Sampling and DNA Extraction from Wastewater **Activated Sludge**

Standard Protocol

Version 7.1
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 device (i.e. Bürkle, 5354-1000)
  - > 100 mL sampling volume
  - Sampling depth > 30 cm below water surface.

- Ice and thermobox for transport
- Sampling container (500-1000 mL)
- Tissues, rubber gloves and plastic sleeves.
- Homogenizer, Heidolph RZR 2020 (Heidolph)
- Glass/Teflon tissue grinder (30 mL)

- Cryotubes, 2 mL
- Greiner tubes 15 mL
- EtOH, 99%
- Paraformaldehyde (PFA), 8%
- Nuclease free water (Qiagen)

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 µL to 1000 µL)
- Nanodrop1000 (Thermo Scientific)
- Electrophoresis chamber and power source
- Gel-Doc
- DNAse free tips (10 µL, 300 µL and 1000 µL)
- Gelred (Biotium)
- 1kb Generuler
- TAE-buffer 50x
- Nuclease Free H_2O (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. which 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 waterbodies.
3. Actual sampling
    a. Tie sampling device to the tank fence.
    b. Submerge the sampling container min 30 cm below the water surface, wait 5 seconds and retract the device (avoid sampling foam).
    c. Transfer the **whole** sampling volume to a sampling container.
    d. Close sampling container tightly and transport on ice.
4. Sample handling
    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 the sample
    b. Homogenize samples in portions with the Heidolph RZR 2020 (stirrer) and glass/Teflon tissue grinder (30 mL).
        i. 1 minute, 2nd gear, speed 9, 10 times from top to bottom of the glass tissue grinder.
    c. Make 2 mL aliquots in cryotubes and store them at -80 °C until DNA extraction.[1]
    d. Fixation of samples for qFISH (optional)
        i. Gram positive fixation
            1. 1 ml sample + 1 ml 99 % EtOH
            2. Store at -20 °C
        ii. Gram negative fixation
            1. 1 ml sample + 1 ml 8 % PFA
            2. Incubate for 3 hrs
            3. Wash 3 times with sterile filtered tap water
            4. Remove supernatant and add 10 ml 50 % EtOH/1x PBS solution.
            5. Store at -20 °C
    e. Measure Total Solids (optional, EPA standard method 1684)

DNA Extraction

1. Sample input
    a. Target volume: 500 µL
    b. Target Total Solids (TS): 2 mg

    NB: Never spin sample down to increase concentration!

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 DNAse free tubes
    e. 1 x 15 mL Greiner tube
3. Thaw sample aliquot at room temperature and store on ice until use.

[1] To ensure consistency in sample processing always store the samples for at least 1 day at – 80 °C before DNA extraction.

Bead beating

1. Mix sample before use i.e. with vortexing.
2. Add **480** µL Sodium Phosphate Buffer (PBS), pH 8 and 120 µl MT Buffer to a Lysing Matrix E tube.
3. Transfer sample volume equal to 2 mg TS [2] to a Lysing Matrix E tube and add Sodium Phosphate Buffer (PBS) so the final added volume is 500 µL[3].
4. Perform bead-beating in the FastPrep-24 instrument
    a. Time: 4 x 40s
    b. Speed: 6 m/s
    c. Adaptor: Custom
    d. Remember to balance samples like in centrifuges.
    e. 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

1. Spin down samples at >10,000 g for 10 min, preferably at 4°C.
2. While samples are spinning add 250 µL PPS (Protein Precipitation Solution) to fresh 1.5 mL spintubes.
3. After centrifugation, transfer supernatant to 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).
4. Centrifuge the tubes at 14,000 g for 5 minutes to pellet the precipitate. While the centrifuge is running, resuspend the Binding Matrix and add 1.0 mL to a 15 mL Greiner tube.
5. Transfer the supernatant to the 15 mL tube with Binding Matrix suspension.
6. Invert by hand for 2 minutes to allow binding of DNA to the matrix.
7. Place the tube in a rack for 3-5 minutes (or until the liquid appears clear) to allow settling of the silica matrix.
8. Remove and discard up to 2x750 µL of supernatant being careful to avoid settled Binding Matrix.
9. Resuspend Binding Matrix in the remaining amount of supernatant.

DNA washing and elution

1. Transfer approximately 750 µL of the mixture to a SPIN™ Filter and centrifuge at 14,000 g for 1 minute.[4]
2. Empty the catch tube.
3. Ensure that ethanol has been added to the Concentrated SEWS-M.
4. 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.
5. Centrifuge at 14,000 g for 1 minute.
6. Empty the catch tube and use it again.
7. Centrifuge at 14,000 g for 2 minutes to “dry” the matrix of residual wash solution.
8. Discard the catch tube and replace it with a new tube.[5]

[2] 1-4 mg of TS usually is acceptable.

[3] Use pipette tip with wide orifice to avoid selecting against large granules.

[4] If you have more sample than 750 µL you should repeat this step.

[5] The new tube is the tube that the sample is to be stored in so be sure to label it properly.

9. Allow the SPIN™ Filter to dry for 5 minutes at room temperature with open lid.
10. 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.
11. Centrifuge at 14,000 g for 1 minute to bring the eluted DNA into the clean catch tube. Discard the SPIN filter.
12. Store DNA at -20°C for short-term storage and -80°C for long-term storage.

DNA QC (optional)

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 [6].

2. Run gel electrophoresis
   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 µL 6x loading buffer, 2 µL sample (20-50 ng) and 3 µL nuclease free water. 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 affect the downstream uses.

[6] DNA extracted with 'FastDNA spin kit for soil' is known to contain contamination producing a peak around 230 nm. The contaminant can lead to overestimation of DNA concentration. The contamination does not affect PCR, but might impact ligation/transposome based sequencing library protocols.

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.

V7.01 2015-2-23

- Materials and methods: Changed example of sampling device
- Beadbeating: Changed PBS volume to 480 from 980 uL. 980 uL PBS and 500 uL was too large a volume for the tubes.
- Beadbeating: When increasing the volume of sample to a total of 500 uL the solution to use was changed from H2O to PBS.
- Beadbeating: Elaboration of bead beating settings