**Standard Operating Procedure**
**for**
**Hazardous Chemicals**

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**Building and rooms:** Davison Life Sciences Building, Lab B310

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|---|---|
| **Chemical(s)** | AbSolve™ , cetyltrimethylammoniumbromide (CTAB), chloroform, ethanl, ethylenediamine tetraacetic acid (EDTA), isoamyl alcohol (IAA), liquid nitrogen (LN), lithium chloride (LiCl), β-mercaptoethanol (βME), polyvinylpyrrolidone (PVP, K-30), sodium chloride (NaCl), tris(hydroxymethyl)aminomethane (Tris-base). |
| **Process** | **CTAB RNA extraction method** |
| **Specific Hazards**<br><br>*referred to MSDSs for more detailed information* | AbSolve contains sodium hypochlorite and sodium hydroxide. It is corrosive and may cause burns to skin/eye. Chloroform is a probable human carcinogen, reproductive hazard and skin/eye irritant. BME and IAA are flammable, and may be harmful upon skin/eye contact, ingestion or inhalation. LN is a frostbite hazard. PVP is slightly hazardous as a skin/eye irritant. |
| **Personal protective equipment** | Must wear 3-5 mil nitrile gloves. Chemical safety goggles and lab coat should be worn when splash potential exist |
| **Engineering/ventilation controls** | All operations involving chloroform, IAA and βME must be done in a chemical fume hood. |
| **Special handling procedures and storage requirements** | Store chloroform, IAA and BME in the flammable cabinets under the hood, and AbSolve in the designated cabinet under the electrophoresis bench in Rm B310. Store all the other buffers on bench top. |
| **Spill and accident procedures**<br><br>*for hazardous chemicals only* | Skin exposure: Rinse affected skin with plenty of water while removing contaminated clothing/shoes. Rinse for > 15 minutes.<br>Eye exposure: Wash eyes for > 15 minutes.<br>For both cases, seek medical attention immediately. |
| | 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 PIs and call 2-5801 to request emergency spill assistance from the Environmental Safety Division. |
| **Waste disposal** | Chloroform waste must be collected and labeled as hazardous waste according to the SOP for Hazardous Waste Disposal. |
| **Special approval** | No special authorization needed after SOP training & reading MSDSs. |
| **Prepared by** | Name/date: C-J Tsai, 8/18/08; updated Scott Harding 7/28/09 |
| **Reviewed by** | Name/date: Kate Tay, 8/13/09 |

# CTAB RNA Extraction Method

This method is very effective for RNA isolation from tissues containing high levels of phenolics and/or polysaccharides (especially those failing with the Qiagen RNeasy kit). Works well with *Populus* leaves, apices, stems, xylem, phloem, roots, flowers, pine and *Eucalyptus* xylem.

## Solutions and Reagents needed

**CTAB RNA extraction buffer**:

| | Reagent | Concentration | Amount/Liter |
|---|---|---|---|
| | CTAB** | 2% w/v | 20.0 grams |
| | (** Add CTAB to 800 ml $H_2O$; dissolve with heat while stirring) | | |
| (Listed in order of addition) | EDTA | 25 mM | 50 ml of 0.5 M stk soln. pH 8.0 |
| | Tris base | 0.1 M | 12.1 grams |
| | PVP (K-30) | 2 % w/v | 20.0 grams |
| | NaCl | 2 M | 116.9 grams |

***Check pH-should be 8.5-9.0***

To be added just before use: β-mercaptoethanol 2% (see Procedure step 3)

**Chloroform:isoamyl alcohol** = 24:1
**8 M Lithium chloride**
**3 M Sodium acetate**, pH 5.0 (see rolodex on microwave)

**Precautions:** RNA extraction and handling are technically more challenging than DNA manipulation 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*, and gloves must be worn at all times.

1. All glassware and reuseable plasticware used in solution preparation, storage, and RNA extraction should be treated with 2% AbSolve (shake well before use) in $ddH_2O$ for several hours to remove residual RNase activity. AbSolve-treated labware should be thoroughly rinsed with $ddH_2O$ (2-3 times) before use. You may also autoclave glassware or Oakridge centrifuge tubes (make sure caps are loosely closed) before use. The AbSolve solution can also be used for other cleaning purposes within a few days (see Precaution 6 below).
2. Maintain separate stock solutions, buffers and $ddH_2O$ for RNA work. Prepare all solutions using treated glassware as described above.
3. Disposable plasticware such as centrifuge tubes and pipette tips are usually free of RNase contamination and can be used as is (from sterile packages) or simply autoclaved without any pretreatment. However, it is advised that opened bags of RNA plasticware be tightly closed and sequestered from general lab circulation if they are to be used for future RNA work.
4. Gloves must 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 stage 9 in the protocol below. It is not such a critical matter before that point and gloves are worn mainly to protect your hands.

5. Decontaminate workbench area and pipetter shafts and tip ejectors by wiping thoroughly with 70% ethanol and a kimwipe before RNA work. Electrophoresis apparatus (tanks, trays and combs) should also be treated by wiping out with a Kimwipe wetted with RNase AWAY or similar product.

For large-scale preps, you will need 4 Oakridge tubes/RNA extraction. If you are doing 6 samples, you need to prepare 24 tubes. The last tube to be used for each RNA extraction must be treated with 2% Absolve and rinsed in advance. The other 3 tubes/extraction need to be clean and dd $H_2O$ rinsed, but it is not critical to Absolve them. Absolve is very expensive, and should be conserved when possible by reheating and reusing the same solution if you anticipate multiple sets of RNA extractions over a 2 or 3 day period.

## Large-scale Extraction Procedure

### (> 0.5 g fresh weight or 0.5ml powder per 15ml CTAB)):

1. Prechill a mortar and a pestle with LN. Dispense 15 ml CTAB extraction buffer per gram of tissue into a 50 ml Oakridge centrifuge tube and pre-warm the tube in a 60-65$^{o}$C water bath.

2. Place frozen tissue into the mortar and add LN to immerse tissue. While the LN is evaporating, gently crush the tissue clumps into small pieces with the pestle. Add a spatula of sea sand to aid grinding and add additional LN if necessary. *Addition of liquid N from this point on should be done very carefully to prevent dispersal of frozen tissue from the mortar.* When the LN is nearly evaporated, vigorously grind the tissue to a fine powder.

3. In a fume hood, add 2% (v/v) β-mercaptoethanol to the warmed CTAB buffer and mix. Use a LN-prechilled spatula to **quickly**** transfer frozen powder to the centrifuge tube. Swirl the tube **frequently** to mix contents during the transfer. Cap the tube and vortex at high speed for 1 min to homogenize.

   *** Speed is critically important. To facilitate a rapid transfer, frozen powder can be aliquoted from the mortar into a pre-chilled, 2-ml eppendorf tube kept frozen in an eppendorf-tube caddy that has been equilibrated for several hours in a -80$^{o}$C freezer. The tube can then be held over the Oakridge tube and smartly rapped once or twice with a stainless steel spatula to knock the contents into the lysis buffer all at once. Immediately cap and shake vigorously or vortex. It sometimes helps to wipe off any condensation in the neck of the Oakridge tube before adding your powder.*

4. Allow vortexed sample to **incubate in the CTAB lysis buffer for 15 minutes before proceeding to chloroform extraction**. Shake vigorously by hand every 5 minutes or so.

5. Extract lysate with an equal volume* of chloroform/IAA by vortexing for 1 min. (*use no more than 10 ml of chloroform per extraction. If you have a 15 or 20 ml lysate, 10 ml of chloroform is sufficient) Separate phases by centrifuging at room temp for 20 min at 15,000 g. (spin longer if phases are not well separated). *You can reduce pipetter wear by using 3-ml disposable transfer pipettes to add chloroform. Whenever transferring chloroform, draw and expel chloroform several times before actually dispensing into Oakridge tube being held close to the mouth of the chloroform bottle. This will greatly reduce messes caused by chloroform 'falling out' of the pipette before you reach your tube*

   **When working with multiple samples, let the 1$^{st}$ sample stand at room temp after adding chloroform:IAA and vortexing. Proceed with the 2$^{nd}$ sample etc, until all samples are ready for centrifugation.**

6. Repeat the chloroform/IAA extraction once more. After each extraction, use a 3-ml disposable transfer pipette to transfer the top, normally yellow, layer to a clean Oakridge tube containing chloroform-IAA for the next extraction. After the last extraction, save 10 µL to be checked on a minigel, if you are extracting RNA for the first time.

7. Add 1/3 volume 8M LiCl to the supernatant and mix. The RNA is precipitated at **4°C (in an ice bucket) for 3 to 5 hours**, and harvested by centrifugation at 4°C for 20 min at 16,000 g. Mark the Oak Ridge tube on the side facing out (to help orient the tube and the position of pellet).

8. Carefully remove the supernatant, either by slowly pouring off into a clean beaker, or by using a transfer pipette. The pellet at this stage is often difficult to see at the bottom of the translucent Oakridge tubes. It generally appears as a faint whitish crescent, sometimes with faint yellow or green color due to pigment carry-over.

9. Suspend the pellet in 500 µL RNase-free dd$H_2O$. Complete re-suspension of the pellet is very important. Keep the tube on ice for up to an hour (or even overnight), with occasional mixing. **It is OK to hasten this step by working the pellet into solution, drawing it in and out of a 1-ml pipetter, provided you avoid drawing sample close to the base of the pipetter. Work the pipetter *smoothly* to avoid contamination.** If you see sample frothing, you are going too hard and have probably let your sample contact the base of the pipettor, a serious error during any stage of RNA handling-see step 9. Transfer the resuspended pellet to a 2ml eppendorf tube. If sample contains insoluble debris, add 200 µL chloroform/IAA, vortex 10 seconds, and separate phases in a microcentrifuge at top speed at room temperature, and transfer the top layer to a clean eppendorf tube. Avoid transferring any of the interface. Better to leave a few µl of your sample behind than to touch the interface.

**Note about RNase-free water:** Do not use DEPC-treated water, as incomplete removal of DEPC may interfere with downstream applications. Best source of RNase-free water is directly from the water purification system, after letting it run for ~30 sec. **DO NOT use water from a storage Carboy**. You may autoclave the water, but it's optional.

**Important cautionary: From this point on it is essential that no contaminants enter your RNA. In the previous steps you had the protection of working in lysis buffer which largely inactivates RNases. You no longer have that protection. Never allow pipette tips to touch anything except the surface of your RNA sample. Very counter-productive habits include tip or tube contamination by inadvertent contact from actions as seemingly innocuous as laying things down then picking them up for use etc. Other counter-productive habits include touching pipette tips against the rim or the inside wall of eppendorf tubes when transferring or otherwise processing RNA samples; contacting the inner rims or necks of bottles containing RNAse-free working solution. 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.**

10. Repeat the LiCl precipitation as follows: Add 1/3 volume 8M LiCl (170 µl) to your sample, close cap, mix, and let stand **on ice for 2 hours.** Centrifuge in coldroom at 15,000g for 10 minutes to pellet RNA. When finished centrifuging, look for a translucent light tan or nearly transparent pellet. Once you see that, use a pipettor to remove liquid.

11. Dissolve the pellet in 300 µl ultra-clean, non-DEPC $H_2O$. This can be a tricky step as the LiCl pellet can become completely transparent before it is actually dissolved. Use pipetter to aid

solubilization, using precautions in steps 8 and 9. If the pellet is not completely dissolved before the next step, there will be carry-over of LiCl, which will interfere with downstream use of the RNA-essentially negating your entire effort.

12. Once fully dissolved, add 0.1 Vol 3 M NaOAc (pH 5.0) and 2.5 Vol 100% Ethanol, mix and store at -80°C for 30 min or at -20°C for 3 h to precipitate RNA. Centrifuge in the coldroom microcentrifuge at 15,000 g for 10 minutes. After carefully removing all liquid with a pipettor, add 1 ml 70% EtOH, and centrifuge as above for 1 minute. Remove liquid with a pipettor. Air dry the pellet in a protected area of your lab bench (don't vacuum dry, as the pellet will become difficult to re-suspend) and proceed to step #13.

13. Resuspend RNA in 50 to 200 µl of RNA-only, dd$H_2O$. Be sure to dissolve completely.

14. QC: Assess the quality and the yield by Nanodrop and electrophoresis. Nanodrop readings greater than 1000 ng/µl may not be accurate. Dilute a small portion of your sample and repeat the measurements. Perform gel electrophoresis by loading an equal amount of RNA (0.2-0.5 µg per lane) according to the Nanodrop.

**RNA storage and handling:** RNA should be divided into aliquots **before <u>first</u> freezing**, and the original stock should not be diluted for long-term storage. It is advised that you prepare prepare two 'working' aliquots of each RNA sample based on the NanoDrop concentration, to avoid repeated freeze-and-thaw cycles of the sample. Working stock RNA concentration for the whole group of samples (from the same experiment) will be determined as that of the most diluted 'original' RNA sample of the group, and must have a concentration >500 ng/µl. Extremely dilute 'original' RNA samples should be concentrated by ethanol precipitation.

**<u>Small-scale Extraction Procedure:</u>**

14. Add 1.3 ml CTAB buffer and 30 µl BME to a 2-ml microcentrifuge tube, vortex briefly and heat to 65°C on a dry heater block. Pipetting will not be accurate with warm solution, so you must aliquot the CTAB buffer at room temp, and pre-warm the buffer (in microtubes) at 65°C before starting the extraction.

**Notes: Tissue sample powders for extraction should be in 2-ml microcentrifuge tubes in a -80°C freezer caddy nearby. Sample powder should no occupy more than 1/3 the volume of the micro tube. A vortexer should be in continuous run mode nearby in the hood.**

15. Open lid of tube with powder. Open lid of tube with CTAB. Bring the tubes together and use a small spatula to rap the powder into the CTAB.

16. Close instantly and vortex powder contents into CTAB.

17. Return tube to 65°C heater block and proceed to next sample.

18. Repeat for up to 20 samples.

19. Tubes should incubate at 65°C for 10 minutes with occasional vortexing.

20. Cool tubes to room temperature and add chloroform with a disposable transfer pipette to within 3 mm of the top of the tube. **Do not overfill as very smelly, messy losses will occur during centrifugation.**

21. Vortex several times, and centrifuge at 16,000 g for 5 minutes.

22. Transfer phase above the interface to a new tube and add 1/3 volume of 8M LiCl.

23. Proceed as for large scale prep (steps 7 and on), being sure to precipitate at least twice with LiCl..

**References:**

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

Tsai C.-J., Cseke, L.J., Harding, S.A. (2003) Isolation and purification of RNA. In: Cseke, L.J., Kaufman, P.B., Podila, G.K., Tsai C.-J. (eds) Handbook of Molecular and Cellular Methods in Biology and Medicine. 2nd ed. CRC Press, Boca Raton, FL. pp. 25-44.

Examples:

03062009_KT_Small-scale RNA extraction using 0.25 ml frozen powder in 1 ml CTAB (sample codes: 43,44,83,85,95,97,121,122,4000,4001)

Top row = before LiCl precipitation (genomic DNA co-purification is visible)

Bottom row = Completed RNA extraction