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Frozen Nematode Sample Preparation (Bulk) for Sequencing

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Manuela Kieninger[1]

[1]Blaxter Faculty, Wellcome Sanger

Manuela R. Kieninger
Blaxter Faculty, Wellcome Sanger

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We use this protocol and it's working

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Abstract

This protocol is about preparing frozen nematode bulk samples for sequencing. The protocol involves preparing growth plates and washing the nematodes off the plates. This is followed by a sucrose floating step and additional washing steps before the nematode pellet gets aliquoted and flash frozen in liquid nitrogen. The samples can be stored in a ULT (ultra low temperature) freezer. The frozen samples can be used for HMW DNA extraction, RNA extraction, nuclei isolation, or HiC sequencing.

Guidelines

Make sure your nematode species is growing well before starting the growth plates.
I like to prepare several frozen sample aliquots of the nematode species I am growing. Therefore, I usually grow at least 20 NGM plates. If your species is not growing to full plates, you might want to prepare more plates accordingly.
20 plates should give you at least 8 sample aliquots of about 60 mg nematodes.

Materials

- NGM agar plates 90 mm seeded with 1.5 to 2 ml OP50 bacteria liquid culture.

Note: Plates must be dry to accommodate the volume of OP50. If they are too wet the OP50 will flow all over the plate and it will take very long to dry.

- E. coli strain for feeding for example OP50; grown over night in LB @37°C
- Amcor Parafilm™ M Laboratory Film
- Ice
- 1x M9 buffer (4°C)
- 1x M9 buffer with 0.01% (v/v)Tween20 (4°C)
- 60% (w/v) Sucrose solution (cold)
- 1x PBS buffer
- Transfer pipets e.g. VWR®, Transfer Pipettes, Graduated; VWR Catalog Number: 612-6803
- DNA LoBind® Tubes 1.5 ml Catalog No. 0030108051 or NEB Monarch® Pestle Tubes T3001-1
- Liquid Nitrogen and PPE for liquid nitrogen handling
- long tweezers (about 30 cm long)
- 1 or 2 litre dewar for liquid nitrogen
- Centrifuge for 50 ml and 15 ml conical tubes cooled to 4 degrees e.g. Eppendorf Centrifuge 5910R
- Centrifuge for 1.5 ml tubes e.g. Eppendorf Centrifuge 5425
- 50 ml falcon tubes Polypropylene e.g. CELLSTAR®, BLUE SCREW CAP, NATURAL, GRADUATED, WRITING AREA, STERILE, 25 PCS./BAG; Item No.: 227261-N
- 15 ml falcon tubes Polypropylene e.g. CELLSTAR®, BLUE SCREW CAP, NATURAL, GRADUATED, WRITING AREA, STERILE, 50 PCS./BAG; Item No.: 188271-N
- Low binding tips (1000 µl and 200 µl, best use wide-orifice tips)

Safety warnings

- This protocol requires you to wear a lab coat and nitrile gloves.
- Make sure to wear appropriate PPE when handling liquid nitrogen.
- Please collect the waste in a suitable container and dispose of the waste in accordance with your local regulations.

Before start

Check the required growing conditions for your nematode species. Check what temperature, plates, and food it requires. As standard, we usually use NGM plates and OP50 for food. The nematode source plate for setting up the growth plates should be free of contaminating fungi or other bacteria.

In this protocol we prepare samples containing nematodes of mixed stages. If you require samples containing only a certain stage of nematode species you have to synchronise them or use cell strainers for selecting your required stage by size.

Growing Nematode Plates

1w 3d

1 Put an equal number of adult females or hermaphrodites containing embryos on 9 cm NGM plates seeded with food bacteria. I usually put 20 worms on each plate and I prepare about 20 plates.

2 Seal the plates with Parafilm.
Put the plates in a box and incubate the box at the respective growing temperature for the nematode species.

3 Let the nematodes grow until bacteria is gone or the plate is full of nematodes. The nematodes should not be starved!

Washing the nematodes off the plates

1h 30m

4 Chill a centrifuge which can accommodate 50 ml and 15 ml tubes down to 4°C

5 Add a small amount of **fridge-chilled** M9 to each plate. Gently swirl to release the worms, and carefully pour into a 50 ml Falcon tube (if you have many plates, you can add M9 to the first plate, pour it into the second plate, and so on. So you do not end up with a large volume.)
Keep the falcon tube on ice to keep the solution cool.

6 Repeat the washing step until most of the worms have been washed off. Discard the washed plates.

7 Fill the falcon tube(s) up to 40 ml with additional M9 and invert the falcon tube(s) several times.

8 Spin tubes at 2500 rcf for 8 mins at 4ºC.
(**Important:** Different species might settle at different forces. You might need to go higher with the g-force if you see still a lot of worms floating after the centrifugation.)

9 Remove the supernatant with a transfer pipet. Take care not to disturb the worm pellet.

10 Fill the falcon tube(s) back up with chilled M9 supplemented with 0.01% Tween.

11 Spin tubes @ 2500 rcf for 8 mins @ 4ºC.

12 Take off the supernatant.

13 If the supernatant is clear go to the Sucrose float step (14).
If you still have a lot of bacteria in the supernatant wash again with M9/Tween until the supernatant is clear (7-12).

Sucrose floating step for reducing bacteria and other contaminations 30m

14 Move worm pellet in 15 ml falcon tube and add **cold** (fridge temperature) M9 plus 0.01% Tween to a final volume of 14 ml.

15 Spin down for 2 min @ 800 rcf.

16 Remove supernatant and keep the tubes on ice.
If you have more than 2 ml worm pellet, divide the worms in more 15 ml tubes.
Then top up to 5 ml total volume with **cold** M9/Tween.

17 Add 5 ml **cold** 60% (w/v) Sucrose.

18 Immediately spin the tube(s) for 5 min @ 3500 rcf @ 4°C.
The "clean" worm should float to the top of the liquid and the bacteria and other contaminations are at the bottom of the tube.

19 Wash a new transfer pipet with M9/Tween.
If you are not washing the tip before, the worms will stick to the tip and you will loose a lot of your sample!

20 Take off the floating worms with washed transfer pipet. Put them in a new 15 ml falcon tube.

21 Wash twice with M9/Tween:
Fill the tube with M9/Tween to 14 ml final volume and spin 2 min @ 800 rcf in a cold centrifuge (4°C).

21.1 Note: If you had a lot of contamination in your washed worms, it might be necessary to repeat the Sucrose wash.

Aliquoting and freezing 30m

22 Perform a final wash using PBS.

Fill the tube with PBS to a final volume of 14 ml and spin 2 min @ 800 rcf.
You now have clean worms ready to make pellets for freezing.

23 Remove supernatant. Add 1-2 ml PBS and transfer all of the worms in 1.5 ml or 2 ml LoBind tube(s). I would only fill them half. Use another tube if you have a lot of sample.

24 Spin the worms down for 1 min @ 10.000 rcf (the centrifuge can be at room temperature here).

25 Use low-bind tip to transfer worms and wash the tip in M9/Tween before using.

26 If you want to know the exact weight of your sample, you can weigh the **empty tubes** before and weigh them again after addition of worms. I usually add about 70 to 80 µl which comes to at least 60 mg of worms. I avoid to have more than 100 mg in one tube.

27 Add about 70 to 80 µl of worms in 1.5 ml tubes (can be pestle tubes used in the Monarch HMW DNA extraction kit for tissue from NEB in case you plan HMW DNA extractions).
Make sure you add the worms to the bottom of the tube.
- Note: I like to use a wide-orifice 200 µl tip here which is low bind.

28 Get your PPE for liquid nitrogen handling. Fill a small dewar for liquid nitrogen with a bit liquid nitrogen for flash freezing the tubes with the nematodes.

29 Using long tweezers of about 30 cm, lower the 1.5 ml tubes with nematodes in liquid nitrogen and let them float in there for a bit.
It is important to lower them with tweezers so the sample does not splash all over the tube.

30 After flash freezing in liquid nitrogen, take the tubes with the frozen nematodes out using the long tweezers and immediately store the tubes in a ULT (ultra low temperature) freezer.