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Protocol for SPLiT-seq

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We use this protocol and it's working

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Abstract

Protocol for SPLiT-seq library preparation from Robbins et al. 2025

Materials

Materials List:

SPLiTseq_MaterialsList.xlsx

Oligonucleotides to order from IDT:

96 well barcode plates

- All plates were ordered from IDT with the following specifications:
  - 100 nmole DNA Plate Oligo (96 Well)
  - Purification: Standard Desalting
  - Plate Type: Deep Well
  - Normalized Yield: 14 nmol
  - Number of Additional Replicates: 1

Round 1 Barcodes (96 barcodes, polydT only):

Round1_oligos.xlsx

Round 2 Barcodes (96 barcodes):

Round2_oligos.xlsx

Round 3 Barcodes (96 barcodes):

Round3_oligo.xlsx

TruSeq Index Primers (1 universal 5' index + 24 3' indices):

SPLiTseq_TruSeqIndices_v2.xlsx

Additional oligos/primers:

	A	B	C
	BC_0108	PCR Forward Primer	AAGCAGTGGTATCAACGCAGAGT
	BC_0062	PCR Reverse Primer	CAGACGTGTGCTCTTCCGATCT
	BC_0215	Round 2 barcode linker	CGAATGCTCTGGCCTCTCAAGCACGTGGAT
	BC_0216	Round 2 blocking strand	ATCCACGTGCTTGAGAGGCCAGAGCATTCG
	BC_0060	Round 3 barcode linker	AGTCGTACGCCGATGCGAAACATCGGCCAC

	A	B	C
	BC_0066	Round 3 blocking strand	GTGGCCGATGTTTCGCATCGGCGTACGACT
	BC_0217	Template switching primer, HPLC purified	AAGCAGTGGTATCAACGCAGAGTGAATrGrG+G
	BC_0243	Adapter duplex 1	ACACTCTTTCCCTACACGACGCTCTTCCGATC*T
	BC_0244	Adapter duplex 2	GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT

*phosphorothioate bond

Starting Notes

1 This protocol is best split into a 3 day process:

Day 1: Sections 1-6
Day 2: Sections 7-9
Day 3: Sections 10-11

1. Barcode Plate Generation

2 Briefly spin down the IDT 96-well barcode plates prior to re-suspension.

3 Dilute the IDT 96-well barcode stock plates to **100 uM** with RNase-free, DNase-free H2O.

4 Dilute the barcode linker oligos (BC_0215 & BC_0600) to **1 mM** with RNase-free, DNase-free H2O.

5 Prepare Round 1 Barcode Plates:

5.1 Using a multichannel P100 pipette, transfer 25 µL of Round 1 barcode oligos from the IDT 96-well barcode stock plate to each corresponding wells of a 96-well LoBind PCR plate.

5.2 Add 75 µL of RNase-free, DNase-free H2O to each well of the 96-well LoBind PCR plate. Mix thoroughly.

5.3 Aliquot 25 µL into (3) 96-well LoBind PCR plates. Store at -20°C until use.

6 Prepare Round 2 Barcode Plates:

6.1 Using a multichannel P100 pipette, transfer 12 µL of Round 2 barcode oligos from the IDT 96-well barcode stock plate to each corresponding wells of a 96-well LoBind PCR plate.

6.2 Add 138.6 µL of BC_0215 (1mM) to 10.9494 mL of RNase-free, DNase-free H2O to a sterile pipette basin. Mix thoroughly.

6.3 Using a multichannel P100 pipette, transfer 88 µL of the diluted BC_0215 from the basin to the 96-well LoBind PCR plate. Mix thoroughly.

6.4 To anneal the barcode and oligo linkers, incubate in a thermocycler with the following protocol: 2m

1. 95 °C for 00:02:00
2. 20 °C at a rate of **-0.1C/s**
3. Hold at 4 °C

6.5 Aliquot 10 µL into (6) 96-well LoBind PCR plates. Store at -20°C until use.

7 Prepare Round 3 Barcode Plates:

7.1 Using a multichannel P100 pipette, transfer 12 µL of Round 3 barcode oligos from the IDT 96-well barcode stock plate to each corresponding wells of a 96-well LoBind PCR plate.

7.2 Add 163.8 µL of BC_0060 (1mM) to 10.6722 mL of RNase-free, DNase-free H2O to a sterile pipette basin. Mix thoroughly.

7.3 Using a multichannel P100 pipette, transfer 86 µL of the diluted BC_0060 from the basin to the 96-well LoBind PCR plate. Mix thoroughly.

7.4 To anneal the barcode and oligo linkers, incubate in a thermocycler with the following protocol: 2m

1. 95 °C for 00:02:00
2. 20 °C at a rate of **-0.1C/s**
3. Hold at 4 °C

7.5 Aliquot 10 µL into (6) 96-well LoBind PCR plates. Store at -20°C until use.

2. Nuclei Counting and Loading

8 Thaw fixed cells/nuclei samples in a 37°C water bath. Once samples are fully thawed, transfer to an ice bucket.

9 Aliquot 5 uL of each sample into 200uL eppendorf tubes or 8-strip PCR tubes.

10 Add 10 uL of 15 ug/mL DAPI in PBS to dilute the samples 1:3.

11 Count on hemocytometer with DAPI fluorescence on EVOS.

12 Dilute nuclei suspensions to 300-1,250 nuclei per uL.

3. Round 1: Reverse Transcription

13 Thaw the Round 1 Barcode plate at RT. Centrifuge briefly. 5m

14 Aliquot 4 µL of the R1 barcodes into a 96-well LoBind PCR plate with a P10 multichannel pipette.

15 Prepare Reverse Transcription mix on ice: 5m

	A	B	C	D	E
	Reagent	**Stock Concentration**	**Desired Concentration**	**Per Reaction (uL)**	**Volume in Mix (uL) (96 wells + 10%)**
	5X RT Buffer	5X	1X	4	422.4
	RNaseOUT	40 U/uL	0.25 U/uL	0.125	13.2
	SUPERase•In	20 U/uL	0.25 U/uL	0.25	26.4
	dNTPs	10 mM	500 µM	1	105.6

	A	B	C	D	E
	Maxima H Minus Reverse Transcriptase	200 U/uL	20 U/uL	2	211.2
	H2O	N/A	N/A	0.625	66
	Total Volume			**8**	**844.8**

16 Add 8 µL of the RT mix to each well of the 96-well plate. Total volume = 12 uL/well. 5m

17 Add 8 µL of nuclei suspension into each well of the 96-well plate, mix up and down 2-3X to ensure the nuclei are resuspended. Total volume = 20 uL/well 15m

In total, load 2,400 to 10,000 nuclei per well.

18 Place the plate in the thermocycler and run the following protocol for reverse transcription: 40m

	A	B	C
	Step	**Time**	**Temperature**
	1	10 min.	50°
		Begin Cycling	
	2	12 sec.	8°C
	3	45 sec.	15°C
	4	45 sec.	20°C
	5	30 sec.	30°C
	6	2 min.	42°C
	7	3 min.	50°C
	8	**Go to Step 2, repeat 2X**	
	9	5 min.	50°C

	A	B	C
	10	HOLD	4°C

Lid Temperature: 70°C; Sample Volume: 20 uL

19 Immediately place the 96-well plate on ice.

20 Prepare 2 mL of 1X NEB buffer 3.1 with 20 µL RNaseOUT RNase inhibitor.

21 Transfer the sample from each well into a 15 mL conical tube, making sure to wash the sides of each well twice to retain nuclei.

22 Add 9.6 µL of 10% Triton X-100 (final conc. 0.1%) to the nuclei suspension.

23 Centrifuge at RT for 00:10:00 at 500 x g in a swinging bucket rotator. 10m

24 Carefully aspirate the supernatant as to not disturb the nuclei pellet.

25 Re-suspend in 2 mL of 1X NEB buffer 3.1 + RNase Inhibitor from Step 12.

4. Round 2: Ligation Barcoding

26 Prepare Round 2 ligation mix on ice:

	A	B	C	D
	Reagent	**Stock Conc.**	**Final Conc.**	**Volume (uL)**
	H2O	N/A	N/A	1337.5
	T4 Ligase Buffer	10X	1X	500

	A	B	C	D
	RNaseOUT	40 U/uL	0.32 U/uL	40
	SUPERase•In	20 U/uL	0.05 U/uL	12.5
	rAlbumin	20 mg/mL	0.2 mg/mL	50
	T4 DNA Ligase	400 U/uL	8 U/uL	100
	Total Volume:			**2040**

27 Add the 2 mL nuclei suspension from Step 17 to the ligation mix. Total volume: 4.04 mL

28 Add nuclei:ligation mix to a sterile pipette basin.

29 With a P50 multichannel pipet, add 40 µL of the nuclei:ligation mix to each well of the 96-well R2 Ligation Plate.

30 Cover the plate with an adhesive seal and incubate for 00:30:00 at 37 °C in a pre-heated thermocycler. 30m

31 Prepare the Round 2 blocking solution and add to a sterile pipette basin.

	A	B	C	D
	Reagent	**Stock Conc.**	**Final Conc.**	**Volume (uL)**
	BC_0216	100 µM	26.4 µM	316.8
	T4 Ligase Buffer	10X	2.5X	300
	H2O	N/A	N/A	583.2
	Total Volume:			**1200**

32 Remove the Round 2 Ligation Plate from the incubator and remove the adhesive seal.

33 Using a P20 multichannel pipet, add 10 uL of the Round 2 blocking solution to each well.
Total volume: 60 uL/well

34 Cover the plate with an adhesive seal and incubate for 00:30:00 at 37 °C in a pre-heated thermocycler. 30m

5. Round 3: Ligation Barcoding

35 Remove the Round 2 Ligation Plate from the incubator and pool the nuclei from each well into a sterile pipette basin with a P200 multichannel pipet.

36 Pass the pooled nuclei suspension through a 40 µM cell strainer into a new basin.

37 Add 100 µL of T4 DNA Ligase to the nuclei suspension (in the basin) and mix by pipetting ~20X.

38 With a P200 multichannel pipet, add 50 µL of the nuclei suspension (+ ligase) to each well of the Round 3 Ligation Plate.

39 Cover the plate with an adhesive seal and incubate for 00:30:00 at 37 °C in a pre-heated thermocycler. 30m

40 Prepare the Round 3 blocking and termination solution and add to a sterile pipette basin:

	A	B	C	D
	Reagent	Stock Conc.	Final Conc.	Volume (uL)
	BC_0066	100 µM	11.5 µM	369
	EDTA	0.5 M	125 mM	800
	H2O	N/A	N/A	2031
	Total Volume:			**3200**

41 Remove the Round 3 Ligation Plate from the incubator and remove the adhesive seal.

42 With a P50 multichannel pipet, add 20 µL of the Round 3 blocking and termination solution to each well of the plate.

43 Pool the nuclei from each well into a sterile pipette basin with a P200 multichannel pipet.

44 Pass the pooled nuclei suspension through a 40 µM cell strainer into 15 mL conical tube.

6. Nuclei Sub-library Lysis

45 Prepare 2X lysis buffer:

	A	B	C	D
	Reagent	**Stock Conc.**	**Final Conc.**	**Volume (mL)**
	Tris, pH 8.0	1 M	10 mM	0.5
	NaCl	5 M	400 mM	2
	EDTA, pH 8.0	0.5 M	100 mM	5
	SDS	10%	4.4%	11
	H2O	N/A	N/A	6.5
	Total Volume:			**25**

46 Incubate the 2X lysis buffer at 37 °C for 00:10:00 (or until the solution is clear and there is no precipitate present). 10m

47 Prepare the wash buffer:

4 mL 1X PBS
40 µL 10% Triton X-100
10 µL SUPERase•In RNase Inhibitor

48 Add 70 µL of 10% Triton X-100 to the nuclei suspension.

49 Centrifuge at RT for 00:10:00 at 600 x g in a swinging bucket rotator. 10m

50 Remove the supernatant with a P1000 pipet, being careful not to disturb the pellet.

You may leave ~20-30 uL in favor of preserving the pellet, using a P20 pipet to remove as much supernatant as possible.

51 Add 1 mL of wash buffer to the nuclei pellet, allow to sit on ice for 00:02:00 2m

52 Re-suspend the nuclei pellet and add an additional 3 mL of wash buffer.

53 Centrifuge at RT for 00:10:00 at 600 x g in a swinging bucket rotator. 10m

54 Remove the supernatant with a P1000 pipet, being careful not to disturb the pellet.

55 Add 50 uL of 1X PBS + RNase inhibitor to the nuclei pellet, let sit on ice for 2 min. prior to re-suspension.

56 Dilute 5 uL of the nuclei suspension in 15 uL of 1X PBS + DAPI. Count on a hemocytometer.

57 Add X uL of nuclei to 8-strip PCR tubes and bring volume to 25 uL with PBS + RNase inhibitor.

58 Add 25 uL of 2X lysis buffer to each tube.

59 Add 5 uL of Proteinase K (20mg/mL) to each tube. Total volume: 30 uL

60 Incubate in a thermocycler with the following protocol:

	A	B	C
	Step	Time	Temperature
	1	120 min.	55°C
	2	HOLD	4°C

Lid Temperature: 80°C; Sample Volume: 55 uL

61 Freeze lysates at 80°C and store for up to 6 months.

7. cDNA Bead Purification

62 Make stock solutions of bead wash buffers (can be made ahead of time and stored at 4°C):

62.1 **2X B&W Stock Solution:**

	A	B
	Reagents	**Volume**
	1M Tris-HCl pH 8.0	500uL
	5M NaCl	20ml
	EDTA, 0.5M	100ul
	Nuclease Free Water	29.4ml
	Total	**50mL**

62.2 **1X B&W-T Stock Solution:**

	A	B
	Reagents	Volume
	1M Tris-HCl pH 8.0	100uL
	5M NaCl	4ml
	EDTA, 0.5M	20ul
	Tween 20 10%	100ul
	Nuclease Free Water	15.78ml
	Total	20mL

63 Prepare working solutions on ice, adding RNase inhibitors:

63.1 **1X B&W-T + RNase Inhibitors**

	A	B	C	D	E	F	G
	Sample #:	**2**	**4**	**6**	**8**	**10**	**12**
	Reagent	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)
	1xB&W-T	3600	4200	4800	5400	6000	6600
	SUPERase In	5	5.8	6.7	7.5	8.3	9.2
	Total Volume:	**3605**	**4205.8**	**4806.7**	**5407.5**	**6008.3**	**6609.2**

63.2 **2X B&W + RNase Inhibitors**

	A	B	C	D	E	F	G
	Sample #:	2	4	6	8	10	12
	Reagent	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)

	A	B	C	D	E	F	G
	2X B&W	110	220	330	440	550	660
	SUPERase In	2	4	6	8	10	12
	Total Volume:	112	224	336	448	560	672

63.3 **Tris-T + RNase Inhibitors**

	A	B	C	D	E	F	G
	Sample #:	2	4	6	8	10	12
	Reagent	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)
	10mM Tris-HCl, pH 8.0	600	1200	1800	2400	3000	3600
	Tween-20 (10%)	6	12	18	24	30	36
	SUPERase In	1.5	3	4.5	6	7.5	9
	Total Volume:	607.5	1215	1822.5	2430	3037.5	3645

64 For each sublibrary add 22 uL of Dynabeads™ MyOne™ Streptavidin C1 beads to a 1.5 mL LoBind Eppendorf tube (2 sublibraries = 44uL, 4 sublibraries = 88uL, etc.)

65 Add 100 uL of 1X B&W + RI buffer to the beads per sublibrary (2 sublibraries = 200uL, 4 sublibraries = 400uL, etc.)

66 Place sample against a magnetic rack and wait until liquid becomes clear (1-2 min).

67 Remove supernatant and repeat steps 58-60 twice for a total of 3 washes.

68 Re-suspend the beads in 55 uL of 2X B&W + RI buffer per sublibrary(2 sublibraries = 110uL, 4 sublibraries = 220uL, etc.).

Leave the beads in 2X B&W + RI buffer on ice.

69 Remove sublibraries from the -80°C and incubate at 37°C for 5-10 minutes (until there is no precipitate present).

70 Add 2.5 uL of 25X cOmplete™ Protease Inhibitor Cocktail to each sublibrary. Gently vortex to mix sample and spin down.

71 Incubate at RT for 10 min.

72 Add 50 uL of beads in 2X B&W + RI buffer to each sublibrary.

73 Incubate the sublibraries for 60 min with gentle agitation (~600 RPM) on a plate shaker to allow the beads to bind the cDNA.

74 Place the tubes against a magnetic rack and wait until liquid becomes clear (1-2 min).

75 Remove the supernatant and re-suspend the beads in 125 uL of 1X B&W + RI buffer.

76 Incubate the sublibraries for 1-2 min. at RT.

77 go to step #74 and repeat once for a total of 2 washes.

78 Place the tubes against a magnetic rack and wait until liquid becomes clear (1-2 min).

79 Remove the supernatant and re-suspend the beads in 125 uL of 10 mM Tris-T + RI buffer.

80 Incubate the sublibraries for 1-2 min. at RT.

81 Briefly spin down the sublibraries and place on ice.

8. Template Switching

82 Prepare the TSO mix on ice:

	A	B	C	D	E	F	G
	Sample #:	**2**	**4**	**6**	**8**	**10**	**12**
	Reagent	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)
	Water	88	176	264	352	440	528
	Maxima RT Buffer	44	88	132	176	220	264
	Ficoll PM-400 (20%)	44	88	132	176	220	264
	10mM dNTPs (each, total is 40mM)	22	44	66	88	110	132
	RNase Inhibitor	5.5	11	16.5	22	27.5	33
	TSO (BC_0127)	5.5	11	16.5	22	27.5	33
	Maxima RT RnaseH Minus Enzyme	11	22	33	44	55	66
	Total Volume:	**220**	**440**	**660**	**880**	**1100**	**1320**

83 Place the tubes against a magnetic rack and wait until liquid becomes clear (1-2 min).

84 With the beads still on the magnetic rack, remove the supernatant and wash with 125 uL of UltraPure H2O.

DO NOT RE-SUSPEND THE BEADS!

85 Re-suspend the beads in 100 uL of TSO mix.

86 Incubate at RT for 30 min.

87 With a P200 pipet mix the samples 5X to re-suspend. Spin down briefly.

88 Incubate at 42°C for 90 min in a thermocycler, lid set to 70°C.

89 (OPTIONAL) If stopping prior to cDNA Amplification:

89.1 Place the tubes against a magnetic rack and wait until liquid becomes clear (1-2 min).

89.2 Remove the supernatant and re-suspend the beads in 125 uL of 10 mM Tris-T + RI buffer.

89.3 Store sublibraries overnight at 4°C.

9. cDNA Amplification

90 Prepare the following PCR mix on ice:

	A	B	C	D	E	F	G
	Sample #:	**2**	**4**	**6**	**8**	**10**	**12**
	Reagent	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)	Volume (uL)
	Kapa Hifi 2x Master Mix	121	242	363	484	605	726
	BC_0108 (10uM)	9.68	19.36	29.04	38.72	48.4	58.08
	BC_0062 (10uM)	9.68	19.36	29.04	38.72	48.4	58.08
	Water	101.64	203.28	304.92	406.56	508.2	609.84
	Total Volume:	**242**	**484**	**726**	**968**	**1210**	**1452**

91 Place the tubes against a magnetic rack and wait until liquid becomes clear (1-2 min).

92 With the beads still on the magnetic rack, remove the supernatant and wash with 125 uL of UltraPure H2O.

DO NOT RE-SUSPEND THE BEADS!

93 Re-suspend beads in 100 uL of the PCR mix.

94 Run the following thermocycler protocol:

	A	B	C
	Step	Time	Temperature
	1	3 min.	95°C
		Begin Cycling	
	2	20 sec.	98°C
	3	45 sec.	65°C
	4	3 min.	72°C
	5	Go to Step 2; Repeat 4X	
	6	20 sec.	98°C
	7	20 sec.	67°C
	8	3 min.	72°C
	9	Go to Step 8; Repeat for variable # of cycles depending on nuclei/cell count	
	10	5 min.	72°C
	11	Hold Forever	4°C

95 Place the tubes against a magnetic rack and wait until the liquid is clear (1-2 min.)

96 Transfer 90 uL of the supernatant from each sub library into a new tube of an 8-tube PCR strip.

97 Add 72 uL of SPRI beads to each tube for a 0.8X cleanup.

98 Vortex briefly to mix and incubate for 5 min. to bind the cDNA.

99 Place the tubes against a magnetic rack and wait until the liquid is clear (1-2 min.)

100 With the tubes still on the magnetic rack, wash with 180 uL of 85% ethanol.

DO NOT RE-SUSPEND THE BEADS!

101 Repeat once for a total of 2 washes.

102 Remove any residual ethanol with a P20 pipette and air dry beads for 3-5 minutes on the magnetic rack.

Do not let the beads over dry (they will turn light brown and crack).

103 Remove the beads from the magnet and re-suspend in 22 uL of RNase, DNase free H2O.

104 Incubate for 10 min. at 37°C in a thermocycler.

105 Place the tubes against a magnetic rack and wait until the liquid is clear (1-2 min.)

106 Transfer 20 uL of the eluting into a new PCR tube.

107 Evaluate quality of cDNA libraries via Qubit and the Agilent Tapestation or Bioanalyzer.

Expected concentration will vary: ~10-50 ng.ul
Expected size range: 500-2500bp

10. Fragmentation, End Repair, A-tailing and Adapter Ligation

108 Make annealing buffer (10X).

	A	B	C	D
	Component	Final Conc.	Stock Conc.	Volume (mL)
	Tris-HCL, pH 8.5	100 mM	1M	1 mL
	NaCl	500 mM	5M	1 mL
	EDTA	10 mM	0.5M	0.2 mL
	Water	NA	NA	7.8 mL
	Total Volume:			10 mL

Stock can be stored at RT.

109 Re-suspend BC_0243 & BC_0244 in 1X annealing buffer to a concentration of 100 µM.

110 Pool adapters prior to annealing:

	A	B
	Component	Volume
	BC_0243 (100 uM)	100 uL
	BC_0244 (100 uM)	100 uL
	Total Volume:	200 uL

111 Anneal adapter oligos: 2m

1. Heat to 94 °C for for 00:02:00
2. Ramp down to 20 °C to at a rate of **-0.1C/s**
3. Hold at 4 °C

112 Aliquot annealed adapter in 25 uL aliquots and store at -20°C.

113 Dilute desired amount (ng) of sample in 10 mM Tris-HCl, pH 8.5 to a final volume of 35 uL.

Note: 25 - 100 ng of cDNA can be used as input for fragmentation (per sublibrary).

114 Pre-cool a thermocycler to 4°C with the lid set to 50°C.

115 Assemble fragmentation reaction on ice:

	A	B
	Component	Volume
	cDNA	35 uL
	KAPA Frag Buffer (10X)	5 uL
	KAPA Frag Enzyme	10 uL
	Total Volume:	

116 Vortex gently and spin down. Place the tubes immediately back on ice and proceed to the next step.

117 Place tubes in the pre-cooled thermocycler and start the following protocol:

	Step	Temp	Time
	Pre-cool block	4°C	N/A
	Fragmentation	32°C	10 min.
	HOLD	4°C	forever

118 Transfer reactions to ice, and proceed immediately to end repair and A-tailing.

119 In the same tubes in which enzymatic fragmentation was performed, assemble each end repair and A-tailing reaction as follows:

Component	Volume
Fragmented cDNA	50 uL
ER & A-tailing Buffer	7 uL
ER & A-tailing enzyme mix	3 uL
Total Volume:	60 uL

120 Vortex gently and spin down briefly. Return the reaction tubes to ice. Proceed immediately to the next step.

121 Incubate in a thermocycler programmed as outlined below. A heated lid is required for this step. If possible, set the temperature of the heated lid to ~85°C (instead of the usual 105°C).

Step	Temp	Time
End Repair and A-tailing	65°C	30 min.
HOLD	4°C	forever

122 Transfer tubes to ice. In the same tubes assemble the adapter ligation reaction as follows:

A	B
Component	Volume
ER and A-tailing rxn	60 uL

	A	B
	Adapter Stock (50 uM)	5 uL
	PCR-grade water	5 uL
	Ligation Buffer	30 uL
	DNA Ligase	10 uL
	Total Volume	110 uL

123 Mix 10X with a P100 pipette and spin down briefly.

124 Incubate the tubes in a thermocycler at 20°C for 15 min. (set the lid to "off")

125 Add 88 uL of SPRI beads to each tube for a 0.8X cleanup.

126 Mix thoroughly by vortexing and/or pipetting up and down multiple times (10X).

127 Incubate the tube at room temperature for 5 min to bind DNA to the beads.

128 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear. (1-2 min.)

129 Carefully remove and discard the supernatant without disturbing the beads.

130 Keeping the tubes on the magnet, add 200 µL of 85% ethanol.

131 Incubate the tubes on the magnet at room temperature for ≥30 sec.

132 Carefully remove and discard the ethanol.

133 Repeat Steps 128-130 for a total of two washes.

134 Remove any residual ethanol with a P20 pipette and air dry beads for 3-5 minutes on the magnetic rack.

Do not let the beads over dry (they will turn light brown and crack).

135 Remove the beads from the magnet and re-suspend in 23 uL of RNase, DNase free H2O.

136 Incubate the tubes at 37°C for 10 min. in a thermocycler.

137 Place the tubes against a magnetic rack and wait until the liquid is clear (1-2 min.)

138 Transfer 21 uL of the eluting into a new PCR tube.

11. Sublibrary Indexing and Amplification

139 Set up PCR reaction as follows:

Component	Volume
2X KAPA HiFi Master Mix	25 uL
BC_0027 (10 uM)	2 uL
BC_0070-BC_0093 (10uM)	2 uL
cDNA	21 uL
Total Volume	50 uL

140 Run thermocycler protocol:

	A	B	C
	Step	Temp	Time
	1	95°C	3 min.
	2	98°C	20 sec.
	3	67°C	20 sec.
	4	72°C	3 min.
		Repeat 2-4	*10-16 cycles
	5	72°C	5 min.
	6	4°C	Forever

*The number of cycles will need to be optimized based on input (ng).

141 Add 30 uL of SPRI beads to each sublibrary. (Total volume: 80 uL)

142 Mix thoroughly by vortexing and/or pipetting up and down multiple times (10X).

143 Incubate at RT for 5 min.

144 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear. (1-2 min.)

145 Transfer 75 uL of clear supernatant into new PCR tubes. Discard beads.

DO NOT DISCARD SUPERNATANT!

146 Add 10 uL of SPRI beads to each tube. (Total Volume: 95 uL)

147 Mix thoroughly by vortexing and/or pipetting up and down multiple times (10X).

148 Incubate at RT for 5 min.

149 Place the tubes on a magnet to capture the beads. Incubate until the liquid is clear. **(2-3 min.)**

150 With the tubes still on the magnetic rack, wash with 180 uL of 85% ethanol.

DO NOT RE-SUSPEND THE BEADS!

151 Repeat once for a total of 2 washes.

152 Remove any residual ethanol with a P20 pipette and air dry beads for 3-5 minutes on the magnetic rack.

Do not let the beads over dry (they will turn light brown and crack).

153 Remove the beads from the magnet and re-suspend in 22 uL of RNase, DNase free H2O.

154 Incubate for 10 min. at 37°C in a thermocycler.

155 Place the tubes against a magnetic rack and wait until the liquid is clear (1-2 min.)

156 Transfer 20 uL of the eluting into a new PCR tube.

157 Evaluate quality of cDNA libraries via the Agilent Tapestation or Bioanalyzer.

Expected size range: 300-600 bp (peak 400 bp)

12. Sequencing Requirements

158 Recommended minimum sequencing depth: 20,000 read pairs/cell
Required sequencing parameters:

	A	B	C	D	E
		Read 1	i7 index	i5 index	Read 2
	Purpose	Insert	Sample index	Sample index	Cell Barcode &

	A	B	C	D	E
					UMI
	Length	100	6	0	94

It is recommended to add 5% phiX to the final sequencing libraries to increase library diversity and improve sequencing quality.

Protocol references

Rosenberg AB, Roco CM, Muscat RA, Kuchina A, Sample P, Yao Z, Graybuck LT, Peeler DJ, Mukherjee S, Chen W, Pun SH, Sellers DL, Tasic B, Seelig G. Single-cell profiling of the developing mouse brain and spinal cord with split-pool barcoding. Science. 2018 Apr 13;360(6385):176-182. doi: 10.1126/science.aam8999. Epub 2018 Mar 15. PMID: 29545511; PMCID: PMC7643870.

Kuchina A, Brettner LM, Paleologu L, Roco CM, Rosenberg AB, Carignano A, Kibler R, Hirano M, DePaolo RW, Seelig G. Microbial single-cell RNA sequencing by split-pool barcoding. Science. 2021 Feb 19;371(6531):eaba5257. doi: 10.1126/science.aba5257. Epub 2020 Dec 17. PMID: 33335020; PMCID: PMC8269303.