MAY 02, 2023

A cost-effective way of extracting high molecular weight (HMW) DNA from low amounts of fresh or herbarium lichen material

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Protocol status: Working
We use this protocol and it's working

Created: Jun 27, 2022

Last Modified: May 02, 2023

PROTOCOL integer ID: 65476

Keywords: HMW extraction, lichen, long reads, short reads, SPRI beads extraction, low input, fungal DNA, lichen DNA, DNA extraction, PEG NaCl, enzymatic digestion, symbiont DNA, bacterial DNA, algae DNA, lichen fungi

ABSTRACT

Presented method allows the extraction of high molecular weight (HMW) DNA of all lichen symbionts using low input (5 - 100 mg) lichen material. Extractions using fresh and over 50-year-old herbarium material have been successful, yielding HMW DNA fragments from 50 to 200 kbp in length suitable for both short- and long-read sequencing. The protocol includes an initial acetone cleaning step to dissolve secondary lichen metabolites, which can later be identified using thin-layer chromatography (TLC). Apart from an overnight incubation during the enzymatic digestion of fungal, algal, and bacterial cells, this protocol provides a relatively fast and easy extraction method. A final size selection is achieved using Solid Phase Reversible Immobilization (SPRI) magnetic beads and a 20%-PEG-0.75M-NaCl buffer.

GUIDELINES

Handle samples very gently. Avoid vortexing, instead gently flick the tubes. Use wide-pored pipettes, which can be made by carefully cutting off the tip of a pipette with a sterile razor blade in a clean bench. During extraction, the number of tubes will double. In the last step, they will be combined again.
For long-read sequencing of HMW DNA use fresh lichen material. To test HMW DNA quantity and quality, capillary electrophoresis is recommended. For short-read sequencing, a quantification using a fluorometer (and gel electrophoresis) is recommended.
DNA purity can be examined using a UV-VIS spectrophotometer - an absorbance ratio of A260/A280 in the range of 1.8 – 2.0 and A260/A230 higher than 2.0 indicates good quality and purity of the DNA.

MATERIALS

To extract DNA from fresh or herbarium lichen material (20-100 mg), you will need thefollowing equipment and reagents:

Equipment:

- 2.0 ml **Protein LoBind tubes** (storage vessels) *[Eppendorf]*

- 1.5 ml **DNA LoBind** tubes *[Eppendorf]*
- Wide-pore **pipettes**
- **Magnetic stand** or plate
- **Thermomixer**, -shaker or heated orbital incubator
- **Centrifuge**
- Optional: **lab rocker** (useful for numerous samples)

Liquid stocks:

- 2.0 M **Sorbitol** $C_6H_{14}O_6$ (M_r = 182.17 g/mol)
- 0.5 M **EDTA** $C_{10}H_{16}N_2O_8$ pH 8 (M_r = 372.24 g/mol)
- 0.1 M **Citric Acid** $C_6H_8O_7$ (monohydrate M_r = 210.14 g/mol)
- 0.1 M **Sodium Citrate** $C_6H_7NaO_7$ (Monosodium citrate, anhydrous M_r = 214.11 g/mol)
- 1.0 M **DTT** (DL-Dithiothreitol) $C_4H_{10}O_2S_2$ (M_r = 154.25 g/mol)
- 1.0 M **Tris-HCl** (tris-HCl hydrochloride) $C_4H_{13}Cl_2NO_3$ pH 9.5 (M_r = 194.05 g/mol)
- 5.0 M **NaCl** (M_r = 58.4428 g/mol)

Enzymes:

- **Lysozyme** ≥20,000 U/mg solid, resuspend in 1 x PBS at 20 mg/ml *[from chicken eggwhite, MP Biomedicals]*
- **Chitinase** ≥200 U/g solid, dissolve the lyophilized powder in nuclease-free water (solubility: 0.90 - 1.10 mg/ml water) *[from Streptomyces griseus, Sigma -Aldrich]*
- **Beta-Glucuronidase** 1784 U/mg protein *[from Helix pomatia, MP Biomedicals]*
- **Cellulase** endo-1,4-ß-D-glucanase 60 U/mg protein at pH 6.0 and 40°C *[from B. amyloliquifaciens, Megazyme]*
- **Protoplast F -** lysing enzyme mixture containing cell wall degrading enzymes used for generating protoplasts: exo-1,3-β-Glucanase ~ 1000 U/mL, endo-1,3-β-Glucanase ~ 45 U/mL *[Megazyme]*
- **Proteinase K -** lyses the tissue and releases the DNA/RNA: >600 mAU/ml *[Qiagen]*

Reagents:

- **2-Mercaptoethanol -** disrupts disulfide bonds in proteins and prevents polyphenols from oxidizing (Proteinase K treatment is recommended) > 99.0% pure liquid (M_r = 78,13 g/mol, 14.3 mol/L) *[merck]*
- Buffer **ATL -** tissue lysis buffer for purification of nucleic acids *[QIAGEN]*
- Buffer **AL -** for DNA isolation *[QIAGEN]*
- only for long-read sequencing: **PEG-NaCl-Buffer**

  20% PEG 8000 Polyethylen glycol/oxide - hydrophile, applies osmotic pressure, no interactions with biological material

  0,75 M NaCl (M_r = 58.4428 g/mol)

Other:

- nuclease-free **water**

- 96-100% **ethanol**
- cold **acetone** (stored at -20 °C)
- **SPRIselect** magnetic beads *[Beckman Coulter]*
- only for a low amount of input material: **carrier RNA**

SAFETY WARNINGS

ß-Mercaptoethanol is a **toxic**, **corrosive**, and **environmentally hazardous** chemical. When handling it, always wear gloves and work in a fume hood. Pierce the safety seal using a syringe to access the chemical.

BEFORE START INSTRUCTIONS

- Prepare and store enzymes according to the manufacturer's instructions. Thaw enzymes just before use
- If Buffer ATL contains precipitates, dissolve by heating to 70 °C with gentle agitation
- Prepare liquid stocks ($m[g] = M_r * c[mol/L] * V[L]$) and fill up with nuclease-free water to desired volume
- For long-read sequencing (fragments > 1 kbp) prepare PEG-NaCl-Buffer:
  0.75 M NaCl
  20% PEG: V% = (v/v)% = volume of solute / volume of solution * 100

SAMPLE PREPARATION

1

- If necessary clean lichen material using nuclease-free water and dry the sample
- Cut lichen material into small pieces (µm) using a sterile razor blade or scalpel
- Transfer samples into 2.0 ml Eppendorf Protein LoBind tubes and store them at -80 °C
- Add **400 µL cold acetone** (stored at -20 °C), shake gently and remove the supernatant. Supernatant can be stored for use in thin-layer chromatography (TLC)
- Repeat until supernatant is clear
- Put the tubes in a heating block set at 30 °C under the fume hood until the samples are dry

BUFFER PREPARATION

2

Buffer 1: prepare 1 mL/sample (plus overhang) using liquid stocks

- **100 mM Tris-HCl**
- Add **10 mM DTT** solution just before use

Buffer 2: prepare 500 µL/sample (plus overhang) under the fume hood using liquid stocks

- **1 M Sorbitol**
- **25 mM EDTA**

- **5.9 mM Citric Acid**
- **4.1 mM Sodium Citrate**
- **14 mM ß-Mercaptoethanol**

ENZYMATIC DIGESTION - DAY 1

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- Add **1 mL Buffer 1** to each sample
- Mix thoroughly by flipping the tube
- Incubate for 15 minutes at RT
- Centrifuge at 3000 x g for 2 minutes. If necessary repeat at a higher speed (e.g. for crust lichens) until a precipitate forms
- Discard supernatant
- Add **500 µL Buffer 2** to each sample
- Mix enzymes well before taking an aliquot. Then add **5 µL** each of **Glucuronidase**, **Cellulase**, **Protoplast F**, and **Lysozyme** to each sample
- Add **90 µL Chitinase** to each sample
- Place the tubes in a thermomixer overnight at 35 °C and rotate at 750 rpm for 9 seconds every 5 minutes

ENZYMATIC DIGESTION - DAY 2

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- Set thermomixer to 56 °C
- Centrifuge samples with 3000 x g for 2 minutes
- Discard supernatant while working under the fume hood
- Add **600 µL Buffer ATL** and mix slowly and carefully
- Add **20 µL Proteinase K** and mix slowly and carefully
- Incubate in a thermomixer at 56 °C with shaking at 600 rpm for at least 1 hour
- Remove samples and set the thermomixer to 70 °C
- Briefly centrifuge tubes to remove drops from inside the lid
- Add **600 µL Buffer AL**, close the lid, and mix gently using the lab rocker at 5 rpm, or by light shaking. Sample and Buffer AL have to be thoroughly mixed to yield a homogeneous solution
- For low-input samples, an optional step is to dissolve **1 µg** of **carrier RNA** in **1 µL** of **buffer ATL**. Then, add **1 µL** of the **dissolved carrier RNA** to each sample
- Incubate tubes at 70 °C in a thermomixer with shaking at 600 rpm for 10 minutes
- Briefly centrifuge tubes to remove drops from inside the lid
- Add **300 µL ethanol** (96-100%), close the lid and mix by light shaking or on the lab rocker at 5 rpm for at least 10 seconds. Mix samples and ethanol thoroughly to ensure efficient binding
- Briefly centrifuge tubes to remove drops from inside the lid
- Transfer **700 µL** of the supernatant into a labelled 1.5 mL DNA LowBind tube. Transfer the remaining **~800 µL** into a second labelled tube, resulting in two storage vessels per sample

EXTRACTION AND SIZE SELECTION

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- Thoroughly shake the **SPRIselect** bottle to resuspend the SPRI beads
- Add ***1.2 SPRIselect** to the sample (sample volume [µL] * 1.2 = SPRIselect [µL])
- For long-read sequencing, an optional step is to add ***0.65** of **PEG-NaCl-Buffer** (sample volume [µL] * 0.65 = PEG-NaCl-Buffer [µL])
- Mix samples gently using the lab rocker, or rock the storage vessels from side to side and incubate at RT for 30 seconds. Mix well, as insufficient mixing will lead to inconsistent results.
- Repeat the mixing and incubating step
- Place tubes into the magnetic stand and allow the SPRI beads to settle to the magnets (settle times will vary)
- Remove the clear supernatant. Be aware that significant bead loss will result in reduced yield
- Add **700 µL 5 M NaCl** without removing the tubes from the magnetic stand, incubate for 1 minute, and discard the supernatant
- Repeat the previous washing step
- Remove the tubes from the magnetic stand and add **≥ 20-70 µL of nuclease-free water** (or standard buffer like Tris or TE)
- Carefully resuspend the beads by slowly mixing them on the lab rocker or by pipetting ten times using wide-pore pipettes until homogeneous
- Incubate at RT for 1 minute
- Place the tubes on a magnetic stand, allowing the SPRI beads to settle to the magnets. If the elution volume is too low so that beads cannot settle to the magnet, flip the magnetic stand while holding the reaction vessels in place
- Transfer the eluate (HMW DNA) into a labelled 1.5 ml DNA LowBind storage vessel, and combine divided samples
- Check DNA quantity and fragment size. If DNA fragments are too short, repeat the size selection part using ***0.8 SPRIselect** and ***1.0 PEG-NaCl-Buffer**