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Nematode Barcoding

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We use this protocol and it's working

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Abstract

This protocol is for barcoding nematode species. The primers used in this protocol are tested and work for various genera of nematodes. Final analysis is done with Sanger sequencing (not described here).

Guidelines

This protocol works for single nematodes or multiple nematodes as input. It is recommended to wash the nematodes in water before transferring them into the lysis buffer mix to minimise the amount of non-nematode (bacterial) DNA in the lysis.

Materials

Lysis buffer: **10x Worm lysis buffer (20ml):**

10 ml 1 M KCl,
2 ml 1 M Tris pH 8.2,
0.5 ml 1 M MgCl2,
0.9 ml Igepal,
0.9 ml Tween 20
fill to 20 ml with water

Filter sterilise (0.2 µm) and freeze -20 to prevent contamination
Do not forget: you have to dilute it to **1x for use**!

- PCR tubes
- Proteinase K 20 mg/ml e.g. Proteinase K, Molecular Biology Grade, NEB, Cat# P8107S
- Thermocycler
- Minicentrifuge with adapter for 1.5 ml tubes and PCR tubes
- Q5® High-Fidelity DNA Polymerase, NEB, Cat# M0491S
- Deoxynucleotide (dNTP) Solution Mix 10mM
- Nuclease free water
- QIAquick PCR Purification Kit for PCR Cleanup, Qiagen Cat# 28106
- Ethanol
- 1.5 ml microtubes
- optional: Qubit™ 1X dsDNA High Sensitivity (HS) assay kit, Invitrogen, Cat# Q33230
- optional: Qubit™ Assay Tubes, Invitrogen, Cat# Q32856
- NanoDrop™ One Microvolume UV-Vis Spectrophotometer, Thermo Scientific, e.g. Cat# ND-ONE-W
- Agarose gel with SYBR green to visualise PCR product and electrophoresis chamber system with power supply (I use 2% gel; 1% is also fine)
- DNA ladder e.g. Quick-Load® 100 bp DNA Ladder, NEB, Cat# N0467S
- pipette tips for 10 µl, 200 µl and eventually 1000 µl pipettes
- set of pipettes (e.g. 1-10 µl, 20-200 µl and 100-1000 µl)
- centrifuge e.g. Eppendorf Centrifuge 5420
- barcoding primers:

nSSU_F_04: 5' GCTTGTCTCAAAGATTAAGCC 3'
nSSU_R_26: 5' CATTCTTGGCAAATGCTTTCG 3'

Safety warnings

- This protocol requires you to wear a lab coat and nitrile gloves.
- Please collect the waste in a suitable container and dispose of the waste in accordance with your local regulations.

Lysis

2h 50m

1 Lysis is performed in the worm lysis buffer which is mixed with Proteinase K. Take 600 µl 1x lysis buffer and add 20µl Proteinase K. This solution can be stored at -20 °C for 3 months and used when needed.

5m

2 Add **20 µl worm lysis buffer mix** to PCR tube (for **10** nematodes *C. elegans* size) and spin down (You can also use **5 µl with 1 worm**).

1m

3 Add 10 worms (adult preferred) to the lysis buffer mix with a worm pick. Then check for their presence under a stereoscope. Make sure not to add too much bacteria. You can wash the worms in water or M9 buffer before adding them to the PCR tube with lysis buffer mix.

2m

4 Shortly spin the tubes in a microcentrifuge.

5 Put the PCR tubes in -70°C for 30 min or longer.

30m

6 Take the tubes out of the freezer and let them thaw. After the solution with the worm is thawed, add the tubes again in the -70 °C freezer for another 30 min.

30m

7 Place PCR tubes in a thermocycler.
The program for lysis is: 90 min @ 65 °C followed by 10 min @ 95 °C.

1h 40m

8 After the thermocycler has finished, check the lysis for the presence of worms with a stereoscope (the worms should be gone in the best case, sometimes you see empty ghost cuticles which is fine).

9 If you have done a lysis with 10 worms, add 80µl nuclease-free water and use 5 µl for PCR. Mix well with pipette! If you used a single worm, use 2 µl per PCR of the pure lysis for PCR.

2m

PCR

50m

10 I use the Q5 polymerase for barcoding. If using another polymerase, follow the manufacturers recommendations for PCR and adjust the temperatures for annealing and amplification.

11 Make a master mix for the number of PCRs. I usually prepare 1 additional reaction for the master mix.

12 For **PCR Using Q5® High-Fidelity DNA Polymerase (M0491)** 10m

Per 50µl reaction:
28.5 µl nuclease-free water
10 µl 5x Q5 buffer
1 µl 10mM dNTPs
2.5 µl primer 1 10 mM (see materials)
2.5 µl primer 2 10 mM (see materials)
0.5 µl Q5 polymerase
→ 45 µl in PCR tube then add 5 µl of the worm lysis.

12.1 Mix all master mix ingredients except polymerase and vortex before adding the Q5 polymerase. After adding the Q5 polymerase invert several times until the "Schlieren" are gone. Add 45 µl to the PCR tube and add 5 µl worm lysis. Pipette mix the reactions.

13 Add to the thermocycler and select the PCR cycle program. 40m
For the standard primers ssuF04/ssuR26 use this program in the thermocycler:
98 °C 30 sec/ cycles: 98 °C 10 sec/ 55 °C 20 sec/ 72 °C 30 sec <-35x cycles/ final 72 °C 2 min → 4 °C hold

14 To visualise the PCR product by gel electrophoresis, use a DNA ladder to check the length of the PCR product. For the primers **nSSU_F_04 / nSSU_R_26** the fragment should be about 900 bp.
I usually run 7 µl of the 50 µl PCR on the gel and mix with 5 µl loading dye. I load 10 µl in a gel pouch.
There should be a single band at 900 bp. If you see no band or multiple bands, the PCR conditions need to be adjusted.

PCR clean-up and sample prep for Sanger sequencing 30m

15 If you have a single product of the expected size proceed to PCR clean-up.

16 Pipette the remaining PCR reaction (43 µl) in a 1.5 ml microtube.

17 Perform Qiagen PCR clean-up:

18 Add 10 µl 3M Na Acetate

19 Add 5X Buffer PB to the mix and mix well. Spin down.

20 Put the mix in a QIAquick column and centrifuge 1 min @ 18.000 rcf

21 Discard flow-through and add 750 µl wash buffer to the column.
Make sure EtOH is added to the wash buffer before use!

22 Centrifuge 1 min @ 18.000 rcf

23 Discard flow through and spin again for 1 min 18.000 rcf to remove all of the wash buffer.

24 Remove the flow through tube and place the column in a 1.5 ml microtube.

25 Add 30 µl elution buffer directly on the column membrane.

26 Let it stand for 1 min and spin for 30 sec 18.000 rcf

27 Measure the concentration and purity with Nanodrop and Qubit 1X dsDNA High Sensitivity (HS) assay using 1 µl each. Record the results.
Make sure you vortexed the Qubit tube before measuring for 5 sec and let it incubate for 2 min.

28 Elutes can be stored in minus 20 °C until sent for sequencing.
The same PCR primers can be used for sequencing. I usually sequence from both sides. Prepare sequencing primers and the cleaned-up PCR product according to the requirements of your sequencing service provider.

Protocol references

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