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RNA extraction & depletion V.1

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We use this protocol and it's working

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Abstract

This protocol provides a detailed methodology for the extraction and purification of RNA from both enrichment cultures and sampels from anaerobic digesters. The targeted microbial communities include organisms relevant to the anaerobic digestion process.

Protocol overview:

1. Sample preparation
Protocol includes description for;
a) Samples from enrichment/pure cultures.
b) Samples from anaerobic digesters.

2. RNA extraction & purification
RNA is extracted using TriZol/Chloroform extraction.
Extraction is followed by purification using Quick-RNA™ Fecal/Soil Microbe Microprep kit, excluding the cell lysis and extraction step included in the kit. Kit protocol is followed from step 4.

3. Quality control
Agilent 2100 BioAnalyzer used for quality control and concentration measurement.
The assay "*Prokaryote Total RNA Nano Series II*" is used at this step.

4. rRNA depletion
Ribosomal RNA is removed using the Pan-Prokaryote riboPOOL kit, followed by ethanol precipitation.

5. Concentration measurement
Agilent 2100 BioAnalyzer used for quality control and concentration measurement.
The assay "*mRNA Nano Series II*" is used at this step.

It is recommended that Sample preparation, RNA extraction & purification, and Quality control is carried out without pause. After the quality control, samples can be stored at -80ºC until further processing.

Materials

For materials needed for RNA depletion see section 25

For each RNA extraction sample the following material is needed:

Tubes/filters/columns
1x ZR BashingBead Lysis Tube
2x Zymo-Spin™ IIICG Column
1x Zymo-Spin™ III-HRC Filter
1x Zymo-Spin™ IC Column
5x Collection tubes (Could be reused at some steps)
3× 2.5ml RNAse free Eppendorf tubes

Solutions/Buffers:
1 ml TriZol Reagent/750 µl TriZol LS
200 µl Chloroform
1-2 ml Ethanol (95-100%)
800 µl RNA Prep Buffer (kit)
115 µl DNase/RNase-Free Water (kit)
600 µl Prep Solution (kit)
200 µl RNA Binding Buffer (kit)
400 µl RNA Prep Buffer (kit)
1100 µl RNA Wash Buffer (kit)

Enzymes/buffers for enzymes(DNA depletion)
DNase I and DNA Digestion Buffer Set (Zymo Research)
Stored in -20°C freezer

35 µl DNA Digestion Buffer
5 µl DNase I (1U/µL)

Equipment and Instruments used:
Bead-beater: MP FastPrep-24 (MP Biomedicals)
Bioanalyzer: Agilent 2100 Bioanalyzer (Agilent Technologies)
Refrigerated Microcentrifuge: Centrifuge 5424 R (Eppendorf AG)
Heat block: Thermal Shake lite (VWR International)
Refrigerated Centrifuge for 50ml falcon tubes: Jouan CR322 (Thermo Fisher Scientific)

Protocol materials

ZR BashingBeadTM Lysis Tubes (0.1 & 0.5 mm) **Zymo Research**

Safety warnings

Warning: Trizol and chloroform are toxic and must be handled in a fume hood. Contaminated materials should be disposed of as hazardous waste in accordance with safety regulations. Due to the strong odor of contaminated materials, it is preferable to store them in a fume hood until disposal.

RNA extraction preparations

1 **Preparations to do before starting the RNA extraction (in the morning of extraction):**

- Chill centrifuge to 4° C
- Chill ethanol, Trizol LS, and chloroform at -20^{O}C.
- Clean workstation and equipment with ethanol and RNAse Away (or any other RNAse decontamination solution)
- Get a box of ice to keep samples on

Sample Preparation

2 **Sample Preparation**

Sample extraction procedure differs slightly depending on sample type.

1. For sample from pure culture or enrichment culture ⇒ do step 2.1-2.5
2. For sample from anaerobic digester ⇒ do step 2.6-2.8

Extraction from pure cultures/enrichment cultures

2.1

Note

The sample volume required to obtain sufficient RNA yield varies significantly depending on the sample type. The amounts provided here are based on mesophilic batch cultivation of syntrophic acid degrading enrichment cultures at the final stage of substrate degradation.

For each sample 3×50 ml were taken and put into 3 50ml falcon tubes while flushing with N_2

Tip: Try to be as quick as possible and keep samples on ice when possible.

2.2 Centrifuge in pre-cooled centrifuge at 4° C, 5500 RPM for 30 minutes 30m

5500 rpm, 4°C, 00:30:00

2.3 Discard the supernatant by pouring/gently flicking the tube.
Put the pellets on ice.

#Tip, try to remove as much liquid as possible without losing the pellet.

2.4 Resuspend the pellet in 750 µl chilled Trizol LS, mix by pipette.
Transfer solution to the next 50ml falcon tube from the same sample, redo mixing and lastly transfer to the last 50ml falcon tube and mix througly.

750 µL Trizol LS

Note

#Discussion
In the case of flocculating enrichment cultures, the final pellet volume is quite large after pooling. Therefore the more concentrated **Trizol LS** is used which requires a lower ratio between the sample and Trizol LS as compared to normal Trizol.

The guideline is either 0.75ml **Trizol LS** or 1ml **Trizol** for 0.25ml of Sample Volume.

You also have to make sure make sure that the final volume should fit in the bead beating tube at the next step. The final volume of pellet + Trizol should be ≈ 1000µl.
The absolute maximum that is possible to add to the bead beating tube is ≈1400µl.

2.5 Transfer the mixed sample to a ZR BashingBead Lysis Tube

ZR BashingBeadTM Lysis Tubes (0.1 & 0.5 mm) **Zymo Research**

Note

Continue at step 3.

Extraction from digestate samples (from anaerobic digestion process)

2.6 Sample the process at a specified time-point.

Note

Ensure the workstation is clean and ready before sample collection. As RNA degrades quickly in the samples, try to work fast and immediately proceed to the next step.

2.7 Add 200-250 µl of the sample to a ZR BashingBead Lysis Tube.
Put tube on ice.

ZR BashingBeadTM Lysis Tubes (0.1 & 0.5 mm) **Zymo Research**

Note

Depending on the viscosity and particle size, samples can either be transferred by pipetting or scooping. Determine the volume using a scale.

2.8 Add 1 ml chilled TriZol Reagent (alt. 750 µL TriZol LS)

1 mL TriZol Reagent

Note

Continue at step 3.

RNA extraction

6m 30s

3 Homogenize samples using a bead-beater at speed 6.0 for 40 s

40s

00:00:40 Speed 6.0

Note

Warning: Mark the side of the BashingBead Lysis Tube as the bead-beating will wear away any markings made on the top of the tube.

4 Add 200 µl chilled chloroform to the cell mixture.
Mix well by inversion.

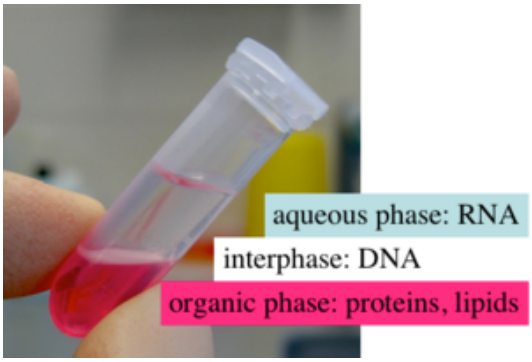
200 µL Chloroform

5 Centrifuge the mixture in pre-cooled centrifuge at 4° C, 14000 rcf for 10 minutes. 10m

14000 rcf, 4°C, 00:10:00

6 (The following steps follow the Quick-RNA™ Fecal/Soil Microbe Microprep protocol, starting from step 4.)

OBS#####
All centrifugation for RNA extraction should be done at 10,000 -16,000 rcf at room temperature unless stated otherwise.



Phase separation after centrifugation. Example of sample from enrichment culture.
Note: For samples from digesters, the aqueous phase will have a brown color.

Transfer 400 µl (or as much as available) of the aqueous phase into an RNase-free tube (not provided) and add **1 volume** of RNA binding buffer. Mix well.

X(400) µL Supernatant
X(400) µL RNA binding Buffer

#Tip: If more than 400 µl can be extracted do that, but you will need to repeat step 7 in order to filter all of the liquid.

7 Transfer up to 800 µl of the mixture into a Zymo-Spin™ **IIICG** Column in a clean 2.5 ml Eppendorf tube and centrifuge at 5,000 x g for 60 seconds. **Save the flow-through!** 1m

5000 rcf, 00:01:00

#Tip: Column might need to be loaded multiple times depending on sample volume.

8 To the flow-through, add an equal volume ethanol (95-100%) (1:1) and mix well.

1 µL 95-100% Ethanol

#Tip: A 2.5ml Eppendorf tube may be too small if the supernatant volume was large. In this case just transfer to RNase free 5ml Eppendorf tube or divide the volume in half and use two 2.5ml tubes.

9 Transfer the mixture into a new Zymo-Spin™ IIICG Column in a Collection Tube and centrifuge. **Discard the flow-through.** 30s

10000-16000 rcf, 00:00:30

#Column might need to be loaded multiple times depending on sample volume. Just repeat this step in that case.

10 Add 400 µl RNA Prep Buffer to the column and centrifuge fo 30s. Discard the flow-through. 30s

400 µL RNA Prep Buffer

10000-16000 rcf, 00:00:30

11 Transfer the column into a nuclease-free tube (not provided).
Add 100 µl DNase/RNase-Free Water directly to the column matrix and centrifuge for 2 minutes at 5000 rcf. 2m

100 µL DNase/RNase-Free Water

5000 rcf, 00:02:00

12 **Zymo-Spin™ III-HRC Filter preparation:** 3m

Place a Zymo-Spin™ III-HRC Filter in a new Collection Tube and add 600 µl Prep Solution. Centrifuge at 8,000 x g for 4 minutes.

600 µL Prep Solution

8000 rcf, 00:03:00

13 Transfer the eluted RNA (step 11) into the prepared filter in a nuclease-free tube (not provided) and centrifuge at 16,000 x g for 4 minutes. 4m

100 µL Eluted RNA

16000 rcf, 00:04:00

14 Add 200 µl RNA Binding Buffer to the filtered RNA (step 13) (2:1) and mix well.

200 µL RNA Binding Buffer

15 Add an equal volume ethanol (95-100%) (1:1) and mix well.

300 µL 100% ethanol

16 Transfer the mixture into a new Zymo-Spin™ **IC Column** in a Collection Tube and centrifuge. Discard the flow-through. 30s

10000-16000 rcf, 00:00:30

Note

IMPORTANT: At this point, when the RNA has bound to the IC Column, DNA can be removed using DNase I treatment. See 16.1 - 16.3

In-column DNase I treatment 6m 30s

16.1 Following RNA binding step (step 16), add 400 µl RNA Wash Buffer to the column, centrifuge and discard the flow-through. 30s

400 µL RNA Wash Buffer

10000-16000 rcf, 00:00:30

16.2 For each sample to be treated, prepare DNase I Reaction Mix (see table below) in an RNase-free tube (not provided) and mix by gentle inversion.

For each sample:
35 µl DNA Digestion Buffer
5 µl DNase I

16.3 Add 40µl of the DNase I Reaction Mix to the column matrix and incubate at room temperature (20-30°C) for 15 minutes. Proceed with the purification (Step 17) 15m

40 µL DNase I reaction mix
00:15:00
Room temperature

TIP: If you are quantifying RNA concentration using Bioanalyzer take out the kit to let it come up to room temperature at this point.

RNA extraction 6m 30s

17 Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow-through. 30s

400 µL RNA Prep Buffer
10000-16000 rcf, 00:00:30

18 Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flow-through. 30s

700 µL RNA Wash Buffer
10000-16000 rcf, 00:00:30

19 Add 400 µl RNA Wash Buffer and centrifuge the column for 1 minute to ensure complete removal of the wash buffer. Then carefully, transfer the column into a nuclease-free tube (not provided). 1m

400 µL RNA Wash Buffer
10000-16000 rcf, 00:01:00

#Tip: The centrifugation step can be repeated without addition of Wash Buffer to ensure that all Wash Buffer is removed.

20 Add 15 µl DNase/RNase-Free Water directly to the column matrix, incubate for 2 minutes and centrifuge. 2m 30s

15 µL DNase/RNase-Free Water (Amount can be varied)
00:02:00 Incubation
10000-16000 rcf, 00:00:30

Alternatively, for highly concentrated RNA use ≥ 6 µl elution.
The eluted RNA can be used immediately for QC control or stored frozen.

The RNA can now be frozen in the -80 ° freezer. Its recommended to do a QC control using Bioanalyzer at this point to avoid repeated freeze/thawing of the RNA samples. Alternatively you can stop here and aliquot 2µl of each sample, freeze everything and continue to do a quality check at a later stage.

Quality Control - BioAnalyzer

21 Info: For QC control the BioAnalyzer was used in combination with the Agilent RNA 6000 Nano Kit.
The protocol followed can be found here (**Protocol**).

For each sample to be analyzed 1 µl of eluted RNA is needed.

1 µL RNA sample

Check whether there is fresh filtered Gel prepared or if new gel has to be prepared (Lasts 4 weeks)

22 Decontaminate the Electrodes:

1. Slowly fill one of the wells of an electrode cleaner with 350 µl RNaseZAP.
2. Open the lid and place the electrode cleaner in the Agilent 2100 Bioanalyzer.
3. Close the lid and leave it closed for about 1 minute.
4. Open the lid and remove the electrode cleaner. Label the electrode cleaner and keep it for future use. You can reuse the electrode cleaner for all 25 chips in the kit.
5. Slowly fill one of the wells of another electrode cleaner with 350 µl RNase-free water.
6. Place the water-filled chip in the Agilent 2100 bioanalyzer, close the lid and leave for 10s.
7. Remove the chip and leave the lid open for 10s to dry the electrode.

23 **Run the analysis according to manufacturers instructions.**
Use the assay "**Prokaryote Total RNA Nano Series II**"

Depletion of ribosomal RNA

10m

24 rRNA depletion is performed with the riboPOOL Kit. For more detailed information read the protocol that comes with the kit.
The protocol presented here is based on the kit protocol.
Newest protocols found here:
https://www.sitoolsbiotech.com/protocols.php

The current protocol is based on the following:
https://www.sitoolsbiotech.com/pdf/riboPOOLkitManualv1-6_2023.pdf

25 **Reagents and storage requirements for RNA depletion**

Stored in freezer:

- **LA** - Linear acrylamide
- **RP** - riboPOOL lyophilized,(aliquoted by user)
- **RNAse inhbitor** RiboLock (40u/µl)

Stored in fridge :

- **SMB** - Streptavidin-coated magnetic beads (SMB)

Stored in room temperature:

- **DB** - Depletion buffer
- **HB** - Hybridization buffer
- **SA** - Sodium acetate, 3M
- **H2O** - Nuclease-free water

Reagents that has to be prepared in advance:

- **BRB** - Bead Resuspension Buffer
  0,1 M NaOH
  0.05M NaCl

- **BWB** - Bead Wash Buffer
  0.1M NaCl

26 **Hybridization of riboPOOL to rRNA**

To 14 µl of RNA sample (containing 100 ng - 5 µg of total RNA), add:
If sample volume is > 14 µl, adjust HB volume accordingly to 0.25X total volume. Total volume however should not exceed 40 µl.

- 1 µL resuspended RP
- 5 µL Hybridization Buffer (HB)
- X µL RNase inhibitor (RiboLock)

#Tip:
For RNase inhibitor the final concentration should be 1 U/µl. Given a RiboLock concentration of 40 U/µl and a working volume of 20 µl (14+1+5) → 0.5µl of RiboLock should be added to each sample

26.1 Vortex and spin down droplets

26.2 Incubate at 68°C for 10 min to denature RNA. 10m

- 00:10:00
- 68 °C

26.3 Allow to **slowly cool down** from 68°C to 37°C for optimal hybridization.
To do this turn off the heat block and let it naturally fall down to 37°C

Preparation of beads 10m

27 **Preparation of beads**

Resuspend the streptavidin-coated magnetic beads (SMB) by carefully vortexing tube at medium speed.

27.1 For each sample transfer 90 µl bead suspension into a fresh tube.

For batch washing of beads for multiple samples, aliquot bead suspension for up to 6 (i.e. 540 µl) or 12 samples (i.e. 1080 µl) in a single tube.

90 or X µL bead suspension

27.2 Place tube on magnetic rack and wait for 1 min 1m

00:01:00

27.3 Aspirate and discard all supernatant, while keeping the tube on the magnetic rack.

27.4 Add 80 µl per sample (i.e. 480 µl for 6 samples, 960 µl for 12 samples) of Depletion Buffer (DB) and agitate the tube well to resuspend beads. 1m
Place on magnetic rack (1 min), aspirate and discard supernatant.

80 µL Depletion Buffer (DB)

00:01:00

27.5 Resuspend beads in 80 µl per sample (i.e. 480 µl 6 samples) of Depletion Buffer and agitate the tube well to resuspend beads.

80 µL Depletion Buffer (DB)

Depletion 10m

28 Briefly centrifuge the tube containing ~20 µl hybridized riboPOOL and total RNA (from step 26.3) to spin down droplets.

28.1 Combine 80 µl of prepared beads (from step 27.5) with ~20 µl of the hybridized riboPOOL-RNA solution. Agitate the tube to resuspend well.

80 µL prepared beads

20 µL hybridized RNA

28.2 Incubate at 37°C for 15 min, followed by a 50°C incubation for 5 min.

37 °C 15 min

50 °C 5 min

28.3 Briefly spin down droplets. Place on magnet for 2 min. Carefully transfer the supernatant to a new tube. 2m

00:02:00 Magnetic Rack

Note

OBS: This new tube is where RNA will be precipitated, make sure to use the ones provided by the kit in order to get a clear visible pellet.
If you are running the optional step below make sure to have this kind of tube for step 28.5

28.4 Place the new tube with the supernatant on the magnetic rack for 1 min to get rid of trace amounts of beads. **(optional)** 1m

00:01:00 Magnetic Rack

28.5 Carefully transfer the supernatant to a new tube **(optional)**.

Note

At this point, RNA can be stored at -20°C overnight or -80°C for up to a month according to the procol, however it's advisable to proceed with the RNA Purification step(29).

RNA Purification 10m

29 **Clean up by EtOH precipitation**
Can be done either over night at -20°C, or for 30 min at -80°C.

-20 °C Overnight

Or

-80 °C 00:30:00

29.1 Add 10 µl of 3M sodium acetate (SA), 1 µl of Linear acrylamide (LA) and 333 µl of 100% ethanol to the RNA solution from step 28.5

Sample

10 µL Sodium Acetate (SA)

1 µL Linear acrylamide (LA)

333 µL EtOh (100%)

Vortex the mixture well

29.2 Incubate tube at -80°C for 30 min OR -20°C overnight

29.3 **AFTER INCUBATION:**
Centrifuge at 11 000 x g (or max speed) for 30 min at 4°C 30m

11000 rcf, 4°C, 00:30:00 , (Or at max speed)

29.4 Carefully remove and discard all supernatant, making sure not to disrupt pellet.

29.5 Add 200 µl of 70% ethanol to wash pellet.

200 µL EtOh (70%)

29.6 Centrifuge at 11 000 x g for 5 min at 4°C 5m

11000 rcf, 4°C, 00:05:00

29.7 Carefully remove and discard all supernatant, making sure not to disrupt pellet.

29.8 Repeat ethanol wash (steps 29.6 to 29.8)

29.9 Dry pellet at room temperature for 5 min

29.10 Resuspend the pellet in 32 µl of nuclease-free water (H2O) or appropriate elution buffer for library preparation. If required, elution volume can be decreased down to 10 µl.

X µL (Or whatever your final end volume should be)

29.11 Run a quality control on the depleted RNA.

Quality Control - BioAnalyzer

10m

30 Info: For QC control the BioAnalyzer was used in combination with the Agilent RNA 6000 Nano Kit.
The protocol followed can be found here (**Protocol**).

For each sample to be analyzed 1 µl of dissolved RNA is needed.

1 µL RNA sample

Check whether there is fresh filtered Gel prepared or if new gel has to be prepared (Lasts 4 weeks)

30.1 Decontaminate the Electrodes:

1. Slowly fill one of the wells of an electrode cleaner with 350 µl RNaseZAP.
2. Open the lid and place the electrode cleaner in the Agilent 2100 Bioanalyzer.
3. Close the lid and leave it closed for about 1 minute.
4. Open the lid and remove the electrode cleaner. Label the electrode cleaner and keep it for future use. You can reuse the electrode cleaner for all 25 chips in the kit.
5. Slowly fill one of the wells of another electrode cleaner with 350 µl RNase-free water.
6. Place the water-filled chip in the Agilent 2100 bioanalyzer, close the lid and leave for 10s.
7. Remove the chip and leave the lid open for 10s to dry the electrode.

31 **Run the gel according to manufacturers instructions**
Use the assay "**mRNA Nano Series II**"

Protocol references

This RNA extraction is based on Quick-RNA Fecal/Soil Microbe Microprep Kit
https://files.zymoresearch.com/protocols/_r2040_quick-rna_fecal-soil_microbe_microprep_kit.pdf ("Ensure you use the latest protocol version from the manufacturer's website.)

Quality control and concentration measurement procedure is described in user manual for Agilent RNA 6000 Nano Kit Guide.
https://www.agilent.com/cs/library/usermanuals/Public/G2938-90034_RNA6000Nano_KG.pdf

RNA depletion and RNA precipitation is based on the RiboPool kit (Pan-Prokayote)
https://www.bioscience.co.uk/resources/ribopool-protocol.pdf