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ForBio Course ONT Barcoding Protocol

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

Protocols for ONT Barcoding Course at NTNU August 2024

Making HotSHOT

1 Alkaline lysis solution:

1 g NaOH

0.074 g Na_2 EDTA

1 L H_2O

Combine reagents in a 1 L flask.

2 Neutralizing Buffer:

6.3 g Tris HCl (do not use Tris base!)

1 L H_2O

Combine reagents in a 1 L flask.

HotSHOT Extraction

3 Pipette 10-20 µL of HOTSHOT alkaline lysis reagent into wells of a microplate

4 Remove specimens onto a clean paper towel, dab on towel before loading to remove excess ethanol

5 Load specimens and check to make sure all specimens are submerged. If necessary, carefully submerge any floating specimens with forceps. Leave the last well empty as your negative.

6 Run extraction in a thermocycler for 18 minutes at 65°C followed by 2 minutes at 98°C.

7 Remove the plate from the thermocycler and pipette in 10 µL (or whatever quantity of lysis you used) of HotSHOT Tris HCl buffer to each well.

8 Specimens should be removed as soon as possible to avoid crystal formation.

Preparing for PCR

9 Prepare plate or strip tube with 1 µL of R primer (at 10 µM) in each well (different R primer in every well).

10 Prepare and distribute PCR mix in plate or strip tube wells according to your needs:

STEP CASE

Plate From 2 to 71 steps

Combine in a 1.5 mL tube:
770 µL CWBIO 2xTaq MM
110 µL BSA
110 µL F primer (at 10 µM)
275 µL H2O

--> 158 µL in each well of a strip tube
--> 11.5 µL in each well of a plate

11 Add 5 µL of DNA to each well.

12 Run the thermocycler protocol according to your chosen barcode:

STEP CASE

658 (Standard Folmer) 69 steps

1. 95°C for 5mins
2. 94°C for 1min
3. 45°C for 2mins
4. 72°C for 1min
2-4 repeat 35 cycles
5. 72°C for 5mins
6. 20°C for infinite

Agarose Gel for QC

13 Pick the frame and combs suitable for your samples. Tape the open ends of the frame (tape is in the drawer to the left of the gel station) and position the comb.

14 Pour 100 mL of agarose gel mixture (pre-prepared in the oven) into a flask and add 10 µl of SYBR stain. Swirl to mix.

15 Pour the mixture into the frame and allow to set for 30 minutes. Submerged the gel into the buffer, ensuring it is completely covered.

16 Fill with 2 µl of PCR product/well. If you are not using dyed mastermix, use loading dye (in fridge). Ladder is also in fridge.

17 Run at 110V for 35 minutes.

18 Turn off the electrophoresis and remove the gel from the buffer. Place it on the light panel and turn on the light, close the door. On the computer, open GeneSnap and use the green button to take a photo.

Pooling

19 Pool 3 µL of PCR product from each well, first into a strip tube and then into a vial. Remove 500 µL of pooled product to a 1.5 mL tube in preparation for bead clean-up. The remaining product can be stored in case it is needed in the future. It will also be included in the electrophoresis gel.

Bead clean-up: preparation, binding/separation

20 Make fresh 80% ethanol (eg. 400 mL pure ethanol + 100 mL molecular grade H_2O for 500 mL 80% ethanol).

21 Bring beads to room temperature and re-suspend them by vortexing thoroughly.

22 Add an equal volume (1:1) of beads to the volume of pooled PCR product (e.g. 500 µL of beads to 500 µL of pooled PCR product). Vortex briefly, then spin down in microfuge very briefly.

23 Incubate for 15 min at room temperature with the tube lid open.

24 Put the tube on the magnetic rack and wait until a bead pellet is formed, and the solution is clear* (5 minutes or so) . Carefully aspirate the supernatant, reserving for the electrophoresis gel.
**Blue mastermix will still be blue!*

Bead clean-up: washing

25 Keep the tube on the magnet and wash twice with 500 µL 80 % Ethanol. Specifically:
a. Add 500 µL of 80% ethanol, gently, to the pellet while still in the magnetic rack, wait for 20 secs, then remove and discard the ethanol.
b. Repeat step (a), but this time be sure to remove all the ethanol (use a small pipette tip to remove the last droplets of ethanol).

26 Leave the bead pellet to dry (make sure the tube lid is open) for 10-15 mins, or until the bead pellet looks less shiny and wet.

Bead clean-up: elution

27 Remove the plate from the magnet. To elute the DNA, add 30 µL of molecular grade water. Mix by pipetting in and out to re-suspend the beads, then vortex and briefly spin down.

28 Close the tube and incubate for 5-10 mins at room temperature (not in the magnetic rack).

29 Place tube in the magnetic rack (lid open) and wait for the solution to become clear. Remove the DNA to a clean tube, careful not to disturb the beads.

30 You now have a tube with approximately 30 µL of cleaned DNA. Reserve 2 µL of this cleaned product for the gel.

31 Run a gel with 2 µL of (a) pooled product, (b) supernatant , (c) final cleaned product

Quantification with Qubit

32 Label 5 0.5-mL tubes for (2) standards and (3) pool samples. You will take the average ng/µl from the three replicates as the pool concentration.
Note: label only the tops of the tubes and use Qubit approved tubes.

33 You will need approximately 1000 µL of working solution for the standards and samples. Make this by combining 5 µL of Qubit® reagent plus 995 µL of Qubit® buffer.

34 Prepare the tubes for standards with 190 µL of working solution in each tube and 10 µL of standard. Vortex for several seconds to mix.

35 Prepare the tubes for your pool samples with 199 µL of working solution in each tube and 1 µL of the pool. Vortex for several seconds to mix.

36 Allow all the tubes to sit at room temperature for 2 minutes before proceeding.

37 On the Qubit, choose "DNA" on the first screen, then the appropriate assay (we use dsDNA Broad Range).

38 Click "Yes" at the bottom of the next screen to read new standards, then insert Standard 1 and hit "Read". It will then prompt you for Standard 2, and your samples. When you have read the last sample it will give you the average in µg/mL (ng/µL). Depending on your Qubit, this may be the concentration of your sample or the entire solution. If Qubit 2.0, it will give you the entire

solution and you will need to multiply this number by 200 to get your sample concentration (if you input 1 µL).

39 You want to load 200 ng of DNA for a MinION, 100 ng for a Flongle. To get the amount of your cleaned DNA you will need, divide the ng you need by the concentration of your sample (ng/(ng/µL)=µL (eg. 100ng/(5ng/µL)=20µL)

Library Preparation and Loading

40 Important! The following modifications to ONT protocols are made:
1. Omit the DCS from end-prep solution
2. Use 100 ng DNA as input
3. During adapter ligation, use a 1:1 ratio of beads and product

A checklist for end-prep, adapter ligation, clean-up and loading:

Checklist Ligation sequencing amplic… 102KB

For the full protocol with detailed photos and instructions for loading:

Full Ligation-sequencing-amplicons-s… 5.1MB

End Preparation

41 Thaw Ultra II End-prep Reaction Buffer & Enzyme Prep on ice
Do Not Vortex End Prep Enzyme Mix - flick, invert and spin
Vortex End-prep Reaction Buffer and spin

42 Transfer amplicon DNA into 0.2ml PCR tube
Adjust to 24.5µl with nuclease-free water
Flick and spin

43 Additionally, add:
3.5µl End-Prep Reaction Buffer
1.5µl End-Prep Enzyme Mix
30µl Total
Pipette mix and spin

44 Run in a thermal cycler - 5min @ 20°C, 5min @ 65°C

45 Transfer 30µl DNA sample to clean 1.5ml Eppendorf

46 Heavily vortex AXP to resuspend and add 30µl to DNA sample - mix by flicking and spin

47 Incubate on Hula Mixer - 5 minutes at room temperature.

48 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 (~60µl).
Discard supernatant

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

50 Repeat previous step

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

52 Remove the tube from the magnetic rack and resuspend the pellet in 30 µl nuclease-free water.
Incubate for 2 minutes at room temperature.

53 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute

54 Remove and retain 30µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube
Dispose of pelleted beads
It is possible to store this sample at 4°C overnight

Adapter Ligation and Clean-up

55 Spin down LA and T4 Ligase, place on ice

56 Thaw LNB at room temperature
Spin and mix by pipetting, then place on ice

57 Thaw EB and SFB at room temperature
Vortex, spin, place on ice

58 Into DNA Library, add:
12.5µl LNB
5µl T4 Ligase
2.5µl LA
50µl Total
Pipette mix, spin briefly

59 Incubate - 10min @ room temperature

60 Resuspend beads and add 50µl to the reaction
flick and spin

61 Incubate on Hula Mixer - 5min @ room remperature

62 Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant (~100µl).
Discard supernatant

63 Wash the beads by adding 125µl SFB
Flick the beads to resuspend and briefly spin down
Return the tube to the magnetic rack and allow the beads to pellet
Remove the 125µl supernatant using a pipette and discard

64 Repeat the previous step

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

66 Remove the tube from the magnetic rack and resuspend pellet in 7µl EB

67 Incubate for 10 minutes at room temperature

68 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 1 minute

69 Remove and retain 7 µl of eluate containing the DNA library into a clean 1.5ml Eppendorf
Dispose of pelleted beads

70 Quantify 1µl of eluted sample using a Qubit fluorometer - See previous section

71 Make up library to 5µl at 10fmol with EB
Use a calculator such as NEBioCalculator
Mass to Moles
Basepairs are ~700 (for 1-step indexed COI amplicons)

Loading the Flongle

72 Remove Flongle from the fridge, keeping the pack sealed until it reaches room temperature

73 Install Flongle Adaptor into Minion, and then install the Flongle into the Adaptor and run a Flow Cell Check in MinKNOW

74 Thaw SB, LIB, FCT and FCF @ room temp, vortex and spin

75 Create flow cell Primer Mix in a 1.5ml Eppendorf by adding:
117µl FCF
3µl FCT
120µl Total
Mix by pipetting

76 Open Tab on Flongle, and withdraw any air in the port
Add Primer Mix, ensuring no air bubbles are introduced.

77 Add to 5µl DNA library to create to create sequencing mix:
15µl SB
10µl LIB - vortexed immediately before use
30µl Total

78 Add the 30µl Sequencing Mix to the flow cell, ensuring no air bubbles

79 Seal tab, close MinION lid, start sequencing!

After the run

80 After basecalling, you will have a lot of compressed fastq files in folders "pass" and "fail". These can be extracted using 7-zip, and then combined into one file using the cat function (requires Ubuntu if you are on Windows):
1. cd to pass folder
2. cat *.fastq > pass.fastq
3. cd to fail folder
4. cat *.fastq > fail.fastq
5. Place both the fail and pass fastq files in the same folder, make that folder you working directory, and run cat one more time to make a single fastq

81 ONTBarcoder is available for download **here**.

It requires a demultiplexing file (see your handout) and the combined fastq to run.