96 well DNA Extraction Protocol for Plant and Lichen Tissue Stored in CTAB

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Abstract

This protocol is a modified version of the MoBio PowerPlant Pro 96 well DNA extraction kit for plant and lichen tissues that have been stored in CTAB buffer. Prior to being placed in CTAB, photosynthetic tissues from plants and lichens were surface-sterilized with sequential washes in 95% EtOH, 0.5% sodium hypochlorite, and 70% EtOH and air dried under sterile conditions. Twenty-four tissue pieces per host were placed in 750 uL of CTAB buffer. Four tubes per host individual were sampled for a total of 96 tissue pieces.

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Guidelines

- Gloves must be worn at all times. Change gloves frequently and decontaminate often with DNA Away.
- Pipetting must be done extremely carefully to minimize the risk of aerosols that can easily cause contamination between samples.
- Use only unopened, sterile, aerosol-resistant pipette tips (filter tips) to minimize contamination of the pipette shaft and your samples. Clean the body of the pipettes regularly with DNA Away.
- Discard pipette tips after each use to avoid cross-contamination. Eject pipette tips carefully to prevent aerosol formation or other splashing.
- Remember to always clean the hood and any equipment with DNA Away and decontaminate where possible with UV for 30 minutes prior to use to avoid cross-contamination.
- The pipettes used need to be calibrated annually.
- Be careful not to touch the inside lid of tubes as it will cross-contaminate samples.

Before start

You will need the following reagents and equipment for the protocol.

Reagents

-DNase Away

-Stainless Steel Beads, 3.2 mm (Next Advance Cat # SSB32)

-Stainless Steel Bead blend, 0.9-2.0mm (NextAdvance Cat # SSB14B)

-Zirconium Oxide Beads, 2.0 mm (Next Advance Cat # ZRoB20)

-Liquid Nitrogen

-Molecular grade 100% EtOH

-MoBio PowerPlant Pro 96 well DNA extraction kit (each kit processes 4 96 well plates)

Equipment:

-Laminar flow hood

-Lyophilizer

-Centrifuge capable of handling 24 1.5-2.0 mL tubes

-Centrifuge capable of spinning 96 well (deep) plates from MoBio at 4,000 x g.

-Next Advance Bullet Blender Storm or comparable bead beater

-Heat block and water bath at 65C

-Parafilm

-Sterile needle

-50 mL Falcon tubes (DNA/RNA free)

-1000 uL filter tips

-200 uL filter tips

-300 uL filter tips (best for transferring supernatant with multichannel pipette)

-Aluminum sealing tape

-Reagent reservoirs

-Two multichannel pipettes. One capable of small volumes (<100 uL) and another for pipetting large volumes (>200 uL)

-Two freezer blocks (-20C)

Protocol

SAMPLE PREPARATION

Step 1.

For each host individual, remove 4 CTAB tubes (each containing 24 tissue pieces) from -80 freezer and thaw at room temperature.

SAMPLE PREPARATION

Step 2.

Clean the laminar flow hood, pipettes, and tip boxes with EtOH and DNase Away. In the laminar flow hood, remove as much CTAB buffer as possible using a 200 ul pipette with filter tips. Discard CTAB buffer.

SAMPLE PREPARATION

Step 3.

Quickly after removing CTAB buffer from the tissue (don't let samples sit at room temperature for very long because the DNA will degrade) parafilm the opening of tube so that the lid remains open.

Use a sterile needle to poke 1-3 holes in the top of the parafilm so that the water can escape during lyophilization. Place tubes in the -20 or -80 freezer.

LYOPHILIZATION

Step 4.

Once all of your sample are cold, turn on the refrigerator for the lyophilizer and place the glass plate on the front (make sure there is no water in the chamber or drain tube).

LYOPHILIZATION

Step 5.

Once the lyophilizer temperature is at -60C, turn on the vacuum pump. Make sure that there is a vacuum seal on the lid and keep an eye on the pressure gauge. It must be below 150 miliTorr to work properly. If the pressure doesn't drop within an hour do not use the machine.

LYOPHILIZATION

Step 6.

Once pressure is at the appropriate level, release the pressure by turning the white port 180 degrees Quickly take frozen tubes out of freezer and place inside the lyophilizer chamber.

Close the lid and close the port. Make sure there is a vacuum seal and the pressure returns to <150 miliTorr.

LYOPHILIZATION

Step 7.

Lyophilize samples for a minimum of 48 hours. To remove the samples, first turn the white port on the lyophilizer 180 degrees to release the pressure in the chamber.

Once the pressure is released and the gauge reads 0, turn off the vacuum pump and then the refrigerator.

Remove the glass plate form the front and wipe out the water after it has melted (also shake out the drain tube).

DNA EXTRACTION PREP

Step 8.

In a sterile laminar flow hood, remove the parafilm from the tubes (being careful not to touch the inside lip of the cap) and add beads for homogenization.

For all plant samples except mosses, add 100 ul (3-4 beads) of the autoclaved 3.2 mm stainless steel beads and 100 ul of the autoclaved stainless steel bead blend using a sterile scoop.

For mosses and lichens, add 100 ul of the autoclaved 2 mm zirconium oxide beads to each tube with a sterile scoop. Close the lids securely.

DNA EXTRACTION PREP

Step 9.

Tubes with beads can be placed in a -20 freezer until ready to extract DNA.

DNA EXTRACTION

Step 10.

DNA EXTRACTION (all steps except bead beating and centrifugation done in sterile laminar flow hood)

**Always write down product #s for each kit used and perform a minimum of one extraction blank per kit.

Before beginning, turn on water bath to 65C (check temperature on a thermometer and not display); clean the laminar flow hood, pipettes, and tip boxes with EtOH and DNase Away; and pour liquid N into small dewer.

Note: the following protocol requires 106 mL of 100% EtOH per 96 well plate (in addition to what is added to solution PD5).

DNA EXTRACTION

Step 11.

Before starting, add 106 mL of 100% EtOH (molecular grade) to Solution PD5. Check the box and write the date on the label.

DNA EXTRACTION

Step 12.

Add RNase A solution to solution PD1 based on the number of samples you need to prep. (For each 96 well plate, add 300 uL of RNase A to 45 mL of PD1 in a sterile 50 mL tube.)

DNA EXTRACTION

Step 13.

To dissolve precipitates, heat solution PD2 at 60C on heating block prior to use.

DNA EXTRACTION

Step 14.

Drop 20 tubes containing beads and tissue into the liquid N for a minimum of 15 sec (until liquid N stops bubbling). Quickly remove each tubes with a forceps and place in the bead beater. *Do not allow tubes to thaw.*

At level 12, beat lichens for 30 sec and plants for 1 minute (FYI: some plants require > 1 min to grind tissue. If this is necessary, refreeze tubes liquid N and beat for an additional 1 minute. Repeat until tissue is ground). *Make sure that the tissue has been homogenized to a fine powder*. Place tubes in -20 freezer block.

DNA EXTRACTION

Step 15.

DNA EXTRACTION

Step 16.

Tubes might be frozen from -20 freezer block. Allow to thaw prior to bead beating an additional 15-30 sec to completely mix the tissue and reagents and remove the clump of tissue from the bottom of the tubes. Place tubes in a rack in the refrigerator until ready to proceed to step 21.

DNA EXTRACTION

Step 17.

Repeat steps 14 through 16 in sets of 20 tubes until 96 tubes have been processed.

DNA EXTRACTION

Step 18.

Remove 48 tubes from refrigerator and invert to mix. Place tubes in water bath at 65C for 15 minutes, inverting every 5 minutes during incubation.

DNA EXTRACTION

Step 19.

Centrifuge tubes at 13.2 rpm (13,000 x g) for 2 minutes.

Step 20.

Place another set of 48 tubes in water bath while other set is in centrifuge.

DNA EXTRACTION

Step 21.

DNA EXTRACTION

Step 22.

After incubation in water bath, centrifuge the 48 remaining tubes at 13.2 rpm (13,000 x g) for 2 minutes.

DNA EXTRACTION

Step 23.

Transfer the supernatant from each tube to a single well of a clean 1 mL Collection Plate. This will fill the remaining 48 wells on the plate.

**Be very careful not to touch pipette tips to the wrong wells. Change tips between samples.

DNA EXTRACTION

Step 24.

Seal the plate with aluminum sealing tape. Store at 4C (suitable for short-term storage, but do not leave longer than one hour).

Repeat steps 15-24 for an additional 96 samples (*i.e., two full plate).

DNA EXTRACTION

Step 25.

Begin this step once two 96 well plates are ready. Using a multichannel pipette, add 200 uL of PD3 to each well. Place Sealing Tape onto plate. Vortex plate for 5 seconds. Repeat for both plates. Incubate plates at 4C for 10 minutes.

DNA EXTRACTION

Step 26.

Centrifuge plates at 4,500 x g for 9 minutes (if centrifuge doesn't go this fast, calculate the amount of time to spin; see MoBio's user guide). Make sure that plates are balanced in centrifuge!

DNA EXTRACTION

Step 27.

Carefully remove plates from the centrifuge (do not tip, which will cross-contaminate your samples). In the hood, remove and discard Sealing Tape.

DNA EXTRACTION

Step 28.

Avoiding the pellet, transfer up to 600 uL of supernatant to a clean 2 ml Collection Plate. Repeat this for second 96 well plate.

(Optional: After this step, seal plates with aluminum sealing tape and freeze at -20 until two additional 96 well plates are ready to proceed to the next step. This way four plates can be processed at once).

DNA EXTRACTION

Step 29.

Using multichannel pipette, add 600 uL of solution PD4 (poured into a reagent reservoir) to the first row of the 2 mL Collection Plate. Very slowly pipette up and down to mix. Repeat for all remaining wells. Repeat for each 96 well plate.

**If you don't have a multichannel pipette that will pipete 600 uL at a time, you can do this in smaller (i.e., 200-300 uL) increments.

DNA EXTRACTION

Step 30.

Using multichannel pipette, add 600 uL of 100% EtOH to the first row of the 2 mL Collection Plate.

Gently pipette up and down to mix. Repeat for all remaining wells. Repeat for each 96 well plate.

DNA EXTRACTION

Step 31.

Place Spin Plate onto a new 0.5 mL Collection Plate. Repeat for each 96 well plate.

DNA EXTRACTION

Step 32.

Using multichannel pipette, load 550 uL of lysate from row 1 to corresponding row 1 of the spin column. Repeat for all remaining wells. Apply Centrifuge Tape to plate. Repeat for all 96 well plates.

DNA EXTRACTION

Step 33.

Centrifuge at 5 min at 4,500 x g. Very carefully remove plates from centrifuge, making sure not to tip the plates. In the sink, discard the flow-through from the 0.5 mL collection plates. Replace the same 0.5 mL Collection Plate beneath the same spin plate.

DNA EXTRACTION

Step 34.

In the hood, discard the Centrifuge Tape from all plates. Repeat steps 32-33 3X until all of the supernatant has been processed for all plates.

DNA EXTRACTION

Step 35.

In the hood, place the Spin Plate on a new 0.5 mL Collection Plate. Add 500 uL of Solution PD5 to each well of the Spin Plate (make sure 100% EtOH was added to PD5 prior to this step). Apply Centrifuge Tape. Repeat for each plate.

DNA EXTRACTION

Step 36.

Centrifuge at 4,500 x g for 3 minutes. Make sure all plates are balanced.

Discard flow-through and replace the same 0.5 mL Collection Plate beneath the Spin Plate. Repeat for each plate.

DNA EXTRACTION

Step 37.

Add 500 uL of 100% EtOH to each well of the Spin Plate. Apply a new piece of Centrifuge Tape. Repeat for each plate.

Step 38.

Centrifuge plates at 4,500 x g for 3 minutes. Make sure all plates are balanced.

DNA EXTRACTION

Step 39.

Discard the flow-through the replace the same 0.5 mL Collection Plate beneath the Spin Plate. Centrifuge for 7 minutes at 4,500 x g.

DNA EXTRACTION

Step 40.

In the hood, very carefully place the Spin Plate on a new Microplate. Remove the Centrifuge Tape and discard.

DNA EXTRACTION

Step 41.

Allow all plates to air dry in the hood for 10 minutes at room temperature.

DNA EXTRACTION

Step 42.

Using multichannel pipette, add 75 uL of PD7 (10 mM Tris, pH 8.0) to the center of the white filter membrane of each well. (Note: you can add 100 ul of PD7 if you want less concentrated DNA.) Apply a new piece of Centrifuge Tape. Incubate plate for 5 minutes at room temperature. Repeat for all plates

DNA EXTRACTION

Step 43.

Centrifuge plates at 4,500 x g for 3 minutes. In the hood, remove and discard Centrifuge Tape.

DNA EXTRACTION

Step 44.

Seal the Microplates with the Elution Sealing Mat (provided). DNA in the microplates is now ready for PCR.

DNA EXTRACTION

Step 45.

Store DNA at -20 or -80 until ready for PCR.

**Some samples may still need an additional processing with MoBio PowerClean Pro to remove PCR inhibitors.

DNA EXTRACTION

Step 46.

Optional: Once all 4 tubes from a single host individual have been extracted, pool into a single tube (total volume per host individual will be 200 uL).