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S. palustris leaves sampling and RNA extraction

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We use this protocol and it's working

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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)

1. 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

2. 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

3. Phenol:chloroform:isoamylalcohol (250 ml), store in 4°C ; Nippongene 311-90151

4. DNAse-RNAse-free water for molecular biology, store in RT (not DEPC-treated water)

5. 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

6. 80% ethanol, store in -30°C; Wako, 057-00456
   add 40 ml 99.5% ethanol to 10 ml molecular grade water

7. 3M NaOAc solution (pH 5.2), store in RT ; Nippongene, 316-90081

8. 99.5% ethanol, store in -30°C ; Wako, 057-00456

9. DNase I (RNase free), store in -30°C ; Nippongene, 314-08071

10. Recombinant RNase Inhibitor ver.2.0, store in -30°C; Takara, 2315A

11. Mortar, pestle, spatula (clean with 70% ethanol or RNase Knockout (Fujifilm Wako, 181-03381) or other RNAse removal spray

12. RNAlater stabilization solution, store in RT ; Invitrogen, AM7020

Protocol materials

RNAlater **Thermo Fisher Scientific Catalog #**AM7020 Step 1

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

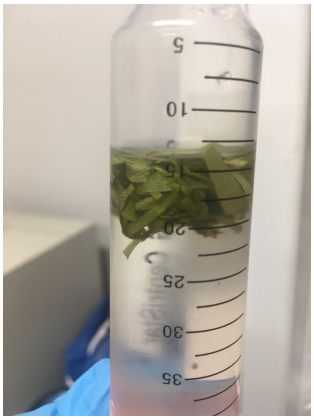
Collecting *S. palustris* from field

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 µL 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 µL 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 µL of 3M NaOAc and 700 µl 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 µL 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 µL 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