3-level sci RNA-Seq with FACS

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ABSTRACT

This protocol is a variation on "3 level sci RNA-Seq", presented on protocols.io at (https://www.protocols.io/view/sci-rna-seq3-9yih7ue).

The variation presented here notably includes a FACS sorting step before the PCR step. This notably decreases background in the library prep.

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GUIDELINES

Note that this protocol is a modification to the method previously distributed at https://www.protocols.io/view/sci-rna-seq3-9yih7ue. The main changes include:

Addition of FACS sorting between ligation and second-strand synthesis steps. (This reduces background for nuclei sci RNA-Seq when using nuclei obtained from primary tissue)

Skipping of the USER enzyme reaction step (this was found to be unnecessary)

A modified reverse transcription reaction temperature ramp (this was found to modestly increase UMIs recovered per nucleus)

MATERIALS TEXT

Nuclease free water (Ambion, AM 9937)
Snap Cap FACS Tube (Corning, 08-771-23)
SUPERase In RNase Inhibitor 20 U/uL (Thermo Fisher Scientific, AM2696)
BSA 20 mg/ml (NEB, B9000S)
1M Tris-HCl (pH 7.4)
5M NaCl (Thermo Fisher Scientific, AM9759)
1M MgCl2 (Thermo Fisher Scientific, AM9530G)
Triton X-100 for molecular biology (Sigma Aldrich, 93443-100ML)
10mM dNTP (Thermo Fisher Scientific, R0192)
384 indexed oligo-dT primers (100uM, 5'- /5Phos/CAGAGCNNNNNNNN[10bp barcode]TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3', where "N" is any base; IDT)
Superscript IV reverse transcriptase with 100mMDTT and buffer(Invitrogen, 18090200)
RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, 10777019)
Quick ligation kit (NEB, M2200L)
384 indexed ligation primers (100uM, 5'- GCTCTG[9bp or 10bp barcode A]/ideoxyU/ACGACGCTCTTCCGATCT[reverse complement of barcode A]-3')
Elution buffer (Qiagen, 19086)
NEBNext® Ultra II Non-Directional RNA Second Strand Synthesis Module (NEB, E7550S)
DNA binding buffer (Zymo Research, D4004-1-L)
AMPure XP beads (Beckman Coulter, A63882)
Ethanol (Sigma Aldrich, 459844-4L)
10 µM P5 primer (5'-AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3', IDT)
10 µM P7 primer (5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3', IDT)
NEBNext High-Fidelity 2X PCR Master Mix (NEB, M0541L)
Select-a-Size DNA Clean & Concentrator (Zymo Research, D4080)
Qubit dsDNA HS kit (Invitrogen, Q32854)
Qubit tubes (Invitrogen, Q32856)
Nextseq 2000 P2, 100 cycle kit (Illumina)
Falcon Tubes, 15 ml (VWR Scientific, 21008-936)
Falcon Tubes, 50 ml (VWR Scientific, 21008-940)
Green pack LTS 200ul filter tips (GP-L200F) (Rainin Instrument, 17002428)
Green pack LTS 20ul filter tips (GP-L20F) (Rainin Instrument, 17002429)
Bright-Line™ Hemacytometer (Sigma Aldrich, Z359629-1EA)
DNA LoBind Tube 1.5 ml, PCR clean (Eppendorf North America, 22431021)
LoBind clear, 96-well PCR Plate (Eppendorf North America, 30129512)
Reagent reservoirs (Fisher Scientific, 07-200-127)
Microseal 'B' Adhesive seal (Bio-Rad Laboratories, MSB1001)

N7-loaded Tn5

Nextera N7 adaptor loaded Tn5
The original use of the protocol involved use of custom Tn5 loaded with Nextera N7 adapters.
The closest commercial equivalent is available from Illumina (FC-121-1031).
Alternately, unloaded Tn5 can be purchased from Diagenode (C01070010-10) and loaded with adapters per the methods described at (https://www.biorxiv.org/content/10.1101/2019.12.17.879304v1.full.pdf)

Equipment:

FACS Aria II Sorter with 96 well plate holder
Ice buckets
Refrigerated centrifuge with 15 mL tube holders
ScreenTape (Agilent)
Qubit (Thermo)

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

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1 Note: All amounts listed here assume using 1 RT plate (96 wells) and 1 ligation plate (96 wells). Buffer volumes to prepare will vary for larger/smaller experiments.

Buffer Preparation

2 Nuclei Buffer: 10 mM Tris HCl, 10 mM NaCl, 3 mM MgCl2 in nuclease-free water

Reagent	Stock (mM)	Final Conc. (mM)	Volume (mL)
Tris HCl (pH 7.4)	1000	10	5
NaCl	5000	10	1
MgCl2	1000	3	1.5
Water			492.5

Store at 4C.

3 Nuclei suspension buffer (NSB).

Prepare at least 1.7 mL per sample, plus 3 mL per RT plate and 1 mL per ligation plate.

For every 1 mL.
- 1 mL Nuclei Buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2)
- 10 uL Bovine Serum Albumin (BSA)
- 10 uL SuperaseIn

Chill on ice

4 Nuclei buffer with BSA (NBB)

Make at least: 6 mL per RT plate and 17 mL per ligation plate.

For every 1 mL.
- 1 mL Nuclei Buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2)
- 10 uL Bovine Serum Albumin (BSA)

Chill on ice

5 10% Triton X-100 Stock

Combine 1.0 mL of Triton X-100 with 9.0 mL nuclease-free water. Gently rock overnight to mix, store at 4C.

6 Permeabilization Buffer:

Make 500 uL per sample. For this, combine:
12.5 uL of **10%** stock of Triton X-100
487.5 uL NSB

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Pre-chill on ice.

Permeabilization

7 Thaw frozen aliquots of all samples using a 37C water bath. Watch carefully, and remove from water bath and place on ice as soon as the frozen buffer + nuclei mixture has thawed.

8 For each sample, add 400 uL permeabilization buffer. Mix gently.

9 Incubate 3 minutes on ice.

10 Pellet the nuclei at 500g for 5min (4C) and remove the supernatant; Resuspend the nuclei in 1 mL NSB

11 Pellet the nuclei at 500g for 5min (4C) and remove the supernatant.

12 Resuspend the nuclei in 300ul NSB and count the nuclei concentration with a hemocytometer.

Reverse Transcription

13 For each well of 4 x 96 well plates, add
30,000 nuclei in 22ul nuclei buffer
2ul 10mM dNTP
2ul indexed oligo-dT primer (100uM).

Note: Up to 80,000 nuclei can be loaded per well. However, typical UMIs-per-nucleus decrease above ~30,000 nuclei per well.

14 Incubate the plates at 55 °C for 5 min. Immediately place the plates on ice.

15 Prepare the reverse transcription reaction mix. For each well, combine

8 uL of 5X Superscript IV First-Strand Buffer
2 uL of 100mM DTT
2 uL of SuperScript IV reverse transcriptase
2 uL of RNaseOUT Recombinant Ribonuclease Inhibitor

Mix well and distribute 14ul to each well.

Note: Make a master mix with a few extra wells worth to space. For example, consider making 105 or 110 wells worth of master mix in order to readily add 14 uL to each well of a 96 well plate.

16 Incubate the RT reaction for the following times/temperatures:

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4°C for 2 minutes
10°C for 2 minutes
20°C for 2 minutes
30°C for 2 minutes
40°C for 2 minutes
50°C for 2 minutes
53°C for 15 minutes
55°C for 10 minutes

17 After the reaction, add 60ul ice-cold NBB into each well with a multichannel pipette.

18 Pool the nuclei from all wells.

19 Pellet the nuclei (600 RCF, 10min, 4C), and remove the supernatant.

Ligation

20 Resuspend the nuclei pellet in 1 mL NSB, then distribute 10 uL to each well of a 96 well plate.

21 Add 8ul indexed ligation oligos (100 uM) into each well with a multichannel pipette.

22 Prepare ligation master mix. For each well, combine:
2 uL Quick Ligase
20 uL Quick Ligase buffer

23 Distribute 22 uL of ligation master mix to each well. Mix by gently pipetting up/down 5 times.

24 Ligate by incubating at 25C for 10 minutes.

25 Add 60 uL of NBB to each well. Pool all wells.

26 Add an additional 10 mL NBB. Spin down nuclei (600 RCF, 10 minutes, 4C) and discard supernatant.

27 Resuspend nuclei in 1 mL ice cold Elution Buffer ("EB", Qiagen).

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28 Add 10 uL of 300 uM DAPI (diluted in water), gently mix.

29 Filter through the cap of a FACS tube (35 uM).

FACS Sorting

30 Add 4 uL of EB to each well of 96 well plates for sorting.

31 Sort singleton nuclei based on DAPI profiles. Sort the number of nuclei to give an acceptable doublet rate.

The number of nuclei sorted per well here depends on the acceptable doublet rate and the number of RT and ligation wells used in previous steps. If a single 96 well plate was used for both RT and ligation, for instance, sorting 750 nuclei per well at this step corresponds to a ~92% singleton rate.

The singleton rate is found as:

(1-[1/(RT_wells * ligation_wells)])^(#sorted per PCR well)

Second Strand Synthesis

32 First, confirm final volume in wells of FACS-sorted nuclei. Volume should be ~12 uL (from 4 uL EB plus buffer from sorted nuclei). If this volume is markedly different, scale **all input volumes for every step from here up until the elution step in the "bead cleanup" step.** So if wells have 25 uL in them, in your plate, all volumes below should be doubled up until the elution step from Ampure beads. That volume should remain constant, as should all following volumes.

33 Prepare second strand master mix. For each well, prepare:
1.33 uL second strand buffer
.67 uL second strand enzyme mix

Add 2 uL per well, mix thoroughly.

34 Incubate for 16 °C for 3 hours.

35 Briefly spin plates.

Optional stopping point. Store at 4C overnight, or freeze at -20C for longer periods.

Tagmentation

36 Make 2x TD Buffer:

1.2 mL tagmentation salt buffer (20 mM Tris pH 7.4, 10 mM $MgCl_2$)
300 uL dimethyl formamide

37 Make tagmentation mix. For each well:
12.5 uL 2x TD buffer

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.02 uL Tn5 (N7-only)

38 Add 12.5 uL of second tagmentation mix to each well. Mix thoroughly.

39 Incubate for 5 minutes at 55C.

40 Immediately add 25 uL DNA binding buffer per well and incubate the mix at room temperature for 5 minutes.

Ampure bead purification

41 Add 50 uL of Ampure Beads (pre-equilibrated to room temperature for at least 30 minutes, and mixed well) to each well.

42 Incubate at room temperature for 5 minutes. Transfer to magnet and incubate for 3 minutes more.

43 Wash beads, using a multichannel pipette.

Wash twice. Each time:
Remove supernatant.
Wash each well with ~150 uL of 80% ethanol (made fresh from 100% ethanol stock).

44 After the second wash of ethanol is removed, remove the plate from the magnet and let air-dry briefly.

Note: Typically the time that it takes to remove all ethanol from all wells means that the first wells to be emptied will have beads fairly well-dried by the time the last wells in a plate have been emptied. The most essential part is to make sure all supernatant has been thoroughly removed from all wells before removing the plate from the magnet.

45 Resuspend beads with a multichannel pipette, adding 17 uL EB to each well (Qiagen). Mix well.

46 Incubate for 3 minutes at room temperature, then transfer to the magnet. Incubate 5 minutes.

47 Transfer 16 uL from each well into a new plate (making sure to keep all wells separate at all times, still).

PCR

48 For each well, add:
2ul indexedP5 PCR primer (10uM)
2ul P7 primer (10uM)
20ul NEBnext master mix for PCR reaction

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49 Carry out PCR using the following cycle settings:

72°C for 5 min, 98°C for 30 sec, 17 cycles of (98°C for 10 sec, 66°C for 30 sec, 72°C for 30 sec) and a final 72°C for 5 min.

Post-PCR Cleanup

50 Pool all PCR wells. For 1 plate of PCR product (40 uL * 96 wells) add 8 mL of DNA binding buffer, and mix well.

51 Clean DNA using a Qiagen "Clean and Concentrate" kit. Use 4 tubes per 1 PCR plate of product being processed to speed the process.

52 Load ~600 uL of DNA binding buffer/PCR product mix at a time. Spin 12,000 RCF for 30s, and discard flow-through. Repeat until all volume has been spun through filter tubes.

53 Wash 2x by adding 200 uL DNA wash buffer, and spinning 12,000 RCF for 30s.

54 Transfer filter tubes to sit in clean Eppendorf tubes. Add 52 uL EB carefully but directly onto the filter of each tube.

55 Incubate 1 minute at room temperature, then spin 12,000 RCF for 1 minute.

56 Combine all flow-through into a single tube (should be ~200 uL, from ~50 uL from each of 4 filter tubes in the previous step).

57 Carry out an Ampure bead cleanup using .7x volumes of bead volume (check volume eluted from column. For example, if 200 uL is eluted in the previous step add 140 uL of well-mixed, room temperature beads).

58 Incubate 5 minutes, move to magnet, incubate 3 minutes.

59 Remove supernatant and wash 2x with 80% fresh made ethanol. 1 mL each wash.

60 After removing second ethanol wash, air dry beads for 5 minutes.

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61 Resuspend beads by adding 52 uL EB. Incubate for 5 minutes, transfer to magnet and incubate 3 minutes more. Remove 50 uL of solution, transfer and store as library.

Quantify and Sequence

62 Libraries were quantified by Qubit and fragment sizes/molar concentration was found using a D1000 kit on the Agilent ScreenTape.

63 Libraries were diluted and sequenced on an Illumina Nextseq 2000, 100 bp kit

Read settings:

Read 1: 34 bases
Read 2 66 bases
Index 1: 10 bases
Index 2: 10 bases

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