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RNA Extraction from Cecum Contents of Gnotobiotic Mice

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We use this protocol and it's working

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Abstract

RNA extraction from the cecum contents of gnotobiotic mice colonized with extremely minimal communities (single yeast and/or single bacterial community) can be difficult due to excess complex carbohydrates. These carbohydrates are not broken down in the same way as they are in conventional mice and their presence can significantly decrease yield and purity of your RNA sample. In this protocol, special considerations are taken, including extra phenol/chloroform extractions and lithium chloride precipitations, to reduce the contamination of carbohydrates. RNA resulting from this protocol is extremely clean ($A_{260/280}$ = > 2.0, $A_{260/230}$ = 2.0-2.3).

Guidelines

This protocol is designed for RNA extraction from the cecum contents of gnotobiotic mice colonized with few (1-2) species, specifically colonized with *Candida albicans* and/or *Enterococcus faecalis*.

Materials

Note: For all materials, similar products from different brands will very likely work, but have not been tested. The protocol was developed and vetted using the exact products listed below. If substituting in alternative products, check their manufacturer protocol and adjust as necessary.

1. ThermoFisher RNAlater stabilization solution (ThermoFisher **AM7021**)
2. 1000µL Wide-Bore Filtered Pipette Tips (ThermoFisher **2079G**)
3. 5ml Snap-Cap Centrifuge Tubes (VWR 1002-728)
4. 2ml RNAse-free screw cap tubes (VWR 89004-302)
5. 1.5ml RNAse-free Microfuge Tubes (ThermoFisher AM12450)
6. Biopic 0.5mm Zirconia/Silica Beads (Biospec **11079105Z)**
7. **Nuclease-free Water (ThermoFisher AM9939)**
8. **0.5M EDTA, pH 8.0 (ThermoFisher AM9260G)**
9. 20% SDS solution (ThermoFisher AM9820)
10. Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1) (ThermoFisher AM9720)
11. Isopropanol
12. Ethanol
13. NEB Monarch RNA Clean-up (500ug) (NEB T2050)
14. TURBO DNA-free™ Kit (ThermoFisher AM1907)
15. Lithium Chloride Precipitation Solution, 7.5M (ThermoFisher AM9480)
16. Ambion Glycoblue Coprecipitant, 15 mg/ml (ThermoFisher AM9515)

Solutions to be made:
Lysis Buffer

- 200mM NaCl
- 20mM EDTA

Troubleshooting

Safety warnings

Phenol/Chloroform is a known carcinogen, irritant and reproductive hazard. Wear proper PPE (lab coat, eye goggles) when working with Phenol/Choloroform and perform work inside properly functioning fume hood.

Before start

Germ-free Mice have been pre-colonized via gavage with distinct gnotobiotic community of yeast and/or bacteria.

At time of euthanization

30m

1 Harvest cecum and cecum contents. Drop ½ of cecum contents into 10 ml RNAlater. Leaves tubes at 4^{O}C for 1 day minimum (7 day maximum).

2 Pre-weigh 2ml tubes. Add 1ml of RNAlater-cecum contents to each tube (~200mg contents) using wide-bore 1000µL tips and centrifuge at 3500RPM for 5 min to pellet contents. Remove supernatant.

3 Weigh pellets. Subtract weight of empty tube to find weight of cecum pellet. Store pellet aliquots at -80^{O}C.

RNA Extraction

2h

4 To 2ml RNase-free screw cap tube, add ~500µL ice-cold 0.5µM zirconia beads (1 tube per 100mg cecum contents). Keep tubes on ice until use.

5 Place cecum contents aliquots on ice to thaw.

6 Re-suspend aliquot in the following per 100mg (200mg – multiple all volumes by 2):
- 500µL Lysis Buffer (200 mM NaCl, 20mM EDTA)
- 210µL 20% SDS
- 500µL phenol:chloroform:isoamyl alcohol (PCI) (pH 4.5, 125:24:1)

7 Transfer resuspended cecum contents to screw-cap tube containing beads. Split total volume between 2 bead-beating tubes (each has ~1210uL)

8 Bead-beat tubes 5min using Mini-beadbeater

5m

9 Centrifuge @ 10000xg for 5 minutes to separate phases

10 Transfer aqueous phase to new Eppendorf tube. Add equal volumes phenol:chloroform:isoamyl alcohol (PCI) and vortex 20s. Spin @ 10000xg for 5 minutes.

11 Transfer aqueous phase to phase-lock tube (heavy). Add equal volume of PCI, shake well to mix and spin @ 10000xg for 3 minutes.

12 Add equal volume PCI to aqueous above the phase-lock layer, mix well by shaking and spin @ 10000xg for 3 minutes.

13 go to step #12 two more times (total of 6 Phenol chloroform extractions). Move aqueous to new phase-lock tube as needed.

Isopropanol Precipitation

1h

14 Decant aqueous phase from phase-lock tube to new RNase-free 1.5ml eppendorf tube. Add 1 volume RT isopropanol and place at -20C for 20 minutes.

- Note: Any amount of time over 20 minutes will cause increased crashing out of the complex carbohydrates present and will reduce yield and purity.

15 Centrifuge at max speed for 25 min, 4^{o}C. You will see a disk of RNA form between two layers (not a pellet like traditional precipitations).

16 Carefully remove ALL liquid from tube (above and below disk of nucleic acid). If you don't remove the liquid below the disk, the carbohydrates will begin to crash out and crystalize when doing the EtOH washes and your sample will be less pure.

17 Wash 2X with 750µl 75% ethanol. Invert to wash tube. Centrifuge max speed for 5 minutes, 4^{o}C.

18 Remove all ethanol from pellet (remove supernatant, quick spin tube, remove residual ethanol with aspirator). Air dry pellet for <5 min if a lot of residual ethanol remains.

19 Resuspend pellet in 50 µL 45^{o}C nuclease-free water

19.1 Make sure pellet is completely resuspended before moving forward! Place at 45^{o}C for a few minutes to fully resuspend

NEB Monarch RNA Clean-up (500ug) (T2050)

15m

20 Follow NEB Monarch RNA Clean-up as documented in product's protocol and detailed below:

21 Add 2X RNA Cleanup Buffer (100ul to 50ul sample)

22 Add 1 vol 100% ethanol to sample (150ul)

23 Invert/flick to mix. Transfer to Spin-cartridge. Spin at 12000xg for 1min. Discard flow-through

24 Add 700µL wash buffer I. Centrifuge and discard flow-through

25 Wash 2X with 500µL wash buffer II. Centrifuge and discard flow-through.

26 Centrifuge spin-cartridge ~ 12000xg for 1 min to dry column and membrane.

27 Place dried column in clean Eppendorf tube.

28 Add 50ul nuclease-free water to center of membrane. Let incubate at RT for 1 min. Centrifuge to collect flow-through.

TURBO DNase Treatment

35m

29 To 50µL sample, add and mix gently: 5 µL 10X buffer; 1 µl TURBO DNA

30 Incubate at 37^{O}C for 20-30 minutes

31 Resuspend DNAse Inactivation Reagent by vortexing. Add 6 µL inactivation reagent to samples and mix by flicking.

32 Centrifuge tube @ 10000xg for 1.5 minutes. Carefully transfer 50ul into new tube.

33 Incubate at RT for 5 minutes. Flick tube occasionally to resuspend inactivation reagent.

Lithium Chloride Precipitation

1h

34 Add 25ul 7.5M Lithium chloride precipitation solution (final= 2.5M) and 1ul glycoblue co-precipitant. Store at -20^{o}C overnight.

35 Centrifuge at max speed for 25 min, 4^{o}C. You should see large, gelatinous pellet form at bottom of tube.

36 Wash 2X with 750µl ice-cold 75% ethanol. Vortex tubes ~30s to completely wash tube and pellet. Centrifuge max speed for 5 minutes, 4^{o}C.

37 Remove all ethanol from pellet (remove supernatant, quick spin, remove residual ethanol with aspirator). Air dry pellet for ~5 min if a lot of residual ethanol remains.

38 Resuspend pellet in 30 µL 50^{o}C nuclease-free water. Make sure pellet is completely resuspended before moving forward!