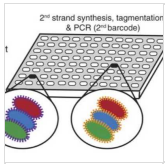

Feb 13, 2021

Single cell RNA sequencing library preparation (2-level sci-RNA-seq)

In 1 collection

Sanjay Srivatsan[1], Junyue Cao[1]

[1]University of Washington

1 *Works for me* This protocol is published without a DOI.

Sanjay Srivatsan

SUBMIT TO PLOS ONE

ABSTRACT

Sci-RNA-seq is a protocol developed by Junyue Cao in the Shendure Lab at the University of Washington and adapted by others in the Shendure and Trapnell labs. The protocol It is among a family of protocols that leverages split-pool barcoding for single cell sequencing.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://science.sciencemag.org/content/357/6352/661

PROTOCOL CITATION

Sanjay Srivatsan, Junyue Cao 2021. Single cell RNA sequencing library preparation (2-level sci-RNA-seq). **protocols.io** https://protocols.io/view/single-cell-rna-sequencing-library-preparation-2-l-bquamwse

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

https://science.sciencemag.org/content/357/6352/661

COLLECTIONS

Single Cell Combinatorial Indexing (SCI) - seqeuncing

KEYWORDS

single cell sequencing, RNA seq, split-pool

CREATED

Dec 16, 2020

LAST MODIFIED

Feb 13, 2021

PROTOCOL INTEGER ID

45666

PARENT PROTOCOLS

Part of collection
Single Cell Combinatorial Indexing (SCI) - seqeuncing

GUIDELINES

This is a long protocol, please read over the entire thing before beginning.

DISCLAIMER:

Preparation

1 Reagents to prepare prior to beginning protocol:

1. Prepare 5mL of NSB

NSB - Nuclei Buffer + Superase In + BSA

10mM Tris/HCl pH 7.4
10mM NaCl
3mM MgCl2
1% (v./v) Superase-Inhibitor

☒SUPERase• In™ RNase Inhibitor (20 U/μL) **Thermo Fisher Catalog #AM2696**

☒BSA 20

1% (vol./vol.) BSA mg/ml **NEB Catalog #B9000S**

Solvent: Nuclease Free Water

2. Prepare 5mL of NBB

NBB - Nuclei Buffer + BSA

10mM Tris/HCl pH 7.4
10mM NaCl
3mM MgCl2

☒BSA 20

1% (vol./vol.) BSA mg/ml **NEB Catalog #B9000S**

Solvent: Nuclease Free Water

2 Reagents purchased prior to beginning protocol:

☒Superscript IV **Thermo Fisher**

3. **Scientific Catalog #18090050**

Each Superscript IV tube is sufficient for 192 barcoded RT reactions (2 plates). To increase the cell barcode space, more unique barcoded RT reactions must be performed.

Each kit includes 5x SSIV buffer master mix and 0.1mM DTT necessary for performing RT reaction.

☒RNaseOUT Recombinant Ribonuclease Inhibitor **Thermo Fisher**

4. **Scientific Catalog #10777019**

Each tube is sufficient for ~495 barcoded RT reactions (4.5 plates).

☒Illumina DNA Prep (M) Tagmentation (96 Samples) **Illumina,**

5. **Inc. Catalog #20018705**

Alternatively, Tn5 can be produced and loaded with sequencing adapters in house.

6. ☒NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**

7. ☒Agencourt AMPure beads **Beckman Coulter**

☒NEBNext High-Fidelity 2X PCR Master Mix - 250 rxns **New England**

8. **Biolabs Catalog #M0541L**

☒Zymo DNA Binding Buffer **Zymo**

9. **Research Catalog #D4003-1-25**

☒dNTP Mix (10 mM each) **Thermo**

10. **Fisher Catalog #R0191**

3 Specific consumables needed:

☒Ring Magnet Plate - Agencourt SPRIPlate 96R **Fisher**

1. **Scientific Catalog #A29164**

2. Multi-channel pipettes (12 channel or 8 channel; 1uL range 20uL range and 200uL range all very useful)

☒Microseal® 'B' Adhesive Seals **BioRad**

3. **Sciences Catalog #MSB-1001**

☒twin.tec® PCR plate 96 LoBind skirted 150 µL PCR

4. clean **Eppendorf Catalog # Catalog No.**

5. Thermocyler with 96 well heating block

6. Temperature controlled swinging bucket bench top centrifuge

4 ⚠

The likelihood of two cells receiving the same set of barcodes is defined by the birthday problem; the probability that two people in a group of a certain size will have the same birthday. Analogous to the birthday problem, the more cells that are sorted into a PCR well, the higher the likelihood that two or more cells came from the same indexed reverse transcriptase well.

This means that increasing the number of RT wells used, will decrease the likelihood of a barcode collision and control the doublet rate.

Prior to beginning an experiment, choose a target number of cells you would like to recover upo n sequencing. The attached image from Cusanovich et. al. 2015 details how to calculate the likelihood of a collision.

A good rule of thumb for 2-level sci-RNA-seq is that every 96 RT-barcodes allows you to sort 25 cells/well at a 10% doublet rate. Therefore after library preparation we would expect 25*96 cells (2400) cells per sequenced plate.

In practice this number turns out to be between 65%-75% of this value, due to the loss of cells that form poor libraries.

Data Analysis
Calculating the barcode collision rate

An important parameter of our experimental design is the barcode collision rate (the fraction of barcodes that represent more than one nucleus out of all barcodes observed in a given experiment). The expected rate can be calculated in a straightforward manner based on the classic birthday problem (28) as a function of the total number of barcodes available to draw from (96 tagmentation barcodes in our experiments) and the number of nuclei sorted into each well of the PCR plate. If we assume that we are equally likely to sort nuclei from any of the tagmentation reactions into any given well of the PCR plate, we can calculate the expected collision rate as 96 – the expected number of barcodes representing one or zero nuclei. The expected number of barcodes not representing any nuclei in an experiment can be calculated as:

$$(\#\ of\ barcodes) * (1 - \frac{1}{\#\ of\ barcodes})^{(\#\ of\ nuclei\ sorted)} \quad (1)$$

And the number of barcodes expected to represent exactly one cell is:

$$(\#\ of\ nuclei\ sorted) * (1 - \frac{1}{\#\ of\ barcodes})^{(\#\ of\ nuclei\ sorted-1)} \quad (2)$$

Calculation of the collision rate from Cusanovich 2015

Washing Nuclei 5m

5 Thaw the flash frozen nuclei on ice. 5m

To speed this step up hold tube in your hand and move the liquid by flicking the tube.

6 Spin nuclei down at 800g for 6 minutes in a chilled (4C) swinging bucket centrifuge. 6m

6.1 Prepare permeabilization solution: 30s
390uL of NSB + 10uL of 10% Triton-X

Keep this solution (and all others) cold on ice.

7 Remove supernatant and resuspend nuclei in 100uL of NSB buffer. After the cells have been resuspended, add 400uL of permeabilization solution and pipette up and down gently to mix. Allow the cells to sit on ice for 3 minutes. 5m

8 Spin nuclei down at 800g for 6 minutes in a chilled (4C) swinging bucket centrifuge.

9 Remove supernatant and resuspend nuclei in 400uL of NSB buffer. Use a sonicator to dislodge any aggregated nuclei or tissue. We use the bioruptor (https://www.diagenode.com/en/p/bioruptor-plus-sonication-device) on low for 12 seconds.

10 Remove 10uL of nuclei and count cells using a hemocytometer. Using trypan blue is fine if the suspension is without significant cellular debris. Otherwise using DAPI as a nuclear stain in conjunction with a fluorescent microscope is very helpful.

11 After counting adjust the nuclei concentration to 2500 nuclei/uL (or 2.5million nuclei per mL).

We have observed that overloading nuclei at this phase has led to detrimental results.

Performing Reverse Transcription 30m

12 Set aside 220uL of nuclei per RT plate used.

13 dNTP Mix (10 mM each) **Thermo**

Add 0.25uL of dNTPs (**Fisher Catalog #R0191**) for every 2uL of nuclei suspension.

14 Dispense 2.25uL of dNTP-nuclei suspension to each well of a 96 well low-bind PCR plate.

Using a multichannel and 8-strip PCR tube makes this process much easier. Make sure suspension stays cold while aliquoting.

15 Add 1.0uL of 10uM barcoded RT primer to each well.

Spin down barcoded RT-Oligo plate prior to use to bring down any condensate that may have formed.

Each RT primer contains a unique barcode sequence. Be careful when opening sealed plate. Use a different tip to add each RT primer and make sure no cross contamination occurs in stock oligo plate or in sample.

16 Perform an annealing step on a thermocycler:

1. 5 minutes at 55°C (heated lid).
2. Forever at 4°C

After this cycle is finished, move back onto ice immediately.

17 Prepare RT Master Mix for every well:

Per Well:
1uL 5x SSIV Master Mix
0.25uL of 0.1mM DTT
0.25 uL RNseOUT
0.25 uL of SSIV RT

Per Plate (includes 10% overage):
110uL 5x SSIV Master Mix
27.5 uL of 0.1mM DTT
27.5 uL RNseOUT
27.5 uL of SSIV RT

Mix well after combining. Solutions are very viscous.

18 After annealing, dispense 1.75uL of RT Master Mix (made in step 16), into each well and mix. Seal with a plate sealer.

Again, it is important to keep every well completely independent. Reagent can be added robotically and mixed with a pulse on a benchtop vortexer.

19 Start the reverse transcription reaction on the thermocycler:

1. 2 minutes at 4°C,
2. 2 minutes at 10°C
3. 2 minutes at 20°C
4. 2 minutes at 30°C
5. 2 minutes at 40°C
6. 2 minutes at 50°C
7. 15 minutes at 55°C
8. Forever at 4°C

-- Heated Lid

20 After reverse transcription is finished, place plate on ice and add 15uL of NBB (Nuclei Buffer + BSA) to every well. Pool all the wells into a trough. Place pooled suspension into a FACs tube and stain nuclei by adding 5uL of 100uM DAPI.

Note: It takes 5-10minutes for DAPI to fully intercalate.
While reverse transcription is happening thaw reagents for second strand synthesis.

FACs Sorting (Option 1)

21 Set up low-bind 96 well plates prior to FACs sorting. Each well in the prepared plates should contain 5uL of Elution Buffer AE, Elution buffer,

Buffer. 240mL **Qiagen Catalog #19077**

For a subset of the plates, if you are planning on performing second strand synthesis, we recommend mixing 0.5uL of Second Strand Synthesis Buffer and 0.25uL of Second Strand Synthesis enzyme mix with EB prior to addition to a 96 well plate

NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**

22 Calibrate the flow sorter to dispense into a 96 well plate. We normally sort on a BD Aria 3 and we use an 80um nozzle, and a 50um filter on the inlet. We also have the sample chilled.

Using the same type of PCR plate that you will be sorting into with a seal on, calibrate the machine so that you can sort into the center of each well.

23 After calibrating the sorter, find the DAPI stained nuclei. We adjust the voltage to set the main DAPI peak (marking cells in G1/S) at 50. Then, while viewing the nuclei in DAPI - Area versus DAPI - height, we draw a polygon to capture singlets, or those cells that are on-diagonal (nuclei for which the DAPI area is equal to the DAPI height)

24 Sort the number of cells/well determined in step#4

25 ⏸

After sorting, spin plates down briefly to bring down any cells that may be stuck to the walls of the 96-well plate.

Begin second strand synthesis on any plates that contain second strand synthesis reagent. Otherwise plates can be stored at 4C for 2-3 days or at -80C for 2 weeks to a month.

Dilution (Option 2)

26 Diluting nuclei is faster way of seeding nuclei, but dilution can lead to more background RNA and a higher number of physical doublets. The latter is dependent on how completely cells were disassociated

27 After pooling nuclei from RT PCR, spin nuclei down in a swinging bucket centrifuge at 650g for 5minutes with the temperature set to 4C.

28 Carefully remove the supernatant, being careful not to disturb the pellet.

29 Resuspend nuclei in 250uL of NSB and count the concentration of nuclei per uL using a hemocytometer and trypan blue. If the nuclei are small or hard to localize, try staining with DAPI prior to counting. Finally, if the concentration of nuclei is too high repeat procedure with a 1:10 or 1:100 dilution

30 If there are a lot of clumps or aggregates present, pass the nucleus suspension through a mesh strainer to remove large clumps. We prefer to use BD FACs tubes that contain a 40um mesh built into the cap. Passing the nuclei through this strainer should separate out large clumps.

31 With the final count in hand resuspend nuclei to the desired final concentration, using NSB as the diluent. Each well will receive 5uL of the nucleus suspension so the concentration of the diluted solution should be one-fifth the number of nuclei desired per well.

For example if 25 nuclei/well is the target, the desired concentration is 5nuclei/uL

32 For a subset of the plates, if you are planning on performing second strand synthesis right away, we recommend mixing 0.5uL of Second Strand Synthesis Buffer and 0.25uL of Second Strand Synthesis enzyme mix with the diluted nucleus suspension prior to addition to a 96 well plate

NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**

If you choose this route load 5.75uL of Second Strand Synthesis + Nucleus suspension into each well of a 96 well plate and proceed to step #33

33 ⏸

Aliquot out 5uL of diluted nucleus suspension in 96 well low-bind plates.

Plates can be stored at 4C for 2-3 days or at -80C for 2 weeks to a month.

Second Strand Synthesis

34 Thaw plates on ice, and prepare a mixture of 0.5uL of Second Strand Synthesis Buffer and 0.25uL of Second Strand Synthesis enzyme mix for every well.

NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module - 100 rxns **New England Biolabs Catalog #E6111L**

Making 10% overage can prevent frustration when adding mix to the final wells of a plate.

35 Add 0.75uL of the second strand synthesis enzyme + buffer mix to each well. Seal with a plate seal

Adhesive PCR Plate Seal **Bio-rad**

Laboratories Catalog #MSB1001 and mix by gently vortexing. Spin nuclei down prior starting the next step.

36

Perform second strand synthesis at 16C (with an unheated lid) for 150 minutes on a thermocycler.

Plates can be stored at -20C after second strand synthesis for up to one month.

Tagementation 10m

37 Prepare tagementation mix:

NEBNext DNA Library Prep Reagent Set for Illumina - 12 rxns **New England Biolabs Catalog #E6000S**

5.75uL of 2x TD buffer (can be made at home)
0.01uL of Tn5 n7 (made in house) or dual loaded Tn5 from Illumina

Prepare 10% overage (enough for 110 wells) to account for loss.

38 Keeping the tagmentation mix on ice, remove the nuclei from the thermocycler and add 5.75uL of the tagmentation mix to every well.

Make sure to mix throughly.

39 Start a 5 minute thermocycler program to tagment cDNA

1. 5 minutes at 55C
2. Forever at 4C
-- Heated Lid

SPRI cleanup and PCR

40 After tagmentation, remove 96-well plate from the thermocycler and add 12uL of Zymo binding buffer to every well

Zymo DNA Binding Buffer **Zymo Research Catalog #D4003-1-25** . Mix well and allow the plate to sit at room temperature for 5 minutes

41

All wells must be kept independent from one another. Cross contamination between wells can lead to cross contamination of RNA molecules between different cells. Use a separate pipette for every well.

To reduce tip waste we generally save the tip used for each well by placing the tip back into the tip box. This allows for the tip to be used during mixing the beads, removal of the unbound DNA, ethanol washes and transfer of the eluted DNA.

42 Prior to using SPRI beads, make sure they have been warmed to room temperature

43 Add 36uL of SPRI beads to each well, mixing to make sure that the solutions are completely mixed.

Allow this to sit for 5 minutes before placing on a magnetic stand.

44 Remove unbound material from each well by removing the supernatant from every well.

45 Add 100uL of 75% ethanol to wash beads. Wait 30 seconds, and then remove.
Perform this wash twice.

46 After removal of the second ethanol wash allow wells to dry for 3-4 minutes.

Be careful at this stage, wet beads lead to the carryover of ethanol a PCR inhibitor when present at sufficiently high quantities. On the other hand overly dried beads crack and do not reconstitute as well.

47 After 3-4minutes of drying add 17uL of pre-PCR molecular grade water to each well. Resuspend each well getting all the beads back into solution.

48 Place 96-well plate on a magnet and transfer 16uL of eluted, tagmented cDNA into a new 96 well plate.

PCR

49 To each well add 2uL of indexed P7 and 2uL of indexed P5 PCR primers.

The combination of P5 and P7 indices used for each well must be unique. Crucially this forms the second level of molecular indexing

50 Add 20uL of 2x NEB Next MasterMix to everywell and mix
NEBNext High-Fidelity 2X PCR Master Mix - 50 rxns **New England Biolabs Catalog #M0541S**

51 Each well should contain:

2µL of 10 µM P5 primer (5'-AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'),
2 µL of 10 µM P7 primer (5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG-3'),
20 µL NEBNext High-Fidelity 2X PCR Master Mix (NEB)

52 Seal the plate and briefly centrifuge

53

PCR amplification was carried out using the following program:
1. 72°C for 5 min,
2. 98°C for 30 sec
3. 98°C for 10 sec

4. 66°C for 30 sec
5. 72°C for 30 sec
6. Return to step 3 for 18-22 cycles
7. 72°C for 5 min.

54 PCR product can be stored at -20C or 4C for a reasonable amount of time.

Purifying Library and Sequencing

55 After PCR, pool samples and purify using a 1x AMPure bead cleanup

56 Run samples on a TapeStaion, BioA or PAGE gel to estimate the average library size

57 Use a Qubit to calculate the concentration of DNA

58 Using the Qubit concentration and the size from the TapeStation, calculate the molarity

59 Samples can be sequenced on any sequencing platform with the following read lengths:

R1: 18
I1: 10
I2 :10
R2 : 52

For NextSeq550 and NextSeq2000 runs we recommend a loading concentration of 2pM and 650pM respectively.