mRNA extraction

At GEUS we use two different protocols for RNA extraction form soil:

1. Jacobs method (based on Griffith but heavily modified)
2. MOBIOs PowerSoil RNA kit with DNA co extraction

How do we chose between the two protocols?
With some soils Jacobs methods completely fail while MOBIOs method works with all soils hitherto tested. The swift handling of samples in Jacobs method makes us believe more in this method for mRNA studies. Studies of rRNA for deep sequencing or DGGE analysis are more securely done with mobio's kit.

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RNA/DNA extraction ML01.002.002

Author:	JBAE/CSJ/PBJ	**Valid from:**	2011-03-18	**Expired:**	
Responsible:	CSJ				
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RNA/DNA extraction protecol

After Griffiths et al 2000 AEM 66:5488-5491 with several modification including the use of a RNA CleanUp kit replacing the final ethanol precipitation.

All surface areas are wiped with UltraClean Lab Cleaner (MOBIO 12095) prior to start.
Centrifuge is cooled down to 4C.
All plastic-ware is RNAse free
All reagents from the protocol are placed in front of you in the hood.

- Ceramic bead tubes 1.4 mm (MOBIO 13113)
- Phenol-Chloroform-Isoamylalcohol (25:24:1), (Sigma P3803) 100ml, pH 8;
- CTAB (One volume 10% (wt/vol) CTAB (Sigma 52365) in 0.7M NaCl (DEPC treated) is mixed with one volume of 240mM phosphate buffer pH 8.0 (DEPC treated) (may pellet after storage, dissolve by stirring) Store wrapped in aluminium-foil)
- Chloroform-isoamyl alcohol (24:1)(Sigma 25666)
- PEG 30% wt/vol polyethylene glucol 6000 in 1,6 M NaCl (Sigma 81253) (DEPC-treat)
- Glycogen, (keep on ice) Roche 10 901 393 001 (20 mg/ml)
- DEPC treated DNase/RNase free water
- NucleoSpin RNA Clean-up kit (Macherey Nagel 740 903)

TO do:

1. Samples taken from environmental sources should always be snapshot frozen in liquid nitrogen and stored at -80 ^{o}C prior to extraction.
2. Samples are freeze dried to avoid liquid when melting.
3. 0.5 gram of frozen (-80°C) soil is transferred to Ceramic Bead Tubes (MOBIO).
4. 0.5 ml Phenol-chloroform-isoamyl alcohol (lower phase) is added to the soil followed by 0.5 ml CTAB extraction buffer (Don't keep on ice - CTAB will crystallize at 4°C. On the other hand, the tubes shouldn't be kept at 20°C to long).
5. Lyse samples 2 x 20 sec.(with 60 s on ice in between) at speed 5 in BIO101 Fast prep FP120.
6. Centrifuge samples at 16..000 g for 5 min at 4°C.
7. (Optional) If the upper phase is still considerable brown, it may help to put the samples on ice for another 10 min. and recentrifuge.
8. Approximately 425 µl of the supernatant (aqueous phase) is carefully transferred to new tubes on ice.
9. Remove phenol totally by slowly mixing (no vortex) with 425 µl (equal vol) chloroform-isoamyl alcohol (24:1) (Sigma 25666).
10. Centrifuge at 16.000 g for 5 min at 4°C.
11. Transfer the aqueous phase (it should be a little less than the aliquot transferred in step 6 – It is important that the same amount is transferred for all the samples - approx 400 µl) to new tubes on ice (carefully avoiding the interphase).

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12. Precipitate total nucleic acids from aqueous phase with two volume (800 µl) of 30% PEG and 1 µl glycogen (mix by carefully pipetting) and place on ice for 2 hours (use a styroform box with lid).
13. Centrifuge tubes 30 min at 16.000 g at 4°C (put the tubes with the lids in the same direction in the centrifuge, to ease spotting the pellet afterwards).
14. Pipet away the entire supernatant. Be careful not to remove the pellet, it may be difficult to see.
15. Resuspend in 150µl DEPC treated dH_2O by shaking in eppendorf shaker at 4°C at 1400rpm until dissolved.
16. Add 150µl Clean up solution from kit (Macherey Nagel) and vortex 2x5 sec.
17. Transfer the entire volume to filter tubes and centrifuge 1 min at 10.000 g.
18. Transfer filter to new collection tube and add 400 µl Wash solution and centrifuge 1 min at 10.000 g.
19. Discard flow-through and add 200µl wash solution and centrifuge 1 min at 10.000 g.
20. Discard flow-through and centrifuge 2 min at 16.100 g.
21. Transfer filter to new collection tube and elute RNA in 20µl DEPC treated water. Centrifuge 1 min at 10.000 g.
22. Transfer 8 µl to a 200 µl RNase free tube for DNase treatment and dilute an other 10 µl 10x in 90 µl DEPC water in 200 µl RNase free tubes. The ladder should be used for DNA-PCR.
23. Place all tubes at -80°C.

Remember, when working with RNA and especially mRNA you should always be a bit paranoid. Everything around you may contain RNases on the surface, which will possible ruining your experiment. So therefore, be careful always to work on a “clean” spot, reduce the time for extraction as much as possible and keep samples on ice. mRNA is very labile and only the cDNA product is stable.

Therefore its recommended to proceed with DNase treatment and reverse transcriptase the same day.

DNase treatment

Materials:
- RNA samples from yesterday
- One RNase free 2 ml micro centrifuge tube
- DNase I (we are using a kit from Fermentas)
- 10× reaction buffer
- 25 mM EDTA solution

To do:

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1. Mix on ice a mastermix of 1 µl DNase I and 1 µl 10×reaction buffer per reaction (make enough for a couple of extra reactions, to be sure to have enough)
2. Add on ice 2 µl of the mastermix to each of your RNA samples and centrifuge
3. Place the samples in the PCR cycler and incubate at 37°C for 30 min, add 1µl EDTA solution, and terminate the reaction by incubation at 65°C for another 10 min
4. Keep the samples on ice

Reverse transcription (RT)

Materials:
- DNase treated RNA samples from the DNase treatment step
- One RNase free 2 ml micro centrifuge tube
- RNase free 0.2 ml tubes in strips
- Hexamer primer
- dNTP mix
- DEPC treated water
- 5×reaction buffer
- Ribolock RNase inhibitor
- RevertAid premium reverse transcriptase

To do:

1. Mix on ice a mastermix of 0.5 µl hexamer primer, 0.5 µl dNTP mix, 0.25 µl DEPC treated water, 2 µl 5×reaction buffer, 0.25 µl Ribolock RNase inhibitor, and 0.5 µl RevertAid premium reverse transcriptase for each reaction (make one reaction too many)
2. Add on ice to new 0.2 µl reaction tubes 4 µl mastermix followed by 6 µl of the DNase treated RNA sample and centrifuge
3. Place the tubes in the PCR machine and incubate at 25°C for 10 min, 50°C for 30 min, followed by 85°C for 5 min
4. Place the samples in the freezer

MOBIOs PowerSoil RNA isolation kit with DNA co-extraction kit

ML01.003.001

Author:	CSJ	**Valid from:**	2011-06-14	**Expired:**	
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All surface areas are wiped with UltraClean Lab Cleaner (MOBIO 12095) prior to start.
Centrifuge is cooled down to 4C.
All plastic-ware is RNAse free
All reagents from the protocol are placed in front of you in the hood.

- MOBIO RNA PowerSoil isolation kit 12866-25
- MOBIO RNA PowerSoil DNA elution accessory kit – 12867-25
- Phenol-Chloroform-Isoamylalcohol (25:24:1), (Sigma P3803) 100ml, pH 8;

1. Add up to 2 g of soil to the 15 ml Bead Tube.

2. Add 2.5 ml of Bead Solution to the Bead Tube and vortex to mix.

3. Add 0.25 ml of Solution SR1 to the Bead Tube and vortex to mix.

4. Add 0.8 ml of Solution SR2 and place the Bead Tube on the Vortex Adapter (MO BIO Catalog # 13000-V1-15 for Vortex Genie 2) and vortex at maximum speed for 5 minutes.

5. Remove the Bead Tube from the Vortex Adapter and add 3.5 ml of phenol: chloroform:isoamyl alcohol (pH 6.5 – 8.0,]) and vortex to mix until the biphasic layer disappears.

6. Place the Bead Tube on the Vortex Adapter and vortex at maximum speed for 10 minutes.

7. Remove the Bead Tube from the Vortex Adapter and centrifuge at 2500 x *g* for 10 minutes at room temperature.

8. Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to a clean 15 ml Collection Tube (provided). The thickness of the interphase will vary depending on the type of soil used. Discard the phenol:chloroform:isoamyl alcohol in an approved waste receptacle. Note: The biphasic layer will be thick and firm in soils high in organic matter and may need to be pierced to remove the bottom phenol layer.

9. Add 1.5 ml of Solution SR3 to the aqueous phase and vortex to mix. Incubate at 4°C for 10 minutes.

10. Centrifuge at 2500 x g for 10 minutes at room temperature.

11. Transfer the supernatant, without disturbing the pellet, to a new 15 ml Collection Tube (provided).

12. Add 5 ml of Solution SR4 to the Collection Tube containing the supernatant, invert or vortex to mix, and incubate at -20°C for 30 minutes.
Note: For samples with high salt content, incubation in Solution SR4 at room temperature

will result in higher RNA yields.

13. Centrifuge at 2500 x *g* for 30 minutes at room temperature.

14. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 minutes. Note: Depending on soil type, the pellet may be large and/or dark in color.

15. Shake Solution SR5 to mix. Add 1 ml of Solution SR5 to the 15 ml Collection Tube and resuspend the pellet completely. (Note: Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45°C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.)

16. Prepare one RNA Capture Column (provided) for each RNA Isolation Sample:
a. Remove the cap of a 15 ml Collection Tube (provided) and place the RNA Capture Column inside the 15 ml Collection Tube. The column will hang in the 15 ml Collection Tube.
b. Add 2 ml of Solution SR5 to the RNA Capture Column and allow it to gravity flow through the column and collect in the 15 ml Collection Tube. Allow Solution SR5 to completely flow through the column (Optional: The Collection Tube may be emptied after Solution SR5 has completely flowed through the column. Note: DO NOT ALLOW THE COLUMN TO DRY OUT PRIOR TO LOADING THE RNA ISOLATION SAMPLE.)

17. Add the RNA Isolation Sample from Step 15 onto the RNA Capture Column and allow it to gravity flow through the column. Collect the flow through in the 15 ml Collection Tube.

18. Wash the column with 1 ml of Solution SR5. Allow it to gravity flow and collect the flow through in the 15 ml Collection Tube.

19. Transfer the RNA Capture Column to a new 15 ml Collection Tube (provided). Shake Solution SR6 to mix and then add 1 ml of Solution SR6 to the RNA Capture Column to elute the bound RNA into the 15 ml Collection Tube. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube. Note: A new kit is available for DNA elution. See *DNA Elution Procedure in the Hints and Troubleshooting Guide or c*ontact MO BIO for details at technical@mobio.com

20. Transfer the eluted RNA to a 2.2 ml Collection Tube (provided) and add 1 ml of Solution SR4. Invert at least once to mix and incubate at -20°C for 10 minutes.

21. Centrifuge the 2.2 ml Collection Tube at 13,000 x *g* for 15 minutes at room temperature to pellet the RNA.

22. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.

23. Resuspend the RNA pellet in 100 µl of Solution SR7. (Note: Although DNA carryover does not

occur with the majority of soil types, certain soils high in organic matter may present unique carryover situations. In situations where the absence of DNA contamination is critical, the purified RNA should be tested for potential DNA carryover by performing PCR with qualified primers on the isolated RNA without performing prior reverse transcription amplification. The absence of a detectable amplification fragment by agarose electrophoresis indicates the absence of detectable carryover DNA. In the event DNA is detected, DNase treatment of the isolated RNA is recommended; see Additional Information Section for instruction).

DNA Elution Procedure

1. Transfer the RNA Capture Column from step 19 of the RNA PowerSoil® Total RNA Isolation Kit to a 15 ml Collection Tube (provided) and add 1 ml of Solution SR8 to the RNA Capture Column to elute the bound DNA into the 15 ml Collection Tube. Allow Solution SR8 to gravity flow into the 15 ml Collection Tube.

2. Transfer the eluted DNA to a 2.2 ml Collection Tube (provided) and add 1 ml of Solution SR4. Invert at least once to mix and incubate at -20°C for 10 minutes.

3. Centrifuge the 2.2 ml Collection Tube at 13,000 x *g* for 15 minutes at room temperature to pellet the DNA.

4. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.

5. Resuspend the DNA pellet in 100 µl of Solution SR7.

MOBIOs PowerSoil RNA isolation kit with DNA co-extraction kit **ML01.003.001**

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DNase treatment

Materials:
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- One RNase free 2 ml micro centrifuge tube
- DNase I (we are using a kit from Fermentas)
- 10× reaction buffer
- 25 mM EDTA solution

To do:

1. Mix on ice a mastermix of 1 µl DNase I and 1 µl 10×reaction buffer per reaction (make enough for a couple of extra reactions, to be sure to have enough)
2. Add on ice 2 µl of the mastermix to each of your RNA samples and centrifuge
3. Place the samples in the PCR cycler and incubate at 37°C for 30 min, add 1µl EDTA solution, and terminate the reaction by incubation at 65°C for another 10 min
4. Keep the samples on ice

Reverse transcription (RT)

Materials:
- DNase treated RNA samples from the DNase treatment step
- One RNase free 2 ml micro centrifuge tube
- RNase free 0.2 ml tubes in strips
- Hexamer primer
- dNTP mix
- DEPC treated water
- 5×reaction buffer
- Ribolock RNase inhibitor
- RevertAid premium reverse transcriptase

To do:

1. Mix on ice a mastermix of 0.5 µl hexamer primer, 0.5 µl dNTP mix, 0.25 µl DEPC treated water, 2 µl 5×reaction buffer, 0.25 µl Ribolock RNase inhibitor, and 0.5 µl RevertAid premium reverse transcriptase for each reaction (make one reaction too many)
2. Add on ice to new 0.2 µl reaction tubes 4 µl mastermix followed by 6 µl of the DNase treated RNA sample and centrifuge
3. Place the tubes in the PCR machine and incubate at 25°C for 10 min, 50°C for 30 min, followed by 85°C for 5 min
4. Place the samples in the freezer