3-Level scRNA kit

Laboratory Protocol

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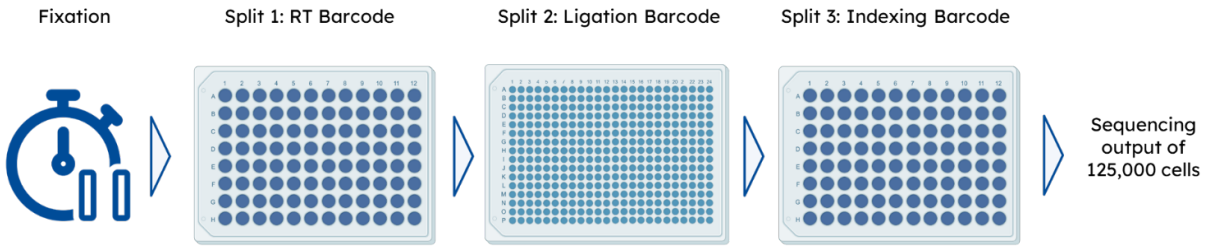
Introduction

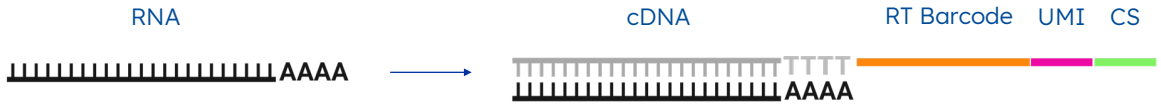
The ScaleBio scRNA Kit provides an instrument-free workflow that increases sample and cell throughput, while reducing cost and bench time. Upstream sample fixation with the integrated FixKit allows for storage for up to one month before processing and enables multiplexing of up to 96 samples from multiple sources, reducing potential biases or experimental artifacts. This entirely plate-based assay utilizes fixed cells as the reaction compartment during a 3-level combinatorial indexing process, with a final output of 125,000 cells and a multiplet rate of less than 5%. This assay can be performed start to finish in just 2 days and includes multiple safe stopping points.

Figure 1. Overall 3L procedure



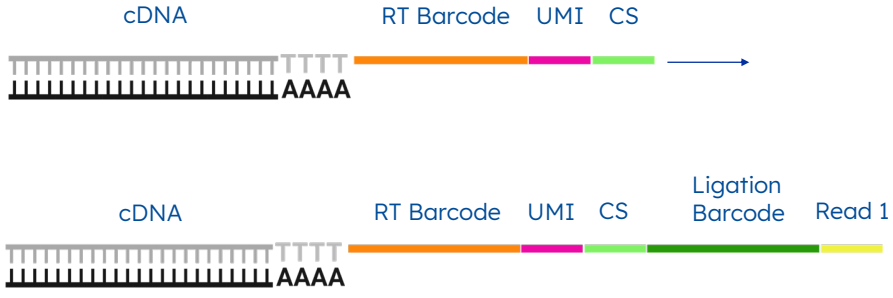
Cell fixation accommodates up to an input of up to 2.5 million cells and multiple samples can be fixed simultaneously in 2 hours or less. These samples can be fed directly into the assay or stored at -80°C until ready for use. Fixed cells are distributed across the 96-well RT plate, with up to 20,000 cells per well for cDNA synthesis and addition of the RT Barcode.

Figure 2. Split 1: RT Barcode



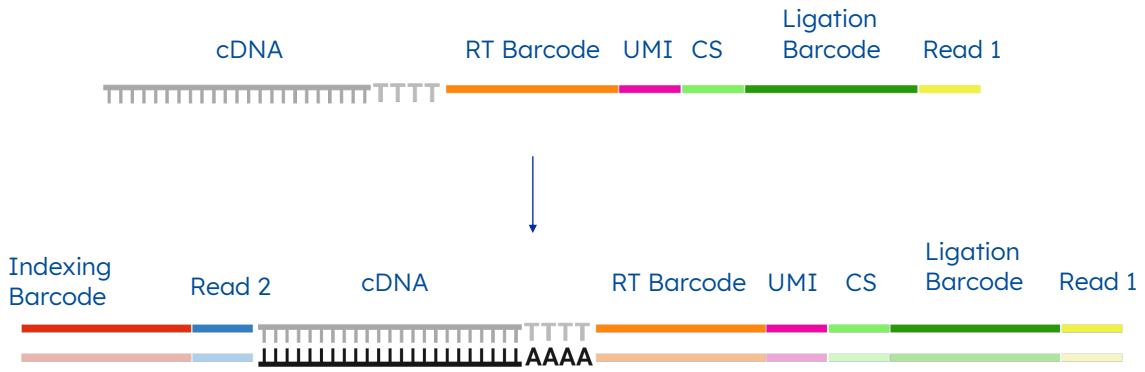
Cells are then pooled by centrifugation using the provided collection funnel, which significantly decreases bench time and cell loss. Pooled cells are then distributed across the 384-well ligation plate for addition of ligation adaptors containing the Ligation Barcode.

Figure 3. Split 2: Ligation Barcode



Cells are pooled again and distributed across the final 96-well plate. Second strand synthesis is then performed, followed by a protease step to break down cells. This is followed by tagmentation and an indexed PCR to add the Tagmentation Barcode. Libraries from each well can then be pooled to target the desired number of cells and purify prior to sequencing.

Figure 4. Split 3: Tagmentation and Index PCR



ScaleBio scRNA libraries are compatible with standard Illumina sequencing. The ScaleBio bioinformatics pipeline can be used to both demultiplex and assign reads to individual cells. Output of this pipeline includes basic scRNA-seq QC metrics and files required for further downstream analysis.

Required Materials

Consumables and reagents manufactured by Scale Biosciences for the R3L sciRNA kit

Consumable	Label	Box	Storage Temp
WRS Buffer	R3L-WRS	R3L-Box 1	4°C
Elution Buffer	R3L-EBS	R3L-Box 2	-20°C
Indexed RT Oligo Plate	R3L-RTP	R3L-Box 2	-20°C
Indexed Ligation Oligo Plate	R3L-LGP	R3L-Box 2	-20°C
RT Enzyme	R3L-RTE	R3L-Box 2	-20°C
RT Buffer	R3L-RTB	R3L-Box 2	-20°C
dNTPs	R3L-RTN	R3L-Box 2	-20°C
Ligation Buffer	R3L-LGB	R3L-Box 2	-20°C
Ligase Enzyme	R3L-LGE	R3L-Box 2	-20°C
Second Strand Synthesis Buffer	R3L-2SB	R3L-Box 2	-20°C
Second Strand Synthesis Enzyme	R3L-2SE	R3L-Box 2	-20°C
Protease	R3L-PRT	R3L-Box 2	-20°C
Tagment Buffer	R3L-ETB3	R3L-Box 2	-20°C
Transposome Mix	R3L-TSM	R3L-Box 2	-20°C
Tagment Stop Component B	R3L-TSB	R3L-Box 2	-20°C
Tagment Stop Component C	R3L-TSC	R3L-Box 1	4°C
Index PCR Additive	R3L-AMA	R3L-Box 2	-20°C
Indexed PCR Master Mix	R3L-AMM	R3L-Box 2	-20°C
Indexed P7 Primer Plate	R3L-AMP7	R3L-Box 1	4°C
Indexed P5 Primers	R3L-AMP5	R3L-Box 1	4°C
Scale Collection Funnel	-	R3L-FNL	RT
Final Distribution Plate (96-well plate)	-	-	RT

Consumables and reagents manufactured by other vendors

Consumable or reagent	Supplier	Part Number(s)
Nuclease Free Water	Various	Various
Sterile, filtered, low retention tips for P1000, P200, P20 pipettes	Various	Various
0.2-mL PCR tube strips	Axygen	PCR-0208-CP-C
1.5-mL LoBind Eppendorf tubes	Eppendorf	0030108418
5-mL LoBind Eppendorf tubes	Eppendorf	0030108310
15-mL conical tubes	VWR	10025-686
Microseal 'B' Adhesive seals	Bio-Rad	MSB1001
Cell counting dye: AO/PI, Trypan Blue, YOYO-1, etc.	Various	Various
SPRIselect size selection beads	Beckman Coulter	B23317
Pure ethyl alcohol	Various	Various
Qubit dsDNA HS Assay Kit	ThermoFisher	Q32851, Q32854, Q33230, Q33231
Agilent High Sensitivity DNA Kit for Bioanalyzer 2100 -or- equivalent high sensitivity DNA analysis kit for an equivalent fragment analyzer	Agilent	5067-4626
NEBNext Library Quant Kit for Illumina	NEB	E7630

Equipment List

Item	Supplier	Part Number(s)
Temperature-controlled centrifuges for 1.5-mL tubes, 15-mL conical tubes, and 96-well plates	Various	Various
Vortex mixer	Various	Various
P1000, P200, P20, P10, P2 pipettes	Various	Various
P200, P20, P10, multi-channel pipettes	Various	Various
96-well aluminum cooler blocks	Various	Various
Thermocycler that can accept full-skirted plates	Various	Various
96-well plate shaker, such as: MixMate –or- ThermoMixer with 96-well plate and 384-well plate adapters	Eppendorf	5353000529 -or- 5382000023 with adapters 5306000006, 5307000000
96-well plate magnet	ThermoFisher	12331D
1.5-mL tube magnet	ThermoFisher	12321D
Qubit 4 Fluorometer	ThermoFisher	Q33238
2100 Bioanalyer Instrument or equivalent fragment analyzer	Agilent	G2939BA

Workflow diagram

Best Practices

For general laboratory best practices:

- Calibrate and service pipettes every 12 months to ensure accurate sample volume transfer at each step.
- Store all reagents at the storage conditions recommended by the supplier.
- Unless otherwise specified, thaw reagents on ice.
- Never reuse pipette tips or tubes.
- Keep pipette tip boxes, reagent containers, and sample tubes closed when not in use.
- Wear suitable protective clothing, eyewear, and gloves.

For RNase-free sample processing:

- Use low-retention, RNase-free pipette tips and low-binding reaction tubes to prevent adsorption to plastic surfaces.
- Routinely wipe work surfaces with RNase AWAY to remove RNases, and with a 10% bleach cleaning solution to remove DNA amplicon contaminants.
- Wear disposable gloves and change them frequently.
- Keep pipette tip boxes, reagent containers, and sample tubes closed when not in use.
- Routinely wipe work surfaces with a 10% bleach solution.

For prevention of amplicon cross-contamination in sequencing libraries:

- Thaw and prepare reagent mixes in pre-amplification workspaces.
- Perform amplification in post-amplification workspaces.
- Perform PCR purification steps in post-amplification workspace
- Never bring material or equipment from post-amplification workspaces into pre-amplification workspaces.
- Regularly clean post-amplification workspaces with a 10% bleach solution.

SciRNAseq Workflow

Split 1: Initial Distribution and Reverse Transcription

Please review the table below to prepare reagents for starting this section:

Material	Place at:	Brief Vortex	Brief Spin
WRS Buffer (WRS)	On ice	-	-
Fixed cell samples, prepared with the ScaleBio FixKit	On ice	✗	✗
96-well Indexed RT Oligo Plate (RTP)	On ice	✗	✓
RT Enzyme (RTE)	On ice	✗	✓
RT Buffer (RTB)	On ice	✓	✓
dNTPs (RTN)	On ice	✓	✓
Scale Collection Funnel	RT	-	-
Cell counting dye	Variable	Variable	✓
5-mL Eppendorf tubes or 15-mL conical tubes	RT	-	-
Wide bore pipette tips	RT	-	-

Before you begin:

- If frozen, thaw cells on ice and do not vortex.
- Bring centrifuges that accommodate 96-well plates and 15-mL conical tubes to 4°C.
- Set a thermocycler to 55°C with a lid temperature of 65°C.
- Fully chill a 96-well metal block on ice.

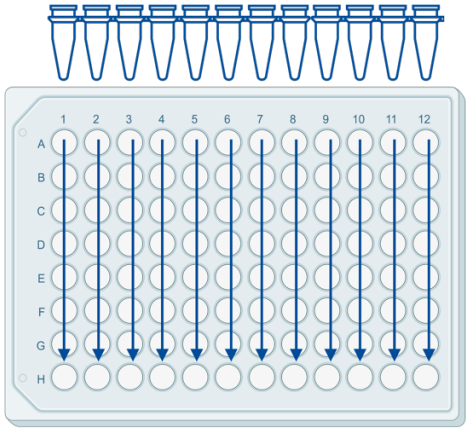
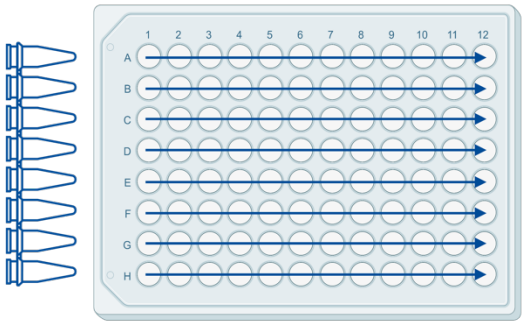
Procedure:

1. Place the thawed RTP on a 96-well metal block on ice.
2. Determine the concentration of the cell suspension prepared with the Scale FixKit using a viability dye and a hemocytometer, Nexcelom Cellometer K2, or similar cell counting equipment. For accurate cell counting, use >=2 µL of cell suspensions and appropriate dilution factors to obtain cell counts that are recommended for accuracy for your cell counting method. Perform cell counts in duplicate, adding additional cell counts if the measurements are >20% different.

Note: Fixed cells may settle at the bottom of the tube. To ensure even distribution of cells, flick the tube 10-15 times until pellet has dispersed before counting cell suspensions.

3. For the initial distribution of cells across the RTP, cell suspensions are diluted in WRS buffer to achieve a final concentration of 2000 cells/µL in a volume that is dependent on the number of wells that sample will occupy. 5 µL of cell suspensions will be distributed to the wells on the RTP, however, guidance below has the user prepare cell suspensions in excess to ensure all intended wells receive 5 µL of cells per well on the RTP. On ice, dilute the fixed cell suspension in WRS to achieve a concentration of 2000/µL in a final volume based on the recommendations below:

Table 1. Dispensing guidelines for Initial Distribution and Reverse Transcription

For distribution down columns, using a 12-channel multichannel pipette and a 12-tube strip	
For full columns: Use a multi-channel pipette • Prepare 50 µL of the cell suspension per strip tube to distribute across a single column. • If you are distributing to multiple columns, multiply 50 µL by the number of columns and 1.2x to generate sufficient overage for aliquoting the cell suspension across a 12-tube strip. **For partial columns: Use a single channel pipette** • Prepare 6 µL of diluted cells per well and single pipette 5 µL per well from this stock.	
For distribution down rows using an 8-channel multichannel pipette and an 8-tube strip	
For full columns: Use a multi-channel pipette • Prepare 75 µL of the cell suspension per tube to distribute across a single row. • If you are distributing to multiple rows, multiply 75 µL by the number of rows and 1.2x to generate sufficient overage for aliquoting the cell suspension across 8-tube strip. **For partial columns: Use a single channel pipette** • Prepare 6 µL of diluted cells per well and single pipette 5 µL per well from this stock.	

4. With the RTP on ice, immediately distribute 5 µL of cells to each well of the RTP.

Caution: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

5. Seal the RTP and place on a plate shaker (see equipment recommendations in the Equipment List).
6. Shake the plate for 30 seconds at 2,000 rpm.
7. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
8. Place the RTP on a thermocycler and incubate at 55°C for 5 min with a lid temperature of 65°C.
9. Remove the RTP from the thermocycler and immediately place on the pre-chilled metal block on ice.
10. Incubate on ice for 5 min or until the top of the RTP is fully chilled.
11. On ice, prepare the reverse transcription master mix by combining the components specified in the following table:

Table 2. Reverse Transcription (RT) Master Mix formulation

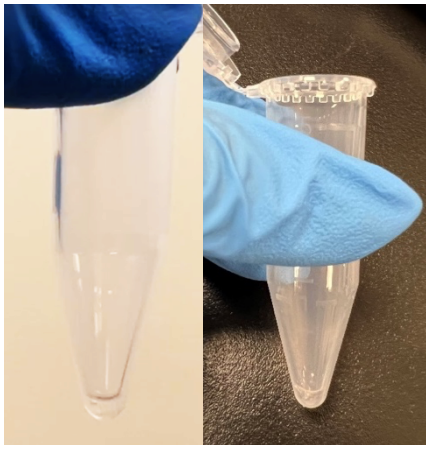
Reagent	Volume (µL)
RTB	240
RTN	60
RTE	60
Total volume	360

12. Gently pipet mix the RT master mix until the solution is homogeneous and briefly spin down.
13. Aliquot the RT master mix into each tube of an 8- or 12- tube strip, using the volumes below:
    a. For an 8-tube strip, aliquot **42** µL into each tube.
    b. For a 12-tube strip, aliquot **28** µL of the mix into each tube.
14. Unseal the RTP and use a multichannel pipette to distribute 3 µL of the RT master mix into each well of the plate, dispensing the master mix to the bottom of the well.

Caution: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

15. Seal the RTP and place on a plate shaker.
16. Shake the plate for 30 seconds at 2,000 rpm.
17. Briefly spin down the plate at 100 x g for 30 seconds.
18. Incubate the RTP at 55°C for 10 min with a lid temperature of 65°C. Remove the RTP from the thermocycler and immediately place the plate on a pre-chilled metal block on ice.

19. Keep the plate on ice until the top of the RTP is fully chilled. This may take up to 10 min.
20. Unpack Scale collection funnel and place on ice. Do not touch the inside of the funnel.
21. Unseal the RTP, place the collection funnel on top, and quickly invert the assembly.
22. Centrifuge the collection funnel with an appropriately weighted balance in tabletop centrifuge at 300 x g for 3 min at 4°C.
23. Transfer pooled material from collection funnel into a 5-mL Eppendorf tube and place on ice.
24. Add 5 mL of WRS to the now empty **collection funnel**, rinsing the sides of the funnel 2-3 times to collect the residual liquid on the funnel into the center. Leave the wash in the funnel on ice, this volume will be used in step 30.
25. Centrifuge the 5-mL tube containing the pooled material from the RTP at 500 x g for 5 min at 4°C.
26. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of residual volume as shown below:



Note: After centrifugation, the pellet will be looser at this point. Take extra precaution when removing supernatant to avoid cell loss.

27. Gently flick the tube several times to resuspend the pellet in the residual volume.
28. Collect the WRS from the collection funnel and add it to the loosened cell pellet.
29. Centrifuge the tube at 500 x g for 5 min at 4°C.
30. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of residual volume as shown above.
31. Gently flick the tube several times to resuspend the pellet in the residual volume.
32. Add 3,082 µL of ice-cold WRS to the tube with pooled cells.

Split 2: Ligation

Please review the table below to prepare reagents for starting this section:

Material	Place at:	Brief Vortex	Brief Spin
384-well Indexed Ligation Oligo Plate (LGP)	On ice	✗	✓
Ligase Buffer (LGB)	On ice	✓	✓
Ligase Enzyme (LGE)	On ice	✗	✓
WRS Buffer (WRS)	On ice	-	-

Procedure:

1. On ice, prepare the ligation master mix by adding the components in the following table to the pooled and washed cells in the specified order:

Table 3. Ligation master mix formulation

Reagent	Volume (µL)
Pooled cells	3,082
LGB	460
LGE	138
Total volume	3,680

2. Using a P1000 fitted with a wide-bore tip, pipet mix the ligation master mix until the solution is homogeneous and quickly proceed to the next step.
3. On a chilled metal block on ice, immediately distribute the Ligation master mix into each tube of an 8- or 12- tube strip, using the volumes below:
    a. For an 8-tube strip, aliquot **225** µL into each tube.
    b. For a 12-tube strip, aliquot **150** µL of the mix into each tube.

    This is enough volume for distribution for half the LGP.
4. Using a multichannel pipette, add 8 µL of Ligation master mix to each well of **half** of the LGP on ice.

! **Caution: DO NOT pipette mix.** Change pipette tips between wells to avoid cross-contamination.

5. Repeat steps 3-4, re-mixing and distributing the remaining ligation master mix to the same 8- or 12-tube strip and dispensing 8 µL of the mix to the remaining half of the LGP.
6. Seal the LGP and place on a plate shaker.
7. Shake the plate for 30 seconds at 2,000 rpm.
8. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
9. Incubate the LGP for 30 min at room temperature.

10. Place the LGP on ice and incubate until top of the plate is fully chilled. This may take up to 5 min.
11. Unpack a Scale collection funnel and place on ice. Do not touch the inside of the funnel.
12. Unseal the LGP, place the collection funnel on top, and quickly invert the assembly.
13. Centrifuge the collection funnel with an appropriately weighted balance in tabletop centrifuge at 300 x g for 3 min at 4°C.
14. Transfer pooled material from collection funnel into a 5-mL Eppendorf tube and place on ice.
15. Add 5 mL of cold WRS to the now empty **collection funnel**, rinsing the sides of the funnel 2-3 times to collect the residual liquid on the funnel into the center. Leave the wash in the funnel on ice.
16. Centrifuge the 5-mL tube containing the pooled material from the LGP at 500 x g for 5 min at 4°C.
17. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of residual volume as shown in the previous section.

Note: After centrifugation, the pellet will be looser at this point. Take extra precaution when removing supernatant to avoid cell loss.

18. Gently flick the tube several times to resuspend the pellet in the residual volume.
19. Collect the WRS from the collection funnel and add it to the loosened cell pellet.
20. Centrifuge the tube at 500 x g for 5 min at 4°C.
21. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of volume.
22. Gently flick the tube several times to resuspend the pellet in the residual volume.
23. Resuspend pellet in 100 µL of WRS.

Split 3: Final Distribution

Please review the table below to prepare reagents for starting this section:

Material	Place at:
WRS Buffer (WRS)	On ice
Cell counting dye	Variable
Final Distribution plate	RT

Before you begin:

- Fully chill a 96-well metal block on ice.
- Place the Final Distribution plate on ice.

Procedure:

1. Determine the concentration of the cell suspension using a viability dye and a hemocytometer, Nexcelom Cellometer K2, or similar cell counting equipment. For accurate cell counting, use >=2 µL of cell suspensions and appropriate dilution factors to obtain cell counts that are recommended for accuracy for your cell counting method. Perform cell counts in duplicate, adding additional cell counts if the measurements are >20% different.
2. Dilute cells in WRS to achieve a final concentration of 400 cells per µL in enough volume to dispense 4 µL per well across the 96-well plate, for a total of 1600 cells per well.
3. Gently pipet mix the tube of cells and distribute 4 µL of the suspension to all the wells of the 96-well Final Distribution plate.
4. Seal the plate and place on ice.

Note: Excess cells processed through ligation can be aliquoted to additional 96-well plates for future processing. Follow steps 2-4 to prepare additional plates.

Safe stopping point. The Final Distribution plate can be stored at -80°C for up to 1 month before proceeding with second strand synthesis.

Second strand synthesis, protease digestion

Please review the table below to prepare reagents for starting this section:

Material	Place at:	Brief Vortex	Brief Spin
96-well Final Distribution plate	On ice	✗	✓
Nuclease-free water	On ice	-	-
Second Strand Synthesis Buffer (2SB)	On ice	✓	✓
Second Strand Synthesis Enzyme (2SE)	On ice	✗	✓
Protease (PRT)	On ice	✓	✓

Before you begin:

- Fully chill a 96-well metal block on ice.
- Set a thermocycler to 16°C (no heated lid).

Procedure:

1. On ice, prepare the second strand synthesis master mix by combining the components in the order specified in following table:

Table 4. Second Strand Synthesis (2SS) master mix formulation

Reagent	Volume (µL)
Nuclease-free water	35
2SB	70
2SE	35
Total volume	140

2. Thoroughly and gently pipet mix the master mix until it is homogeneous, briefly spin down, and place on ice.
3. On a pre-chilled metal block on ice distribute prepared 2SS master into each tube of an 8- or 12- strip tube, using the volumes below:
    a. For an 8-tube strip, aliquot **16** µL into each tube.
    b. For a 12-tube strip, aliquot **11** µL into each tube.
4. Using a multichannel pipette add 1 µL of the 2SS master mix to each well of the Final Distribution plate.

! **Caution: DO NOT pipette mix.** Change pipette tips between wells to avoid cross-contamination.

5. Seal the Final Distribution plate and place on a plate shaker.

6. Shake the plate for 30 seconds at 2,000 rpm.
7. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
8. On a thermocycler, incubate the plate at 16°C for 1 hr (no heated lid).

Safe pause point. The plate can be stored overnight at 4°C after incubation.

9. Remove Final Distribution plate from the thermocycler and place on ice.
10. Distribute PRT into each tube of an 8- or 12- strip tube, using the volumes below:
    a. For an 8-tube strip, aliquot **16** µL into each tube.
    b. For a 12-tube strip, aliquot **11** µL into each tube.
11. Using a multichannel pipette add 1 µL of PRT to each well of Final Distribution plate.

Caution: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

12. Seal Final Distribution plate and place on plate shaker.
13. Shake the plate for 30 seconds at 2,000 rpm.
14. Briefly spin down the plate at 100 x g for 30 seconds.
    Incubate the plate for 37°C (85°C heated lid) for 30 min followed by 75°C for 20 min with a 4°C hold.

Safe pause point. The plate can be stored overnight at 4°C after incubation.

15. Briefly centrifuge the plate and place on a pre-chilled metal block on ice. Proceed with Tagmentation and Index PCR.

Tagmentation and Index PCR

Please review the table below to prepare reagents for starting this section:

Material	Place at:	Brief Vortex	Brief Spin
Nuclease-free water	On ice	✓	✓
Tagment Buffer (ETB3)	On ice	10 sec at max speed*	✓
Transposome Mix (TSM)	On ice	10 sec at max speed*	✓
Tagment Stop Component B (TSB)	RT	✓	✓
Tagment Stop Component C (TSC)	RT	✓	✓
Index PCR Additive (AMA)	On ice	✗	✓
Indexed P5 primer (AMP5)	On ice	✓	✓
Index PCR Master Mix (AMM)	On ice	✓	✓
Indexed P7 primer plate (AMP7)	On ice	✗	✓

*Be sure to vortex for the full 10 seconds at maximum speed.

Before you begin:

- Set a thermocycler to 55°C with a 65°C heated lid.

Procedure:

1. Prepare the Tagmentation master mix by combining the components in the order specified in the following table:

Table 5. Tagmentation master mix formulation

Reagent	Volume (µL)
Nuclease-free water	108
ETB3	444
TSM	48
Total volume	600

2. Mix Tagmentation master mix by vortexing for 10 sec at maximum speed, briefly spin down, and place tube on ice.
3. Aliquot the Tagmentation master mix into each tube of an 8- or 12- tube strip, using the volumes below:
    a. For an 8-tube strip, aliquot **70** µL into each tube.
    b. For a 12-tube strip, aliquot **45** µL into each tube.

4. Using a multichannel pipette add 5 µL of the Tagmentation master mix to each well of the protease-digested Final Distribution plate.

Caution: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

5. Seal Final Distribution plate and place on plate shaker.
6. Shake the plate for 30 seconds at 2,000 rpm.
7. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
8. Incubate at 55°C for 10 min (65°C heated lid). Afterwards, place the plate on the benchtop.
9. Prepare the Tagment Stop solution at room temperature by combining the components in the order specified in the following table. After formulation, **DO NOT** place on ice.

Table 6. Tagment Stop Solution formulation

Reagent	Volume (µL)
Nuclease-free water	270
TSB	60
TSC	60
Total volume	390

10. Vortex the Tagment Stop Solution for 10 sec at maximum speed and briefly spin down.
11. Distribute Tagment Stop Solution into each tube of an 8- or 12- tube strip, using the volumes below:
    a. For an 8-tube strip, aliquot **45** µl into each tube.
    b. For a 12-tube strip, aliquot **30** µl into each tube.
12. Using a multichannel pipette add 2.6 µL of Tagment Stop Solution to each well of the tagmented Final Distribution plate.

Caution: DO NOT pipette mix. Change pipette tips between wells to avoid cross-contamination.

13. Shake the plate for 30 seconds at 2,000 rpm.
14. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
15. Incubate at 55°C for 15 min (65°C heated lid). Afterwards, place the plate on ice.
16. On ice, prepare the Index PCR master mix in a 5 mL tube by combining the components in the order specified in the following table:

Table 7. Index PCR master mix formulation

Reagent	Volume (µL)
Nuclease-free water	264
AMA	240
AMP5	24
AMM	2,400
Total Volume	2,928

17. Briefly vortex the Index PCR master mix and briefly spin to collect contents at bottom of tube.
18. Distribute 235 µL of Index PCR master mix into each tube of a 12-tube strip.
19. Using a multichannel pipette add 24.4 µL of Index PCR master mix to each well of the Final Distribution plate.
20. Using a multichannel pipette, add 2 µL of primers from the 96-well Indexed P7 primer plate (AMP7) to the corresponding well of the Final Distribution plate.
21. Seal the Final Distribution plate and place on plate shaker.
22. Shake the plate for 30 seconds at 2,000 rpm.
23. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
24. Place the Final Distribution plate on a thermocycler and run the following program with 105°C lid and 40 µL volume:

Table 8. Index PCR thermal cycling program

Temperature	Duration	Cycles
70°C	5 min	1
98°C	30 sec	1
98°C	10 sec	13 cycles
63°C	30 sec	
72°C	1 min	
72°C	3 min	1
4°C	∞	

Safe pause point. The plate can be stored overnight at 4°C prior to purification.

Index PCR Purification and QC

Please review the table below to prepare reagents for starting this section:

Material	Place at:
SPRIselect bead	RT
Pure ethyl alcohol	RT
Nuclease-free water	RT
Elution Buffer (ESB)	RT
Qubit dsDNA HS Assay Kit	RT
Agilent High Sensitivity DNA Kit for the 2100 Bioanalyzer instrument -or- a reagent kit for high sensitivity DNA fragment analysis for an equivalent fragment analyzer	RT
NEBNext Library Quant Kit for Illumina	RT

Before you begin:

- Prepare 1 mL fresh 80% ethanol.

Procedure:

1. Pool 5 µL from each well of the Indexed PCR plate into a 1.5 mL tube for cleanup and sequencing.
2. Vortex the SPRI beads at high speed for 1 min. The beads should appear homogeneous and uniform in color.
3. Transfer 384 µL of SPRIselect beads (0.8X) to the tube containing Indexed PCR products.
4. Vortex to mix.
5. Incubate at room temperature 5 min.
6. Place the tube on a magnetic stand for 5 min.
7. Once the solution is clear, briefly spin the tube and place the tube back on the magnetic stand.
8. Remove and discard the supernatant, being careful not to disturb the beads.
9. Keep the tube on the magnetic stand and add 500 µL of 80% ethanol to the side of the tube opposite the pellet.
10. Incubate the tube on the magnetic stand for 30 s.
11. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
12. Repeat the 500 µL 80% ethanol wash (steps 8–10) for a total of two washes.
13. Briefly spin the tube to collect residual 80% ethanol at the bottom of the tube and place tube back on the magnetic stand.
14. Remove residual 80% ethanol being careful not to disturb the beads.
15. Air dry the beads for 2 min or until the bead pellet appears matte instead of glossy but not cracked.
16. Remove the tube from the magnetic stand and add 30 µL ESB.

17. Vortex to mix and briefly spin down to collect liquid at the bottom of the tube.
18. Incubate the tube off the magnetic stand for 5 min and then briefly spin down.
19. Place the tube on a magnetic stand until the solution is clear.
20. Transfer the supernatant to a new tube.
21. Determine the average fragment size of the library using Agilent High Sensitivity DNA Kit for the 2100 Bioanalyzer instrument or equivalent reagent kit for high sensitivity DNA fragment analysis for an equivalent fragment analyzer.
    a. Optional: Quantify the sequencing library concentration using the Qubit dsDNA HS assay kit.
22. Determine library concentration for sequencing with *NEBNext Library Quant Kit for Illumina* (NEB# E7630) according to manufacturer's protocol. Perform library dilution and clustering according to sequencing manufacturers parameters.

Sequencing Parameters

Target >20,000 read pairs per cell

- Read 1: 34
- Index 1: 10
- Read 2: 76

Final product for sequencing:

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