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sci-RNA-seq 3 level protocol

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We use this protocol and it's working

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Abstract

Single-cell combinatorial indexing RNA sequencing (sci-RNA-seq) is a powerful method for recovering gene expression data from an exponentially scalable number of individual cells or nuclei.

Materials

Reagents

- DSP (Lomant's reagent; Thermo Fisher, cat. no. 22586 or PG82081)
  CRITICAL DSP is sensitive to water and should be used immediately after dissolving in dimethyl sulfoxide (DMSO).
- Methanol (Millipore Sigma, cat. no. 494437-2L)
- DMSO (Millipore Sigma, cat. no. D2438-5X10ML)
  CRITICAL DMSO used for dissolving DSP should be new and unopened so that water is not introduced. These smaller bottles are useful for this reason.
- Sodium phosphate dibasic (Millipore Sigma, cat. no. S3264-250G)
- Sodium phosphate monobasic monohydrate (Millipore Sigma, cat. no. 71507-250G)
- Potassium phosphate monobasic (Millipore Sigma, cat. no. P9791-100G)
- Sodium chloride (Millipore Sigma, cat. no. S3014-500G)
- Potassium chloride (Millipore Sigma, cat. no. P9541-500G)
- Magnesium chloride solution, 2 M (Millipore Sigma, cat. no. 68475-100ML-F)
- Igepal CA-630 (Millipore Sigma, cat. no. I8896-50ML)
- BSA, 20 mg/ml (New England Biolabs, cat. no. B9000S)
- DEPC (Millipore Sigma, cat. no. D5758-25ML)
  CAUTION Handle DEPC, and samples containing it, in a fume hood.
- Sucrose (VWR, cat. no. 97061-428)
- Triton X-100 (Millipore Sigma, cat. no. T8787-100ML)
- Tween 20 (Thermo Fisher, cat. no. BP-337-100)
- 10× Dulbecco's PBS (10× DPBS; Thermo Fisher, cat. no. 14200075)
- Superscript IV reverse transcriptase (Thermo Fisher, cat. no. 18090200)
  CRITICAL This protocol has not been tested with other reverse transcriptases.
- T4 DNA Ligase (New England Biolabs, cat. no. M0202L)
  CRITICAL The previous version of this protocol used Quick Ligase, but the buffer that is included with that enzyme interferes with the pelleting of the nuclei during centrifugation.
- Tagmentase (Tn5 transposase), unloaded (Diagenode, cat. no. C01070010-20)
  CRITICAL The amount of tagmentase added to Step 57 has been determined for this brand only.
- Tn5-N7 oligo (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3′, high-purity salt-free; Eurofins)
- Mosaic End (ME) oligo (5′-/5Phos/CTGTCTCTTATACACATCT-3′, high-purity salt-free; Eurofins)
- NEBNext mRNA second-strand synthesis module (New England Biolabs, cat. no. E6111L)
- NEBNext high fidelity 2× PCR master mix (New England Biolabs, cat. no. M0541L)
- dNTP mix (New England Biolabs, cat. no. N0447L)
- Agencourt AMPure XP (Beckman Coulter, cat. no. A63882)
- Yoyo dye (Thermo Fisher, cat. no. Y3601)
- RNaseAlert kit (IDT, cat. no. 11-02-01-02)
- RNaseZap (Thermo Fisher, cat. no. AM9780)
- Elution buffer (EB, 10 mM Tris pH 8.5; Qiagen, cat. no. 19086)
- Protease (Qiagen, cat. no. 19157)
  CRITICAL Do not use any other protease/proteinase. This one can be heat-inactivated at the temperature and

time listed in the protocol.

- 6% TBE Novex PAGE gels (10 wells; Thermo Fisher, cat. no. EC6262BOX)
- UltraPure agarose (Thermo Fisher, cat. no. 16500-500)
- Qubit double-stranded DNA high-sensitivity quantitation kit (Thermo, cat. no. Q32851)
- sci-RNA-seq3 indexed primer plates at 10 µM dilution (standard desalting for purification; random bases do not need hand-mixing). The complete list of primers is found in Supplementary Table **1**. The plates include the following: plate(s) of indexed oligo-dT RT primers (5′-/5Phos/CAGAGCNNNNNNNN[10bpRTindex]TTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′, where 'N' is any base; IDT), plate(s) of indexed ligation primers (5′- GCTCTG[9- or 10-bp ligation index]TACGACGCTCTTCCGATCT[reverse complement of ligation index]-3′), plate of indexed PCR P7 primers (5′-CAAGCAGAAGACGGCATACGAGAT[PCR P7 index]GTCTCGTGGGCTCGG-3′; IDT); and PCR P5 primers (this primer does not need to be indexed if you do only one plate of PCR) (5′-AATGATACGGCGACCACCGAGATCTACAC[PCR P5 index]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′; IDT).

Troubleshooting

Nuclei Isolation ~2hours

1 Remember everything is cold all the time. Prepare two ice buckets with wet ice, a bucket with crushed dry ice to hold your frozen tissues, and a thick, flat slab of dry ice for smashing tissues. Precool a centrifuge that will hold 50ml tubes, and a microfuge, to 4C.

2 Determine how much lysis buffer you will need for the tissue you will be processing. A E13.5 mouse embryo (~200mg) works with 5ml of lysis buffer. A E16.5 embryo (~500mg) will need 10ml. An adult mouse heart needs 5ml. Adult mouse kidneys need 5 ml per kidney. Adult mouse liver 20ml. Adult mouse pancreas 15ml. Adult tissues and tissues high in Rnases will necessitate a bigger lysis volume. The buffer is cheap so err on the side of more volume for lysis.

3 For every 1 ml of lysis buffer needed, add 2.5 µl 10% igepal (vol/vol), 10 µl DEPC to the hypotonic lysis buffer solution (and 40µl BSA if using lysis buffer B), then vortex solution to disperse the DEPC throughout. Have complete lysis buffer in a 50 ml tube for each sample on ice ready to go.

CAUTION DEPC is flammable and toxic. Avoid breathing vapors. The following steps should be performed in the chemical hood from this point until the DEPC is washed from the sample (step 17).

4 Fold a piece of aluminum foil so that you have a small pouch with 4 layers of foil on each side. Place this on a slab of dry ice to chill.

5 Place your frozen tissue inside this foil and hold it firmly closed on the dry ice and smash it with a hammer. You want to be gentle enough not to tear the foil, but thorough enough to make a powder of the tissue. Do not let the tissue thaw.

6 Use the foil to guide your powdered tissue into the tube of lysis buffer. It will stick a bit, pipet some of the lysis buffer from the tube to rinse the sample from the foil into the tube. Try to make sure that the sample is only thawing if it's in lysis buffer.

7 Cap the 50ml tube and shake to disperse the chunks in the buffer. Let sit on ice 10min. Triturate the chunks with a 1ml pipette tip to help tease them apart a bit.

8 Set up another 50 ml tube on ice with a 40 µm cell strainer on top. Pour your lysate through that – there will still be a lot of chunks. Use a disposable pestle to coax the tissue through the filter. Don’t worry about getting all of it through.

9 Take 45ul sample of the filtered lysate and check for Rnase activity with the IDT RnaseAlert kit. The RNaseAlert will guide you on whether to proceed or not. There should not be any RNase detected at this point, and if there is, you will have to restart with a new sample and adjust either the sample size or the volume of lysis buffer, so that there is enough DEPC to inactivate the RNases. You cannot continue with a sample that has RNases detected at this point, the damage is already done.

10 While the RNaseAlert sample is incubating, spin the remainder of the lysate. 500xg, 3min, 4C. Resuspend the nuclei in 1ml 0.3M SPBSTM (with DEPC added - 10ul per ml of SPBSTM). Last time you'll need to add DEPC to this buffer.

Nuclei Fixation ~ 1hour

11 For each sample, prepare fixative: add 100ul of 50mg/ml DSP stock solution to 4ml of ice cold methanol for every 1ml of nuclei that you are starting with.

12 Add the fixative to the nuclei gradually and swirl to mix.

13 Fix on ice 15min, swirling occasionally.

14 Add 2 volumes 0.3 SPBSTM gradually, 2–3 milliliters at a time, swirling in-between additions to rehydrate the nuclei. For instance, with 1 ml of nuclei and 4 mls of fixative, you would need 10ml of buffer to rehydrate.

15 Spin down the nuclei at 500×g 3min 4°C.

16 Carefully remove supernatant and dispose properly. The nuclei pellet is at the bottom and should look a little white-ish from the DSP.

17 Resuspend the nuclei in 1 ml (or more) 0.3M SPBSTM. Triturate gently with a pipette tip to separate nuclei.

18 Divide fixed nuclei into aliquots in microfuge tubes. Spin 500×g 3 min 4°C and remove supernatant. Snap freeze tubes in LN2 and store at −80°C. Fixed samples may be stored at −80C up to 6 months.

19 **OPTIONAL:** If there are obvious clumps at this point that won't tease apart, you will need to sonicate them to break them up. Sonicate (Diagenode Bioruptor Plus) on low intensity for 12s only at 4°C. Spin and resuspend the nuclei in 1 ml 0.3M SPBSTM.
Can stop at this point.

Reverse Transcription ~2-3hours

20 Follow the chart below to determine how many starting nuclei you need and their volume. If you are only doing sci on one sample (filling all 12 columns of the 96-well plate with that sample), then follow the volumes outlined by the box. If you are dividing the plate among multiple samples, then the chart will outline the cell number you need depending on how many columns in the plate each sample will occupy. For example, if you will have two samples on one plate, each one will take 6 columns and you will need 1M cells of each sample in a tube to start with in a volume of 250µl each, and you will add 28µl of dNTPs to each before distributing to their RT plate wells.

	A	B	C	D	E	F	G
	cell number:	**2M**	**1M**	**800K**	**500K**	**400K**	**200K**
	number of columns:	12	6	4	3	2	1
	nuclei volume	500ul	250ul	170ul	125ul	85ul	42.5ul
	10mM dNTP	56ul	28ul	19ul	14ul	9.5ul	4.75ul

21 Resuspend an aliquot of frozen nuclei in 500 µl of 0.3M SPBSTM to start. Count. Dilute nuclei if necessary to get an accurate count. If nuclei are clumpy, even after sonicating, and can't be teased apart with pipetting, then put them over a 40µm Flow-mi pipette tip filter before counting. Flow-mi filter is a last resort as it results in nuclei loss, but is helpful if you have an excess of nuclei.

22 Pull out the desired amount of nuclei into a new tube and spin. Remove supernatant and resuspend nuclei in the necessary volume determined by the chart and add the appropriate amount of dNTPs.

23 Aliquot 5ul nuclei+dNTP mix to each well of the plate on ice.

24 Quick spin the plate of 3-level RT primers (10uM). (5'-/5Phos/CAGAGCNNNNNNNN[10bpRTindex]TTTTTTTTTTTTTTTTTTTTTTTTTTTTT -3', where "N" is any base)

25 Add 2ul of primer to each well. Don't pipet up and down to mix, just stir gently with the pipet tips.

26 Incubate plate 55C for 5min (heated lid set to 65C) and then immediately place on ice.

27 While this is incubating, make the reaction mix. Note: we are not including DTT in this mix, as it will undo the DSP crosslinks (it is not necessary for the RT to work).

	A	B	C
	RT mix per plate:	each	X120
	5X Superscript IV buffer	2ul	240ul
	Superscript IV (200u/ul)	0.5ul	60ul
	water	0.5ul	60ul
	total		360

28 Put 3ul of reaction mix into each well (45ul mix x8 in strip tube for multichannel), stirring gently with tips. 10ul total now.

29 Incubate 55C 10min (heated lid at 65C) and then immediately place on ice.

30 Ice plates until they are cold (10–15min). Add 5 µl cold 0.3M SPBSTM per well. To maximize recovery, pool wells by using a 12-multichannel with 200µl tips to pipet gently up and down (the pipetting up and down is important to dislodge the nuclei, but try to avoid creating excessive bubbles), and combine each row of the plate into the bottom row. You can use the same tips for the whole plate. Then collect these wells into 2 cold microfuge tubes. (It will be bubbly so it's difficult to squeeze into 1 tube).

31 Spin 500xg 3min 4C. Pellet will be small but you should be able to see it. Remove supernatant.

32 Combine tubes and wash once more in 1ml cold 0.3 SPBSTM. Spin 500xg 3min 4C. Remove supernatant.

Ligation ~1hour

33 Resuspend nuclei in 1200ul 0.3M SPBSTM.

34 Distribute 11ul to each well of a new plate on ice.

35 Quick spin the plate of 3-level ligation primers (10uM) (5′- GCTCTG[9bp or 10bp ligation index]TACGACGCTCTTCCGATCT[reverse complement of ligation index]-3′)

36 Add 2ul of primer to each well. Don't pipet up and down.

37 Make a 3:1 mix of 10X T4 ligation buffer and T4 DNA ligase. (195ul 10X buffer + 65ul T4 DNA Ligase)

38 Add 2ul ligase mix to each well. (32ulx8 in strip tube for multichannel). 15ul total now.

39 Incubate 20 min at room temperature.

40 Ice plates until cold.

41 Add 10ul cold SPBSTM to each well. This helps keep the cells from clumping and allows for more cell recovery.

42 Pool wells by using a 12-multichannel to pipet gently up and down (the pipetting up and down is important to dislodge the nuclei), and combine each row of the plate into the bottom row. Then collect these wells into 2 cold microfuge tubes.

43 Spin 500xg 3min 4C. Remove supernatant.

44 Combine the two tubes and wash twice more with 1ml 0.3M SPBSTM per wash.

45 Resuspend in 1ml 0.3M SPBSTM to count. If they are clumpy and can't be teased apart with gentle pipetting, sonicate for 6s only. You can also use a flow-mi filter but you will lose a lot of nuclei.

Final Distribution ~30minutes

46 **In the final plate you will want 1000 nuclei/well (or 4000/well if you've scaled up the experiment to 384×384×384). You should have enough nuclei to make multiple plates if you like.**

Make 400ul 1X Second Strand Synthesis for each plate in the final distribution: Dilute 40ul 10X Second Strand buffer in 360ul water to get 1X concentration.

47 Spin down 100K nuclei for each plate desired for the final distribution. (400K per plate if this is a 384×384×384 experiment). For each plate/100K, resuspend in 400ul 1X Second Strand Synthesis buffer.

48 Put 4ul nuclei into each well of a regular, not lo-bind, plate on ice.

49 Cover with foil seal and freeze plates at -80C or proceed with second strand synthesis. Plate may be kept frozen at -80C for up to 6 months.

Second Strand Synthesis ~3hours or O/N

50 Thaw plate on ice.

51 Make second strand synthesis mix as follows:

	A	B	C
			1 plate
	reaction mix per plate:	each	X140
	water	0.675ul	94.5
	second strand buffer (10X)	0.075ul	10.5
	second strand enzyme (20X)	0.25ul	35
	total		140

52 Put 1ul of second strand synthesis mix into each well (17ul mix x8 in strip tube for multichannel). 5ul total now.

53 Incubate 16C 2.5hours. (No heated lid)

Can stop here, keep plate at 4C.

Protease Digestion ~2hours

54 Add 1ul protease to each well. This is NOT the same as proteinaseK. It's important to use Qiagen protease (#19157) because it can be heat-inactivated.

55 Incubate 37C for 30min (47C heated lid). Check 1ul on microscope: mix 1ul sample with 2ul of diluted yo-yo1 dye and put this on a slide (no coverslip) and check on the GFP channel. You should see whisps of DNA instead of intact nuclei.

56 Heat-inactivate the protease 75C 20min (85C heated lid). Critical, do not lower this temperature, do not shorten this time. Qiagen lists different conditions for heat-inactivating their protease, but it is not sufficient. Put plate on ice after inactivating.

Can store plate at 4C for up to a week.

Tagmentation ~1hour

57 On ice, make tagmentation mix as follows:

	A	B	C
	reaction mix per plate:	each	X110
	TD buffer	5ul	550
	N7-loaded Tn5	0.125ul	13.75

58 On ice, add 5ul tagmentation mix to plate. ~10-11ul total now.

59 Incubate 55C 5min, do not put on ice afterwards, just keep on the bench at room temperature as you can add the next reaction mix, or else the SDS will come out of solution at the next step.

60 Remove the transposases with this buffer (keep at room temperature):

	A	B	C
	reaction mix per plate:	each	X120
	1% SDS	0.4ul	48ul
	BSA	0.4ul	48ul
	water	1.8ul	216ul
	total	2.6ul	

61 Add 2.6ul to each well and mix (39ulx8 into a strip tube for multichannel).

62 Incubate 55C 15min.

63 **Quench SDS by adding 2ul 10% Tween 20(vol/vol) to each well.** (bolding this because it is a very easy step to forget)

PCR ~1hour for PCR, 2-3hours for gel purification

64 PCR is done with 96 indexed P7 primers. Alternatively, you can also add an index on the P5 end for multiplexing multiple plates.

Assemble the PCR master mix. PCR is done with 96 indexed P7 primers and 1 P5 primer. You can also add an optional index sequence on the P5 primer for multiplexing multiple plates.
Plate of 96 Indexed PCR P7 primers (5′- CAAGCAGAAGACGGCATACGAGAT[PCR P7 index]GTCTCGTGGGCTCGG-3′)
P5 primer(s) (5′- AATGATACGGCGACCACCGAGATCTACAC[PCR P5 index]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3′)

	reaction mix per plate:	each	X110
	2X NEBNext	20ul	2200ul
	TruSeqP5-noindex primer (100uM)	0.2	22ul
	water	3.2ul	352ul
	total	23.4ul	2574ul

65 Add 2ul of indexed P7 primers (10uM) to each well.

66 Add 23.4ul of PCR master mix to each well.

67 1. Amplify 16 cycles with a pre-extension step in the following program:

	1	70C	3min
	2	98C	30s
	3	98C	10s
	4	63C	30s
	5	72C	1min
	6	go to step 3, 15 more times	
	7	72C	5min

68 Run 1.5ul of a few wells on a 6% PAGE gel to check. You should see a smear of products with primer-dimers underneath. You should see a bright upper smear, but what you want is the fainter smear centered on 400bp.

69 Concentration of library and agarose gel purification: Pool 3ul of each well and do a 0.8X ampureXP cleanup (230ul beads). (Save the remaining plate in case you need to redo cleanup or if you anticipate needing more library for a large Novaseq run). Wash the ampure bead pellet twice gently with 70% EtOH (vol/vol) and elute pool in 50ul. Load this into a single 1cm well on a 1% agarose gel (wt/vol). Cut out the smear between 250-600bp and use the NEB gel extraction kit, using extra dissolving buffer since it will be bigger than a normal slice and run it all through the same purification column. Wash twice with 200ul NEB wash buffer, elute in 20ul EB. Quantitate library with Qubit dsDNA HS.

70 Run library on NextSeq (or Novaseq depending on final cell numbers or sequencing depth desired), using standard primers. Read1 34 cycles, Index 10 cycles, Read2 48 cycles. If you've also used the P5 primers for PCR, then add a second Index read of 10bp.

71 Reagents and troubleshooting can be found: doi: **10.1038/s41596-022-00752-0**