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Entomophthorales Culturing to Sequencing v1 V.1

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We use this protocol and it's working

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Abstract

This protocol has been developed for sequencing the genomes of Entomophthorales fungi from mycosed cadavers. This includes all steps from collection of sample up to, but not including, DNA extraction. This entire protocol should take approximately 3 weeks.

Guidelines

Prepare before starting:

1. Have mycosed insect cadaver.
2. Prepare SDAYEYM media according to protocols.io.
3. Prepare sterile rings of filter paper.
4. Have sterile 150mL flasks with bevel lids.

Safety precautions:

1. Wear PPE (nitrile gloves and lab coat).
2. 70% Ethanol is flammable, so keep away from open flames.
3. Do all work in biological safety cabinet to protect from fungal spores and contamination.

Materials

Required

- Grace’s insect media (50mL)
- SDAYEYM media (~5 plates per sample)
- Sterilized filter paper
- Sterile empty petri dishes

Optional

- Fetal bovine serum (FBS)
- PDA media plates (for contamination issues)

Set up

1 Prepare biological safety cabinet by turning on the blower, and sterilizing with the UV light. After 30 minutes, turn off the UV light and wipe down the BSC with 70% ethanol.

2 Bring in insect cadavers from field collection, gather metadata, and take dissecting scope photos of the cadaver.

2.1 Metadata includes location coordinates, date, host ID, substrate, and description.

3 Microscopically search and document cadaver for rhizoids, conidiophores, and resting spores.

Collecting spores from cadaver

1d

4 Remove mycosed cadaver from substrate (leaf, bark, rock, etc.). If this is not possible, remove as much substrate as possible surrounding the cadaver.

5 Place insect cadaver in the center of a sterile empty petri dish.

6 Place sterile ring of filter paper around the edge of the plate, avoiding contact with cadaver.

7 Use 1000ml pipette tip to add water to filter paper, not too much that it starts to pool but enough to coat the paper. Be careful to not allow the water to touch the insect cadaver.

8 Parafilm the plate, be careful not to jostle it, and keep it in the incubator for around 24 hours.

1d

9 Continue monitoring the dish until spores are shot out from the cadaver and are visible on the lid of the petri dish.

Culturing fungal spores

3d

10 In the BSC, cut a small square out of a plate of SDAYEYM media with a sterile scalpel.

11 Use a sterile tool to rub the media along the lid of the petri dish where there are spores. Do this step quickly, try to keep the plates open as briefly as possible.

11.1 The spore pattern will often be directly above where the cadaver is on the plate, but look at the plate under a microscope first to confirm.

12 Place media back onto previous plate in a new area, sandwiching the spore collecting side between the side you had cut out and the plate media.

13 Keep plate in incubator, it should show signs of growth between 48 and 96 hours. 3d

14 Continue to transfer as needed to maintain culture, approximately monthly.

15 Before continuing on to DNA extraction, transfer one clean plate that has not been touched since the original transfer and input the sample and metadata into ARSEF.

Liquid culturing 1w

16 When plates have substantial growth along the surface of the media, they are ready for transfer to liquid culture. Be sure to maintain the solid media plate (and a backup) just in case.

17 Prepare two 150mL flasks: add 25mL of Grace's insect media and 1mL of liquid FBS to each.

18 Cut a square into the culture plate and scrape/peel culture from the media. It is ok to also take some SDAYEYM media, if necessary, but as minimal as possible.

19 Cut the piece of fungus into 3-4 smaller pieces, then deposit using sterile tool into the flask of liquid media.

20 Repeat for the other flask.

21 Leave both flasks in shaker at room temperature for ~1 week. 1w

Lyophilization

22 When liquid culture has developed enough to see significant hyphal growth, remove cultures from shaker.

23 Pour both 25mL flasks into one 50mL Falcon tube.

24 Centrifuge at 4000g for 15 minutes.

25 Once culture has pelleted at the bottom, pour off Grace's media supernatant.

26 Transfer pellet to 1.5 mL Eppendorf tubes, using sterile tool or pipette tip. The pellet should be large enough to fill three or four tubes about halfway.

27 Drop Eppendorf tubes into liquid nitrogen to freeze instantly and maintain gDNA quality.

28 Remove from liquid nitrogen, open lid of Eppendorf tubes and cover with foil, poking five holes in the foil with sharp forceps.

29 Place onto tube rack and into lyophilizer according to fungal protocols for 48 hours.

29.1 Instructions are listed in manual on top of machine but should run under "AUTO" option.

30 Check appearance of tissue after 48 hours, if light in color and dry then remove from lyophilizer and store at -80°C until ready for extraction.

Extraction

31 Use lyophilized material for high molecular weight gDNA extraction protocol of choice.

31.1 Our lab follows the Lovett lab CTAB protocol along with NEB Monarch HMW gDNA Extraction Kit.

32 Use one tube for extraction, maintaining a backup of the material in the -80°C freezer.

Relaxation

33 Once DNA has been extracted and stored at 4°C overnight, relax it once daily for three days.

34 Pipette up and down once VERY SLOWLY using a wide bore 1000uL tip.

35 Incubate DNA at 37°C for one hour.

36 Repeat step 2.

37 Return to 4°C until the next day, then repeat steps 2-5.

38 After three days, run Broad Range Qubit according to protocols with 2ul of sample each from the top, middle and bottom of Eppendorf tube.

38.1 This step is to test that the DNA is fully relaxed evenly throughout the tube.

Shipping

39 After Qubit, calculate how much sample is necessary to follow sequencing facility guidelines and transfer that amount to a screw-cap sequencing tube for transport.

40 Double seal tubes in a Ziploc bag inside of an empty pipette tip box.

41 Include an ice pack and scrap paper to keep sample from jostling during transit.

42 Print shipping label and cover in clear packing tape on top of box, deliver to FedEx.

Contamination

43 If contamination appears, remove a small clean section of culture and place culture side down onto a plate of PDA media.

44 Entomophthorales should be “unhappy” on this media and shoot spores toward the lid within a few days.

45 Repeat steps 10-13 of the Culturing section using the PDA plate lid, transferring back onto SDAYEYM media.

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