Sampling and DNA Extraction from Wastewater Activated Sludge

Standard Protocol

Version 7.0

Skill Prerequisites: DNA handling

Introduction

This protocol explains sampling and DNA extraction from activated sludge from wastewater treatment plants. The protocol is based on the FastDNA spin kit for soil protocol (MP biomedicals) with some modifications, mainly streamlining and longer bead beating.

The key in sampling and DNA extraction is consistency and hence this protocol should be followed to the letter. If you choose to deviate from the protocol do it consistently throughout your experiment.

Materials

Sampling
- Sampling rod with container
- Ice and thermobox for transport
- Sampling container (500-1000 mL)
- Tissues, rubber gloves and plastic sleeves.
- Heidolph RZR 2020 (Heidolph)
- Glass/Teflon tissue grinder (30 mL)
Cryotubes, 2 mL
- Greiner tubes 15 mL
- EtOH, 99%
- Paraformaldehyde (PFA), 8%
- Ultrapure water

**DNA Extraction and QC**
- FastDNA Spin kit for soil (MP Biomedicals)
- FastPrep-24 (MP Biomedicals)
- Spintubes (DNAse free), 1.5 mL
- Greiner tubes, 15 mL
- Ice
- Pipettes (Range 1 uL to 1000 uL)
- Nanodrop1000 (Thermo Scientific)
- Electrophoresis chamber and power source
- Gel-Doc
- DNAse free tips (10uL, 300uL and 1000 uL)
- Gelred (Biotium)
- 1kb Generuler
- TAE-buffer 50x
- Nuclease Free H₂O (Qiagen)

**Method**

**Sampling**

1. Investigate plant operation
   a. Inform the plant operators of your arrival (and remember to sign-out when you leave)
   b. Ask plant operator about operation conditions the last couple of days (operation stops, excessive use of chemicals etc. will impact the analysis).
2. Location
   a. The sample should be taken from the aeration tank. Aeration and mixing should be
turned on when sampling.
   b. Avoid sampling positions in the tank with a lot of foam or static water.
3. Actual sampling
   a. Tie sampling device to the tank fence.
   b. Submerge the container >1 m below the water surface and sample more > 1 L of
activated sludge (avoid sampling foam).
   c. Shake the activated sludge vigorously and transfer some to a sampling container. Pour
surplus sludge back into the aeration tank.
   d. Close sampling container tightly.
4. Transport
   a. Store sample on ice or in the fridge.
   b. Process within 4 hrs of sampling
   c. Remember proper labeling (date, content, plant of origin)
5. Storage preparation
   a. Shake sample
   b. Homogenize samples in portions with the Heidolph RZR 2020 (stirrer) and glass/Teflon
tissue grinder (30 mL).
      i. 1 minute, 2 nd gear, speed 9, 10 times up/down
   c. Make 2 mL aliquots in cryotubes and store them at -80 °C until DNA extraction. NB: To
ensure consistency in sample processing always store the samples for at least 1 day at – 80 °C before DNA extraction.
   d. Fixation of samples for qFISH (optional)
      i. Gram positive fixation
         1. 5 ml sample + 5 ml EtOH
         2. Store at -20 °C
      ii. Gram negative fixation
         1. 5 ml sample + 5 ml 8% PFA
         2. Incubate for 3 hrs
         3. Wash 3 times with water
         4. Remove supernatant and add 10 ml 50% EtOH/1x PBS solution.
         5. Store at -20 °C
   e. Measure Total solids (google: EPA standard method 1684)

DNA Extraction
1. Sample input
   a. Target volume: 500 µL
   b. Target Total solids: 2 mg
      NB: Never spin sample down to upconcentrate!
2. Prepare tubes for the whole workflow (pr. Sample):
   a. 1 x Lysing Matrix E tube (from kit)
   b. 1 x SPIN filter (from kit)
   c. 1 x Catch tube (from kit)
d. 3 x 1.5 mL tubes

e. 1 x 15 mL greiner tube

3. Thaw sample aliquote at room T immediately vortex/homogenise it.

Bead beating

4. Transfer sample volume equal to 2 mg TS to a Lysing Matrix E tube and dilute to a final volume of 500 uL. NB: Use pipette tip with wide orifice to avoid selecting against large granules.

5. Add 980 uL Sodium Phosphate Buffer, pH 8 and 120 μl MT Buffer.

6. Homogenize in the FastPrep-24 instrument (4 x 40s at 6 m/s). Between each 40s interval the samples should be kept on ice for 2 min to cool down.

Protein Precipitation and binding of DNA to matrix

7. Spin down samples at >10 000 g for 10 min.

8. While samples are spinning add 250 μL PPS (Protein Precipitation Solution) to fresh 1.5 mL spintubes.

9. After spindown transfer supernatant to fresh 1.5 mL spintubes with PPS and then shake the tubes 10 times by hand (keep the tubes on ice until all samples are processed).

10. Centrifuge at 14,000 x g for 5 minutes to pellet precipitate. While the centrifuge is running, resuspend the Binding Matrix and add 1.0 mL to a clean 15 mL tube.

11. Transfer the supernatant to the 15 mL tube with Binding Matrix suspension.

12. Invert by hand for 2 minutes to allow binding of DNA.

13. Place the tube in a rack for 3-5 minutes (until the liquid appears clear) to allow settling of silica matrix.

14. Remove and discard up to 2x750 µL of supernatant being careful to avoid settled Binding Matrix.

15. Resuspend Binding Matrix in the remaining amount of supernatant.

DNA washing and elution

16. Transfer approximately 750 µL of the mixture to a SPIN™ Filter and centrifuge at 14,000 x g for 1 minute. NB: If you have more sample than 750 µL you can repeat this step.

17. Empty the catch tube.

18. Ensure that ethanol has been added to the Concentrated SEWS-M.

19. Add 500 µL prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip - or by stirring with a pipet tip.

20. Centrifuge at 14,000 x g for 1 minute.

21. Empty the catch tube and replace.

22. Centrifuge at 14,000 x g for 2 minutes to “dry” the matrix of residual wash solution.

23. Discard the catch tube and replace with a new, clean tube. NB: The new tube is the tube that the sample is to be stored in so be sure to label it properly.

24. Allow the SPIN™ Filter to drive for 5 minutes at room temperature with open lid.

25. Gently resuspend Binding Matrix (above the SPIN filter) in 60 μL of DES. - Use a pipet tip to stir the matrix until it gets liquid. Make sure not to disrupt the filter.
26. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN filter.
27. Store DNA at -80°C.

DNA QC

1. Quality and Concentration Check
   a. Nanodrop DNA measurement. Use guidelines provided by the vendor. A260/280 should be 1.8 to 2.0. A260/230 2.0 to 2.2. Also compare the UV-vis absorbance curve to that of pure DNA. 

   NB: DNA extracted with 'FastDNA spin kit for soil' is known to contain contamination producing a peak around 230 nm. The contaminant does not effect this protocol but may result in overestimation of the DNA concentration.

2. Preliminary dilution
   1. Based on the Nanodrop concentrations measurements dilute all samples to 20 ng/ul and in volume of at least 15 ul in a 96-well plate using DNA H2O.

3. Run gel electrophoresis (optional)
   1. Prepare a 1% agarose gel
      1. 1 g agarose, 100 mL 1xTAE-buffer, heat in microwave and gently swirl to completely dissolve agarose. After slight cooling add 2 uL gelred and cast gel.
      2. Prepare samples by mixing 1 uL 6x loading buffer, 2 uL sample (20-50 ng) and 3 uL DNA H2O. Prepare ladder according to vendor recommendations.
      3. Run electrophoresis approximately 80 min at 120V (6 V/cm)
      4. Capture gel image on gel-doc, save and analyze. The majority of DNA should be in the range 3-10+ kb. If it is below the DNA is heavily degraded and it might effect the analysis.

Literature

Instruction Manual, FastDNA® SPIN Kit for Soil, Revision # 6560-200-07DEC, MP Biomedicals

Revision History

V7.00 2013-2-14
- Protocol format redesigned
- Added Materials paragraph
- Made clarifications in sampling paragraph
- Removed pelleting step before DNA extraction. Pelleting potentially removes DNA from cells lysed due to freeze/thaw
- Reduced DNA QC to Nanodrop measurement and Gel electrophoresis.