)

200mM Na_2HPO_4 (dibasic) MW 142 142g/1L =1M 5.68g/200mL = 200mM

To make up 200mL lysis buffer

39mL 200mM NaH_2PO_4

61mL 200mM Na_2HPO_4

17.54g NaCl

2g CTAB

4g PVP K30

+ ddH20 to make up to 200ml

Adjust to pH 7.0 (using NaOH – try couple of mL of 10M NaOH)

Lysozyme

Proteinase K – 20mg/ml

From FastDNA™ Spin Kit for Soil (MP Biomedicals)

MT buffer

From PowerWater® Sterivex™ DNA Isolation Kit (MoBio Laboratories, Inc)

Columns and sample recovery tubes, 3ml and 20ml syringes, 5ml tubes

Buffer ST4 (warmed to 65 °C before use)

Buffer ST5 and ST6

Inlet and outlet caps for Sterivex filters

Phenol:Chloroform:Isoamyl (25:24:1) (PCI)

Chloroform:Isoamyl (24:1) (CI)

TE buffer

Protocol

(Processing limited by capacity of horizontal vortexer, 2 vortexers x 6 filters = 12 filters per extraction set)

1. weigh 125 mg lysozyme into 50 mL falcon tube and add 25 mL Lysis Buffer to dissolve (lysozyme final conc. 5 mg/ml).
2. remove filters from -80 °C, remove inlet cap and using a pipette add 1.875 ml Lysis buffer (containing 5mg/ml final concentration of lysozyme) and 0.125 ml MT buffer. *(if filters are covered with RNAlater, remove RNAlater with back pressure from 3 ml syringe)*
3. recap the Sterivex filter and attach filter (with inlet end facing out) to the horizontal vortexer, Speed 5-7 for 60 min (turning the filter a couple of times during the hr)
4. using 3 ml syringe, draw back plunger and attach to inlet end of filter until pressure builds up – release plunger and buffer in filter should flow into syringe. Divide approx 2 ml of buffer evenly into 2 × 2.0 ml tubes *(do not use the 2 ml collection tubes that come in the PowerWater kit – they don't tolerate PCI) (may need to use syringe several times to get all buffer out of filter, should be about 0.800-1.00 ml per tube)*
5. in fume hood, add 900 ul PCI to each tube, invert several times, spin down 13000/10 min/RT
6. combine the aqueous phases from both tubes into one 2.0 ml tube (which will be between 1.2 – 1.5 ml), add 20 ul Prot K, onto heat block for 2 hr at 60 °C
7. in fume hood, add 500 ul CI, spin down 13000/10 min/RT – put aqueous phase into new tube
8. in fume hood, add a further 500 ul CI, spin down 13000/5 min/RT – put aqueous phase into new tube
9. after 2nd spin, take out 1 ml of aqueous phase, add to 5 ml tube
10. add 3 ml of warmed ST4 buffer (65 °C), mix by inversion
11. attach column to barrel of 20 ml syringe and attach to vacuum manifold
12. pour contents of 5 ml tube into barrel while still warm
13. using vacuum, pull contents through the column
14. while keeping column attached to the manifold, remove barrel and add 800 ul ST5 to column
15. using vacuum, pull contents through the column
16. add 800 ul ST6 to column
17. using vacuum, pull contents through the column, then keep on vacuum for 2 mins
18. turn vacuum off, put column into new 2.0 ml tube and let air dry on bench for 10 mins
19. add 80 ul TE to column, incubate at 37 °C for 45 min
20. spin down column and tube at 13000/2 min/RT to elute DNA
21. Quantify 2 µl on the Nanodrop and record concentration and 260:280 ratio in spreadsheet
22. Proceed to whole genome amplification

Storage and Downstream Applications

1. Transfer DNA samples (total volume approx. 78 µl) to 96 well plate and record the well location of each sample in the spreadsheet
2. Using a multi-channel pipette aliquots into further 4 x 96 well plates suitable for storage at -80 °C (Eppendorf…) in the following way:

    i) 5 µl (for 454 sequencing)
    ii) 5 µl (for in-house array, Q-PCR, ARISA)
    iii) 10 µl (for storage)
    iv) 10 µl (for storage)

3. Dry down in vacuum centrifuge at 30 °C. Seal with AlumaSeal CS™ foil film. Place plates ii), iii) and iv) at -80 °C (bottom shelf in plastic box provided). Send plate i) for 454 sequencing with sample submission sheet filled in:

GS FLX+ Sequencing Service Sample Submission Form.doc

Attention: Jason Koval
The Ramaciotti Centre
Lowy Cancer Research Centre
Loading Dock, Lower Ground Goods Store (C25)
via Gate 11 Botany Street
University of New South Wales
Randwick, NSW 2052
Australia

Ph: +61 (2) 93851144

4. Of the remaining template DNA, use 1-2 µl (10 ng) for whole genome amplification as described below.

5. Transfer the remaining DNA samples (approx. volume 45 µl) to QIAsafe DNA 96-well Plates (Qiagen) for long-term storage at -80 °C as outlined in manufacturers protocol:
    a. Remove the seal from the QIAsafe plate and and using a multi-channel pipette carefully transfer the DNA samples to the center of the wells.
    b. Mix the sample thoroughly with the QIAsafe matrix by gentle pipetting up and down (sample turns a pink colour)
    c. Dry down in vacuum centrifuge at 30 °C
    d. Cover the plate with the seal provided in the kit and with the plate lid. Place plate at -80 °C (bottom shelf in plastic box provided).

Whole Genome Amplification Protocol

using Illustra GenomiPhi V2 DNA Amplification Kit (Product Code 25-6600-31 ; GE Healthcare Life Sciences)

NB. V3 of this kit is currently available and could be used in future studies

1. Thaw sample buffer and reaction buffer on ice (kit components stored at -80 °C)
2. Add 9 µl sample buffer to 1 µl template DNA (10 ng)
3. Denature samples 95 °C x 3 min and cool to 4 °C in thermocycler then place on ice for 5 min.
4. Prepare master mix of 9 µl reaction buffer and 1 µl enzyme mix on ice for n + 0.5 samples (can only be done immediately prior to use and remains discarded)
5. Add 10 µl master mix to samples on ice
6. Incubate samples at 30 °C x 1.5 hr
7. Deactivate enzyme at 65 °C x 10 min
8. Move immediately to purification

Purification of Whole Genome Amplification

using illustra ProbeQuant G-50 Micro Columns (Product Code 28-9034-08; GE Healthcare Life Sciences)

1. Adjust the volume of your samples to 50 µl using Probe Buffer 1 (add 30 µl buffer to the 20 µl amplification reaction)
2. Prepare column by resuspending the resin in the column with vortexing
3. Loosen cap by a ¼ turn and twist off bottom closure then place in a collection tube (provided in kit)
4. Spin 735 g x 1 min
5. Discard collection tube and place the column into a new DNase-free 1.5 mL tube (user supplied)
6. Slowly apply 50 µl sample to the top center of the resin (without disturbing the resin bed with the tip)
7. Spin 735 g x **2 min**
8. Discard column and cap tube
9. Quantify 2 µl on the nanodrop and record concentration and 260:280 ratio in spreadsheet
10. Transfer purified amplified samples to 96 well plates (as per original DNA templates; (v) genomiphi)), dry down in vacuum centrifuge at 30 °C. Seal with AlumaSeal CS™ foil film and place at -80 °C.

NB. For full whole genome amplification and purification protocols and trouble-shooting see the manufacturers protocols.

Ordering information

From Sigma-Aldrich:

AlumaSeal CS™ sealing films with special adhesive for cold storage x 100 (Product Code Z722634 - 100) $137

From VWR:

GE Healthcare Life Sciences

illustra GenomiPhi V2 DNA Amplification Kit x 100 rxns (Product Code 25-6600-31) $634

illustra ProbeQuant G-50 Micro Columns x 30 rxns (Product Code 28-9034-08) $331