RNA extraction protocol for organic and mineral soils

From Pourslavati et al. 2023 based on Sharma et al. 2012, Griffiths et al. 2000, and Angel et al. 2012: https://apsjournals.apsnet.org/doi/10.1094/PBIOMES-12-22-0108-TA

Modified by Hannah Holland-Moritz from Naser Poursalavati's protocol (more detail added). 2023-09-29

Total unchangeable time: 2h 5 minutes
Minimum time: Likely 2h 30 minutes

******Warnings: This protocol involves the use of phenol and chloroform. Phenol chloroform is highly toxic. Read the SDS and SOP for phenol and chloroform. Take appropriate precautions. One should not do this extraction when pregnant or breastfeeding. Change gloves regularly, and check for spills on gloves after handling either phenol or chloroform. Double-layering nitrile gloves or using ChemTek Viton/Butyl gloves are recommended for this protocol as chloroform can penetrate nitrile gloves in 3-5 minutes. Phenol and chloroform should always be handled in a chemical fume hood, gloves should be used, thorough cleaning of the area should be done immediately following extraction so there is no risk of exposure of anyone else and hands should be washed immediately following extraction. Please refer to the SDS for full necessary safety precautions.******

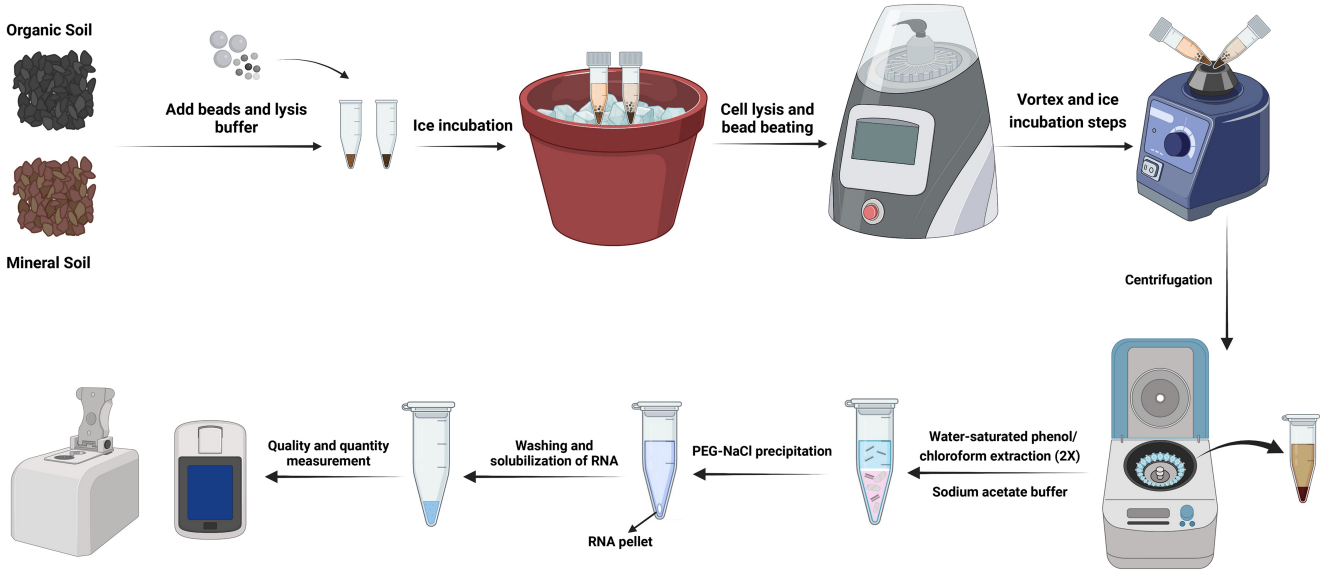

Fig. 1. Overview of workflow of total RNA extraction from two types of soils (mineral and organic) using the improved RNA extraction method. From Poursalavati et al. 2023

Quick Protocol (Detailed is below)

Supplies needed

*Refer to end of protocol for recipes for stock solutions (indicated with a *)*

1. Decontamination solutions
   a) *Custom Decontamination Solution
      OR
      RnaseZap (Sigma R2020-250ML) (for cleaning surfaces and tools)
   b) 70% Ethanol (for cleaning surfaces)
   c) Diethyl pyrocarbonate (DEPC) OR Nuclease-free water
   d) Pre-autoclaved tools; cleaned with RnaseZap and autoclaved or soaked overnight in DEPC water and autoclaved. 15 min 121°C (this helps remove nucleases). Or bake for several hours at 180°C or higher
2. Soil Samples
   a) 0.2 (organic) 0.25 g (mineral) of stone-free soil
3. Tubes (per extraction)
   a) 1 2ml Screw tube per sample (VWR 10158-556) can also buy .1 mm pre-filled bead tubes (Cole-parmer EW-04577-04 or VWR 10158-606)
   b) 2 2ml centrifuge tubes per sample (VWR 470339-648)
   c) 1 1.5-ml tube (low-binding RNAse-free) per sample (VWR 76332-068).
   d) for >10 extractions; 1 50 ml falcon tube for preparing extraction buffer mix
4. Miscellaneous
   a) Sterile Zirconia/silica beads 0.1mm and 0.3mm (Sigma G1277-10G).
   b) Nitrile gloves (double layered for phenol chloroform); or more powerful Viton, butyl or neoprene gloves
   c) Ice bucket; filled with ice
   d) P1000 and P100 pipettes; Pre-sterilized by being wiped with RnaseZap and nuclease-free water
   e) 2ml tube racks. 3X the number of samples. Tubes will be left in racks in fume hood to off-gas phenol chloroform
   f) Fine-tipped sharpie or tube-labeling pen
   g) Heat block at 60°C
5. Chemicals
   a) *200µL per sample (plus more to account for pipetting error) CTAB extraction buffer
   b) 10µL per sample 2-mercaptoethanol (Sigma 63689)
   c) 900µL per sample water-saturated phenol (Fisher 327105000)
   d) ~800µL per sample chloroform per sample (Sigma 25668-100ML)
   e) *350µL per sample 3M Sodium acetate (pH = 4.6)
   f) *400 µL per sample 150mM phosphate buffer 5.8
   g) ~ 600 µL per sample PEG (polyethylene glycol)-NaCl
   h) *Optional*: 1µL per sample glycogen (RNA-grade)
      OR
      1µL per sample LPA (linear polyacrylamide)
   i) *1 ml per sample chilled 80% ethanol (reagent quality)
   j) Nuclease-Free Water

Preparation of Necessary Substances Prior to extraction (refer to "Stock Solutions" section for instructions with *)

1. Prepare the bead-beating tubes (if not using pre-filled tubes). Add 1 g of 0.1mm silica beads and three 0.3mm beads to each tube
2. Prepare Nuclease Free water (use only nuclease-free water to create buffers or treat with DEPC afterwards; CTAB cannot be treated with DEPC)
3. *Prepare the CTAB extraction buffer
4. *Prepare phosphate buffer
5. *Prepare 3 M Sodium Acetate (NaAc) at pH 4.6

Pre-extraction notes:
Keep tubes as cold as possible during extraction to preserve RNA and reduce humic acids.

Prepare Lysis Buffer and Soil Samples

1. Pre-heat **CTAB extraction buffer** to 60°C for **20 minutes**
2. Pre-cool the centrifuge to 4°C
3. Add 250 mg of mineral soil (200mg of organic soil) to labeled **2-ml bead-filled screw tubes** (or do ahead of time).
4. In a fume hood, prepare the extraction mix by adding the following amounts per-sample.
   - 200 µL of pre-heated extraction buffer
   - 400 µL of 150mM phosphate buffer (pH 5.8)
   - 10 µL of 2-mercaptoethanol
   - 300 µL of water-saturated phenol
   - 200 µL of chloroform
5. Close the **2 ml screw-top tubes** tightly and place on **ice to cool for 2 minutes**. Change your gloves and dispose of them in the hazardous waste.

Lysing the cells

6. Vortex samples for **10 minutes** using a vortex adapter stopping every **2 minutes** to cool the tubes on ice for about **1 minute**.
   OR
   Bead-beat for **20 seconds** at 6.5 m/s or 4.5 m/s for mineral or organic soil, respectively. Place the **2 ml screw-top tubes** on ice to cool for **1 minute**. Do this twice. Vortex the samples for **5 minutes** (high speed) cooling them on ice periodically.
7. While the samples are vortexing, label a set of fresh **2 ml centrifuge tubes.**

Phase separation and Phenol:chloroform extractions

8. Centrifuge **2 ml screw-top tubes** at 10,000 × *g* for 2 min at 4°C.
9. Transfer the aqueous phase into a the labeled empty **2 ml centrifuge tubes**. Place the chloroform and phenol-containing tubes with tops open on a rack in the hood to evaporate the chloroform.
10. Add **350 µl of 3 M Sodium Acetate (NaAc) (pH = 4.6)** to **2ml centrifuge tubes**.
11. Incubate on ice for **2 minutes**.
12. In the hood, add **600 µl of water-saturated phenol**. Close the tubes and mix gently either by shaking by hand, mix by pipetting up and down. Incubate on ice for **3 minutes.**

13. Next, in the hood add **300 µl of chloroform**. Mix vigorously (or vortex briefly). And incubate on ice for **3 minutes.**
14. Next centrifuge for **10 minutes** at 10,000 × *g* at 4°C to separate the phases.
15. While the centrifuge is running, label another set of **2 ml centrifuge tubes**

Final chloroform extraction

16. In the hood, transfer the aqueous phase to the new **2 ml centrifuge tubes**. For each sample, record the volume of aqueous phase on the tube.
17. Add a volume of chloroform equivalent to the aqueous phase volume written on the tube. Incubate on ice for **3 minutes.**
18. Centrifuge for **10 minutes** at 10,000 × *g* at 4°C.
19. While the centrifuge is running, label a set of **1.5ml tubes (low-binding, Rnase-free)**

RNA Precipitation

20. In the hood, transfer the aqueous phase (approximately 500 to 600 µl) into a new **1.5-ml tube (low-binding RNAse-free).**
21. Add an equivalent volume of polyethylene glycol **(PEG)-NaCl precipitation buffer** to the aqueous phase and incubate on **ice for 20 minutes**.
22. (Optional step, but recommended) Add 1µL of RNA-grade glycogen or 1µL of LPA to help visualize the RNA pellet precipitation.
23. Place the tubes in the centrifuge, being certain to put them all in the same orientation, as this will make it easier to see the pellet. Then centrifuge for **20 minutes at 15,000 rpm and 4°C** to precipitate the pellet.
24. Remove tubes from the centrifuge, being careful not to disturb the pellet.

Cleaning the RNA

25. Carefully remove the supernatent by pipetting, or pouring off, the supernatent. Do not lose the pellet. If the pellet becomes detached from the tube wall, re-centrifuge the tube.
26. Add **500µL of 80% cold ethanol**. Gently rinse the pellet by flicking the tube with your fingers several times.
27. Centrifuge the tube at **15,000 rpm for 5 minutes** at 4°C
28. Repeat steps 25, 26, and 27.
29. Pour or aspirate off the ethanol with a pipette tip. Try not to leave any ethanol on the side of the tube as it can cause issues with downstream analysis.

Drying and dissolving RNA

30. Turn the tubes upside down on a clean cloth in a clean laminar flow hood to dry for ~5 minutes or until all ethanol has evaporated.
31. Add 50 µl of water treated with RNAsecure, and gently pipette up and down to resuspend the pellet. Store at -80 °C to prevent degradation of the RNA
32. Optional: If DNA contamination is present, treat with TURBO DNase (Ambion) according to manufacturers instructions. Add 1x Dnase buffer and 10 units of TURBO enzyme. Incubate for 30 minutes at 37C, then remove Dnase enzyme with phenol chloroform extraction

Detailed Protocol

Supplies needed

*Refer to end of protocol for recipes for stock solutions (indicated with an *)*

1. Decontamination solutions
   a) *Custom Decontamination Solution
      OR
      RnaseZap (Sigma R2020-250ML) (for cleaning surfaces and tools)
   b) 70% Ethanol (for cleaning surfaces)
   c) Diethyl pyrocarbonate (DEPC) *OR* Nuclease-free water
      - *What it does:* DEPC is used to treat water and laboratory utensils to remove RNAse enzymes. It modifies the enzymes, inactivating them. When autoclaved it breaks down to CO_2 and ethanol, deactivating the DEPC and removing it from the water.
   d) Pre-autoclaved tools; cleaned with RnaseZap and autoclaved or soaked overnight in DEPC water and autoclaved. 15 min 121°C (this helps remove nucleases). Or bake for several hours at 180°C or higher
2. Soil Samples
   a) 0.2 (organic) 0.25 g (mineral) of stone-free soil
3. Tubes (per extraction)
   a) 1 2ml Screw tube per sample (VWR 10158-556) can also buy .1 mm pre-filled bead tubes (Cole-parmer EW-04577-04 or VWR 10158-606)
   b) 2 2ml centrifuge tubes per sample (VWR 470339-648)
   c) 1 1.5-ml tube (low-binding RNAse-free) per sample (VWR 76332-068).
   d) For >10 extractions – 1 50ml falcon tube for preparing extraction buffer mix
4. Miscellaneous
   a) Sterile Zirconia/silica beads 0.1mm and 0.3mm (Sigma G1277-10G). Can sterilize glass beads if not new from the bottle. Use 1:10 solution of household bleach for 5 minutes. Rinse beads with DI water afterwards. Autoclave 121C for 80 minutes (to fully remove any template DNA on beads). https://www.biospec.com/beads-guide-lines/cleaning-your-beads
   b) Nitrile gloves (double layered for phenol chloroform); or more powerful Viton, butyl or neoprene gloves
   c) Ice bucket; filled with ice
   d) P1000 and P100 pipettes; Pre-sterilized by being wiped with RnaseZap and nuclease-free water
   e) 2ml tube racks. 3 times the number of samples. Tubes will be left in racks in fume hood to off-gas phenol chloroform
   f) Fine-tipped sharpie or tube-labeling pen
5. Chemicals
   a) *200µL per sample (plus more to account for pipetting error) CTAB extraction buffer (See end of protocol for preparation instructions)
      - *What it does:* **CTAB** is a quaternary ammonium surfactant (detergent) that disrupts membranes while maintaining the integrity of DNA. **PVP** reduces humic acid content in extraction buffer (Sharma et al. 2012); **Phosphate buffer** of lower pH helps with humic acid removal (Poursalavati et al. 2023). Salt [**NaCl**] can help burst cells by creating hypotonic solution, it also helps disrupt binding charges of proteins that are bound to DNA
   b) 10µL per sample 2-mercaptoethanol

- *What it does:* 2-mercaptoethanol eliminates ribonuclease release during cell lysis. Ribonucleases (enzymes that degrade RNA molecules) depend on disulfide bonds for stability, 2-mercaptoehtanol reduces these disulfide bonds, denaturing the ribonucleases.

c) 900µL per sample water-saturated phenol (Fisher 327105000)
- *What it does:*Dissolves proteins and lipids separating them in a dense organic layer that sinks to the bottom of the tube away from the aqueous layer containing dissolved nucleic acids

d) ~800µL per sample chloroform per sample
- *What it does:* Dissolves proteins and lipids separating them in a dense organic layer that sinks to the bottom of the tube away from the aqueous layer containing dissolved nucleic acids. Also is more dense than phenol and water, so helps 'sink' the organic layer.

b) *350 µL per sample 3M Sodium acetate (NaAc) (pH = 4.6)

c) *400 µL 150mM phosphate buffer (pH = 5.8)

e) *~ 600 µL per sample PEG-NaCl
- *What it does:* Polyethylene glycol (PEG) is a a large molecule that when added to solution with the right amount of salt (Na^+) will cause nucleic acids to aggregate into long random coils which then precipitate out of solution as the salt disrupts the interaction between the nucleic acid's negatively charged backbone and the water. The PEG works similarly to ethanol or isopropanol by "loosening" the water polar matrix to better allow the salt ions to "find" the nucleic acid.

f) *Optional*: 1µL per sample glycogen (RNA-grade)
OR
1 µL per sample Linear Polyacrylamide (LPA)
- *What it does*: Makes it easier to see the RNA pellet. Can also use linear polyacrylamide (LPA) for this.

g) *1 ml per sample chilled 80% ethanol
- *What it does:* Ethanol is used to clean nucleic acid pellets of salts. The salts dissolve but the nucleic acids remain in a pellet.

h) Nuclease-Free Water
- *What it does:* Nucleases can degrade RNA and DNA, therefore, nuclease-free water must be used to dissolve RNA to keep it from being degraded

Preparation of Necessary Substances Prior to extraction (refer to stock solutions for instructions with *)

1. Prepare the bead-beating tubes (if not using pre-filled tubes). Add 1 g of 0.1mm silica beads and three 0.3mm beads to each tube
2. Prepare nuclease-free water:
   a) Pure nuclease-free water, or DEPC or RNASecure-treated water (Ambion). Add 1 ml of RNASecure to every 24ml of water. Incubate at 60C for10 minutes then cool to room temperature. Store treated water at 4 or -20 until use. Reheating the solution to 60C for 10-20 minutes can re-activate RNASecure product if contamination is suspected.
3. *Prepare the CTAB extraction buffer
4. *Prepare phosphate buffer
5. *Prepare 3 M Sodium Acetate (NaAc) at pH 4.6
   a) *What it does:* this helps keep the extraction aqueous phase at a low pH reducing transfer of humic acids into final RNA (Pourslavati et al. 2023)

Pre-extraction notes:
Angel et al. 2012 found that performing extractions at a low temperature reduced humic acid carryover, protected RNA from degradation, and prevents tubes from accidentally overheating and leaking phenol (which is highly toxic).

Prepare Lysis Buffer and Soil Samples

1. Pre-heat **CTAB extraction buffer** to 60°C for 20 minutes
   - *What it does: Heating the extraction buffer makes it easier to pipette and also better at solubilizing nucleic acids*
2. Pre-set the centrifuge to 4°C
3. Add 250 mg of mineral soil (200mg of organic soil) to labeled **2-ml bead-filled screw tubes** (this step can be done ahead of time and the tubes stored at -80C until the day of processing).

4. In a fume hood, prepare the extraction mix by adding the following amounts on a per-sample basis.
   - 200 µL of pre-heated extraction buffer
   - 400 µL of 150mM phosphate buffer (pH 5.8)
   - 10 µL of 2-mercaptoethanol
   - 300 µL of water-saturated phenol
   - 200 µL of chloroform

   If preparing for a larger batch of samples (>10 extractions), calculate the amount of each ingredient by multiplying by 110% of your total number. This helps account for pipetting inaccuracies. For >10 samples, prepare the extraction buffer mix in a 50ml falcon tube, then pipette 1110µL of the mix into each sample tube. Pipette up and down several times to mix the extraction buffer mix before transferring to a tube.

Example for 24 samples:

24 x 1.1 = 26.4 (this is the number of sample equivalents you'll multiply by)
200µL x 26.4 = 5280µL (this is the amount of extraction buffer you'll need, and also the amount of chloroform you'll need)
10µL x 26.4 = 264 µL (this is the amount of 2-mercaptoethanol you'll need)
400µL x 26.4 = 10560µL = 10.56 ml (this is the amount of phosphate buffer you'll need)
300µL x 26.4 = 7920µL = 7.92 ml (this is the amount of phenol you'll need)

- *What it does: **CTAB** is a quaternary ammonium surfactant (detergent) that disrupts membranes while maintaining the integrity of DNA. **PVP** reduces humic acid content in extraction buffer (Sharma et al. 2012); **Phosphate buffer** of lower pH helps with humic acid removal (Poursalavati et al. 2023). Salt [**NaCl**] can help burst cells by creating hypotonic solution, it also helps disrupt binding charges of proteins that are bound to DNA. **2-mercaptoethanol** eliminates ribonuclease release during cell lysis. Ribonucleases (enzymes that degrade RNA molecules) depend on disulfide bonds for stability, 2-mercaptoehtanol reduces these disulfide bonds, denaturing the ribonucleases. Phosphate from the **phosphate buffer** has an added benefit of helping release nucleic acids from soil particles. In clay-rich soils, increasing the molarity of the extra phosphate buffer may help dissociate nucleic acids from mineral surfaces. **Phenol** and **Chloroform** dissolve proteins and lipids separating them in a dense organic layer that sinks to the bottom of the tube away from the*

aqueous layer containing dissolved nucleic acids. Other phenols (citrate-phenol, Trizol, Tris-saturated phenol) can be used, but their salts result in lower quality nucleic acids. The exact ratio of phenol and chloroform can favor DNA or RNA alternatively. Additionally chloroform is more dense than phenol and water, so helps 'sink' the organic layer.

5. Close the **2ml screw-top tubes** tightly and place on ice to cool for 2 minutes. Change your gloves and dispose of them in the hazardous waste.
   - *What it does: RNA extraction efficiency increases when samples are kept cold. Partially due to the disabling of ribonucleases, partially because RNA is unstable and more stable in the cold*

Lysing the cells

6. Vortex samples for **10 minutes** using a vortex adapter. Set breaks every **2 minutes** to cool the tubes on ice for about **1 minute**. (**15 minutes** total time). Extraction efficiency improves with colder processing.
   OR
   If a bead-beating system is available, bead-beat for **20 seconds** at 6.5 m/s or 4.5 m/s for mineral or organic soil, respectively. Place the **2 ml screw-top tubes** on ice to cool for **1 minute**. Do this twice. Vortex the samples for **5 minutes (high speed)** cooling them on ice periodically (~every 2 minutes).
   - *What it does: violently shaking the tubes with beads helps rip open the cells, particularly those with strong cell walls. It also increases the efficiency of the surfactant action of the extraction buffer.*
7. While the samples are vortexing, label a set of fresh **2 ml centrifuge tubes.**

Phase separation and phenol:chloroform extractions

8. Centrifuge **2 ml screw-top tubes** at 10,000 × *g* for 2 min at 4°C.
   - *What it does: The denser organic layer of phenol and chlorofom (along with cellular proteins and humic acids) will sink to the bottom of the tube. The top phase will be an aqueous layer containing the DNA, RNA, salts, and other polar substances.*
9. Transfer the aqueous phase containing nucleic acids (approximately 550 µl per sample) into a the labeled empty **2 ml centrifuge tubes**. Place the chloroform and phenol-containing tubes with tops open on a rack in the hood to evaporate the chloroform (they can be disposed of in the hazardous waste trash after phenol and chloroform have evaporated ~overnight).
10. Add **350 µl of 3 M Sodium Acetate (NaAc) (pH = 4.6)** to **2ml centrifuge tubes**. Note: The NaAc can be added to the empty 2ml centrifuge tubes ahead of time at step 6, if desired.
    - *What it does: **Sodium acetate** buffer decreases humic substances and also allowed extraction of DNA-free total RNA; The pH of the NaAc solution was important in determining amount of DNA vs. RNA yield. The lower the pH the less humic acids, but the more the nucleic acids absorbed into the soil. pH 5.8 was a good compromise. Adjusting*

this pH allows the co-extraction of RNA and DNA. See Poursalavati et al 2023 for more information.

11. Incubate the tubes on ice for **2 minutes**.
12. Perform a second phenol chloroform extraction punctuated by a chilling period. In the hood, Add **600 µl of water-saturated phenol**. Close the tubes and mix either by shaking by hand, mix by pipetting up and down, or briefly vortexing, and incubate on ice for **3 minutes.**
    - *What it does: The second extraction helps remove any left-over organic substances that were not removed in the first round. It also helps isolate DNA-free RNA since more acidic phenol chloroform extractions favor RNA in the aqueous phase, while DNA is partially or completely partitioned into the phenol phase or interphase (Xu et al. 2019). DNA and RNA quality and length can be better preserved by gentle mixing of the phases by hand rather than vortexing. The ratio of chloroform to phenol changes during this round of extraction.*
13. Next, in the hood add **300 µl of chloroform**. Mix vigorously (or vortex briefly). And incubate on ice for **3 minutes.**
14. Next centrifuge for **10 minutes** at 10,000 × *g* at 4°C to separate the phases.
15. While the centrifuge is running, label another set of **2 ml centrifuge tubes**

Final chloroform extraction

16. In the hood, transfer the aqueous phase (~5**00-600µl**) to the new **2 ml centrifuge tubes**. For each sample, record the volume of aqueous phase on the tube.
17. Add a volume of chloroform equivalent to the aqueous phase volume written on the tube. Incubate on ice for **3 minutes.**
18. Centrifuge for **10 minutes** at 10,000 × *g* at 4°C.
19. While the centrifuge is running, label a set of **1.5ml tubes (low-binding, Rnase-free)**

RNA Precipitation

20. In the hood, transfer the aqueous phase (approximately **500 to 600 µl**) into a new **1.5-ml tube (low-binding RNAse-free).**
21. Add an **equivalent volume** of polyethylene glycol **(PEG)-NaCl precipitation buffer** (0.6 M NaCl and 30% PEG-8000) to the transferred aqueous phase and incubate on ice in a refrigerator (4°C) for 20 min.
22. (Optional step, but recommended) Add 1 µL of RNA-grade glycogen or 1 µL of LPA to help visualize the RNA pellet precipitation.
23. Place the tubes in the centrifuge, being certain to put them all in the same orientation, as this will make it easier to see the pellet. Then centrifuge for **20 minutes at 15,000 rpm and 4°C** to precipitate the pellet.
24. Remove tubes from the centrifuge, being careful not to disturb the pellet.

- *Helpful tip: As you remove tubes from centrifuge, mark the location of the pellet on the tube with a fine-tipped sharpie.*

Cleaning the RNA

25. Carefully **remove the supernatent** by pipetting, or pouring off, the supernatent. **Do not lose the pellet.** If the pellet becomes detached from the tube wall, re-centrifuge the tube.
26. Add **500 µL of 80% cold ethanol**. Gently rinse the pellet by flicking the tube with your fingers several times.
27. Centrifuge the tube at 15,000 rpm for **5 minutes** at 4°C
28. Repeat steps 25 and 26.
29. Pour or aspirate off the ethanol with a pipette tip. Try not to leave any ethanol on the side of the tube as it can cause issues with downstream analysis.

Drying and dissolving RNA

30. Turn the tubes upside down on a clean cloth in a clean laminar flow hood to dry for ~**5 minutes** or until all ethanol has evaporated.
31. Add **50 µl of nuclease-free water treated with RNAsecure**, and gently pipette up and down to resuspend the pellet. Store at **-80 °C to prevent degradation of the RNA**
32. Optional: If DNA contamination is present, treat with TURBO DNase (Ambion) according to manufacturers instructions. Add 1x Dnase buffer and 10 units of TURBO enzyme. Incubate for 30 minutes at 37C, then remove Dnase enzyme with phenol chloroform extraction

Preparation of Stock Solutions

Nuclease-free Materials/Reagents
Notes: Prepare all stock solutions in RNAse and DNAse free bottles. Details about battling RNAses can be found here: https://cshprotocols.cshlp.org/content/2019/2/pdb.top101857.full.

- Wherever reagents are not RNAse-free and if possible (not possible with Tris-buffer and other amine buffers), treat solutions with 0.1% DEPC for at least 1 h at 37°C, and then autoclave for 15 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

- Autoclaving glassware and plasticware may not be sufficient to inactivate RNase. Bake glassware for 4 h at 300°C. Treat plasticware either with DEPC or commercially available products that inactivate RNase upon contact (e.g., RNaseZap from Ambion).

- DEPC is water-sensitive. Store in a dry place and allow to come to room temperature slowely to prevent condensation that can facilitate it's conversion into diethylcarbonate.

Decontamination Solution (500 mL)
In an Erlenmeyer flask containing 200ml of deionized water, mix:
- 50 ml sodium hypochlorite (bleach), [10% vol/vol] (Grainger 56HN13)
- 5 g sodium dodecyl sulfate (SDS) [1% wt/vol] (Sigma 822050 - this grade only appropriate for decontamination use not as reagent)
- 5 g sodium hydroxide (NaOH) [1% wt/vol] (Sigma S5881-500G)
- 5 g sodium bicarbonate (baking soda $NaHCO_3$) [1% wt/vol] (Grainger 33X680 – this grade only appropriate for decontamination not as reagent)

Fill with deionized water up to 500ml

Rnase-free water (1 L)
Note: often, it's only slightly more expensive to purchase nuclease-free water
- add 1ml of Diethyl pyrocarbonate (DEPC – Sigma D5758-5ML) to 1L of milliq water
- autoclave for 15 minutes at 121C to degrade the DEPC and inactivate any left-over RNases.

1M phosphate buffer 5.8 (500ml)
(Helpful buffer calculator here – to modify protocol if needed
https://www.sigmaaldrich.com/US/en/support/calculators-and-apps/buffer-calculator)
- Add 60 g sodium phosphate monobasic (anhydrous) (Sigma S3139-250G) to 500ml milliq water or 78 g of sodium phosphate monobasic (dihydrate) (Sigma 71505-250G) to 500 ml milliq water
- After dissolving, test the pH (it is likely more acidic than necessary). Adjust the pH to 5.8 with drops of NaOH (base) or HCl (acid).

150mM phosphate buffer 5.8 (100ml)
- Add 15ml of 1M phosphate buffer 10 85ml milliq RNAse-free water
- Re-check the pH; adjust if necessary with NaOH or HCl

CTAB extraction buffer (100ml) – 200µL per extraction

CTAB is hard to dissolve in solution. Prepare a heat block to 65C to help with dissolving. Put a beaker with a stir bar on the block and add the following in order (do not add next ingredient until previous one is fully dissolved, CTAB can take several hours to overnight to dissolve):

- 50ml of milli-Q Nuclease-free water
- dissolve 4.1 g NaCl (molar mass 58.44), final concentration 0.7M
- 10 g CTAB [cetyltrimethylammonium bromide] (10% weight/volume) – Sigma H6269-100G
- 3.4 g PVP [Polyvinylpyrrolidone] (3.4% weight/volume) Sigma P0930-50G
- 24 mL 1M phosphate buffer (pH = 5.8, see above for how to make) (final concentration 240mM)

Add water until the solution is 100ml (~26ml)

Store at room temperature for up to 6 months.

Notes: CTAB is very sticky and hard to pipette. For best results, pipette 1mL aliquots into microcentrifuge tubes. Heat to 55-60C for accurate pipetting. After adding to sample, pipette up and down several times to make sure all the CTAB is in the sample and not left on the pipette tip.

3M Sodium Acetate (NaAc), pH = 4.6 (200ml)

- Prepare 160 mL of distilled nuclease-free water in a erlynmyer flask.
- Add 40.01 g of Sodium Acetate to the solution.
- Add 27.56 g of Acetic Acid to the solution.
- Adjust solution to final desired pH using HCl or NaOH.
- Add distilled water until volume is 200ml

Polyethylene glycol (PEG-NaCl) Precipitation Buffer (100ml)

- 3.51 g NaCl in 100ml = 0.6M
- 30 g PEG 8000
- fill with sterile, milli-q, RNAse free water to 100ml

80% cold ethanol (100ml) – cleaning pellet

Do NOT use lab-cleaning ethanol. Make a new solution for this purpose from high quality stock

- in a graduated cylinder, mix 80ml of high quality ethanol and fill to 100ml with RNAse-free miliq water.
- store cold at 4C

Notes on Safety

Many of the chemicals used in this protocol are quite hazardous. Please make sure to visit the CEMS Hazardous chemical fact sheet before you work with them. Here's some quick notes:

Phenol

- acutely toxic; corrosive, causes severe burns to skin and eyes
- affects central nervous system and can damage liver and kidneys
- has local anaesthetic effect so no pain may be felt on initial contact. Usually the contact area whitens first and then later severe burns may develop.
- can be absorbed through skin, toxic to fatal amounts can be absorbed in a relatively small area
- phenol is combustible, and can react with strong oxidizing agents (like bleach!) - do not store in same location
- Phenol must be handled with Viton, butyl or neoprene gloves, nitrile gloves are insufficient; Safety goggles should be worn along with protective clothing (lab coat) and all transfers of substances containing phenol should be conducted in a fume hood!
- Wash hands immediately after working with phenol.
- dispose as hazardous waste, appropriately.

Chloroform

- carcinogen; skin and eye irritant;
- inhalation can cause dizziness, headache, drowsiness,
- reacts violently with alkalimetals such as sodium and potassium; Strong bases should also be avoided mixing with chloroform
- light sensitive. In light autooxidizes to phosgene which is acutely toxic and should not be inhaled as it is acutely toxic; To mitigate keep exposure to light and air minimal.
- Viton gloves and chemical safety goggles are necessary. Chlorofom can soak through nitrile gloves in 2.5 minutes or less. If wearing nitrile gloves and chloroform gets on them, remove them immediately.

2-mercaptoethanol (also called beta-mercaptoethanol)

- toxic if swallowed or inhaled. Can be fatal if absorbed through skin;
- corrosive and can cause severe burns to skin and eyes
- extremely unpleasant odor that smells like natural gas at very low concentrations
- goggles, and lab coat are required. Nitrile gloves are fine. Wearing two pairs is recommended. Contaminated gloves (even just a few drops) must be disposed of as hazardous waste (due to odor).
- wash hands after removing PPE;
- must be used in a hood

When using Phenol and Chloroform in the Frey lab, at a minimum follow these protocols:

- Only use chloroform and phenol in the chemical fume hood
- Properly collect any chloroform or phenol waste in a labeled hazardous waste bottle, and keep in secondary containment in the chem fume hood (this is already set up).
- Double glove when using these solvents. You can tell right away if you dripped onto your glove as it gets cold. You have 3-5 minute before the solvent breaks through the glove time once you feel that, so good to have an outer set of gloves to immediately remove.
- Wear a lab coat.
- Any pipette tips or falcon tubes that have a chloroform or phenol residue can off-gas in the chem hood for a day before discarding into the biohazardous collection box.