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Ligation sequencing V14 —single-cell transcriptomics with 5' cDNA prepared using 10XGenomics on PromethION(SQK-LSK114)

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We use this protocol and it's working

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Abstract

This protocol describes how to carry out sequencing of cDNA from single cells using the LigationSequencing Kit V14 (SQK-LSK114). You will need to have reverse-transcribed single-cell mRNA into cDNA using the 10X Genomics Next GEM Single Cell 5' Kit (V2) before performing PCR amplification of your cDNA with custom-ordered oligos. Finally, a standard Ligation Sequencing Kit V14 library preparation is completed to prepare the cDNA ends for sequencing on a PromethION device.

Attachments

PDF

ligation-sequencing-...
4.4MB

Guidelines

♦Requires cDNA amplicons produced using the 10X Genomics Next GEM Single Cell 5' Kit (V2)
♦Library preparation time ~135 minutes
♦High output
♦PCR required

Materials

Materials

10 ng of cDNA amplicons prepared using 10X Genomics Next GEM Single Cell 5'Kits (V2)
Custom-ordered oligo at 10 µM: Fwd_3580_partial_read1_defined_for_5'_cDNA
Custom-ordered oligo at 10 µM: Rev_PR2_partial_TSO_defined_for_5'_cDNA
Ligation Sequencing Kit V14 (SQK-LSK114)

Consumables

PromethION Flow Cell (FLO-PRO114M)
LongAmp Hot Start Taq 2X Master Mix (NEB, M0533)
NEBNext® Ultra II End Repair / dA-tailing Module (NEB, E7546)
Salt-T4® DNA Ligase (NEB, M0467)
Qubit 1x dsDNA HS Assay Kit (ThermoFisher, Q33230)
Agilent Technologies DNA 12000 Kit
Agencourt AMPure XP beads (Beckman Coulter™, A63881)
Freshly prepared 80% ethanol in nuclease-free water
Nuclease-free water (e.g. ThermoFisher, AM9937)
15 ml Falcon tubes
1.5 ml Eppendorf DNA LoBind tubes
0.2 ml thin-walled PCR tubes
Qubit™ Assay Tubes (Invitrogen, Q32856)

Equipment

PromethION Flow Cell Light Shield
PromethION device
Agilent Bioanalyzer (or equivalent)
Hula mixer (gentle rotator mixer)
Magnetic rack (e.g. Invitrogen DynaMag-2 Magnet, Cat # 12321D)
Microfuge
Vortex mixer
Thermal cycler
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
Qubit fluorometer (or equivalent for QC check)

PCR amplification

7m 30s

1 **Prepare the cDNA amplicons in nuclease-free water**

1.1 Transfer 10 ng of cDNA amplicons into a 0.2 mL thin-walled PCR tube.

1.2 Adjust the volume to 21 µL with nuclease-free water.

1.3 Mix thoroughly by flicking the tube to avoid unwanted shearing.

1.4 Spin down briefly in a microfuge.

2 **In the same 0.2 ml thin-walled PCR tube, set up the following reaction:**

Reagent	Stock	Final	Volume
cDNA template	0.48 ng/µl	0.2 ng/µl	21 µl
Fwd_3580_partial_read1_defined_for_5'_cDNA	10 µM	0.4 µM	2 µl
Rev_PR2_partial_TSO_defined_for_5'_cDNA	10 µM	0.4 µM	2 µl
LongAmp Hot Start Taq 2X Master Mix	2X	1X	25 µl
Total	-	-	**50 µl**

3 **Mix by pipetting and spin down.**

4 **Amplify using the following cycling conditions:**

Cycle step	Temperature	Ramp rate	Time	No. of cycles
Initial denaturation	94°C	max	3 min	1
Denaturation	94°C	max	30 sec	
Annealing ramp-down	66°C down to 58°C	0.2°C/s	40 sec	8
Annealing	58°C	max	50 sec	
Extension	65°C	max	6 mins	
Final extension	65°C	max	10 min	1
Hold	4°C	-	∞	-

5 **Resuspend the AMPure XP beads by vortexing.**

6 **Add 40 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.**

7 **Incubate on a Hula mixer (rotator mixer) for** 00:05:00 **at** Room temperature **.** 5m

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

9 **Spin down the samples and pellet the beads on a magnet until the eluate is clearand colourless. Keep the tubes on the magnet and pipette off the supernatant.**

10 **Keep the tube on the magnet and wash the beads with** 200 µL **of freshly prepared 80% ethanol in nuclease-free water without disturbing the pellet. Remove the ethanol using a pipette and discard.**

11 **Repeat the previous step.**

12 **Briefly spin down and place the tubes 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

13 **Remove the tube from the magnetic rack and resuspend the pellet in** 15 µL **nuclease-free water. Spin down and incubate for** 00:02:00 **at** Room temperature **.** 2m

14 **Pellet the beads on a magnet until the eluate is clear and colourless.**

15 **Remove and retain** 15 µL **of eluate into a clean** 0.2 mL **Eppendorf DNA LoBind tube.**

16 **Quantify** 1 µL **of eluted sample using a Qubit fluorometer - recovery aim >50 ng total.**

17 **Quantify 500 fmol of cDNA from the average fragment size identified using an Agilent Bioanalyzer**

End-prep 18m 30s

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

Note

For optimal performance, NEB recommend the following:

Thaw all reagents on ice.

Ensure the reagents are well mixed.Do not vortex the Ultra II End Prep Enzyme Mix.

Always spin down tubes before opening for the first time each day.

The NEBNext Ultra II End Prep Reaction Buffer may contain a white precipitate. If this occurs,allow the mixture(s) to come to room temperature and pipette the buffer several times to breakup the precipitate, followed by a quick vortex to mix.

19 **Transfer 500 fmol of cDNA amplicons into a clean** 0.2 mL **thin-walled PCR tubeand adjust the volume to** 50 µL **with nuclease-free water.**

20 **In the same** 0.2 mL **thin-walled PCR tube, mix the following:**

Reagent	Volume
cDNA amplicons	50 µl
Ultra II End-prep Reaction Buffer	7 µl
Ultra II End-prep Enzyme Mix	3 µl
Total	**60 µl**

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

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

23 **Transfer the DNA sample to a clean** 0.2 mL **Eppendorf DNA LoBind tube.**

24 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

25 **Add** 60 µL **of resuspended the AMPure XP Beads (AXP) to the end-prep reactionand mix by flicking the tube.**

26 **Incubate on a Hula mixer (rotator mixer) for** 00:05:00 **at** Room temperature **.** 5m

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

28 **Spin down the sample and pellet on a magnet until supernatant is clear andcolourless. Keep the tube on the magnet, and pipette off the supernatant.**

29 **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.**

30 **Repeat the previous step.**

31 **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

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

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

34 **Remove and retain** 61 µL **of eluate into a clean** 1.5 mL **Eppendorf DNA LoBind tube.**

Adapter ligation and clean-up 26m 30s

35 **Spin down the Ligation Adapter (LA) and Salt-T4® DNA Ligase, and place on ice.**

36 **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 afterthawing and mixing.**

37 **Thaw the Elution Buffer (EB) and Short Fragment Buffer (SFB) at** Room temperature **and mix by vortexing. Then spindown and place on ice.**

38 **In a** 0.2 mL **Eppendorf DNA LoBind tube, thoroughly mix the reaction in the following order by gently pipetting and briefly spinning down:**

Reagent	Volume
DNA sample from the previous step	60 µl
Ligation Adapter (LA)	5 µl
Ligation Buffer (LNB)	25 µl
Salt-T4® DNA Ligase	10 µl
Total	**100 µl**

39 **Incubate the reaction for** 00:10:00 **at** Room temperature **.** 10m

40 **Resuspend the AMPure XP Beads (AXP) by vortexing.**

41 **Add** 40 µL **of resuspended AMPure XP Beads (AXP) to the reaction and mix byflicking the tube.**

42 **Incubate on a Hula mixer (rotator mixer) for** 00:05:00 **at** Room temperature **.** 5m

43 **Spin down the sample and pellet on a magnet. Keep the tube on the magnet, andpipette off the supernatant when clear and colourless.**

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

45 **Repeat the previous step.**

46 **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

47 **Remove the tube from the magnetic rack and resuspend the pellet in** 34 µL **ElutionBuffer (EB).**

48 **Spin the sample down briefly and incubate for** 00:10:00 **at** Room temperature **.** 10m

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

50 **Remove and retain** 34 µL **of eluate containing the DNA library into a clean** 0.2 mL **Eppendorf DNA LoBind tube.**

51 **Prepare 150 fmol of your final library to** 32 µL **with Elution Buffer (EB).**

Priming and loading the PromethION Flow Cell

5m

52 **Thaw the Sequencing Buffer (SB), Library Beads (LIB) or Library Solution (LIS, ifusing), Flow Cell Tether (FCT) and Flow Cell Flush (FCF) at** Room temperature **before mixing by vortexing. Then spin down and store** On ice **.**

53 **To prepare the flow cell priming mix, combine Flow Cell Tether (FCT) and Flow CellFlush (FCF), as directed below. Mix by vortexing at** Room temperature **.**

54 **For PromethION 2 Solo, load the flow cell(s) as follows:**

54.1 Place the flow cell flat on the metal plate, leaving at room temperature for at least 30 minutes before use.

54.2 Slide the flow cell into the docking port until the gold pins or green board cannot be seen.

55 **Slide the inlet port cover clockwise to open, draw back a small volume to remove any air bubbles:**

55.1 Set a P1000 pipette tip to 200 µL .

55.2 Insert the tip into the inlet port, turn the wheel until the dial shows 220-230 µl, or until you see a small volume of buffer entering the pipette tip.

56 **Load** 500 µL **of the priming mix into the flow cell via the inlet port, avoiding the introduction of air bubbles. Wait** 00:05:00 **. During this time, prepare the library for loading using the next steps in the protocol.**

5m

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

58 **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)	100 µl
Library Beads (LIB) thoroughly mixed before use, or Library Solution (LIS)	68 µl
DNA library	32 µl
Total	**200 µl**

59 **Complete the flow cell priming by slowly loading** 500 µL **of the priming mix into the inlet port.**

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

61 **Load** 200 µL **of library into the inlet port using a P1000 pipette.**

62 **Close the valve to seal the inlet port.**

63 **If the light shield has been removed from the flow cell, install the light shield as follows:**

63.1 Align the inlet port cut out of the light shield with the inlet port cover on the flow cell. The leading edge of the light shield should sit above the flow cell ID.

63.2 Firmly press the light shield around the inlet port cover. The inlet port clip will click into place underneath the inlet port cover.

64 **Start sequencing**