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An optimized CTAB based RNA extraction from rhizosphere, followed by universal rRNA depletion for metatranscriptomics

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Kipa Tamrakar[1,2], Maureen Dolan[1,2], Asela Wijeratne[1]

[1]Arkansas State University; [2]Arkansas Biosciences Institute

Kipa Tamrakar
Arkansas State University, University of Florida

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We use this protocol and it's working

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Abstract

Metatranscriptomics is an approach to analyze the gene expression dynamics to find the functional microbial population in soil. This protocol describes an **optimized CTAB based Phenol Chloroform method** to extract good-quality RNA from soybean rhizosphere. Furthermore, it incorporates **Zymo-Seq RiboFree Total RNA Library Kit** to remove highly abundant ribosomal RNA (rRNA) that impedes the isolation of messenger RNA (mRNA)needed for transcriptomics analysis.

Guidelines

The step duration is calculated based on time required to process 3 rhizosphere samples.

Materials

Reagents & Equipment:

- RNaseZap® **Thermo Scientific Catalog #**AM9780
- Diethyl pyrocarbonate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5758

RNA Extraction:

1. 0.1 mm Zirconia/Silica Beads **Bio Spec Products Inc. Catalog #**11079101z
2. 0.5 mm Zirconia/Silica Beads **Bio Spec Products Inc. Catalog #**11079105z
3. CTAB (Hexadecyltrimethylammonium bromide) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**52365-50G
4. Sodium Chloride **Fisher Scientific Catalog #**S271
5. Polyvinylpyrrolidone **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**PVP40
6. Sodium phosphate monobasic monohydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S9638
7. Sodium Phosphate Dibasic Heptahydrate, ACS Reagent Grade, 500g Poly Bottle **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**7914-04
8. 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
9. UltraPure™ Phenol **Thermo Fisher Scientific Catalog #**15509-037
10. OmniPur® Chloroform **Thermo Fisher Scientific Catalog #**31-509-50ML
11. Isoamyl Alcohol (Clear, Colorless/Certified ACS), Fisher Chemical™ **Thermo Fisher Scientific Catalog #**A393-4
12. Sodium acetate, anhydrous, 99%, Thermo Scientific Chemicals **Thermo Fisher Scientific Catalog #**AAA1318430
13. Glacial acetic acid **Fisher Scientific Catalog #**A38-500
14. PEG 8000 Powder (Polyethylene Glycol), 500gm **Promega Catalog #**V3011
15. Deionized water - DI water

RNA Clean up:

1. RNA Clean & Concentrator™-5 **Zymo Research Catalog #**R1015
2. DNase I Set (RNase-free) **Zymo Research Catalog #**E1010
3. Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500

RNA Quality Control:

1. Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #**Q32852
2. RNA ScreenTape **Agilent Technologies Catalog #**5067-5576

3. RNA ScreenTape Sample Buffer **Agilent Technologies** **Catalog #**5067-5577
4. RNA ScreenTape Ladder **Agilent Technologies** **Catalog #**5067-5578

RNAseq Library Preparation:

1. Zymo-Seq RiboFree Total RNA Library Kit **Zymo Research** **Catalog #**R3000

Consumables:

RNA extraction:

1. 1 L Glass bottles
2. 100 mL Glass bottles
3. 50 mL Nuclease free tubes
4. Weighing boats
5. 2 mL Screw-capped tubes
6. 0.1 mm & 0.5 mm zirconium beads
7. 2 mL Nuclease free microfuge tubes
8. 1.5 mL Nuclease free microfuge tubes
9. 50 mL Serological pipettes
10. 10 mL Serological pipettes
11. 20 µL pipette and tips
12. 200 µL pipette and tips
13. 1000 µL pipette and tips

RNA Clean-up:

1. 1.5 mL Nuclease free microfuge tubes
2. 20 µL pipette and tips
3. 200 µL pipette and tips
4. 1000 µL pipette and tips

RNA quality Control:

1. Qubit assay tubes
2. Strip tubes and caps
3. Loading tips
4. 20 µL pipette and tips
5. 2.5 µL pipette and tips

RNAseq Library Preparation:

1. 0.2 mL PCR tubes
2. Magnetic stand
3. 200 µL pipette and tips
4. 20 µL pipette and tips
5. 2.5 µL pipette and tips

Reagent Preparation:

1. DEPC-treated water

- Add 1 mL of Diethyl Pyrocarbonate (DEPC) to 1 L DI water in a 1 L glass bottle.
- Mix by stirring and treat the DEPC overnight.
- Next day, autoclave at 121ºC at 15psi for 30 mins.

2. Cetyltrimethylammonium bromide (CTAB) extraction buffer:

A. **100ml of 5M Sodium Chloride (NaCl) stock**

- Dissolve 14.61 g of NaCl in 70 mL DI water. Stir to mix
- Bring the volume to 100 mL by adding DI water.
- Autoclave to sterilize.

B. 100ml stock of 500mM Sodium Phosphate (NaP) Buffer, pH 5.8

- Prepare [M] 500 millimolar (mM) sodium phosphate monobasic by dissolving 6.8995 g of sodium phosphate monobasic (monohydrate, M.W. 138.0) in 100 mL of DI water.
- Prepare [M] 500 millimolar (mM) sodium phosphate dibasic by dissolving 13.4035 g of sodium phosphate dibasic (heptahydrate, M.W. 268.0) in 100 mL of DI water.
- Take 95 mL of 1M sodium phosphate monobasic in a beaker.
- Adjust the pH to 5.8 by adding 1M sodium phosphate dibasic (usually around 5ml).
- Autoclave to sterilize.

C. Mix all reagents as described in the following table to prepare 500mM NaP buffer, pH 5.8:

Reagents	Stock	Final	50ml
CTAB	--	10%	5g
NaCl	5M	0.7M	7ml
PVP	--	3.40%	1.7g
NaP Buffer (pH 5.8)	500mM	240mM	24ml
DEPC treated water			~ 19ml

Preparation of CTAB extraction buffer

3. Water-saturated Phenol:

- Thaw the bottle in water bath set at 50 °C
- Add DI water to fill the amber bottle to the base of the neck. (A saturated solution of phenol contains 12.36 mL of deionized water per 100 g of phenol.)
- Cap securely then mix the organic and aqueous phases until they form a fine emulsion.
- Store at 4 °C overnight until phases separate.
- Aspirate the upper aqueous layer.

- Once saturated, store with some water phase on top of the phenol.
- Store at 4^{o}C. Reagent is typically stable for a month or two.

NOTE: The phenol phase will eventually turn pink upon oxidation – DO NOT USE when pink!

4. 100 mL of Chloroform: Isoamyl alcohol (49:1) solution:

- Take 98 mL of chloroform in a glass amber bottle.
- Add 2 mL of Isoamyl alcohol to it. Mix properly.

5. 100 mL of 3M Sodium acetate (NaOAc), pH 4.6:

- Prepare [M] 3 Mass Percent acetic acid solution by mixing 8.62 mL of glacial acetic acid (17.4N) in 41.38 mL DI water.
- Prepare [M] 3 Mass Percent NaOAc solution by dissolving 24.61 g NaOAc (82.03 g/mol) in 100 mL DI water)
- Mix 21 mL 3M acetic acid with 79 mL 3M NaOAc solution.
- Autoclave to sterilize.

5. Precipitation Buffer:

A. Oven bake glass bottle at 225 °C Overnight. For caps, perform DEPC treatment Overnight

These bottle will be used for preparing 2M NaCl, 50% PEG, and precipitation buffer.

B. Prepare 100 mL **of** [M] 2 Mass Percent **NaCl:**

- Dissolve 11.68 g of NaCl in 60 mL deionized water in a beaker. Stir to mix
- Bring the volume to 100 mL by adding DI water.
- Add 100 µL DEPC to solution. Treat Overnight .
- Transfer the solution to an oven baked glass bottle.
- Autoclave to inactivate DEPC.

C. Prepare 100 mL **of** [M] 50 % (v/v) **PEG solution:**

- Weigh 50 g of PEG-8000.
- Dissolve in 50 mL DEPC treated water. Stir to mix.
- Transfer the solution to an oven baked glass bottle using serological pipette.
- Add DEPC water to bring volume to 100ml.

D. Mix the solution as per the following table to prepare NaCl-PEG precipitation buffer:

Reagents	Stock	Final	10ml
NaCl	2M	0.6M	3ml
PEG - 8000	50%	30%	6ml
DEPC treated water			~ 1ml

Preparation of PEG-NaCl precipitation buffer

6. [M] 75 % (v/v) **Ethanol:**

- Dispense 37.5 mL Absolute ethanol in a nuclease free 50ml tubes.
- Add 12.5 mL DEPC treated water to bring the total volume to 50ml.
- Store in -20°C freezer until use.

Safety warnings

DEPC, Phenol, Chloroform, Isoamyl alcohol and 2-Mercaptoethanol are hazardous chemical, which should be handled under fume hood.

Before start

- Clean the workspace with RNase decontamination solution (i.e. RNaseZap)
- Treat pipettes, tip boxes and racks used during experiment with RNase decontamination solution.

RNA Extraction

4h

1 Prepare bead beating tubes by adding 950 mg 0.1 mm and 50 mg 0.5 mm silica beads in 2 mL screw-capped tube

5m

2 Weigh 250 mg of rhizosphere in a clean weigh boat and transfer sample to bead tubes.

10m

3 Add the following reagents to each sample:

10m

- 600 µL water-saturated phenol
- 400 µL 500 mM NaP buffer, pH 5.8
- 200 µL Chloroform: Isoamyl alcohol (49:1)

Note

Set water bath at 60°C and place tube containing CTAB extraction buffer.

- 200 µL pre-heated CTAB extraction buffer
- 10 µL 14.3 M 2-Mercaptoethanol
- Vortex to mix

4 Incubate samples on ice for 2 mins.

2m

5 Homogenize using Mini beadbeater (Biospec, Cat# 112011) in two bead-beating steps (3.5 × 1000 strokes per min for 40 secs) with one intermediate cooling step on ice for 1 min.

5m

6 Completely mix samples using a series of vortex cycles (one cycle with 20 secs vortexing/40 secs cooling on ice; followed by two cycles of 10 secs vortexing/30 secs cooling on ice)

5m

7 Centrifuge at 10,000 xg for 10 mins at 4ºC. Transfer the aqueous phase to a new 2 mL microfuge tube.

15m

8 Add 350 µL 3M Sodium acetate, pH 4.6 to the tube and mix by vortexing. Incubate on ice for 3 mins.

5m

9 Add 600 µL water-saturated phenol and vortex to mix. Incubate on ice for 3 mins. 5m

Note

STOP POINT: Samples can be stored on ice bath for few hours

10 Add 300 µL Chloroform: Isoamyl alcohol and pulse vortex to mix till milky solution is obtained. Incubate on ice for 3 mins. 5m

11 Centrifuge at 10,000 xg for 10 mins at 4ºC. Transfer (~800 µL) aqueous phase into a new 2 mL microfuge tube. 10m

12 Add 800 µL Chloroform: Isoamyl alcohol (49:1) and pulse vortex to mix sample until a milky consistency is attained. Incubate on ice for 3 mins. 5m

13 Centrifuge samples at 10,000 xg for 10 mins at 4ºC. 15m

Note

After centrifugation, carry out the rest of the steps in a clean area. An isolated area properly sterilized with RNase decontamination solution can be considered as a clean area.

14 Transfer aqueous phase into a new 1.5 mL microfuge tube.

15 Add one volume of PEG-NaCl precipitation buffer and quick vortex. 3m

16 Incubate on ice bath in a 4°C refrigerator for 20 mins. 20m

17 Centrifuge samples at 20,000 xg for 20 mins at 4°C. 20m

18 Add 1500 µL of 70% ice-cold ethanol. Centrifuge at 20,000 xg at 4 °C for 5mins. Discard ethanol and retain pellet. 10m

19 go to step #18 10m

20 Minifuge samples to enable removal of any remining ethanol.

21 Air dry pellet under laminar hood and resuspend pellet in 50 µL Nuclease free water. 15m

> Note
>
> Do not leave the tubes to dry for more than 15 mins

22 RNA samples can be stored at -80°C until further use or proceed directly to RNA clean-up step (go to step #23)

RNA Clean up using ZYMO RNA Clean & Concentrator Kits-5 45m

23 Add 100 µL of RNA binding buffer to each 50 µL total RNA sample and mix by pipetting. 3m

24 Add 150 µL of absolute ethanol and mix by pipetting. 3m

25 Transfer 300 µL of this mixture to the Zymo-Spin IC Column placed in a collection tube. 5m

26 Centrifuge 10,000 xg for 30 secs at room temperature and discard the flow-through. 5m

27 Add 400 µL RNA Wash Buffer to the column. Centrifuge at 10,000 xg for 30 secs at room temperature and discard the flow-through. 5m

28 Prepare DNase I Reaction Mix as follows in a Nuclease free tube: 20m

Component	Amount
DNase I (reconstituted; 1 U/ul)	5 µl
DNA Digestion Buffer	35 µl
Total	40 µl

ZYMO DNase Mix

- Mix by gentle inversion.
- Add 40 µL of the mixture directly onto the column matrix.
- Incubate column at room temperature for 15 minutes.

29 Add 400 µL RNA Prep Buffer to the column and centrifuge at 10,000 xg for 30 secs at room temperature Discard the flow-through. 3m

30 Add 700 µL RNA Wash Buffer to the column and centrifuge at 10,000 xg for 30 secs at room temperature. Discard the flow-through. 3m

31 Add 400 µL RNA Wash Buffer to the column and centrifuge at 10,000 xg for 1 min at room temperature to ensure complete removal of the wash buffer. Carefully, transfer the column into a RNase-free 1.5 mL tube. 5m

32 Add 15 µL DNase/RNase-Free Water directly to the column matrix. Incubate for 1 min and centrifuge at 10,000 xg for 30 secs at room temperature 3m

33 Purified RNA samples can be stored at -80°C until further use.

RNA Quality Control 30m

34 Measure RNA quantity using 1 µL of each purified total RNA sample and a Qubit RNA HS kit assay in Qubit fluorometer (ThermoFisher, Cat# Q33238). 10m

35 Measure RNA quality with 1.5 µL of sample on a Nanodrop spectrophotometer (ThermoFisher, Cat# ND-ONE-W). 5m

36 Confirm RNA samples were intact (not degraded) using an Agilent Tapestation (Cat# G2992AA) with 1 µL of each RNA sample. 10m

RNAseq Library Preparation using Zymo-Seq RiboFree® Total RNA Library Kit

2d

37 cDNA Synthesis

1h

Program	Stage	Temperature	Time
Primer Annealing	1	98°C	3 min
	2	4°C	Hold
Reverse Transcription	3	25°C	5 min
	4	48°C	15 min
	5	4°C	Hold

ZYMOseq cDNA Synthesis Cycle

- Transfer 250 ng RNA to a clean PCR tube and adjust the total volume to 8 µL with Nuclease free water.
- Add 2 µL of **cDNA Synthesis Reagent 1** to the sample tube for a total of 10 µL. Mix thoroughly by pipetting and spin down.
- Place the tube in the thermal cycler and run Primer Annealing program (Stage 1-2); see detail settings in Table below.
- Add 10 µL of **cDNA Synthesis Reagent 2** to each sample during the 4°C hold of Stage 2. Mix thoroughly by pipetting and spin down.
- Resume the Reverse Transcription program on the Thermal Cycler (Stage 3-5); see detail settings in Table below.

Note

Proceed directly to RiboFree Universal Depletion for the depletion of ribosomal RNA.

38 RiboFree Universal Depletion

2h 30m

Program	Stage	Temperature	Time
Pre-Depletion Incubation	1	98°C	3 min
	2	68°C	5 min
	3	68°C	Hold
Depletion Reaction	4	68°C	15 min
Stop Depletion	5	68°C	Hold
	6	98°C	2 min
	7	25°C	Hold
Depletion Cleanup	8	55°C	15 min
	9	95°C	5 min
	10	25°C	Hold
DNA Elution	11	95°C	5 min
	12	25°C	Hold

ZYMOseq Ribo-depletion Cycle

- Following cDNA synthesis, transfer sample tubes from Step 37 to ice and add 10 µL of **Depletion Reagent 1** for a total of 30 µL. Mix thoroughly by pipetting and spin down.
- Return sample tubes to the thermal cycler and run PreDepletion Incubation program (Stage 1-3); see detail settings in Table above.

Note

Do not remove the tube from the thermal cycler at the 68°C hold of Stage 3.

- Add 10 µL of **Depletion Reagent 2** to the tube in the thermocycler. Mix thoroughly by pipetting.
- Close the thermal cycler lid and continue Depletion Reaction program (Step 4); see detail settings in Table above

Note

DO NOT remove the tube from the thermal cycler at the Stage 5 hold.

- Add 10 µL of **Depletion Reagent 3** to each sample tube in the thermocycler. Mix thoroughly by pipetting.
- Close the thermal cycler lid and continue through Stop Depletion (Stage 6-7).
- Add 2 µL of **Depletion Reagent 4** to each sample tube. Quickly remove the tube from the thermal cycler and gently flick to mix. Spin down briefly to collect the reaction. Immediately return the tube to the thermal cycler to continue and continue through Depletion Cleanup program (Stage 8-10).
- Remove the tube from the thermal cycler and spin down. Add 26 µL of 95% ethanol to the sample for a total of 78 µL. Mix thoroughly by pipetting.
- Add 156 µL of Select-a-Size MagBeads to the tubes and incubate at room temperature for 5mins.
- Pellet the beads by placing the tubes on a magnetic stand. Wash the beads with 200ul of Zymo-Seq Wash Buffer.
- Repeat the washing step.
- Remove the tube from the magnetic stand. Spin down and place back on the magnetic stand. Without disturbing the pellet, aspirate any residual Zymo-Seq Wash Buffer.
- Air dry all sample pellets while tubes remain on the magnetic stand
- Resuspend the beads in 11 µL of DNA Elution Buffer and transfer the tube to the thermal cycler to complete DNA Elution program (Stage 11-12).
- Remove the tube from the thermal cycler, collect sample by centrifugation and place each tube on the magnetic stand to elute.

- Transfer 10 µL of the eluate Air dry all sample pellets while tubes remain on the magnetic standto a new 0.2 mL PCR tube.

Note

STOP POINT: Purified cDNA can be safely stored at -20°C short term until proceeding to next step in the process.

39 Adapter Ligation 2h 30m

- Preheat a thermal cycler to 98°C (lid temperature at 105°C).
- For each sample from step 28, combine the following components (see Table below) in a 0.2 mL PCR tube, on ice. Mix by pipetting thoroughly and spin down.

Component	Volume
Eluate from Step 28	10 µL
Adapter Ligation Buffer 1	2 µL
DNase/RNase-Free Water	8 µL
Total Volume	20 µL

ZYMOseq Adapter Ligation Mix 1

- Incubate the tube on ice for 2 minutes.
- Heat shock by immediately placing the tube at 98°C (lid temperature at 105°C) for 3 minutes.
- Immediately return the tube to ice and incubate for at least 2 minutes.

Note

During this incubation, reset the thermal cycler to 37°C (lid temperature at 50°C) for next step in the process. Leave the lid open to allow for faster cooling.

- Mix the Adapter Ligation Master Mix by vortexing for 30 secs before use. For each sample, add the following components, in the order listed in the Table below. Carry this step out on ice.

Component	Volume
Reaction from previous step	20 µL
Adapter Ligation Buffer 2	2 µL
Adapter Ligation Buffer 3	2 µL
Adapter Ligation Master Mix	26 µL
Total Volume	50 µL

ZMOseq Adapter Ligation Mix 2

- Mix by vortexing for 1 min and spin down.
- Incubate the sample tubes at 37°C (lid temperature at 45°C) for 1 hour.
- Remove the tube from the thermal cycler. At room temperature, add 85 µL of DNA Elution Buffer to the sample and mix thoroughly by pipetting.
- Add 48.6 µL of Select-a-Size MagBeads to the tubes and incubate at room temperature for 5mins.
- Pellet the beads by placing the tubes on a magnetic stand. Wash the beads with 200 µL of ZymoSeq Wash Buffer.
- Repeat the washing step.
- Remove the tube from the magnetic stand. Spin down and place back on the magnetic stand. Without disturbing the pellet, aspirate any residual Zymo-Seq Wash Buffer.
- Air dry all sample pellets while tubes remain on the magnetic stand.
- Resuspend the beads in 16 µL of DNA Elution Buffer and spin down. Incubate at room temperature for 1 min.
- Place the tube on the magnetic stand to elute.
- Transfer 15 µL of the eluate to a new 0.2 mL PCR tube.

Note

STOP POINT: Purified cDNA can be safely stored at -20°C short term until proceeding to next step in the process.

40 RNAseq Library Amplification 1h 30m

- Add 10 µL of the appropriate Zymo-Seq UDI Primers to each sample tube (15 µL eluate from Step 39) for a total volume of 25 µL. Mix thoroughly by pipetting.
- Add 25 µL of Amplification PreMix to the tube for a total volume of 50 µL. Mix thoroughly by gently pipetting and spin down.
- Place the tube in the thermal cycler and run the following program:

Step	Temperature	Time	Cycle
1	98°C	45 sec	1
2	98°C	15 sec	10
3	60°C	30 sec	
4	72°C	1 min	
5	72°C	1 min	1
6	4°C	∞	Hold

ZYMOseq Library Amplification Cycle

- Remove the tube from the thermal cycler and spin down. Add 50 µL of DNA Elution Buffer to the tube.
- Add 80 µL of Select-a Size MagBeads to the tube. Mix thoroughly by pipetting until homogenous. Incubate for 5 mins at room temperature.
- Place the sample tube on a magnetic stand until the beads have fully separated from the solution. Without dislodging the bead pellet, aspirate slowly and discard the supernatant.
- Remove the sample tube from the magnetic stand. Add 100 µL of DNA Elution Buffer to the beads and mix thoroughly by pipetting until homogenous.
- Add 80 µL of Library Binding Solution to the tube. Mix thoroughly by pipetting. Incubate for 5 mins at room temperature.
- Pellet the beads by placing the tubes on a magnetic stand. Wash the beads with 200 µL of ZymoSeq Wash Buffer.
- Repeat the washing step.
- Remove the tube from the magnetic stand. Spin down and place back on the magnetic stand. Without disturbing the pellet, aspirate any residual Zymo-Seq Wash Buffer.
- Air dry all sample pellets while tubes remain on the magnetic stand.
- Resuspend the beads in 21 µL of DNA Elution Buffer and spin down. Incubate at room temperature for 1 min.
- Place the tube on the magnetic stand to elute.
- Transfer 20 µL of the eluate to a new 0.2 mL PCR tube.
- Use 1 µL of each generated cDNA library sample for quantification using a Qubit DNA HS kit assay.
- The eluate is the final RNA-Seq library. Libraries may be stored at ≤ 4°C overnight or ≤ -20°C for long-term storage.

Protocol references

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