

Oct 04, 2024

S. palustris leaves sampling and RNA extraction

DOI

<https://dx.doi.org/10.17504/protocols.io.6qpvr85jzlmk/v1>

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Protocol Citation: Maria Stefanie Dwiyanti 2024. *S. palustris* leaves sampling and RNA extraction . protocols.io
<https://dx.doi.org/10.17504/protocols.io.6qpvr85jzlmk/v1>

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Protocol status: Working

We use this protocol and it's working

Created: October 02, 2024

Last Modified: October 04, 2024

Protocol Integer ID: 108810

Keywords: Stenochlaena palustris, fern, field, CTAB buffer, RNA extraction, rna extraction method, rna extraction, protocol for leaf sampling, leaf sampling, extraction, rna

Funders Acknowledgements:

Heiwa Nakajima Foundation

Abstract

This is a protocol for leaf sampling of *S. palustris* in natural habitats as well as RNA extraction method using CTAB+PVP buffer.

The extraction will require 3 days.



Materials

REAGENTS (the brands are the ones were used, but can be replaced with other brands)

- 2% CTAB Extraction buffer containing 4% PVP (1 Liter) - store at room temperature (RT)
1 M Tris-HCl (pH 8.0) 100 ml (100 mM) ; Fujifilm Wako, 015-20093 (1 kg) or Fujifilm Wako, 019-20091 (100 g)
0.5 M EDTA (pH 8.0) 40 ml (20 mM) ; Nippongene, 311-90075
NaCl 71.9 g (1.4 M) ; Fujifilm Wako, 191-01665
Add MilliQ water up to 1 liter
Autoclave 120°C, 30 min
When still warm, add following CTAB and mix well
CTAB (Hexadecyltrimethyl-ammonium bromide) 20 g ; Fujifilm Wako, 030-02105

Add PVP fresh before each use
Polyvinylpyrrolidone (PVP MW 4.0) 40 g ; Sigma Aldrich, 9003-39-8
- Chloroform • isoamylalcohol (24:1) , store in 4°C (Chloroform: Fujifilm Wako, 67-66-3; isoamylalcohol : Fujifilm Wako, 133-12011)
Add 20.8 mL isoamylalcohol to newly opened 500 ml chloroform. No need to autoclave
- Phenol:chloroform:isoamylalcohol (250 ml), store in 4°C ; Nippongene 311-90151
- DNase-RNase-free water for molecular biology, store in RT (not DEPC-treated water)
- 8M LiCl solution, store in RT ; Fujifilm Wako, 129-05243
Note: Add 24.16 g lithium chloride slowly to 50 mL water. LiCl releases heat when dissolve.
Autoclave 121°C, 30 minutes
- 80% ethanol, store in -30°C; Wako, 057-00456
add 40 ml 99.5% ethanol to 10 ml molecular grade water
- 3M NaOAc solution (pH 5.2), store in RT ; Nippongene, 316-90081
- 99.5% ethanol, store in -30°C ; Wako, 057-00456
- DNase I (RNase free), store in -30°C ; Nippongene, 314-08071
- Recombinant RNase Inhibitor ver.2.0, store in -30°C; Takara, 2315A
- Mortar, pestle, spatula (clean with 70% ethanol or RNase Knockout (Fujifilm Wako, 181-03381) or other RNase removal spray
- RNAlater stabilization solution, store in RT ; Invitrogen, AM7020



Protocol materials

 RNA later Thermo Fisher Scientific Catalog #AM7020

Before start

1. Confirm the SDS (Safety Data Sheet) for safety warnings and hazards for all reagents.
2. Use fumehood when working with phenol:chloroform:isoamylalcohol (PCI) and chloroform:isoamylalcohol (CI).
3. Follow PCI and CI disposal instruction.
4. Use gloves during RNA extraction
5. Wipe equipments with 70% ethanol or RNase removal spray

Collecting *S. palustris* from field

- 1 Prepare RNAlater solution in 50 ml falcon tubes.

 RNAlater Thermo Fisher Scientific Catalog #AM7020

- 2 Choose young leaves without fiber.



Cut leaves into small pieces into the RNAlater solution. Proportion of leaves and RNAlater solution should be maximum 1:5.



Amount of leaves in RNAlater solution

- 3 Store 50 ml tubes inside ice box/ice packs for shipment.
- 4 Leaves in RNAlater storage can be stored at 4°C up to 1 month. After that, leaves will show reddish color. It is difficult to extract RNA from reddish color leaves.

RNA extraction

- 5 Warm CTAB extraction buffer to 65°C (in water bath or beaker with warm water).
- 6 Add leaf materials into mortar. Wipe RNAlater left on leaves using kimwipe.



The amount of leaves from the 50 ml tubes. 6 ml extraction buffer or more will be enough for the leaves.

- 7 Add liquid nitrogen to mortar. Quickly crush plant material to fine powder. Keep adding liquid nitrogen while crushing, to keep plant material frozen.
- 8 Add 1 spatula of sample powder to 1 ml warm CTAB buffer in 2 ml tubes. Vortex to mix the material and buffer, make sure no clumps. Add more CTAB buffer if needed.
- 9 Add 700 μ L chloroform:isoamylalcohol (24:1). Vortex to mix.
- 10 Centrifuge at 15,000 rpm, 10 min, 4C
- 11 Transfer upper phase to new 1.5 ml tube.



- 12 Repeat step 9-11 twice (total 3 times). At this stage the upper phase should be greenish-clear color.
- 13 Add 500 μL of 8 M LiCl, mix by inverting tubes several times.
- 14 Prepare ice in a styrofoam box. Put tubes in ice and store overnight at 4°C (ideally 12-15 hours, before solution turns red)
- 15 Centrifuge at 15,000 rpm, 45 min, 4°C. (Thaw DNaseI buffer while waiting).
- 16 White-transparent pellet should appear at bottom. Discard supernatant using pipette.
- 17 Add 80% cold ethanol 50 μL .
- 18 Centrifuge 15,000 rpm, 5 min, 4°C.
- 19 Discard 80% ethanol by pipette.
- 20 Dissolve pellet in 270 μL DNase-RNase free water. Pellet from 6 tubes can be combined into 1 tube.
- 21 Add 27 μL of 10×DNase I buffer to solution. Add also 2 μL of DNase I (RNase free) and 1 μL of RNase inhibitor.
- 22 Incubate at 37°C, 30 minutes.
- 23 Add 300 μL of phenol:chloroform:isoamylalcohol (25:24:1), invert tubes to mix.
- 24 Centrifuge 15,000 rpm, 3 min, 4°C.



- 25 Transfer supernatant to new 1.5 ml tube, add 300 μ L of chloroform:isoamylalcohol invert tubes to mix.
- 26 Centrifuge 15,000 rpm, 3 min, 4°C.
- 27 Transfer supernatant to new 1.5 ml tube, 30 μ L of 3M NaOAc and 700 μ l of 99.5% ethanol (4°C), mix well by inverting tubes.
- 28 Store in -30°C, overnight.
- 29 Centrifuge 15,000 rpm, 45 min, 4°C.
- 30 Discard supernatant using pipette. White-transparent pellet should appear at the bottom of tube.
- 31 Add 100 μ L of 80% ethanol (4°C),
- 32 Centrifuge 15,000 rpm, 3 min, 4°C .
- 33 Discard solution using pipette, open tubes on ice to dry pellet (a few minutes).
- 34 Add 50 μ L of water to dissolve pellet.
- 35 Quantify with Nanodrop and check quality using gel electrophoresis
- 36 Store RNA at -80°C.



Protocol references

1. Kiss, T., Karácsony, Z., Gomba-Tóth, A. *et al.* A modified CTAB method for the extraction of high-quality RNA from mono-and dicotyledonous plants rich in secondary metabolites. *Plant Methods* **20**, 62 (2024).
<https://doi.org/10.1186/s13007-024-01198-z>