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A modified CTAB method for the simultaneous extraction of host and pathogen total RNA in inoculated conifer plants V.1

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Abstract

High-quality and high-yield RNA is a powerful source of data that find multiple application in biology. Transcriptomics can be utilized to characterize biological process of well studied systems, as well as non-model organism. This can render crucial information that may be an insightful contribution to the field of biology. In this protocol, we describe a method for the extraction of RNA processing recalcitrant samples such as tree bark. A layer of living tissues lies below the bark of trees, and it is the area where many process happen in the development of a tree. In this particular case, we applied this technique for the elucidation of molecular interaction between cypress plants (*Cupressus sempervirens*) and the causal agent of Cypress Canker Disease (the ascomycetes fungus *Seiridium cardinale*). After the inoculation of plants, and sample collection, we attempted to extract RNA with kits from different vendors, but all of them underperformed. With this protocol, were able to obtain high-quality and high molecular weight RNA, suitable for downstream application. Ultimately, this method has proven to be reliable, low-cost and exceptionally successful. The outcome of this procedure allowed us to characterize host-pathogen interaction in a non-model pathosystem. Moreover, we contributed to the field of forest pathology providing insight about the molecular interaction between a conifer plant and a canker-causing pathogenic fungus.

Guidelines

This protocol describes the process necessary for an RNA extraction. The method presented here is based on the protocol described in Chang et al., 1993. This workflow is suitable for obtaining RNA from all plant tissue (e.g. leaves, root, bark), and was specifically designed for conifers trees. It can be used for other plant species and performs well even when attempting to extract RNA from tissue coming from non-plant organism. The total RNA obtained enable the downstream application for transcriptomics analyses, providing a high molecular weight samples. It is suitable for the application of Dual RNA-seq techniques, in which plant tissues are inoculated with a pathogen.

Materials

Lab Equipment

- Refrigerated microcentrifuge
- Set of pipettes (P-1000, P-200, P-20, P-2)
- 50 ml Falcon tubes
- 15 ml Falcon tubes
- 1.5 ml Eppendorf tubes
- 2 ml Eppendorf tubes
- Liquid Nitrogen (LN2)
- Cryo-gloves and goggles for steps involving LN2
- Long forceps for submerging tubes in the LN2
- Autoclave-safe mortar and pestle (we recommend a set of 12 so you can work in rounds of 12 samples if processing a large amount)
- Spinner
- Vortex
- Magnetic rack

Chemical preparation

- DEPC treated H2O
- Chloroform:Isoamyl alcohol (24:1)
- SSTE Buffer:
 1. 1.0M NaCl
 2. 0.5% SDS
 3. 10mM TrisHCl (pH 8.0)
 4. 1mM EDTA (pH 8.0)
- CTAB Extraction Buffer:
 1. 2% PVP (polyvinylpyrrolidone K 30)
 2. 100 mM Tris-HCl (pH 8.0)
 3. 25 mM EDTA
 4. 2.0 M NaCl
 5. 0.5g/L spermidine (mix and autoclave)
 6. 2% β -mercaptoethanol (add just before use)
- 10 M Lithium Chloride solution
- SPRI bead



Safety warnings

⚠ This protocol involves the use of harmful chemicals such as chloroform and Beta-mercaptoethanol and Chloroform-Isoamil Alcohol.

It is strongly advised to always work under a chemical hood when handling such harmful chemicals. In particular, wear a mask and double nitrile gloves when handling the Chloroform-Isoamil Alcohol.

It is recommended to place the centrifuge under the chemical hood to avoid exposure to toxic aerosol.

If spilling occurs, act accordingly to your institution's safety guidelines.

Before start

Make sure to work in a clean environment.

Make sure that your lab has a well functioning chemical hood, it is important to utilize it for steps involving beta-mercaptoethanol and chloroform-isoamil alcohol.

Place your centrifuge under the chemical hood in order to avoid inhalation of harmful aerosols.

Clean all bench-tops, centrifuge, and pipette set, with 70% Ethanol and RNase zap.

Always use filtered pipette tips.

Always use DEPC nuclease-free water for reagent preparations, RNA pellet suspension, and dilutions.



Sample preparation

- 1 Assuming that sampling has been done and that tissue collected has been flash frozen by immersion in liquid nitrogen (LN2), and stored in a -80°C fridge, this first step requires a mortar and pestle as well as liquid nitrogen LN2.
 - 1.1 Before starting: sterilize mortar and pestle in autoclave. We recommend working in batches of 12 or 24 samples so you will need an equal amount of mortar and pestles.
 - 1.2 Place the frozen samples's tissue in mortar and pour LN2 to make the sample bubble, careful to not cause tissue to jump out of the mortar.
 - 1.3 Use the pestle to grind the sample. It is recommended to keep grinding the sample until a very fine powder is obtained. If needed, gradually add LN2 while grinding to maintain low temperature.
 - 1.4 Put the powder in a new sterile vial. A screw cap vial, such as Falcon 15mL tubes is recommended, because microcentrifuge tube cap may suddenly pop when re-exposed to room temperature after staying in very low temperatures. NOTE: Glass tubes are also challenging because ink, tape, or stickers don't work at very low temperatures. Store the ground samples in -80°C fridge.

Extraction

10m

- 2 NOTE: because of the overnight precipitation, it is recommended to start this process in the afternoon.

Before starting: preheat a water bath and a dry thermal bath, both at 65°C.
Heat the CTAB extraction buffer at 65°C.

 - 2.1 Take samples from the -80°C and place them on ice or a cool rack.
 - 2.2 Inside the chemical hood, add beta-mercaptoethanol 2.67% (20 µL) to each sample. 
 - 2.3 Add 750 µL of warm CTAB extraction buffer to each sample. 
 - 2.4 Mix by inverting the tubes, ensuring that the buffer thoroughly makes contact with all of the powdered tissue. Do not shake.



- 2.5 Incubate samples in dry thermal bath for 10 minutes; mix by inverting tubes after 5 minutes. Do not shake.

10m



Washing

20m

- 3 Perform the first washing.
Bring the samples back to the chemical hood. Add 750 μ L of Chloroform:Isoamil alchohol (24:1) (CHISAM) to each sample.
- 3.1 Mix by inverting the tubes. Do not shake.
- 3.2 Centrifuge at 4,500 rpm for 20 minutes at room temperature. It is preferable to place centrifuge in chemical hood for the remainder of the extraction process.
Prepare new set of 2mL microcentrifuge tubes.
- 3.3 Collect the supernatant and transfer into the new 2mL microcentrifuge tubes. At this stage, it's not a problem if some plant material ends up with the collected supernatant. Dispose of the remaining liquid.
- 4 Perform the second washing.
Add 750 μ L of Chloroform:Isoamil alchohol (24:1) (CHISAM) to each sample.
- 4.1 Centrifuge at 4,500 rpm for 20 minutes at room temperature.
Prepare new set of 2mL microcentrifuge tubes.
- 4.2 Collect the supernatant and transfer into the new 2mL microcentrifuge tubes. Again, dispose of the remaining liquid.

20m



Overnight precipitation

- 5 To each sample, add 1/4 volume of Lithium Chloride (LiCl) 10M.
- 5.1 Place each tube in a styrofoam box filled with ice.
- 5.2 Place the box in a 4°C fridge or a 4°C cold room, and leave samples to decant overnight.





Pellet precipitation

30m

6 Before starting:

- 1) Preheat a water bath and a heat block at 60°C.
- 2) Heat the SSTE buffer at 60°C.
- 3) Cool the centrifuge at -4°C.

6.1 Centrifuge samples at 10,000 rpm for 30 minutes at -4°C.

30m



6.2 Remove samples and place them in a styrofoam box filled with ice.

Pellet re-suspension

10m

7 Add 700 µL of warm SSTE buffer. If needed, pipet to mix the buffer inside the tube and re-suspend the pellet by disturbing it. At this stage, the pellet may be a dark visible brown or a very faint, clear smudge. It is not uncommon to not see the pellet at all.

7.1 Incubate samples at 60°C for 5-10 minutes to re-suspend the pellet.

10m



Washing

10m

8 Perform the third washing.
Bring the samples back to the chemical hood. Add 700 µL of CHISAM to each sample.



8.1 Mix by inverting the tubes.

8.2 Centrifuge at 4,500 rpm for 10 minutes at room temperature.
Prepare new 2mL microcentrifuge tubes.

10m



8.3 Collect the supernatant and transfer into the new 2mL microcentrifuge tubes. Dispose of the remaining liquid.

9 Perform the fourth washing.
Add 700 µL of CHISAM to each sample.



- 9.1 Centrifuge at 4,500 rpm for 10 minutes at room temperature. Prepare new set of 2mL microcentrifuge tubes.
- 9.2 Collect the supernatant and transfer into the new 2mL microcentrifuge tubes. Dispose of the remaining liquid.

Ethanol wash

1h 34m

- 10 Add 2 volumes of cold absolute ethanol.
- 10.1 Incubate at -80°C for 1 hour.

NOTE: now is a convenient time to prepare the gel for imaging the extracted product. In the meantime, cool down the centrifuge to -4°C.
- 10.2 Centrifuge samples at 13,000 rpm for 30 minutes at -4°C.
- 10.3 Carefully discard the supernatant and place tubes in ice or cold rack. Tip: avoid tilting the tube too quickly to avoid disturbing the pellet; avoid tilting more than 90°. At this stage, the pellet should appear as a small clear or white smudge.
- 11 Wash with 700 µL of cold 70% ethanol.
- 11.1 Centrifuge samples at 10,000 rpm for 2 minutes at -4°C
- 12 Discard the supernatant and place tubes in ice or cold rack.
- 12.1 Centrifuge samples at 10,000 rpm for 2 minutes at -4°C
- 12.2 Discard the supernatant and let the tubes air dry in the hood. You can use P10 pipette to gently remove the excess ethanol to advance the drying stage.

1h



30m



2m



2m



Elution

13 When the pellet is dry, add 25-50 μL of DEPC H_2O .

Note: The amount of water used to elute the samples depends upon the concentration of RNA you expect to obtain. It is recommended to have at least 100 ng/ μL to start preparing the library.

13.1 Store samples in -80°C

Final notes

- 14
- It is recommended have a rough estimate of the concentrations and quality by measuring with Nanodrop. For more accurate quantifications, use Qubit.
 - It is recommended to make gel images for each extracted samples.
 - Before proceeding with library preparation, it is advisable to measure RNA integrity with bioanalyzer.
 - This is a very lengthy extraction and takes some time to master. Begin with extracting a small sets of just a few samples to familiarize with this procedure before increasing to sets of 24 samples.

Extra: RNA beads clean up

15

Note: This last step explain a cleanup procedure using enzymatic DNA digestion and beads. Make sure to bring SPRI beads to room temperature; vortex thoroughly to ensure that the beads are fully resuspended.

15.1 Dilute your samples to a concentration of 16.6 μL in 100 μL volume. This amount can be adjusted accordingly with the RNA yield that you have obtained. We recommend to have > 1000 ng of total RNA in 100 μL before starting with the clean up.

15.2 Add 1 μL DNase of your choice and 10 μL of 10x buffer from your DNase kit.

15.3 Incubate at 37° for 10 minutes.

15.4 Add 200 μL (2x volume) of SPRI beads. Mix on touch vortex and spin down.

15.5 Incubate at room temperature for at least 5 minutes to maximize recovery.



- 15.6 Carefully place samples on a magnet rack for 5 minutes or until the beads have been fully collected on the tube wall and the solution is visibly clear. You may notice that some beads remain fixed at the bottom of the tube. We recommend gently moving the tube up and down to guide all the beads to the magnet point at the side of the tube.
- 15.7 Remove all supernatant, making sure not to touch the beads. Dispose of the supernatant.
- 15.8 Add 200 μ L of freshly prepared 80% ETOH. Again, take care not to disturb the beads.
- 15.9 Incubate at room temperature for 2 minutes without removing the tubes from the magnet or disturbing the beads.
- 15.10 Carefully remove and discard the ETOH.
- 15.11 Repeat steps 15.8 to 15.10 for a total of 2 washes.
- 15.12 Allow remaining ETOH to evaporate by air drying the beads for 3-5 minutes. The beads are sufficiently dry when cracks begin to appear in the bead pellet and no visible liquid remains.
- 15.13 Re-suspend beads in 32 μ L.
- 15.14 Incubate at room temperature for 2 minutes.
- 15.15 Place samples on magnet until supernatant is clear. Again, you may need to guide remaining beads at the bottom to join at the magnet on the side of the tube.
- 15.16 Transfer 30 μ L of supernatant to new tube.

Final remarks

- 16 At this point the RNA should be purified and ready to be used for library prep.



Protocol references

Chang, S., Puryear, J. & Cairney, J. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Rep* 11, 113–116 (1993). <https://doi.org/10.1007/BF02670468>