RNA extraction - Nymphaea seeds

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Modified from:
Wang et al., 2012. Isolation of High Quality RNA from Cereal Seeds Containing High Levels of Starch. Phytochem. Anal. 23:159-163

Notes:

- *Nymphaea* seeds are extremely starchy – this precludes use of spin-column-based kits. The protocol this is based off of was optimized for seeds of cereal crops, and the authors noted a few key steps for dealing with the starch:

1) the separate extraction buffer incubation followed by a high concentration of SDS detergent helps dissolve starch while preventing gel formation,
2) saturated acid phenol separates RNA from DNA, protein, and most of the starch
3) additional precipitation steps (including sodium acetate) to improve quality of isolated RNA.

- This protocol adds PVP40 to the extraction buffer to help remove polysaccharides.

- *Nymphaea* seed samples processed according to this protocol typical yield at least 250 ug/g (fresh weight), with 260/280 between 1.89 and 2.10, and 260/230 between 1.70 and 1.90, and RIN scores between 8.7 and 9.7.

- Phase lock gel tubes can be useful to simplify phase extractions. The gel will separate the organic and aqueous phases, trapping anything that gets caught at the interphase, and making it easier to pipette the aqueous layer off cleanly.

- RNA work requires constant diligence and care as to the removal of RNases and the prevention of their introduction into your samples. Many guides for working RNA can be found online, here is one from Roche: https://lifescience.roche.com/wcsstore/RASCatalogAssetStore/Articles/lab_faqs_%20workingWithRNA.pdf

Reagents/Equipment:

- Extraction Buffer **+** *(make fresh before extraction)*

	For 5 mL:
100 mM Tris-HCL (pH 9.0)	4.9 mL
2 % 2-Mercaptoethanol (v/v)	0.1 mL
2 % PVP40 (w/v)	0.1 g

- 10% SDS
- TRIzol solution
- Chloroform
- Isopropanol
- Saturated acid phenol: chloroform (1:1 or 5:1, can purchase 5:1 from Sigma)
    *note: 5:1 gives higher yields of RNA
- 3M Sodium Acetate
- 100% ethanol
- 70% ethanol

- H20, RNA-work grade.

- mortar and pestle, small metal spatulas
- liquid nitrogen
- 1.7 mL microcentrifuge tubes
- pipettes and tips
- refrigerated microcentrifuge (4 C)
- freezers, -20 and -80 C
- heatblock (only needed if pellet does not dissolve easily), set to ~50 C
- spec (nandrop/qbit/bioanalyzer)

Protocol:

1) Grind (100 to 50 mg; use less of starchier material) of seeds in liquid nitrogen. Add 0.4 mL (Extraction Buffer **+**). Vortex and incubate at RT for 15 min.

2) Add 40 ul 10% SDS. Invert gently 5-8 times and incubate at RT for 5 min. Centrifuge at 12k g for 10 min at 4 C

3) Transfer aqueous phase to new tube (~400 ul), add 2 volumes of TRIzol (~800 ul). Vortex and incubate at RT for 10 min.

4) Add 1/5 volume of chloroform (~240 ul). Vortex and incubate at RT for 5 min. Centrifuge at 12k g for 10 min at 4 C.

5) Transfer aqueous (upper) phase (~700 ul) to new tube. *If the aqueous phase is at all cloudy, split the aqueous phase into two tubes per sample (~350 ul into each tube), and repeat steps 2-4:*

a) Add 40 ul 10% SDS. Invert gently 5-8 times and incubate at RT for 5 min. Centrifuge at 12k g for 10 min at 4 C

b) Transfer aqueous phase to new tube (~400 ul), add 2 volumes of TRIzol (~800 ul). Vortex and incubate at RT for 10 min.

c) Add 1/5 volume of chloroform (~240 ul). Vortex and incubate at RT for 5 min. Centrifuge at 12k g for 10 min at 4 C. Aqueous phase should be clearer – transfer it (~700 ul) to new tube.

6) Add equal volume (~700 ul) of ice-cold isopropanol. Mix by inversion several times. Precipitate at -20 C for at least 60 min.

7) Centrifuge at 12k g for 10 min at 4 C. Discard supernatant.

8) Resuspend pellet in 400 ul H2O. If pellet does not dissolve, but remains as a jelly-like mass, you'll need to start over and include Steps 5a-c. Add 400 ul of saturated acid phenol: chloroform (1:1 or 5:1) and vortex.

9) Centrifuge at 12k g for 20 min at 4C. *If your samples need higher 260/230 values, try repeating the phenol:cholorform step:*

9a) Transfer aqueous (upper) phase to fresh tube (~350 ul). Add 400 ul of saturated acid phenol: chloroform (1:1 or 5:1) and vortex.

9b) Centrifuge at 12k g for 20 min at 4C.

10) Transfer aqueous (upper) phase to fresh tube (~350 ul). Add 1/10th volume (~35 ul) of 3M sodium acetate (pH 4.8) and 2 volumes (~700 ul) of ice-cold 100% ethanol. Mix by inversion. Precipitate at -80 C for at least 30 min.

11) Centrifuge at 12k g for 20 min at 4C.

12) Discard supernatant. Wash pellet with 500 ul of ice-cold 70% ethanol. Centrifuge at 12k g for 10 min at 4C.

13) Discard supernatant. Wash pellet with 500 ul of ice-cold 70% ethanol. Centrifuge at 12k g for 10 min at 4C.

14) Discard supernatant. Wash pellet with 500 ul of ice-cold 70% ethanol. Centrifuge at 12k g for 10 min at 4C.

15) Discard supernatant, air-dry pellet for 5-10 min.

16) Dissolve pellet in water, ~30-50 ul, incubate ~10 min at RT (can heat briefly to help dissolution). Spec (nandrop/qbit/bioanalyzer) and store at -80 C.