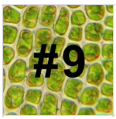

Aug 20, 2019

RNA Isolation from Plant Tissue Protocol 9: CTAB/Acid Phenol/Silica Membrane Method

1 Works for me dx.doi.org/10.17504/protocols.io.4yegxte

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ABSTRACT

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This protocol is a modification of protocol 8. It was developed after protocol 8 and several commercially available plant RNA isolation kits failed to produce a sufficient yield and quality of RNA from *Oenothera* spp. (Onagraceae) for next-generation sequencing. *Oenothera* are rich in polysaccharides, oils, flavonoids and complex ellagitannins that likely interfere with isolation.
We suspect that this protocol will be most useful for species and tissues with complex secondary chemistry and rich in oils (e.g. some Rosaceae and Pinaceae).
The most important modifications to this protocol versus Protocol 8 is the use of less plant tissue, more extraction buffer, and repeating solvent extractions until the interphase is clean of debris. The protocol is regrettably longer and more involved than Protocol 8. We attempted to remove or reduce the replication of the solvent extractions steps without success (i.e. yield and quality are always decreased in *Oenothera* when any steps are removed).

This protocol is part of a collection of eighteen protocols used to isolate total RNA from plant tissue. (RNA Isolation from Plant Tissue Collection: https://www.protocols.io/view/rna-isolation-from-plant-tissue-439gyr6)

journal.pone.0050226.s011-1.pdf

MATERIALS

NAME	CATALOG #	VENDOR
RNaseZap™ RNase Decontamination Solution	AM9780	Thermo Fisher Scientific

MATERIALS TEXT

The solutions are identical to Protocol 8 except we added 2 µl of β-ME to 1 ml of CTAB extraction buffer (instead of 40 µl/1ml) immediately prior to the RNA extraction and deleted the addition of SDS. We also wiped the bench, mortars, pestles with RNaseZap Solution (Ambion, Austin, TX) immediately prior to grinding tissue and rinsed the mortar and pestles with ddH_2O.

SAFETY WARNINGS

Please see SDS (Safety Data Sheet) for hazards and safety warnings.

1 Chill mortar and pestle with liquid nitrogen (fill the mortar carefully with liquid nitrogen, let it evaporate, repeat one more time. Keep the pestle in the mortar when doing this).

2 Fill the mortar again.

2.1 Add **50 mg** – **100 mg** of tissue into the mortar while the liquid nitrogen is still “bubbling”.

3 Carefully grind tissue to a powder in liquid Nitrogen.

Add more nitrogen if needed, but it is important to be careful when you pour the liquid nitrogen. Especially if you are grinding a small amount of tissue in a small sized mortar. The liquid nitrogen tends to “blow” the tissue out of the mortar.

4 Add **2 ml** to **5 ml** (depending on the amount of tissue you start with) of prepared extraction buffer on top of the tissue powder and mix in with the pestle.

The extraction buffer will freeze because the mortar is still very cold from the liquid NITROGEN.
Do not worry about this – start grinding your next sample.
The tissue/CTAB mix will slowly thaw and at some point you will be able to mix it well using the pestle.

Keep in mind that if you use more extraction buffer you will be working in more 2 ml tubes per sample and that the number of tubes per sample will increase once you reach the Qiagen RNeasy part of the protocol. Thus, there will be a lot of pipetting into the Qiagen spin column. One can do the solvent extraction steps in 15 ml Falcon tubes, but keep in mind that you will still have to pass a significantly larger amount of liquid
through the Qiagen spin column.

5 Once thawed and well mixed, pipette or pour the slurry into a 2 ml tube.

If the slurry is too thick, cut the tip of the 1 ml pipette tip with a clean razor blade or scissors.
If youstart with a lot of tissue, you might have to rinse the side of the mortar with an additional ml of extraction buffer (or more if needed.)

6 Vortex each tube until the tissue is mixed with the buffer.

To facilitate mixing, you may have to invert the tube on the vortex, and/or heat it briefly in a **65 °C** water bath.

7 Incubate tubes at **65 °C** for **00:10:00** – **00:15:00** , vortexing briefly (15 seconds) twice during the incubation.

8 Spin the tubes at maximum speed (> 14000 x g) for 00:03:00 in a microcentrifuge.

All of the insoluble matter should form a pellet at the bottom of the tube.

9 Pour or pipette the supernatant into a new 2 ml tube.

Make sure that the total volume ofthe supernatant per tube is ≤ 1 ml. (You will be adding approximately 1:1 volume ratiosin the following steps)

Solvent Extraction #1

10 Add an equal volume (compared to sample) of 24:1chloroform:isoamyl alcohol to each tube.

We attempted to remove solvent extraction #1 and #2, but doing so lowers the quality and quantity of total RNA in evening primrose (*Oenothera*).

11 Vortex the tubes for 00:00:15 or until the phases mix and appear cloudy.

12 Spin the tubes at maximum speed (> 14000 x g) for 00:03:00 in a microcentrifuge.
Most of the chlorophyll will be dissolved in the lower, organic phase.

13 Pipette the upper, aqueous phase to a new 2 ml tube.

Avoid transferring any of the material (usually a white precipitate) from the boundary between the phases.

Solvent Extraction #2

14 Repeat steps 10 to 13 one more time and proceed to step 15. go to step #10

Solovent Extraction #3

15 Add equal volume (compared to the sample) of 5:1 phenol:chlorofom (pH 4.5) to the tubes containing the aqueous phase.

16 Vortex each tube for 00:00:15 or until the phases mix and appear cloudy.

17 Spin the tube at maximum speed (>14000 x g) for 00:03:00 in a microcentrifuge.

18 Pipette the upper aqueous phase to a new 2 ml tube, using a disposable pipette.

Avoid transferring any of the material (usually a white precipitate) from the boundary between the phases.

19 Repeat steps 15 to 18 until the interphase is clean (2-3x for most *Oenothera*) and then proceed to step 20. go to step #15

This is likely the most critical step. Do not proceed to 20 until the interphase appears clean.

Solvent Extraction #4

20 Add an equal volume (compared to sample) of 24:1 chloroform:isoamyl alcohol to each tube containing the aqueous phase.

21 Vortex each tube for 00:00:15 or until the phases mix and appears cloudy.

22 Spin the tubes at maximum speed (>14000 x g) for 00:03:00 in a microcentrifuge.

23 Pipette the upper, aqueous phase to a new 2 ml tube.

Avoid transferring any of thematerial (usually a white precipitate) from the boundary between the phases.

At this point the interphase should be relatively clean, especially if you repeated the phenol/chloroform steps to the point where the interphase appeared to be clean.

24 Repeat steps 20 to 23 one more time and then proceed to step 25. go to step #20

25 Estimate the volume of the aqueous phase based on the markings on the tube.

25.1 Add at least 0.5 volumes of buffer RLT and mix well by briefly shaking.

We attempted excluding this step but doing so decreased the RNA yield andquality

26 Estimate the new total volume in the tube.

26.1 Add 0.5 volumes of 95–100 % ethanol.

26.2 Mix by briefly shaking.

If you hold the tube against the light while mixing, you sometimes see the RNA swirl while it precipitates out.

27 Pipette the contents of the tube into a Qiagen miniRNA spin column (pink), until the column is almost filled with liquid (≤ **750 µl**).

28 Cap the tube and spin for **00:00:15** at > **8000 x g**.

The column should be empty at the endof this spin.

29 Discard the flow-through from the collection tube.

30 Repeat the previous two steps with the same spin column, until all of the liquid in all the tube(s) from the same sample have passed through the column.

The nucleic acid is now bound to the silica membrane in the spin column.

31 Apply **350 µl** of buffer RW1 to the spin column.

32 Cap the tube and spin for **00:00:15** at > **8000 x g**.

The column should be empty at the endof this spin.

33 Discard the flow-through from the collection tube.

34 Apply **80 µl** of DNase digestion solution to the membrane of the spin column.

35 Incubate at **Room temperature** for **00:15:00** .

36 Apply **350 µl** of solution RW1 to the spin column.

37 Cap the tube and spin for **00:00:15** at > **6300 x g**.

The column should be empty at the endof this spin.

38 Discard the flow-through from the collection tube.

39 Apply **500 µl** of solution RPE to the spin column.

40 Cap the tube and spin for **00:00:15** at > **6300 x g**.

The column should be empty at the end of this spin.

41 Discard the flow-through from the collection tube.

42 Apply **500 µl** of solution RPE to the spin column.

43 Cap the tube and spin for **00:00:15** at > **6300 x g**.

The column should be empty at the endof this spin.

44 Discard the flow-through from the collection tube.

45 Transfer the spin column to a new collection tube.

45.1 Spin at maximum speed for **00:03:00** to remove remaining liquid from the silica membrane.

46 Transfer the spin column to a new 1.5 ml conical bottom microcentrifuge tube

47 Add **44 µl** of RNase-free water onto the column.

47.1 Incubate for **00:01:00** to elute at **Room temperature** and spin to to elute.