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Mycelium RNA Extraction and Library Prep

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Abstract

The goal of the Mycelium RNA extraction and sequencing protocol is to sequence mycelium RNA. The protocol begins with a method to extract mycelium from soil samples. Following this process, the mycelium can be stored if needed. The next step is to extract the RNA from the mycelium samples. The RNA collected will be prepared for sequencing using a MinION flow cell. The library preparation and sample loading follows the Ligation sequencing kit for cDNA by Oxford Nanopore Technologies. The RNA is reverse transcribed into cDNA and the cDNA is then sequenced.

The Ligation sequencing kit for cDNA does include third-party items that need to be purchased from outside sources.

Some key considerations for this protocol are to maintain the sterility of the sample and to prevent fungal rot. The fungal samples may have other organic materials like wood chips or plant roots. The extraction procedure should prevent contamination from occurring by filtering out large particles. Fungal rot can be prevented by maintaining a temperature range between 20°C to 30°C.

Attachments

PNG

Screenshot 2024-10-0...

125KB

Materials

General Equipment and Consumables

- centrifuge
- timer
- Glass beakers
- 50 mL conical tubes
- centrifuge tubes
- 3mL Eppendorf tube
- Deionized water
- Qubit and Nanodrop fluorometers
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P10 pipette and tips
- P2 pipette and tips
- Ice bucket with ice
- Timer

Obtaining Mycelium Samples: Extraction of soil fungal mycelium protocol materials

Equipment

- sieve
- magnetic stirrer
- 1000 µm pore nylon mesh
- 50 µm pore metal sleeve
- 50 µm pore nylon mesh

Consumables

- 45.5% sucrose solution

RNA extraction protocol

Consumables

- TRIzol Reagent from Invitrogen
- chloroform
- isopropanol

Ligation sequencing V14 - Direct cDNA sequencing kit materials

- User-supplied VN Primer, 2 µM
- User-supplied Strand-Switching Primer, 10 µM
- User-supplied PR2 Primer, 10 µM
- NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (NEB, E7180S, or E7180L). Alternatively, you can use the NEBNext products below:
- NEBNext Ultra II End Repair / dA-tailing Module (NEB, E7546)

- NEBNext Quick Ligation Module (NEB, E6056)
- 1.5 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water (e.g. ThermoFisher, AM9937)
- Freshly prepared 80% ethanol in nuclease-free water
- 10 mM dNTP solution (e.g. NEB N0447)
- LongAmp Taq 2X Master Mix(e.g. NEB M0287)
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher, cat # EP0751)
- RNaseOUT, 40 U/µl (Life Technologies, cat # 10777019)
- RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286)
- Bovine Serum Albumin (BSA) (50 mg/ml) (e.g Invitrogen UltraPure BSA 50 mg/ml, AM2616)

Equipment
- Magnetic rack, suitable for 1.5 ml Eppendorf tubes
- Microfuge
- Vortex mixer
- Thermal cycler
- Pre-chilled freezer block at -20° C for 200 µl tubes
- Hula mixer

Protocol materials

Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**#EP0741

TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019

Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog #**#EP0741

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019

Troubleshooting

Before start

This kit contains reagents from a third-party site and not included in the Ligation sequencing V14 - Direct cDNA sequencing kit by Oxford Nanopore Technologies.

Obtaining Mycelium Samples: Extraction of soil fungal mycelium

9m

1 Sieve collected soil samples through a 2-mm sieve to remove any root fragments

2 Disperse a 5 g soil sample into 100 mL of deionized water

3 Stir the mixture at 500 rpm using a magnetic stirrer for 00:05:00 5m

4 Filter the mixture through a 1000 µm pore nylon mesh

5 Thorough wash the oversized particles with 100 mL of deionized water

6 Repeat steps 4-5 with the filtrate

7 Filter the filtrate twice through a 50 µm pore metal sleeve containing a 50 µm pore nylon mesh

8 Discard the filtrate and collect the oversized particles in a 50 mL tube by rinsing them using 35 mL of deionized water for storage if needed (store at 4°C)

9 Centrifuge the samples for 00:03:00 at 31000xg 3m

10 Discard the supernatant

11 Disperse the pellet by adding 50 mL of 45.5% sucrose solution and hand-shaking vigorously

12 Centrifuge the solution at 50 x g for 00:01:00 1m

13 Filter the supernatant through a three-layered 50 µm pore size nylon mesh

14 Repeat steps 9-13 four more times

15 Discard the remaining soil pellet

16 Thoroughly wash the particles collected on the nylon mesh with deionized water. Make sure to rinse out the sucrose solution

17 Collect the particles into a 3 mL Eppendorf tube and allow them to dry completely

18 Store between 20-30 °C if needed.

RNA extraction

19 Ground the particles into a fine powder in liquid nitrogen

20 Add 2 mL of TRIzol Reagent **Thermo Fisher Scientific Catalog #**15596026 and ground the sample further until the slurry is thawed

21 Split the sample into two 2 mL Eppendorf tubes

22 Incubate at room temperature for 00:05:00 5m

23 Add 300 µL of chloroform and sahe the tube vigorously for 15 seconds

24 Incubate at room temperature for 00:03:00 3m

25 Centrifuge the samples at 13.000 x g for 00:15:00 at 4 °C 15m

26 Repeat steps 5-7

27 Precipitate the RNA using 500 µL of isopropanol at -20 °C for 02:00:00 2h

28 Collect the precipitated RNA through centrifugation at 13000 x g for 00:15:00 at 4 °C 15m

29 Discard the supernatant and resuspend the RNA pellets

30 Quantify the RNA using the Qubit and Nanodrop fluorometers

Library Preparation: Reverse transcription and strand-switching

31 Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.

	Reagent	1. Thaw at room Temperature	2. Briefly spin down	3. Mix well by pipetting
	User-supplied VN Primer diluted to 2 µM	✓	✓	✓
	User-supplied Strand-Switching Primer diluted to 10 µM	✓	✓	✓
	10 mM dNTP solution	✓	✓	✓
	RNaseOUT	Not frozen	✓	✓
	Maxima H Minus Reverse Transcriptase	Not frozen	✓	✓
	Maxima H Minus 5x RT Buffer	✓	✓	Mix by vortexing

RNaseOUT™ Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific Catalog #**10777019

Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741

32 Prepare the RNA in nuclease-free water.

32.1 Add 1 µg of total RNA to a 0.2 ml PCR tube

32.2 Adjust the volume to 7.5 µL with nuclease-free water

32.3 Mix the tube by flicking

32.4 Spin down in a microfuge

33 Prepare the following reaction in the 0.2 ml PCR tube containing the prepared RNA input:

	Reagent	Volume
	RNA input from previous step	7.5 µl
	VN Primer diluted to 2 µM	2.5 µl
	10 mM dNTPS	1 µl
	Total Volume	11 µl

34 Mix gently by flicking the tube and then spin down

35 Incubate at 60 °C for 00:05:00 and then snap cool on a pre-chilled freezer block for 00:01:00 . 6m

36 In a separate tube, mix the following together:

5x RT Buffer	4 µl
RNaseOUT	1 µl
Nuclease-Free water	1 µl
Strand-Switching Primer diluted to 10 uM	2 µl
Total Volume	8 µl

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37 Mix gently by flicking the tube, and spin down.

38 Add the 8 µL of strand-switching reagents (prepared in steps 6-7) to the 11 µL of snap-cooled mRNA (from steps 2-5). Mix by flicking the tube and spin down.

39 Incubate at 42 °C for 00:02:00 in the thermal cycler. 2m

40 Add 1 µl of

Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741

. The total volume is now 20 µL .

41 Mix gently by flicking the tube, and spin down.

42 Incubate using the following protocol using a thermal cycler:

Cycle Step	Temperature	Time	No. of cycles
Reverse Transcription and Strand-Switching	42 ℃	90 minutes	1
Heat inactivation	85 ℃	5 minutes	1

Hold	4 °C	∞	

Library Preparation: RNA Degradation and Second strand synthesis

43 1. Thaw the following reagents and spin down briefly using a microfuge, before mixing as indicated in the table below, and place on ice.

Reagent	1. Thaw at room Temperature	2. Briefly spin down	3. Mix well by pipetting
User-supplied PR2 Primer diluted to 10 µM	✓	✓	✓
RNaes Cocktail Enzyme Mix	Not frozen	✓	✓
LongAmp Taq 2X Master Mix	✓	✓	✓

44 Thaw the AMPure XP Beads (AXP) at room temperature and mix by vortexing. Keep the beads at room temperature.

45 Add 1 µL RNase Cocktail Enzyme Mix (ThermoFisher, cat # AM2286) to the reverse transcription reaction.

46 Incubate the reaction for 00:10:00 at 37 °C in a thermal cycler. 10m

47 Resuspend the AMPure XP beads (AXP) by vortexing.

48 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

49 Add 17 µL of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube.

50 0 µL Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

51 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

52 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

53 Keep the tubes on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

Note

Note: If the pellet was disturbed, wait for beads to pellet again before removing the ethanol.

54 Repeat the previous step.

55 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

56 Remove the tube from the magnetic rack and resuspend pellet in 20 µL nuclease-free water.

57 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. 10m

58 Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

59 Remove and retain 20 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

60 Prepare the following reaction in a 0.2 ml thin-walled PCR tube:

Reagent	Volume
2x LongAmp Taq Master Mix	25 µl
PR2 Primer diluted to 10 µM	2 µl
Reverse-transcribed sample from above	20 µl
Nuclease-free water	3 µl
Total Volume	50 µl

61 Incubate using the following protocol:

Cycle Step	Temperature	Time	No. of cycles
Denaturation	94 °C	1 minute	1
Annealing	50 °C	1 minute	1
Extension	65 °C	15 minutes	1
Hold	4 °C	∞	

62 Resuspend the AMPure XP beads (AXP) by vortexing.

63 Transfer the sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

64 Add 40 µL of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube.

65 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

66 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

67 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

68 Keep the tubes on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

> Note
>
> If the pellet was disturbed, wait for the beads to pellet again before removing the ethanol.

69 Repeat the previous step.

70 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

71 Remove the tube from the magnetic rack and resuspend pellet in 21 µL nuclease-free water.

72 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. 10m

73 0 µL Briefly spin down the tube and pellet the beads on the magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

74 Remove and retain 21 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

75 Quantify the DNA using a Agilent Bioanalyzer and Qubit Fluorometer

Library Preparation: cDNA repair and end-prep

76 Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

77 1. Combine the following reagents in a 0.2 ml PCR tube:

	Reagent	Volume
	cDNA sample	25 µl
	Nuclease-free water	30 µl
	Ultra II End-prep reaction buffer	7 µl
	Ultra II End-prep enzyme mix	3 µl
	Total Volume	60 µl

78 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

79 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

80 Resuspend the AMPure XP Beads (AXP) by vortexing.

81 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

82 Add 60 µL of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.

83 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

84 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

85 Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.

86 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

87 Repeat the previous step.

88 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

89 Remove the tube from the magnetic rack and resuspend pellet in 61 µL nuclease-free water. Incubate for 00:02:00 at room temperature. 2m

90 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00 1m

91 Remove and retain 61 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

92 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

93 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

94 Resuspend the AMPure XP Beads (AXP) by vortexing.

95 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

96 Add 60 µL of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.

97 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

98 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

99 Spin down the sample and pellet on a magnet until supernatant is clear and colourless. Keep the tube on the magnet, and pipette off the supernatant.

100 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

101 Repeat the previous step.

102 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

103 Remove the tube from the magnetic rack and resuspend pellet in 61 µL nuclease-free water. Incubate for 00:02:00 at room temperature. 2m

104 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

105 Remove and retain 61 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

106 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

107 Using a thermal cycler, incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 . 10m

108 Resuspend the AMPure XP Beads (AXP) by vortexing.

109 Transfer the DNA sample to a clean 1.5 ml Eppendorf DNA LoBind tube.

110 Add 60 µL of resuspended the AMPure XP Beads (AXP) to the end-prep reaction and mix by flicking the tube.

111 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

112 Prepare 500 µL of fresh 80% ethanol in nuclease-free water.

113 Spin down the sample and pellet on a magnet until supernatant is clear and colorless. Keep the tube on the magnet, and pipette off the supernatant.

114 Keep the tube on the magnet and wash the beads with 200 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

115 Repeat the previous step.

116 Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 00:00:30 , but do not dry the pellet to the point of cracking. 30s

117 Remove the tube from the magnetic rack and resuspend pellet in 61 µL nuclease-free water. Incubate for 00:02:00 at room temperature. 2m

118 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

119 Remove and retain 61 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Library Preparation: Adapter ligation and clean-up

120 Spin down the Ligation Adapter (LA) and Quick T4 Ligase, and place on ice.

121 Thaw Ligation Buffer (LNB) at room temperature, spin down and mix by pipetting. Due to viscosity, vortexing this buffer is ineffective. Place on ice immediately after thawing and mixing.

122 Thaw the Elution Buffer (EB) at room temperature and mix by vortexing. Then spin down and place on ice.

123 Thaw the Short Fragment Buffer (SFB) at room temperature and mix by vortexing. Then spin down and place on ice.

124 In a 1.5 ml Eppendorf DNA LoBind tube, mix in the following order:

Between each addition, pipette mix 10-20 times

	Reagent	Volume
	cDNA sample from the previous step	60 µl
	Ligation Adapter (LA)	5 µl
	Ligation Buffer (LNB)	25 µl
	NEBNext Quick T4 DNA Ligase	10 µl
	Total	100 µl

125 Thoroughly mix the reaction by gently pipetting and briefly spinning down.

126 Incubate the reaction for 00:10:00 at room temperature. 10m

127 Resuspend the AMPure XP Beads (AXP) by vortexing.

128 Add 40 µl of resuspended AMPure XP Beads (AXP) to the reaction and mix by flicking the tube.

129 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

130 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when clear and colorless.

131 Wash the beads by adding 250 µL of Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

Note

Take care when removing the supernatant, the viscosity of the buffer can contribute to loss of beads from the pellet.

132 Repeat the previous step.

133 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~ 00:00:30 , but do not dry the pellet to the point of cracking. 30s

134 Remove the tube from the magnetic rack and resuspend the pellet in 15 µL Elution Buffer (EB). Spin down and incubate for 00:10:00 at room temperature. 10m

135 Pellet the beads on a magnet until the eluate is clear and colorless, for at least 00:01:00 . 1m

136 Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

137 Quantify 1 µL of the eluted sample using a Qubit fluorometer

138 Depending on your DNA library fragment size, prepare your final library in 12 µL of Elution Buffer (EB).

	Fragment library length	Flow cell loading amount
	Very short (<1 kb)	100 fmol
	Short (1-10 kb)	35–50 fmol
	Long (>10 kb)	300 ng

Loading and priming MinION

139 Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, if using), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at room temperature before mixing by vortexing. Then spin down and store on ice.

140 1. To prepare the flow cell priming mix with BSA, combine Flow Cell Flush (FCF) and Flow Cell Tether (FCT), as directed below. Mix by pipetting at room temperature.

	Reagent	Volume per flow cell
	Flow Cell Flush (FCF)	1,170 µl
	Bovine Serum Albumin (BSA) at 50 mg/ml	5 µl
	Flow Cell Tether (FCT)	30 µl
	Total volume	1,205 µl

BSA is not required but improves sequencing

141 Open the MinION or GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.

142 Slide the flow cell priming port cover clockwise to open the priming port.

143 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:

143.1 Set a P1000 pipette to 200 µL

143.2 Insert the tip into the priming port

143.3 Turn the wheel until the dial shows 220-230 µl, to draw back 20-30 µl, or until you can see a small volume of buffer entering the pipette tip

144 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes. During this time, prepare the library for loading by following the steps below.

145 Thoroughly mix the contents of the Library Beads (LIB) by pipetting.

146 In a new 1.5 ml Eppendorf DNA LoBind tube, prepare the library for loading as follows:

	Reagent	Volume per flow cell
	Sequencing Buffer (SB)	37.5 µl
	Library Beads (LIB) mixed immediately before use, or Library Solution (LIS), if using	25.5 µl
	DNA library	12 µl
	Total	75 µl

147 Complete the flow cell priming:

147.1 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

147.2 Load 200 µL of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

148 Mix the prepared library gently by pipetting up and down just prior to loading.

149 Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

150 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port and close the priming port.

151 Place the light shield onto the flow cell, as follows:

151.1 Carefully place the leading edge of the light shield against the clip. Note: Do not force the light shield underneath the clip.

151.2 Gently lower the light shield onto the flow cell. The light shield should sit around the SpotON cover, covering the entire top section of the flow cell.

152 Close the device lid and set up a sequencing run on MinKNOW

Protocol references

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