

DNA Extraction from Bacterial Communities – Freeze-Grind Method

There are now three protocols available for DNA extraction from soils and sediments: Freeze-Grind, MoBio PowerSoil kit, and Modified MoBio PowerSoil kit.

Before choosing a protocol, test your samples by extracting DNA from a typical soil in your soil sample set using the MO BIO PowerSoil DNA Isolation Kit (MO BIO, 12888-100). Check DNA shearing with an agarose gel (0.8%, 105 V, 30 min).

If DNA shearing is negligible, continue using the PowerSoil kit for all these type of soil samples.

If DNA shearing is visible but not very serious, you can try the Modified PowerSoil protocol to *reduce DNA shearing*.

If DNA shearing is serious, you should use the classic Freeze-Grind protocol.

Because DNA extraction methods may have a significant effect on the results of microbial community analysis, it is recommended to use the same method for all samples you want to compare.

Freeze-Grind Method:

Two buffers are available to use in DNA extractions: PIPES and Tris. There are slight differences in the protocols for each buffer – these are noted below.

PIPES buffer generally results in a cleaner DNA extract but the DNA is slightly sheared or damaged so the DNA should not be purified using gel extraction (the DNA smears when run on a gel). The column purification method should be used instead.

Tris buffer generally produces a less clean DNA extract, especially for soils with high levels of humic acid. The DNA is in larger fragments and remains in a compact band when run on a gel, so gel purification can be used with this method. Gel purification is ideal for soil or sediment samples with high DNA yields.

Solutions:

Tris buffer

6.8 mL 1 M NaH₂PO₄ (*monobasic*)

93.2 mL 1 M Na₂HPO₄ (*dibasic*)

Combine phosphate sol., pH to 8.0 with NaOH, continue with remaining ingredients

200 mL 0.5 M EDTA, pH 8.0

100 mL 1 M Tris-HCl

300 mL 5 M NaCl

100 mL 10% CTAB [*for filter samples, leave CTAB out*]

Bring to 1 L with DI water, **if CTAB is left out; bring to 900 mL with DI water**

(Final concentrations: 0.1 M NaH₂PO₄, 0.1 M Na₂HPO₄, 0.1 M EDTA, 0.1 M Tris-HCl, 1.5 M NaCl, 1% CTAB)

PIPES Buffer: 100 mM PIPES salt, 100 mM EDTA, 1.5 M NaCl and 1% CTAB, final pH=7.0

Other Chemicals Needed:

Proteinase K

10 mg mL⁻¹ (store at -20C)

20 % SDS (pre-made)

70 % Ethanol (cold, store at -20C)

2-Isopropanol

0.5 M EDTA, pH 8.0

1 M Tris-HCl

5 M NaCl

Chloroform:isoamyl alcohol (24:1)

Combine the chloroform and isoamyl alcohol and store in a dark or foil covered bottle and keep it in the flammables cabinet below the fume hood. *This is a toxic chemical and must be disposed of as hazardous waste.*

[For **filter samples**: 10% CTAB (store at room temperature – heat to redissolve)]

Other Notes:

The Oak Ridge tubes used in this protocol should not be autoclaved. If the tubes are autoclaved, the DNA pellet does not form a tight pellet and can be difficult to see. To sterilize the tubes, rinse them in DI water after cleaning and then boil for at least 30 min in DI water.

Protocol:

For soils and sediments, skip to step 8

- (1) If not already, place **filters** in a 50 mL conical centrifuge tube. If the filters are large use *pre-cleaned scissors to cut the filters*. Add 5 mL of extraction buffer (without CTAB).
- (2) Vortex the tube vigorously for several minutes to remove the biomass.
- (3) Transfer liquid to a fresh centrifuge tube. Remove as much liquid as possible from the tube.
- (4) Repeat steps 1-3 twice (total of 3 washes).
- (5) Check the pH of the liquid using pH paper. The pH should be ~pH 8. If it is too low, add NaOH to bring the pH up.

(6) Centrifuge the collected liquid 6000 x g for 40 min to pellet cells [*use the floor model Sorvall RC5C Plus with the conical tube adaptor (SLA-600TC)*].

(7) Transfer supernatant to a fresh tube, use 1 mL of the supernatant to resuspend the pellet.

(8) For **soils or sediments**, weigh out 5 g of sample into a sterile mortar. *Only take out one sample at a time to minimize DNA degradation from being at room temperature.* [For **filter**, add resuspended pellet to the mortar]. Add 2 g of sterile sand to the mortar. *More sand can be added if needed.* Add liquid N₂ to the mortar to cool the sand and mortar. *Be generous with the N₂.*

(9) Add more N₂ to cover the sand/sample and begin grinding once the N₂ has evaporated. Try to contain sample to a small area of the mortar. Grind until the sample starts to thaw. If possible, keep the sample frozen while grinding (this can be easy if the soil is dry and sandy). *But if the sample is difficult to grind when frozen, you can allow it to thaw, but make sure to add 1-2 mL of extraction buffer to the sample. The buffer will inhibit degradation of the DNA when it warms.*

(10) Repeat freezing and grinding twice (3 times total).

(11) While the sample is still frozen scrape the sides of the mortar with a spatula to collect the sample in the center of the mortar (add more N₂ if necessary to keep the sample frozen). Transfer this to the supernatant collected previously (for **filter samples**) or to a fresh tube (for **soils and sediments**) using a spatula.

At this point the sample can be kept frozen (-80 °C) until ready to proceed with DNA extraction

(12) Remove samples from freezer and thaw. For **soil and sediment samples**, add 16.5 mL Extraction Buffer (with CTAB) and proceed to step 14. *The total volume of buffer should be 16.5 mL, so if you added buffer during the freeze-grinding, remember to subtract that amount from the amount added at this step.*

(13) For **filter samples**, add 1.5 mL 10% CTAB (final concentration, 1%) to the thawed and warmed samples, mix gently.

(14) For **Tris buffer** add 61 µL proteinase K (10 mg mL⁻¹), mix gently. For **PIPES buffer** skip to step 16.

(15) Incubate at 37 °C for 30 min (*keep in a 37 °C water bath and invert every 5-10 min*)

(16) Add 1.83 mL 20 % SDS, mix gently

(17) Incubate at 65 °C for 2 h for **TRIS buffer** with gentle inversion every 15-30 min. Incubate at 65 °C for 1 h for **PIPES buffer** with gentle inversion every 15-30 min.

- (18) Centrifuge 20 min, 6000 x g at 25 °C.
- (19) Transfer liquid to a 50-mL conical centrifuge tube, avoiding the white surface layer. *(Pour the liquid slowly into the new tube, the surface layer will remain in the tube – if the surface layer breaks apart, you can use a pipette)*
- (20) Add 6 mL extraction buffer containing CTAB (or 5.5 mL extraction buffer without CTAB plus 0.55 mL 10 % CTAB) to the remaining sand pellet and mix.
- (21) Add 0.67 mL 20 % SDS, mix gently
- (22) Incubate at 65 °C for 15 min
- (23) Centrifuge 20 min, 6000 x g at 25 °C
- (24) Collect supernatant and combine with previous supernatant, avoiding the surface layer as in step 17
- (25) Extract supernatant with an equal volume of isoamyl:chloroform (1 part isoamyl, 24 parts chloroform) for 5-10 min by continuous inversion *(the rotator located in the fume hood can be used to continuously mix the samples)*
- (26) Centrifuge at 3700 x g, 20 min – use the benchtop centrifuge *(the orange or purple capped conical tubes can withstand this speed).*
- (27) Collect the supernatant into a fresh conical tube. *The isoamyl:chloroform layer should be poured into the chloroform waste container.*
- (28) Repeat steps 23-25, except transfer the supernatant into an oak ridge tube *(Use the translucent oak ridge tubes. Pay attention to the final volume of supernatant transferred.)*
- (29) Add 0.6 volume of 2-isopropanol *(very important that exactly 0.6 volume is added)*
- (30) Incubate at -20 or -80 °C overnight. *The cold will help the DNA to precipitate.*
- (31) Remove the tube from the freezer and warm in a 37 °C water bath. Make sure the sample is warm and all precipitates have dissolved before proceeding. *Warming the sample prior to centrifugation will dissolve any mineral precipitates that may have formed overnight.*
- (32) Centrifuge 10,500 x g (RCF) for 20 min at 25 °C *(Make sure the centrifuge is at RT – if it is too cold, mineral precipitates in the sample will be allowed to form).* Immediately after centrifugation, transfer the supernatant to a fresh tube *(keep the supernatant until you know whether DNA is present).*

(33) Wash the pellet with 1 mL ice-cold 70 % ethanol, if no pellet is visible, centrifuge 15,000 x g, 5 min. If the ethanol becomes dirty after washing, repeat the wash step with fresh 70% ethanol.

(34) Allow the pellet to dry 10-15 min.

(35) Dissolve the pellet with 50-500 μ L nuclease-free water pre-warmed to 50 °C (*start with 50 μ L, if pellet is larger, use a larger volume*). At this point DNA can be stored at -20 °C or -80 °C. If the DNA quality is good, then the DNA can be stored long-term. For low quality/dirty DNA, purification should be performed as soon as possible. For sediment samples, keep the volume as low as possible and immediately begin gel purification.

It is important that gel purification be done using freshly extracted DNA. As the extracted DNA ages, it degrades resulting in a DNA smear on the gel rather than to clean band.

(36) Measure DNA concentration of samples using Picogreen. You can also check the quality of the DNA using NanoDrop and/or run a gel of the DNA (~1 μ L)
The DNA concentration at this point can be used for normalization later.

Keep in mind that the NanoDrop is probably not very accurate at this point. Possible contaminants in the DNA (e.g., humics) have absorbance values over the 230-280 nm range. Based on the curve, 260/280 ratio, and gel image, decide whether purification is necessary. If you have questions about the DNA quality or quantity, please discuss with Liyou before proceeding.

If DNA is from a site that has or is expected to have high microbial activity, check to see if there is a high amount of RNA. If so, this should be removed using an RNase protocol before proceeding. The presence of large amounts of RNA may interfere with subsequent steps.

Note: Extracted DNA should be purified as soon after extraction as possible. As the extracted DNA ages, it degrades, which will make purification difficult.

To cite this method, use:

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