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Automation Protocol for DNA Barcode Sequencing Library Preparation

Nina Alperovich[1], Drew S Tack[1], Olga Vasilyeva[1], Sasha F Levy[2,3], David Ross[1]

[1]National Institute of Standards and Technology; [2]SLAC National Accelerator Laboratory; [3]Joint Initiative for Metrology in Biology

1 Works for me dx.doi.org/10.17504/protocols.io.bjjzkkp6

David Ross

ABSTRACT

Here, we describe a protocol for automated library preparation for DNA barcode sequencing. The protocol uses a Hamilton NGS-STAR automated liquid handler equipped with an automated thermocycler.

This protocol requires an automated liquid handler (Hamilton Robotics, NGS-STAR) equipped with an automated thermocycler.

EXTERNAL LINK

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ATTACHMENTS

PlasmidSequence.txt ReverseIndexPrimers.csv ForwardIndexPrimers.csv

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KEYWORDS

laboratory automation, DNA barcode sequencing

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GUIDELINES

Anticipated Results:

The concentration of DNA in each pooled sequencing sample should range from 10 ng/µL to 16 ng/µL.

MATERIALS TEXT

Reagents:

- Input plasmid DNA in 96 well plate, each sample prepared in nuclease-free water with at least 35 µL volume and up to approximately 1.5 ng/µL concentration
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- ApaI Restriction Enzyme Solution: 80 % FastDigest Buffer (Thermo Scientific, cat. #B64), 20 % FastDigest ApaI (Thermo Scientific, cat. #FD1414)
- 2x PCR Master Mix 1 (Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix, Cat. #F548L)
- PCR Master Mix 2 (volume per reaction): 33 µL 2x PCR Master Mix (Thermo Scientific, Cat. #F548L), 1.5 µL Secondary Primer F (20 µmol/L), 1.5 µL Secondary Primer R (20 µmol/L)
- Sera-Mag SpeedBeads Carboxyl Magnetic Beads (GE Healthcare, cat. #65152105050250)
- Poly (ethylene glycol) PEG-8000 (Sigma Aldrich, Cat. #81268)
- Sodium chloride (Fisher Scientific, Cat. #AM9759)
- TWEN20 (Thermo Scientific, Cat. #3005)
- 1x TE Buffer (Thermo Scientific, cat. #12090015 pH 8.0)
- Elution Buffer: 10 mmol/L Tris-Cl, pH 8.5 (VWR, cat. # MB-027-1000)
- Ethanol, 200 proof (100%) (Fisher Scientific, Cat. #2716TP)
- Nuclease-free water (Thermo Scientific, cat. no. AM9938)

Primers:

- DNA sequence for Secondary Primer F: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
- DNA sequence for Secondary Primer R: CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT
- Forward Index Primer: see supplementary file: “ForwardIndexPrimers.csv”
- Reverse Index Primer: see supplementary file: “ReverseIndexPrimers.csv”

Additional labware:

- PCR plate (Bio-Rad, HSP9645)
- 96-well midi plate (Abgene, AB-0765)

Preparation of Magnetic Bead/PEG-NaCl Stock

1 Add 9 g PEG-8000 to a new 50 mL sterile conical tube.

2 Add 10 mL 5 mol/L NaCl.

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3 Add 500 µL 1 mol/L Tris-HCl, pH8.

4 Add 500 µL 0.5 mol/L EDTA.

5 Fill 50 mL tube to 45 mL using sterile nuclease free water (vendor), mix by vortexing every 1-2 minutes until PEG goes into solution.

6 Add 27.5 µL Tween-20 and mix gently by inverting 4-6 times.

7 Vortex Sera-mag SpeedBeads to resuspend and transfer 1 mL to each of two 2 mL micro-centrifuge tubes.

8 Place SpeedBeads solutions on a magnetic separation rack until beads are drawn to the magnet and solutions are clear.

9 Remove supernatant.

10 Add 1 mL 1x TE Buffer (Thermo Scientific, cat. #12090015 pH 8.0) to each micro-centrifuge tube containing beads, remove from magnetic stand, and mix by vortexing.

11 Repeated steps 8-10 two additional times.

12 After mixing, add both 1 mL SpeedBeads solutions to the 50 mL conical tube containing the PEG/NaCl solution.

13 Fill conical tube to the 50 mL mark with sterile nuclease free water, and gently mix until solution is a uniformed brown color.

14 Wrap in foil (or place in dark container) and store at 4 °C until use.

Preparation of DNA barcode sequencing samples

15 Transfer 35 µL of each plasmid DNA sample to a PCR plate (Bio-Rad, HSP9645).

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16 Add 5 µL ApaI Restriction Enzyme Solution for plasmid DNA linearization; mix 3x by repeated aspiration and dispense.

17 Incubate samples in PCR plate in automated thermocycler at 37 °C for 15 minutes.

18 Add 2.5 µL Forward Index Primer and 2.5 µL Reverse Index Primer to each sample (20 µmol/L stock primer solutions, all primer sequences are listed in supplementary files). The Forward and Reverse Index Primers attached sample multiplexing tags to the resulting amplicons so that the different samples could be distinguished when they are pooled and run on the same sequencing flow cell. Eight different Forward Index Primers and 12 different Reverse Index Primers are used to uniquely label the amplicons from each sample across 24 different chemical environments and four time points used for barcode sequencing.

19 Add 45 µL 2x PCR Master Mix 1 (Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix, Cat. #F548L) to each sample; mix 3x by repeated aspiration and dispense.

20 Run the first PCR reaction in automated thermocycler with the following conditions:
a. Initial denaturation: 98 °C for 60 s
b. 3 cycles:
i. Denaturation: 98 °C for 10 s
ii. Annealing: 58 °C for 20 s
iii. Elongation: 72 °C for 20 s
c. Final Extension: 72 °C for 60 s
d. Cooling: 20 °C for 15 s

21 During the first PCR reaction, pipette 48 µL Magnetic Bead/PEG-NaCl Stock into each of 24 wells in a 96-well midi plate (Abgene, AB-0765); mix bead stock well by repeated aspiration and dispense before each transfer.

22 When the first PCR reaction is finished, transfer 80 µL from each PCR reaction to a well in the midi plate; mix PCR reaction solution and bead stock 10x by repeated aspiration and dispense; wait for 5 minutes. During this step, the ratio of Bead Stock to PCR reaction volume is 0.6x. Consequently, because of the relatively low PEG concentration in the mixture, the 5.5kb plasmid template binds to the beads.

23 Move the midi plate to 96-well magnet base to pull the magnetic beads out of suspension; wait for 2 minutes.

24 Transfer 128 µL of supernatant from each sample to a clean well in the midi plate (still on the magnet base); wait an additional 3 minutes for final bead separation.

25 Transfer 118 µL of supernatant (containing the 218 bp PCR amplicon and primers)from each sample to another clean well in the midi plate; move the midi plate from the magnet base to automated shaker.

26 Add 70.8 µL Magnetic Bead Stock (0.6x of 118 µL supernatant volume) to each sample; mix supernatant and bead stock 10x by repeated aspiration and dispense; wait for 5 minutes. During this step, only the 206 bp PCR amplicon

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binds to the beads; the 70 bp primers remain in the supernatant.

27 Move the midi plate back to the magnet base; wait for 5 minutes.

28 Remove and discard the supernatant from each well.

29 Add 200 µL 80 % ethanol to each magnetic bead pellet; wait 30 s.

30 Remove and discard the ethanol supernatant; then, using 50 µL tips, remove residual supernatant from the bottom of each well.

31 Move the midi plate from magnet base to the automated shaker; allow magnetic bead pellets to dry for 5 minutes at room temperature.

32 Add 35 µL nuclease-free water to each sample; resuspend beads by 5x repeated aspiration and dispense.

33 Mix samples by shaking at 1800 rpm for 10 s; wait for 5 minutes.

34 Move the midi plate back to the magnet base; wait for 5 minutes.

35 During 5-minute wait, pipette 36 µL PCR Master Mix 2 (containing primers) into each of 24 wells in a PCR plate.

36 After 5-minute wait, transfer 30 µL of each supernatant from the midi plate (on magnet base) to the PCR plate.

37 Run the second PCR reaction in the automated thermocycler with the following conditions:
a. Initial denaturation: 98 °C for 60 s
b. 15 cycles:
i. Denaturation: 98 °C for 10 s
ii. Annealing and Elongation: 72 °C for 30 s
c. Final Extension: 72 °C for 120 s
d. Cooling: 15 °C for 20 s

38 During the second PCR reaction, pipette 66 µL (1.1x of the 60 µL PCR volume) Magnetic Bead Stock into each of 24 clean wells in the midi plate (on the automated shaker); mix bead stock well by repeated aspiration and dispense before each transfer.

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39 When the second PCR reaction is finished, transfer 60 µL from each PCR reaction to a well in the midi plate; mix the PCR reaction solution and bead stock 10x by repeated aspiration and dispensing; wait for 5 minutes. During this step, with a Bead Stock to PCR reaction volume ratio of 1.1, the 306 bp PCR amplicon binds to the beads while the 70 bp primers remain in the supernatant.

40 Move the midi plate back to the magnet base; wait for 4 minutes.

41 Remove and discard the supernatant from each well.

42 Add 200 µL 80 % ethanol to each magnetic bead pellet; wait 30 s.

43 Remove and discard the ethanol supernatant; then, using 50 µL tips, remove residual supernatant from bottom of each well.

44 Move the midi plate from the magnet base to the automated shaker; allow magnetic bead pellets to dry for 5 minutes at room temperature.

45 Add 50 µL Elution Buffer to each sample; resuspend beads by 5x repeated aspiration and dispense.

46 Mix samples by shaking at 1800 rpm for 10 s; wait for 5 minutes.

47 Move the midi plate back to the magnet base; wait for 5 minutes.

48 Transfer the supernatant to pooled sequencing sample, one pooled sample per input time point.

49 Transfer pooled sample to a 2 mL micro-centrifuge tube.

50 Place the tube on a magnetic separation rack until the remaining beads are drawn to the magnet (approximately 5 minutes). Transferred the fully clarified samples to a new 2 mL micro-centrifuge tube and store them at 4 °C until needed for sequencing.

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