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We use this protocol and it's working

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RNA Extraction Protocol for Leaves with High Content of Secondary Metabolites

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ABSTRACT

Protocol used for extraction of total RNA from plant material rich in secondary metabolites.

MATERIALS

- Trizol - TRI Reagent Sigma Aldrich T9424;
- Chloroform - CHCl3 GC-MS grade;
- PVPP - Sigma P6755;
- DEPC - Sigma D5758;
- Isopropanol - Sigma Molecular Biology - 200 Proof - >99.45% - Sigma I9516;
- Ethanol - Sigma Molecular Biology - 200 Proof - >99.45% - Sigma E7023;
- Sterile Milli-Q water treated with 0.1% DEPC;
- Ethanol 75% - Prepared with sterile DEPC 0.1% water;
- Pipettes - P1000, P200, P100, P10;
- Tips - P1000, P200, P10;
- Microtubes of 1,5mL;
- Thermomixer;
- Refrigerated centrifuge;
- Freezer -20ºC;
- Liquid nitrogen;
- Styrofoam boxes that fit all the samples;
- Ice;
- Rack for 1.5 mL microtubes;
- Exhaustion hood;
- Nitrile gloves;
- Vortex;
- Bleach;
- TAE or TBE 1X;
- Ball mill.

SAFETY WARNINGS

***BOOK ALL EQUIPMENT IN ADVANCE AND MAKE SURE ALL THE MATERIAL WILL BE AVAILABLE.

***KEEP SAMPLES ON ICE DURING ALL HANDLING, AND STORE FINAL RNA AT -80 °C.

BEFORE START INSTRUCTIONS

Important details:
*Sterilize everything before and ensure that all material is suitable for RNA extraction (molecular grade).
*Aliquot the Trizol, Chloroform and Ethanol 99% for Mol. Bio. in 50 mL Falcons, and leave them cold before starting the extraction (-20ºC).
*Leave all 1.5 mL tubes ready for appropriate tube changes (2 changes).
**Make a maximum of 12-16 tubes per shift (up to 24 with two people).
*Samples taken from the -80 °C freezer should be kept in a bottle of liquid nitrogen. When adding the Trizol, we must take the samples out of the bottle and immediately add the Trizol to avoid enzymatic degradation of the RNA.
**Add as soon as possible the 500 uL of Trizol.
***Before starting, read the protocol and ensure that the necessary volume of each reagent is available.
*Before starting, place a microtube rack that fits all your samples in the -20 °C freezer.
75% ethanol should be prepared with ***NEW DEPC H2O and Ethanol for Molecular Biology 200 Proof >99.45%.

Procedure

21h 28m

1 Collect the plant material and freeze it immediately in liquid nitrogen; 30m

2 Macerate and transfer approximately 20 to 30 mg (max. 50 mg) to a 1.5 mL microtube;

3 Add 500 uL of TRIzol to every 3 tubes at once (with two people, otherwise from tube to tube if alone); 30m

3.1 Add in each tube 30mg of PVPP (2 full aliquot spoons); 2m

4 Homogenize by vortexing briefly until the solution turns brown (5 seconds); 5m

5 Incubate at room temperature for 5 minutes (dissociate ribonucleoproteins); 5m

6 Add 100 uL of cold Chloroform (-20ºC); 3m

7 Vortex vigorously for 15 seconds; 5m

8 Incubate in the thermomixer at 23°C (room T), 700 RPM for 15 min; 15m

9 Centrifuge at 12,000 RPM for 15 min at 4°C to separate the two phases: organic and aqueous 15m

(RNA);

10 Collect the aqueous phase (200 to 300uL - as much as you can get) and transfer to new, labeled microtubes (1.5 mL) (we collected 250uL for safety); 10m

***Don't touch the middle phase with the pipette tips.**

11 Add 1 volume of chloroform (200 to 300 uL) - 250 uL in our case; 3m

12 Invert the tubes 20x using a microtube rack, or gently vortex the tubes for 2 seconds; 2m

13 Incubate in the thermomixer at 23°C (room T), 700 RPM for 10 minutes; 10m

14 Centrifuge at 13,000 RPM for 10 minutes at 4°C; 10m

15 Collect 100 to 200 uL of the supernatant (we collected 175 uL for safety) and transfer to new, labeled microtubes (1.5 mL); 25m

16 Add **1 volume** of ice-cold isopropanol (175 uL) - (leave at -20°C until the time of application). 3m
*Use only Isopropanol Molecular Biology Grade - 200 Proof (>99.45%);

17 Invert the tubes for 30 seconds using a microtube rack or gently vortex the tubes for 2 seconds; 2m

18 Incubate for 30 min at -80 °C (ultra freezer) in a new rack previously placed at -80 °C or leave overnight at -20 °C; 12h

19 Centrifuge at 13,000 RPM for 15 minutes at 4°C; 15m

20 Carefully discard the isopropanol with the aid of pipette tips; 15m

21 Add 500 uL of 75% ethanol to wash the pellet and rehydrate the RNA 3m
- Use Molecular Biology Grade Ethanol - 200 proof to prepare this solution;

***We must add the 75% EtOH solution directly to the RNA pellet, homogenizing by vortexing (lightly tapping the vortex, <1 second), just to *WASH* it, to do this, centrifuge**

the tubes with the hinge facing away from the rotor, the pellet will be at the bottom of the tube in the exact direction of the hinge.

22 Centrifuge at 13,000 RPM for 10 minutes at 4°C; 10m

23 Carefully dispose of the ethanol with the aid of a pipette tip; 10m

24 Repeat the last 3 steps (21,22 and 23); 25m

***Spin the microtubes and remove the excess ethanol using pipette tips, taking care not to aspirate the pellet.**

25 Place the open tubes in the laminar flow cabinet with the glass closed at room temperature for 5 minutes (pay attention to which location the airflow is strongest, and position the rack at this location); 5m

26 Resuspend in 30 uL of autoclaved Milli-Q Water treated with 0.1% DEPC at 60 °C; 30m

***Add water to all tubes immediately after leaving the cabin;**

***Homogenizing with up & down (setting the pipette at 25 uL so that no bubbles form, or homogenizing carefully at 30 uL) for 30 seconds per sample.**

***This step takes *1 minute per tube*!**

27 Treat with DNase - gDNA RNA CleanUp Protocol; 3h

28 Run 1.5% chlorine-agarose gel (3 uL sample + 2 ul loading buffer 6X (Gel Red at 9X) - 80 V - 80 min); 1h 40m

*****The electrophoresis tank should be cleaned well with distilled water and fresh running buffer should be added, and the gel polymerization tray should be cleaned**

well, and the gel should be made with fresh buffer to avoid RNA degradation.
*****Chlorine gel: Use 5% commercial bleach added to TAE 1X buffer.**

29 Quantify RNA purity and concentration in the Nanodrop or Qubit;
-Pure RNA has 260/280 ratio > 1.8 and 260/230 ratio > 1.6.
For RNAseq, Microarray or more sensitive downstream RNA applications, quantify RNA integrity with Bioanalyzer 2100 (RNA 6000 kits) or Tapestation 2200; always using Plant RNA Algorithm (refer to the software version manual)

30 Store the samples in the ultra-freezer (-80 ºC) for up to 6 months.