Standard Operating Procedure
for
Hazardous Chemicals

Principal Investigators: Christopher Frost
Building and rooms: Keating Building, Lab 302

Chemical(s)	CTAB, PVP, Tris-HCl, EDTA (ethylenediaminetetraacetic acid), βME (β-mercaptoethanol), SDS (Sodium Dodecyl Sulfate), Ethanol, KOAc (potassium acetate), Isopropanol, liquid nitrogen (LN), sodium chloride (NaCl).
Process	**Plant RNA extraction - miniprep**
Specific Hazards *referred to MSDSs for more detailed information*	Isopropanol, Ethanol: Flammable. βME: flammable, and may be harmful upon skin/eye contact, ingestion or inhalation. LN: frostbite hazard. KOAc: Hazardous in case of eye contact (irritant).
Personal protective equipment	☒ 3-5 mil nitrile gloves ☒ double gloves (w/ concentrated stock) ☒ lab coat (except when used in microcentrifuge tubes) ☒ chemical safety goggles (when splash potential exists)
Engineering/ventilation controls	All operations involving βME and chloroform must be done in a chemical fume hood.
Special handling procedures and storage requirements	Store isopropanol, βME in the flammable cabinets under the hood B. LN: Store and use with adequate ventilation. Under normal conditions these containers will periodically vent product. Do not plug, remove, or tamper with pressure relief device.
Spill and accident procedures	Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes. Eye exposure: Wash eyes for > 15 minutes. For both cases, seek medical attention immediately.
for hazardous chemicals only	Small (< 2L): Absorb with vermiculite or spill pads and transfer absorbed material to a closed container. Label and date as hazardous waste for disposal. Notify PIs.
	Large (> 2L): Evacuate the room, notify PI and call 626-6850 to request emergency spill assistance from UA's Research Laboratory & Safety Services (RLSS).
Waste disposal	Dispose waste as regular disposing method.
Special approval	No special authorization needed after SOP training & reading MSDSs.
Prepared by	Name/date: Chris Frost, 09/16/2017, updated 1/5/2021
Reviewed by	Name/date: Chris Frost, 1/5/2021

Total RNA Extraction Protocol (CTAB Method)

From: Chang, S., Puryear, J., and Cairney. 1993. A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11(2):113-116

<u>**Note**</u>: This is a modification of the original proposal for using small sample volumes of 100-200 mg tissue. The protocol scales just fine, with the only caveat that below a certain amount of tissue, the pellets can be small and very difficult to see.

Preparation and Precautions:

i. MAINTAIN RNAse-FREE CONDITIONS AT ALL TIMES. RNA isolation is technically more challenging than DNA isolation due to the ubiquity and stability of RNAses, and to the presence of a reactive 2'-OH on RNA ribose residues. Since airbone bacteria and the skin (e.g., hands) are also sources of RNAses, all solutions and labware used in RNA isolation should be treated to remove RNAses.

ii. Wear gloves (latex or nitrile) at all times and don't be afraid to replace them as necessary, don't let tips touch anything before drawing solutions, etc. While this protocol is quite robust, RNA is always vulnerable to degradation at certain stages of the protocol. The two main times of vulnerability are (1) right when buffer is being added to frozen samples, and (2) after step 14.

iii. Try to develop a keen awareness and avoidance of all sorts of fast little moves, sneezing, blowing, touching vulnerable spots (tube rims, pipette tips-even with gloved hands) etc that can introduce contamination into stock or sample solutions.

iv. Gloves <u>must</u> be worn when working with RNA. Touching freezer/refrigerator handles, door knobs, or any surfaces that are not RNase-free will contaminate gloves. It is good to wipe your gloves off with a Kimwipe of 70% ethanol if you contaminate them at the same time you are handling RNA after step 14 in the protocol below. This is not as critical before that point and gloves are worn mainly to protect your hands.

v. Disposable plasticware such as centrifuge tubes and pipette tips are usually free of RNAse contamination and can be used as is (from sterile packages). However, opened bags of RNA plasticware be tightly closed and sequestered from general lab circulation if they are to be used for future RNA work.

vi. Pre-grind all samples before starting RNA extraction.
   a. Use a single mortar/pestle for each prep. This is critical to ensure that there is no cross contamination of samples. See Tissue Grinding SOP.

vii. Prepare buffers before starting, calculating how much CTAB solution will be needed, dispensing just the necessary amount and adding the BME. Also, make sure that the 65°C heat bath and 4°C centrifuge are both on and at temperature. Put the CTAB/BME solution at 65°C to heat up. Have chloroform ready in the hood, pour our necessary amount into an RNAse-free 50 mL tube.

viii. Each sample will require 4 tubes: (1) Initial 2ml tube with -80°C ground tissue, (2) 1.5ml tube with 600µl chloroform, (3) 1.5ml tube with 200µl 10M LiCl, (4) 1.5 ml final collection tube. Pre-filling the exact number of chloroform and LiCl tubes can be done while the samples are incubating in CTAB (Step 9), and then during the first spin (Step 11). Prepare these tubes ahead of time.

ix. RNAse-free water is available in the lab from the Elga Flex water system. Let the system run for 20-30 sec before using water for RNAse-free applications.

Procedure:

Day 1: Start at approximately 3pm, or enough time to end the prep right around 5pm. The LiCl overnight precipitation should occur for a maximum 15 hours, 5pm-8am.

1. Remove samples from -80ºC, and store in a liquid N bath (I use a Styrofoam container lined with aluminum foil with the insert of a freezer box inside).
2. Grind samples as necessary (Grinding SOP), and dispense ~200 mg (~200µl mark of 2 ml tube) of ground tissue into a pre-chilled tube. Or, proceed if tubes with ground tissue for RNA extraction have already been prepared.
3. **CRITICAL STEP**: In a chemical fume hood, one sample at a time, quickly add 1 ml of hot CTAB solution, cap, and shake vigorously and vortex to integrate the extraction buffer with the sample **as quickly as possible**. Place sample into 65 ºC water bath. ***The speed with which the CTAB solution is incorporated in to the sample will affect the RNA quality***.
4. Repeat 4-5 until samples are processed. (8-12 samples are pretty easy to handle, 24 is about the limit). If doing a larger number of preps, divide into two separate groups.
5. After the final extraction, vortex all of the samples and let sit again for 15 min @ 65ºC. Shake samples by hand a few times during this incubation.
6. Still in a chemical fume hood, add 600 µl chloroform to each sample. Vortex 10-20 sec. If working with green leaves, the solution should turn from a forest green to more lime green in color.
7. Spin 10 min @ 10,000 x g, room temperature
8. Remove the aqueous (top) phase to a new tube. This new tube should already contain 600 µl chloroform. Vortex mix 10-20 sec.
9. Spin 10 min @ 10,000 x g, room temperature
10. Transfer upper aqueous phase (this solution should now be clear with a yellow tint if working with leaf material) to a new tube containing 200 µl (¼ volume) 10M LiCl. Do not transfer any interphase material at this point as this will co-precipitate with the RNA. **CRITIAL STEP**: GENTLY invert to mix completely, DO NOT VORTEX OR SHAKE VIGOROUSLY, as this will shear the RNA.
11. Precipitate overnight (or at least 4-6 hr) in on ice in the fridge. For best results, LiCl precipitation should not continue for more than 15 hours.

Day 2: Start at 8am or earlier.

12. Spin 20 min @ 14,000 x g, 4 ºC to pellet the RNA. This pellet should look opaque and off-white.
13. Decant supernatant into waste container. Briefly spin, and use a pipette to remove the remaining liquid from the pellet.
14. **CRITICAL**: From this point on it is essential that no contaminants enter the RNA. Previously, RNA had protection of contact with lysis buffer, which largely inactivates RNAses. This protection is now gone. Never allow pipette tips to touch anything except the surface of your RNA sample. Again, try to be keenly aware of your

surroundings and ALL actions, including seemingly innocuous actions such as laying down pipettors and accidently touching a contaminated surface.

15. Dissolve pellet in 500 µl RNAse-free water. This will require vortexing, or pipetting up and down, and may be facilitated by pre-heating the water to 65 ºC. Make sure the pellet is completely dissolved. If the pellet is not completely dissolved before continuing, there will be carry-over of LiCl, which will interfere with downstream use of the RNA.
16. **Optional**: Add 1/4vol LiCl (125µl), invert to mix, precipitate for 2-3 hr on ice. This extra LiCl precipitation helps remove residual DNA contamination.
17. **Optional**: Repeat steps 12-14 if opting for Step 16.
18. Add 500 µl chloroform, vortex 10-20 sec.
19. Spin 3 min @ 5000 rpm, room temperature.
20. Transfer aqueous layer to a new tube and add 0.1 vol (50µl) 3M NaOAc and 2 vol (1 ml) of **ice-cold** 100% EtOH. **Gently** invert to mix thoroughly.
21. Precipitate RNA for 20 min @ -80ºC. Solutions will be viscous but not frozen when they are ready.
22. Spin 20 min @ 20,000 x g, 4ºC to pellet RNA. This time, the pellet should look opaque white to glassy and will be substantially smaller.
23. Discard supernatant, quick spin, and use a pipette tip to remove remaining supernatant.
24. Air dry pellet on a paper towel for a few minutes.
25. Re-suspend pellet in 50 µl RNAse-free water.
26. Use 1 µl concentration on Nanodrop. The 260/280 ratio should be >1.9, and the 260/230 ratio should be > 2.0.
27. Determine quality by using 1µl to run out on an RNAse-free gel for standard downstream applications like qPCR. Analyze on the Agilent Bioanalyzer if samples are to be submitted for RNA sequencing.
28. The RNA is now ready for downstream applications. For RT-PCR, RNA will need to be treated with DNAse and then an RT reaction to generate cDNA according to the QPCR SOP.
29. **RNA storage and handling**: RNA should be divided into aliquots **before first freezing**, and the original stock should not be diluted for long-term storage. In our lab, it is advisable to prepare 4 aliquots: (1) 5ul for Bioanalyzer, (2) 10-15ul aliquot for RNAseq, (3&4) 2 x 2.5ug total RNA [determined from Nanodrop] for qRT-PCR. This way, individual aliquots are only subjected to one freeze-thaw before use.
30. **NOTE**: This protocol can be done in a single day if the prep is started early, and the precipitation step (step 11) is only 4h. By this method, RNA can be recovered by mid-to-late afternoon. If this is done, it is important to proceed directly with DNAse treatment and cDNA synthesis using the thermal cycler program for one aliquot, and freeze the other aliquots. Then, cDNA will be ready the next morning for qRT-PCR.

Materials

CTAB Extraction Buffer	[Stock]	[Final]	For 50 ml	For 25 ml
CTAB	--	2%	1.0 g	0.5 g
Polyvinylpyrrolidone (PVP)	--	2%	1.0 g	0.5 g
Tris-HCl (pH 8.0)	1M	100mM	5 mL	2.5 mL
EDTA (pH 8.0)	0.5M	25mM	2.5 mL	1.25 mL
NaCl	5M	2M	20 mL	10 mL
β-mercaptoethanol (βME)	--	2% (20ul/ml)	1 mL	0.5 mL

Weigh out CTAB and PVP and put into a 50ml tube and add ~17mL RNAse-free water. Vortex to mix, this initial mixture will foam quite a bit. Put tube in 65°C water bath for 5-10 min while you prepare other parts of the prep. Solution should be clear (all CTAB and PVP dissolved) with dissipating foam on top. Then add Tris and EDTA. You may need then to put back into 65°C bath for a few minutes. Add NaCl and the solution should again become viscous (possibly very viscous). After a few more minutes in 65°C bath, bring to final volume RNAse-free water. Add βME just before starting the extraction. All work with βME should be conducted in the RNA-specific chemical fume hood.

Chloroform (stored in the cabinet under the fume hood)
100% EtOH (Bottle specifically for RNA preps stored in -20°C freezer)

STOCK SOLUTIONS:

Making these solutions RNAse free is critical. If you are making stocks into 50mL tubes, the tubes are RNAse-free and you should just need to use RNAse-free water. To make these solutions in purple-capped bottles, first add a small amount of 3% SDS to a cleaned bottle and mix vigorously, rinse thoroughly ONLY with RNAse-free water. For either, prepare spatulas by cleaning with SDS and rinsing with RNAse-free water.

1M Tris, pH 8.0 (FW 121.13 g/mol; 2-Amino-2-(hydroxymethyl)propane-1,3-diol)

For 1 L	121.13 g
For 500 ml	60.565 g
For 250 ml	30.283 g
For 50 ml	6.0565 g

Add RNAse-free water to volume. Autoclave.

0.5 M EDTA pH 8.0 (FW 292.25 g/mol)

For 1 L	146.125 g
For 500 ml	73.06 g
For 50 ml	7.306 g

EDTA will not go into solution until pH is near 8, so start with a small volume of RNAse-free water and make a 5M NaOH solution with RNAse-free water to titrate the solution until it reaches pH 8.0, then bring to volume if necessary with RNAse-free water. Autoclave.

5 M Sodium chloride (NaCl, FW 58.44 g/mol)

For 1 L	292.2 g
For 500 ml	146.1 g
For 250 ml	73.05 g
For 50 ml	14.61 g

Dissolve NaCl in approximately 85% target volume with RNAse-free water, with vigorous vortexing and heating at 65^oC. When dissolved, add RNAse-free water to volume. Autoclave.

10 M Lithium chloride (LiCl, FW 42.39 g/mol)

For 1 L	423.90 g
For 500 ml	211.95 g
For 200 ml	84.78 g
For 50 ml	21.20 g

Dissolve LiCl in approximately 85% target volume with RNAse-free water, with vigorous vortexing and heating at 65^oC. When dissolved, add RNAse-free water to volume. Autoclave.