TRF Protocol

STEP 1- To extract DNA out of your environmental sample

Extraction
Using MoBio Power Soil DNA Extraction Kit
Please wear gloves at all times

1. To the 2ml PowerBead Tubes provided, add 0.25 gm of soil sample or pulverized half filter. For sand add 1.0 gram, for feces 0.1 gram.
2. Gently vortex to mix.
3. **Check Solution C1.** If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60µl of Solution C1 and invert several times or vortex briefly.
5. Fast Prep for
   - Soil - 5.0 m/s for 45 sec.
   - Feces - 4.5 m/s for 30 sec.
   - Pure culture - 4.5 m/s for 30 sec
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean microcentrifuge tube (provided).
   Note: Expect between 400 to 500µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate in the freezer for 10-15 minutes.
9. Centrifuge the tubes for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to, but no more than, 600µl of supernatant to a clean microcentrifuge tube (provided).
11. Add 200µl of Solution C3 and vortex briefly. Incubate in the freezer for 10-15 minutes.
12. Centrifuge the tubes for 1 minute at 10,000 x *g*.
13. Avoiding the pellet, transfer up to, but no more than, 750µl of supernatant into a clean microcentrifuge tube (provided).
14. Add 1200µl of Solution C4 to the supernatant and vortex for 5 seconds.
15. Load approximately 675µl onto a spin filter and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through and add an additional 675µl of supernatant to the spin filter and centrifuge at 10,000 x *g* for 1 minute. Load the remaining supernatant onto the spin filter and centrifuge at 10,000 x *g* for 1 minute. ***Keep loading until all supernatant from all replicates has been filtered through the same filter.***
16. Add 500µl of Solution C5 and centrifuge for 30 seconds at 10,000 x *g*.
17. Discard the flow through.
18. Centrifuge again for 1 minute.
19. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution C5 onto the spin filter.
20. Add 100µl of Solution C6 to the center of the white filter membrane. Let sit for 15 minutes. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica spin filter membrane at this step (Mo Bio Catalog No. 17000-10).
21. Centrifuge for 30 seconds.
22. Discard the spin filter. DNA in the tube is now application ready. No further steps are required.
    We recommend storing DNA frozen (-20° to -80°C). Solution C6 contains no EDTA.

Detailed Extraction Protocol (This is the same protocol as above, with explanations for each step)

1. To the 2ml PowerBead Tubes provided, add 0.25 gm of soil sample. For sand add 1.0 gram, for feces 0.1 gram
   After your sample has been loaded into the PowerBead Tube, the next step is a homogenization and lysis procedure. The PowerBead Tube contains a buffer that will (a) help disperse the soil particles, (b) begin to dissolve humic acids and (c) protect nucleic acids from degradation.
2. Gently vortex to mix.
   Gentle vortexing mixes the components in the PowerBead Tube and begins to disperse the sample in the PowerBead Solution.
3. **Check Solution C1**. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.
   Solution C1 contains SDS and other disruption agents required for complete cell lysis. In addition to aiding in cell lysis, SDS is an anionic detergent that breaks down fatty acids and lipids associated with the cell membrane of several organisms. If it gets cold, it will precipitate. Heating to 60°C will dissolve the SDS and will not harm the SDS or the other disruption agents. In addition, Solution C1 can be used while it is still hot.
4. Add 60µl of Solution C1 and invert several times or vortex briefly.
5. Fast Prep for
   - Soil - 5.0 m/s for 45 sec.
   - Feces - 4.5 m/s for 30 sec.
   - Pure culture - 4.5 m/s for 30 sec

Note: *The vortexing step is critical for complete homogenization and cell lysis. Cells are lysed by a combination of chemical agents from steps 1-4 and mechanical shaking introduced at this step. By randomly shaking the beads in the presence of disruption agents, collision of the beads with one another and with microbial cells causes the cells to break open.*
We have designed the Mo Bio Vortex Adapter as a simple platform to facilitate keeping the tubes tightly attached to the vortex. It should be noted that although you can attach tubes with tape, often the tape becomes loose and not all tubes will shake evenly or efficiently. This may lead to inconsistent results or lower yields. Therefore, the use of the Mo Bio Vortex Adapter is a highly recommended and cost effective way to obtain maximum DNA yields.

6. Make sure the 2 ml PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean microcentrifuge tube (provided).
   Note: *Expect between 400 to 500µl of supernatant at this step. The exact recovered volume depends on the absorbancy of your starting material and is not critical for the procedure to be effective. The supernatant may be dark in appearance and still contain some soil particles. The presence of carry over soil or a dark color in the mixture is expected in many soil types at this step. Subsequent steps in the protocol will remove both carry over soil and coloration of the mixture.*
8. Add 250µl of Solution C2 and vortex for 5 seconds. Incubate in the freezer for 10-15 minutes.
   Solution C2 contains a reagent to precipitate non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream applications for the DNA.
9. Centrifuge the tubes for 1 minute at 10,000 x *g*.
10. Avoiding the pellet, transfer up to 600µl of supernatant to a clean microcentrifuge tube (provided).
    The pellet at this point contains non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.
11. Add 200µl of solution C3 and vortex briefly. Incubate in the freezer for 10-15 minutes.
    Solution C3 is a second reagent to precipitate additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. It is important to remove contaminating organic and inorganic matter that may reduce DNA purity and inhibit downstream applications for the DNA.
12. Centrifuge the tubes for 1 minute at 10,000 x *g*.
13. Transfer up to 750µl of supernatant to a clean microcentrifuge tube (provided).
    The pellet at this point contains additional non-DNA organic and inorganic material including humic acid, cell debris, and proteins. For the best DNA yields, and quality, avoid transferring any of the pellet.
14. Add 1.2ml of Solution C4 to the supernatant (be careful solution doesn't exceed rim of tube) and vortex for 5 seconds.
    Solution C4 is a high concentration salt solution. Since DNA binds tightly to silica at high salt concentrations, this solution will adjust the salt concentrations to allow binding of DNA, but not non-DNA organic and inorganic material that may still be present at low levels, to the spin filters.
15. Load approximately 675µl onto a spin filter and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through and add an additional 675µl of supernatant to the spin filter and centrifuge at 10,000 x *g* for 1 minute. Load the remaining supernatant onto the spin filter and centrifuge at 10,000 x *g* for 1 minute. **Note**: A total of three loads for each sample processed are required.
    DNA is selectively bound to the silica membrane in the spin filter device in the high salt solution. Almost all contaminants pass through the filter membrane, leaving only the desired DNA behind.
    This is the step where you add your replicates together on the filter.
16. Add 500µl of Solution C5 and centrifuge for 30 seconds at 10,000 x *g*.
    Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the spin filter. This wash solution removes residues of salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.
17. Discard the flow through from the collection tube.
    This flow through fraction is just non-DNA organic and inorganic waste removed from the silica spin filter membrane by the ethanol wash solution.
18. Centrifuge again for 1 minute.
    This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in C5 can interfere with many downstream applications such as PCR, restriction digests and gel electrophoresis.
19. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution C5 onto the spin filter.
    Note: *It is important to avoid any traces of the ethanol based wash solution.*
20. Add 100µl of Solution C6 to the center of the white filter membrane. Let sit for 15 minutes.
    Note: *Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient release of the DNA from the silica spin filter membrane.*
    As the Solution C6 (elution buffer) passes through the silica membrane, DNA is released because it only stays bound to the silica spin filter membrane in the presence of high salt. Solution C6 is 10mM Tris pH 8 and does not contain salt. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica spin filter membrane at this step (Mo Bio Catalog No. 17000-10).
    Note: *Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of buffer C6 for elution of DNA from the spin filter.*
21. Centrifuge for 30 seconds.
22. Discard the spin filter. DNA in the tube is now application ready. No further steps are required.
    We recommend storing DNA frozen (-20° to -80°C).

STEP 2 – To see if your DNA extraction worked. You have three choices

Use the Qubit with the BR DNA Pico Green

STEP 3

PCR (Forward primer is labeled with a Cy5 fluorescent tag)

Now the question is how much DNA to add to the PCR.
If you have spec'ed the DNA use 10 ng of DNA.
If you have run a gel, how bright is the band? Usually 1 uL of the straight DNA or 1uL of a 1/10 dilution works well. If the band is super bright dilute it.

- Two control reactions are needed
    1. a closed negative (master mix, no DNA, not opened outside PCR room),
    2. a positive (DNA known to amplify with PCR conditions).
        - Use E. coli for general 16S eukaryotes
        - Use Pichia farinose for fungal Its
        - Use H. volcanii for archea
        - Ask about other positive controls for other primers sets

- Run three reactions for each sample. The three reactions will be combined in a later step.

- The following volumes are to be used for a 50 uL 16S PCR
  10 µL - 5X Buffer
  3 µL – dNTPs (10mM, 2.5mM of each, A,T,C,G)
  2 µL – BSA (20ug/mL)
  7 µL - $MgCl_2$ (25mM)
  1 µL – 8dF (10 uM) (AGAGTTTGTTCMTGGCTCAG)
  1 µL - K2R- (10 uM) (GTATTACCGCGGCTGCTGG)
  0.3 µL - AmpliTaq Gold (5U/uL)
  Water to bring final volume, after adding DNA, to 50 uL
  Template DNA (this is added last outside the PCR room)

- Use the following cycling parameters.
  94°C for 10 minutes
  30 cycles of (94°C for 1 minute, 46.5°C for 1 minute, 72°C for 2 minutes)
  72°C for 10 minutes
  4°C soak.

STEP 4- to see if your PCR worked

Electrophoresis
Use 3-5 uL of PCR product. Run on a 1.5% gel for 20-25 minutes at ≈80-100V

STEP 5- to combine the PCR replicates that worked and remove leftover salts, dNTPs, and primer

Using MoBio PCR Ultra-Clean kit

1. Add 5 volumes SpinBind solution to each well and pipet up and down well
2. Transfer 750 µl to the spin filter unit. Centrifuge for 30 sec at 10 x kg. Discard eluate.
3. Repeat step 3 until all PCR SpinBind mixture is filtered. You are combining the three PCR replicates at this point.
4. Add 300 µl of SpinClean buffer to spin filter and centrifuge for 30 sec at 10 x kg. Discard eluate.
5. Centrifuge spin filter for 120 sec at 10 x kg to remove any remaining fluid.
6. Transfer spin filter to clean 2.0 ml collection tube.
7. Add 60 µl of PCR water to spin filter and incubate 10 min.
8. Centrifuge for 60 sec at 10 x kg.
9. Discard spin filter and store at –20°C.

STEP 6 – Quantitate PCR product

Using the Bio-Tek Fluorometer determine the PCR product concentration by measuring the Cy5 incorporated fluorescent label from the forward primer

STEP 7 – produce the labeled fragments

Enzyme Digests (Amount of DNA digested varies depending on the samples being prepared. Ask for instructions before proceeding further.)

1. Digest 30 ng - 300 ng DNA.
2. Digest 5-10 ng of a E. coli digest standard. Do not use the E. coli genomic DNA. Use the digest standard which is E. coli PCR product with the fluorescent label.
3. For **DpnII** (10,000 U/mL) use 1.0 uL enzyme and 4 uL buffer per reaction. Add DNA and water to bring the volume 40 uL
   For **HaeIII** (10,000 U/mL) use 1.0 uL enzyme and 4 uL buffer per reaction. Add DNA and water to bring the volume to 40 uL. For HaeIII use Pichia farinose as the digest control.
   For **HhaI** (20,000 U/mL) use 0.5 uL enzyme, 0.4 uL BSA, and 4 uL buffer per reaction. Add DNA and water to bring the volume to 40 uL
4. Place tubes in PCR machine for 4 hours @ 37°C then cycle to either **65°C** for **DpnII** , **65°C** for **HhaI**, or **80°C** for **HaeIII** for 20 minutes to deactivate the enzyme and finally to 4°C for infinity.
5. Store the digests in the -20°C freezer until ready for ethanol precipitation.

STEP 8 – remove excess salts

Ethanol Precipitation (Note: prompt removal of samples from centrifuge will ensure minimal loss of sample.)

1. To the digest, add 100 µl (2.5 x digest volume) of cold 95% ethanol and 2 µl 3M NaAc pH4.6 (5% digest volume) and 1 uL glycogen (20 mg/mL)
2. Invert five times making sure the lids are securely on.
3. Place the tubes in the -20°C freezer for 30 minutes.
4. Centrifuge the tubes for 15 minutes at 5300 RPM to pellet DNA. (program 2)
5. Remove ethanol by inverting the PCR tray on a paper towel.
6. Add 100 µl of cold 70% ethanol.
7. Centrifuge the tubes for 5 minutes at 5300 RPM. (program 3)
8. Remove ethanol by inverting the PCR tray on a paper towel.
9. Centrifuge rack in inverted position on top of a paper towel for 1 min. @ 700 RPM to dry the pellet. (program 4)
10. Store the DNA in the -20°C freezer until ready to proceed to CEQ8000 preparation.

STEP 9 – Separates the labeled digested fragments

CEQ8000-sample preparation

1. Make a master mix of 20 uL formamide and 0.25 uL 600 base pair standard per reaction. Add 20 µl of the master mix to each tube.
2. Add one drop of mineral oil to the top of each well to prevent sample evaporation.
3. Run in the CEQ 8000
4. Look at your beautiful TRF patterns!!