ScaleBio™ Single Cell RNA Sequencing Kit

Laboratory Protocol

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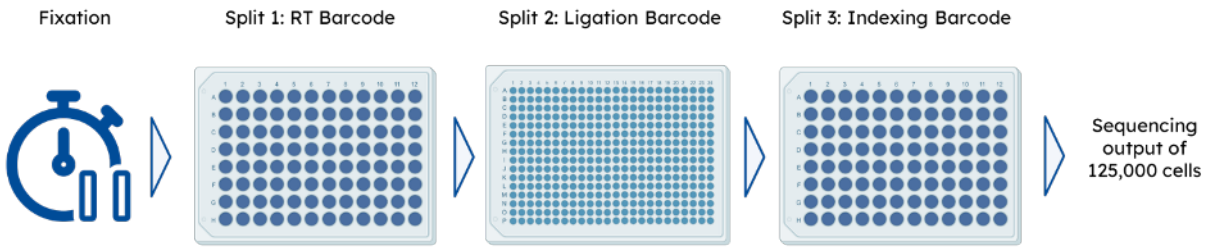
Introduction

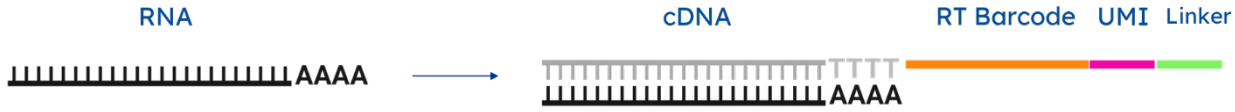
The ScaleBio Single Cell RNA Sequencing Kit provides an instrument-free workflow that increases sample and cell throughput, while reducing cost and bench time. Upstream sample fixation with the integrated ScaleBio Sample Fixation Kit allows for storage for up to one month before processing and enables 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 procedure



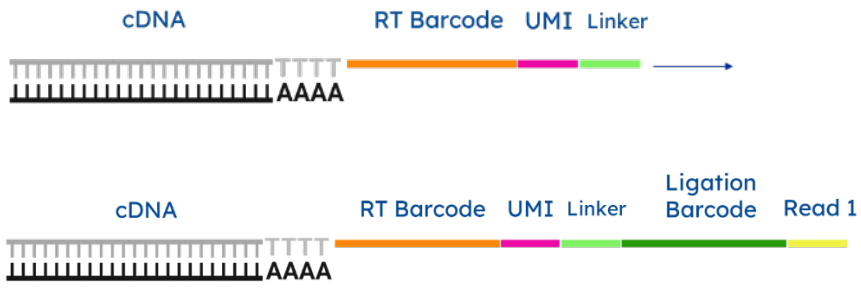
Cell fixation accommodates up to an input of up to 2.5 million cells and multiple samples can be fixed simultaneously in 2 hours or less. These samples can be processed directly into the assay or stored at -80°C until ready for use. Fixed cells are distributed across the 96-well RT Barcode Plate, using 10,000 cells per well for cDNA synthesis and addition of the RT Barcode.

Figure 2. Split 1: RT Barcode



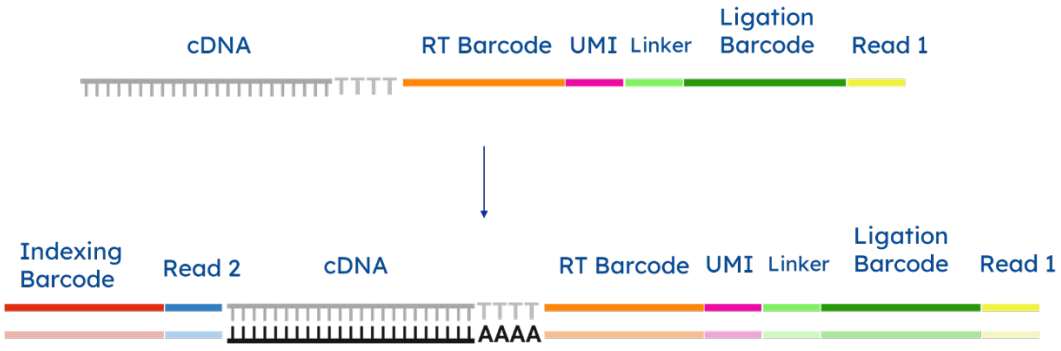
Cells are then pooled by centrifugation using the provided ScaleBio Collection Funnel, which significantly decreases bench time and cell loss. Pooled cells are then distributed across the 384-well Ligation Barcode Plate for addition of ligation adaptors containing the Ligation Barcode.

Figure 3. Split 2: Ligation Barcode



Cells are pooled again and distributed across the 96-well Final Distribution Plate. Second strand synthesis is then performed, followed by a cleanup enzyme digestion step to break down cells. This is followed by tagmentation and an indexed PCR to add the Index PCR Barcode. Libraries from each well can then be pooled to target the desired number of cells and purified prior to sequencing.

Figure 4. Split 3: Tagmentation and Index PCR



ScaleBio Single Cell RNA 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 single-cell RNA sequencing QC metrics and files required for further downstream analysis.

Required Materials

Consumables and reagents contained within the ScaleBio Single Cell RNA Sequencing Kit (PN 2020008)

Kit Module	Consumable	Part Number	Cap Color	Storage Temp
-	Cells fixed with the ScaleBio Sample Fixation Kit		-	-80°C
ScaleBio RT Module (PN 2020004)	Wash Buffer	202100002		-20°C
	RT Barcode Plate	202110002		-20°C
	RT Enzyme Mix	202110003	Green	-20°C
	RT Buffer Conc.	202110004	Green	-20°C
	RT Additive	202110005	Green	-20°C
ScaleBio Ligation Module (PN 2020005)	Ligation Barcode Plate	202110006		-20°C
	Ligation Enzyme Mix	202110007	Blue	-20°C
	Ligation Buffer Conc.	202110008	Blue	-20°C
ScaleBio Tagment and Index PCR Module (PN 2020006)	Second Strand Buffer Conc.	202110009	Purple	-20°C
	Second Strand Enzyme Mix	202110010	Purple	-20°C
	Cleanup Enzyme	202110011	Brown	-20°C
	Tagment Buffer Conc.	202100003	Red	-20°C
	Tagment Enzyme Mix	202100004	Red	-20°C
	Index PCR Enzyme Mix	202110012	Orange	-20°C
	Adaptor Primer	202110015	Orange	-20°C
	Index PCR Barcode Plate	202110013		-20°C
	Elution Buffer	202110014	Clear	-20°C
ScaleBio Workflow Consumables Module (PN2020007)	Tagment Stop Solution	202110017	Black	4°C
	Index PCR Additive	202110016	Orange	4°C
	ScaleBio Sample Collection Funnel	202100005		4°C
	Final Distribution Plate	202110018		4°C

Consumables and reagents manufactured by other vendors:

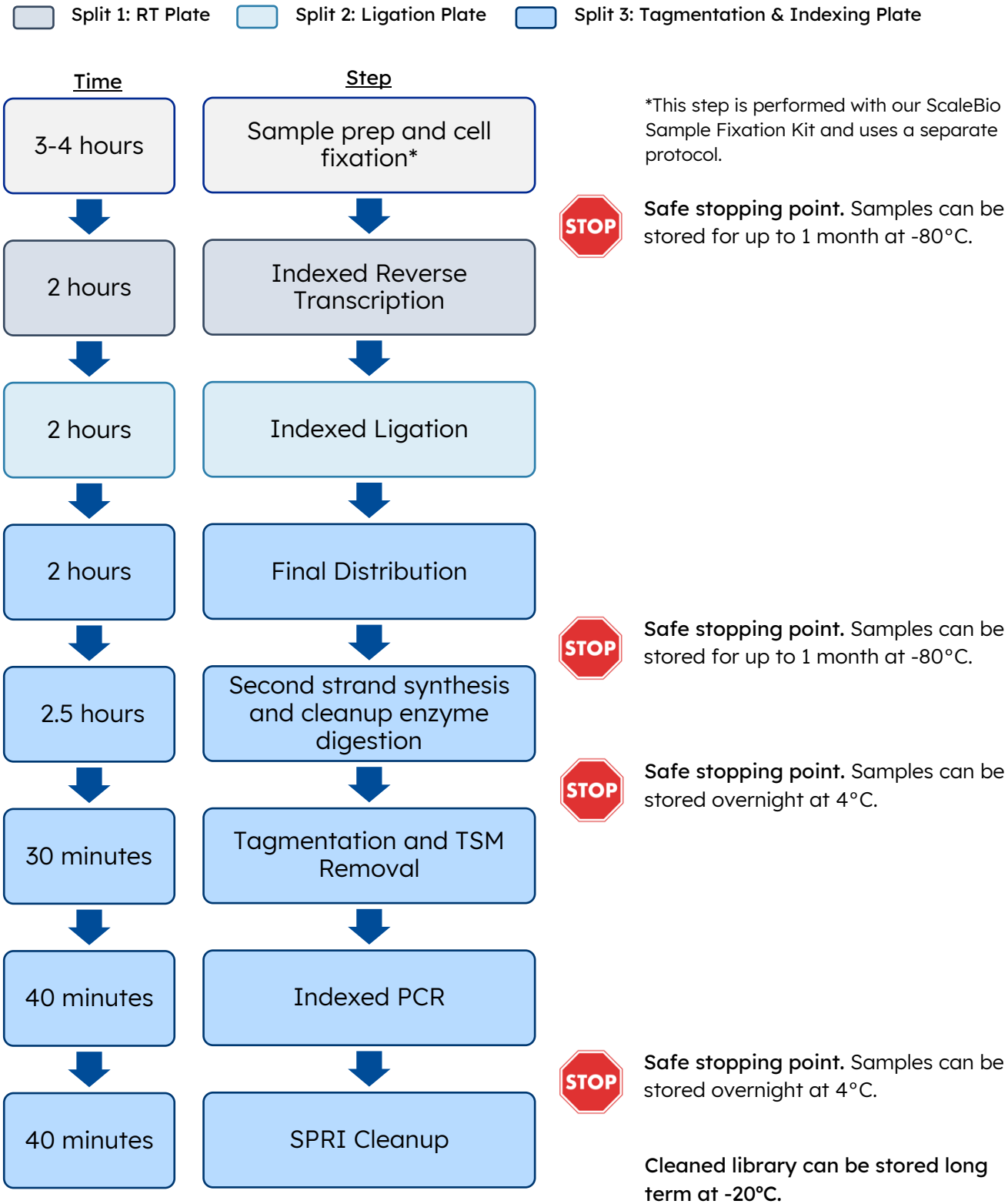
Consumable or reagent	Supplier	Part Number(s)
Nuclease Free Water	Various	Various
Sterile, filtered, low retention tips for P1000, P200, P20 pipettes	Various	Various
0.2-mL PCR tube strips	Axygen	PCR-0208-CP-C
1.5-mL LoBind Eppendorf tubes	Eppendorf	0030108418
5-mL LoBind Eppendorf tubes	Eppendorf	0030108310
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

*We strongly recommend against the use of mixers with foam attachments. Foam particulates can break off, attach to well plates, and potentially interfere with the assay.

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.
- Thaw all reagents on ice, unless otherwise specified.
- 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.

Workflow

Split 1: Initial Distribution and Reverse Transcription

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

Material	Cap Color	Place at:	Brief Vortex	Brief Spin
Wash Buffer	-	Room Temp to thaw, then on ice	-	-
Fixed cell samples, prepared with the ScaleBio Sample Fixation Kit	-	On ice	✗	✗
96-well RT Barcode Plate	-	On ice	✗	✓
RT Enzyme Mix	Green	On ice	✗	✓
RT Buffer Conc.	Green	On ice	✓	✓
RT Additive	Green	On ice	✓	✓
ScaleBio Sample Collection Funnel		Room Temp	-	-
Cell counting dye		Variable	Variable	✓
5-mL Eppendorf tubes or 15-mL conical tubes		Room Temp	-	-
Wide bore pipette tips		Room Temp	-	-

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.
- Once thawed, invert the Wash Buffer to ensure it is fully mixed.

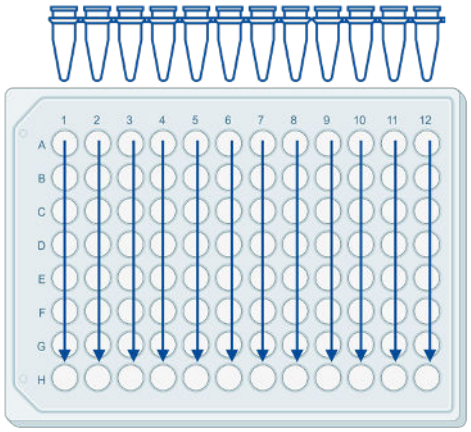
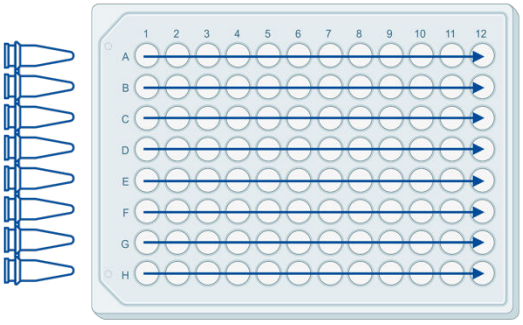
Procedure:

1. Place the thawed RT Barcode Plate on a 96-well metal block on ice.
2. Determine the concentration of the cell suspension prepared with the ScaleBio Sample Fixation Kit 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 recommended 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 RT Barcode Plate, cell suspensions are diluted in Wash Buffer to achieve a final concentration of 2,000 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 RT Barcode Plate, however guidance below can be used to prepare cell suspensions in excess, ensuring all intended wells receive 5 µL on the RT Barcode Plate. On ice, dilute the fixed cell suspension in Wash Buffer to achieve a concentration of 2,000/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 rows: 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 rows: 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 RT Barcode Plate on ice, immediately distribute 5 µL of cells to each well of the RT Barcode Plate.

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

5. Seal the RT Barcode Plate 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 RT Barcode Plate on a thermocycler and incubate at 55°C for 5 minutes with a lid temperature of 65°C.
9. Remove the RT Barcode Plate from the thermocycler and immediately place on the pre-chilled metal block on ice.
10. Incubate on ice for 5 minutes or until the top of the RT Barcode Plate 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)
RT Buffer Conc.	240
RT Additive	60
RT Enzyme Mix	60
Total volume	360

12. Gently pipette 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 RT Barcode Plate 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 RT Barcode Plate 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 and place on ice.
18. Program the RT thermal cycling program on a thermal cycler:

Table 3. Reverse Transcription (RT) Thermal Cycling Program (Lid Temp = 65°C)

Temperature	Time
4°C	∞
4°C	2 min
10°C	2 min
20°C	2 min
30°C	2 min
40°C	2 min
50°C	2 min
55°C	10 min
4°C	∞

19. Once the thermal cycler reaches 4°C, place the RT Barcode Plate on the pre-cooled thermocycler block, then "Skip" to the next step of 4°C for 2 minutes on the thermal cycler program.
20. Check the bottom of the RT Barcode Plate for debris and moisture. If debris or moisture is present, wipe the bottom of the plate with a clean Kimwipe coated in 70% ethanol, followed by a dry Kimwipe until the plate bottom is fully dry.
21. Unpack the ScaleBio Sample Collection Funnel and place on ice. Do not touch the inside of the funnel.
22. Unseal the RT Barcode Plate, place the collection funnel on top, and quickly invert the assembly.
23. Centrifuge the collection funnel with an appropriately weighted balance in tabletop centrifuge at 300 x g for 3 minutes at 4°C.
24. Transfer pooled material from collection funnel into a 5-mL Eppendorf tube and place on ice.
25. Add 5 mL of Wash Buffer 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 28.
26. Centrifuge the 5-mL tube containing the pooled material from the RT Barcode Plate at 500 x g for 5 minutes at 4°C.

27. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of residual volume as shown below:

Note: After centrifugation, the pellet may be loose and more easily dislodged. Take extra precaution when removing supernatant to avoid cell loss. Gently flick the tube several times to resuspend the pellet in the residual volume.

28. Collect the Wash Buffer from the collection funnel and add it to the loosened cell pellet.
29. Centrifuge the tube at 500 x g for 5 minutes 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 Wash Buffer to the tube with pooled cells.

Split 2: Ligation

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

Material	Cap Color	Place at:	Brief Vortex	Brief Spin
384-well Ligation Barcode Plate	-	On ice	✗	✓
Ligation Buffer Conc.	Blue	On ice	✓	✓
Ligation Enzyme Mix	Blue	On ice	✗	✓
Wash Buffer	-	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 4. Ligation Master Mix formulation

Reagent	Volume (µL)
Pooled cells	3,082
Ligation Buffer Conc.	460
Ligation Enzyme Mix	138
Total volume	3,680

2. Using a P1000 fitted with a wide-bore tip, pipette 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 Ligation Barcode Plate.
4. Using a multichannel pipette, add 8 µL of Ligation Master Mix to each well of **half** of the Ligation Barcode Plate 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 Ligation Barcode Plate.
6. Seal the Ligation Barcode Plate 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 Ligation Barcode Plate for 30 minutes at room temperature.

10. Place the Ligation Barcode Plate on ice and incubate until top of the plate is fully chilled. This may take up to 5 minutes.
11. Check the bottom of the Ligation Barcode Plate for debris and moisture. If debris or moisture is present, wipe the bottom of the plate with a clean Kimwipe coated in 70% ethanol, followed by a dry Kimwipe until the plate bottom is fully dry.
12. Unpack a Scale collection funnel and place on ice. Do not touch the inside of the funnel.
13. Unseal the Ligation Barcode Plate, place the collection funnel on top, and quickly invert the assembly.
14. Centrifuge the collection funnel with an appropriately weighted balance in tabletop centrifuge at 300 x g for 3 minutes at 4°C.
15. Transfer pooled material from collection funnel into a 5-mL Eppendorf tube and place on ice.
16. Add 5 mL of cold Wash Buffer 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.
17. Centrifuge the 5-mL tube containing the pooled material from the Ligation Barcode Plate at 500 x g for 5 minutes at 4°C.
18. Carefully remove supernatant without disturbing the pellet, leaving ~50 µL of residual volume as shown in the previous section.

Note: After centrifugation, the pellet may be loose and more easily dislodged. Take extra precaution when removing supernatant to avoid cell loss.

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

Split 3: Final Distribution

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

Material	Place at:
Wash Buffer	On ice
Cell counting dye	Variable
Final Distribution Plate	On ice

Before you begin:

- Fully chill a 96-well metal block 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 recommended for your cell counting method. Perform cell counts in duplicate, adding additional cell counts if the measurements are >20% different.
2. Dilute cells in Wash Buffer 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 1,600 cells per well.
3. Gently pipette mix the tube of cells and distribute 4 µL of the suspension to all the wells of the 96-well Final Distribution Plate using a multi-channel pipette.
4. Seal the plate, spin briefly and place on ice.

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, Cleanup Enzyme Digestion

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

Material	Cap Color	Place at:	Brief Vortex	Brief Spin
96-well Final Distribution Plate	-	On ice	✗	✓
Nuclease-free water	-	On ice	-	-
Second Strand Buffer Conc.	Purple	On ice	✓	✓
Second Strand Enzyme Mix	Purple	On ice	✗	✓
Cleanup Enzyme	Brown	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 5. Second Strand Synthesis (2SS) Master Mix formulation

Reagent	Volume (µL)
Nuclease-free water	35
Second Strand Buffer Conc.	70
Second Strand Enzyme Mix	35
Total volume	140

2. Thoroughly and gently pipette mix the 2SS 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 Mix 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.

Note: If volume of aliquots become hard to work with while distributing, quickly centrifuge to collect full volume at the bottom of the strip tube.

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 stopping 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 Cleanup Enzyme 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 Cleanup Enzyme to each well of Final Distribution Plate.

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

Note: If volume of aliquots become hard to work with while distributing, quickly centrifuge to collect full volume at the bottom of the strip tube.

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.
15. Incubate the plate at 37°C (85°C heated lid) for 60 minutes followed by 75°C for 20 minutes with a 4°C hold.

Table 6. Cleanup Enzyme Digestion (Lid = 85°C)

Temperature	Time
37°C	60 min
75°C	20 min
4°C	∞

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

16. 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 before starting this protocol section:

Material	Cap Color	Place at:	Brief Vortex	Brief Spin
Nuclease-free water	-	On ice	✓	✓
Tagment Buffer Conc.	Red	On ice	10 sec at max speed*	✓
Tagment Enzyme Mix	Red	On ice	10 sec at max speed*	✓
Tagment Stop Solution	Black	Room Temp	✓	✓
Index PCR Additive	Orange	On ice	✗	✓
Adaptor Primer	Orange	On ice	✓	✓
Index PCR Enzyme Mix	Orange	On ice	✗	✓
Index PCR Barcode Plate	-	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 7. Tagmentation Master Mix formulation

Reagent	Volume (µL)
Tagment Buffer Conc.	444
Tagment Enzyme Mix	156
Total volume	600

2. Mix Tagmentation Master Mix by vortexing for 10 seconds 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 Cleanup Enzyme-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 minutes (65°C heated lid). Afterwards, place the plate on the benchtop.
9. Vortex the Tagment Stop Solution for 10 seconds at maximum speed and briefly spin down.
10. 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.
11. 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.

12. Shake the plate for 30 seconds at 2,000 rpm.
13. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.
14. Incubate at 55°C for 15 minutes (65°C heated lid). Afterwards, place the plate on ice.
15. 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 9. Index PCR Master Mix formulation

Reagent	Volume (µL)
Nuclease-free water	264
Index PCR Additive	240
Adaptor Primer	24
Index PCR Enzyme Mix	2,400
Total Volume	2,928

16. Using a P1000 fitted with a wide-bore tip, pipette mix the Index PCR Master Mix until the solution is homogeneous.
17. Distribute Index PCR Master Mix into each tube of an 8- or 12-tube strip, using the volumes below:
    a. For an 8-tube strip, aliquot **176** µL into each tube for distribution to half of the Final Distribution Plate. After the first distribution, add another **176** µL to each tube of the 8-tube strip for distribution to the second half of the Final Distribution Plate.
    b. For a 12-tube strip, aliquot **235** µL into each tube.
18. Using a multichannel pipette add 24.4 µL of Index PCR Master Mix to each well of the Final Distribution Plate.
19. Using a multichannel pipette, add 2 µL of primers from the 96-well Index PCR Barcode Plate to the corresponding well of the Final Distribution Plate.
20. Seal the Final Distribution Plate and place on plate shaker.
21. Shake the plate for 30 seconds at 2,000 rpm.
22. Briefly spin down the plate at 100 x g for 30 seconds at 4°C.

23. Place the Final Distribution Plate on a thermocycler and run the following program with 105°C lid and 40 µL volume:

Table 9. Index PCR thermal cycling program (Lid = 105°C)

Temperature	Duration	Cycles
70°C	5 min	1
98°C	30 sec	1
98°C	10 sec	14 cycles
63°C	30 sec	
72°C	1 min	
72°C	3 min	1
4°C	∞	

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

Index PCR Purification and QC

Instructions are given below for purification of a single library pool from all wells from the Final Distribution Plate. Users may wish to sequence a subset of wells (representing a proportion of the total cells from the workflow) before proceeding to sequencing the entire cell library pool. For purification of a subset of wells from the Final Distribution Plate, see the instructions outlined in **Appendix A: Index PCR Purification of Individual Wells from the Final Distribution Plate**.

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

Material	Place at:
SPRIselect beads	Room Temp
Pure ethyl alcohol	Room Temp
Nuclease-free water	Room Temp
Elution Buffer	Room Temp
Qubit dsDNA HS Assay Kit	Room Temp
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	Room Temp
NEBNext Library Quant Kit for Illumina	4°C

Before you begin:

- Prepare 3 mL fresh 80% ethanol.

Procedure:

1. Pool 5 µL from each well of the Final Distribution Plate into a 1.5 mL tube for cleanup and sequencing, changing tips between each well. If pooling wells using a multichannel pipette, follow the guidance below:

    a. Using an 8-channel multichannel pipette, pipette 6 µL from each column into an 8-tube strip, changing tips between each column. Pipette 60 µL from each of the 8-strip tubes to a 1.5-mL tube.
    b. Using a 12-channel multichannel pipette, pipette 6 µL from each row into a 12-tube strip, changing tips between each row. Pipette 40 µL from each of the 12-strip tubes to a 1.5-mL tube.

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

Note: Optional instructions for further purification of 30 µL from individual wells are outlined in Appendix A: Index PCR Purification of Individual Wells from the Final Distribution Plate.

2. Vortex the SPRI beads at high speed for 1 minute. 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 minutes.
6. Briefly spin the tube and place on a magnetic stand for 5 minutes.
7. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
8. Keep the tube on the magnetic stand and add 1 mL of 80% ethanol to the side of the tube opposite the pellet.
9. Incubate the tube on the magnetic stand for 30 s.
10. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
11. Repeat the 1 mL 80% ethanol wash (steps 8–10) for a total of two washes.
12. Briefly spin the tube to collect residual 80% ethanol at the bottom of the tube and place tube back on the magnetic stand.
13. Remove residual 80% ethanol being careful not to disturb the beads.
14. Air dry the beads for 2 minutes or until the bead pellet appears matte instead of glossy but not cracked.
15. Remove the tube from the magnetic stand and add 50 µL Elution Buffer.
16. Vortex to mix and briefly spin down to collect liquid at the bottom of the tube.
17. Incubate the tube off the magnetic stand for 5 minutes and then briefly spin down.
18. Place the tube on a magnetic stand until the solution is clear.
19. Transfer the supernatant to a new 0.2 mL PCR tube for second sequential purification.
20. Vortex the SPRI beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
21. Transfer 40 µL of SPRIselect beads (0.8X) to the tube containing the purified library.
22. Vortex to mix.
23. Incubate at room temperature for 5 minutes.
24. Briefly spin the tube and place on a magnetic stand for 5 minutes.
25. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
26. Keep the tube on the magnetic stand and add 200 µL of 80% ethanol to the side of the tube opposite the pellet.
27. Incubate the tube on the magnetic stand for 30 seconds.
28. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
29. Repeat the 200 µL 80% ethanol wash (steps 26–28) for a total of two washes.
30. Briefly spin the tube to collect residual 80% ethanol at the bottom of the tube and place tube back on the magnetic stand.
31. Remove residual 80% ethanol being careful not to disturb the beads.
32. Air dry the beads for 2 minutes or until the bead pellet appears matte instead of glossy but not cracked.
33. Remove the tube from the magnetic stand and add 30 µL Elution Buffer.

34. Vortex to mix and briefly spin down to collect liquid at the bottom of the tube.
35. Incubate the tube off the magnetic stand for 5 minutes and then briefly spin down.
36. Place the tube on a magnetic stand until the solution is clear.
37. Transfer the supernatant to a fresh tube.
38. 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.
39. Determine library concentration for sequencing with *NEBNext Library Quant Kit for Illumina* according to manufacturer's protocol. Perform library dilution and clustering according to sequencing manufacturers parameters.

Sequencing Parameters

We recommend a minimum sequencing depth 20,000 read pairs per cell.

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

Final sequencing-ready library product:

Appendix A: Index PCR Purification of Individual Wells from the Final Distribution Plate

Users may wish to sequence a small number of wells (representing a proportion of the total cells from the workflow), to ensure the library is of sufficient quality before sequencing the entire output of the Final Distribution Plate. We recommend users dedicate a minimum of 3,000 reads per cell for shallow sequencing with accurate cell calling.

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

Material	Place at:
SPRIselect beads	Room Temp
Pure ethyl alcohol	Room Temp
Nuclease-free water	Room Temp
Qubit dsDNA HS Assay Kit	Room Temp
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	Room Temp
NEBNext Library Quant Kit for Illumina	4°C

Before you begin:

- Prepare 1 mL of fresh 80% ethanol for each well being purified.

Procedure:

1. Transfer 30 µL from one well of the Final Distribution Plate into a 0.2 mL PCR tube and record the well number used.
2. Vortex the SPRI beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
3. Transfer 24 µL of SPRIselect beads (0.8X) to the tube containing Index PCR products.
4. Vortex to mix.
5. Incubate at room temperature 5 minutes.
6. Briefly spin to collect contents and place the tube on a magnetic stand for 5 minutes.
7. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
8. Keep the tube on the magnetic stand and add 200 µL of 80% ethanol to the side of the tube opposite the pellet.
9. Incubate the tube on the magnetic stand for 30 seconds.
10. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
11. Repeat the 200 µL 80% ethanol wash (steps 8-10) for a total of two washes.
12. Briefly spin the tube to collect residual 80% ethanol at the bottom of the tube and place tube back on the magnetic stand.
13. Remove residual 80% ethanol being careful not to disturb the beads.

14. Air dry the beads for 2 minutes or until the bead pellet appears matte instead of glossy but not cracked.
15. Remove the tube from the magnetic stand and add 50 µL nuclease-free water.
16. Vortex to mix and briefly spin down to collect liquid at the bottom of the tube.
17. Incubate the tube off the magnetic stand for 5 minutes and then briefly spin down.
18. Place the tube on a magnetic stand until the solution is clear.
19. Transfer the supernatant to a new 0.2 mL PCR tube for second sequential purification.
20. Vortex the SPRI beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
21. Transfer 40 µL of SPRIselect beads (0.8X) to the tube containing the purified library.
22. Vortex to mix.
23. Incubate at room temperature for 5 minutes.
24. Briefly spin the tube and place on a magnetic stand for 5 minutes.
25. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
26. Keep the tube on the magnetic stand and add 200 µL of 80% ethanol to the side of the tube opposite the pellet.
27. Incubate the tube on the magnetic stand for 30 seconds.
28. Once the solution is clear, remove and discard the supernatant, being careful not to disturb the beads.
29. Repeat the 200 µL 80% ethanol wash (steps 26–28) for a total of two washes.
30. Briefly spin the tube to collect residual 80% ethanol at the bottom of the tube and place tube back on the magnetic stand.
31. Remove residual 80% ethanol being careful not to disturb the beads.
32. Air dry the beads for 2 minutes or until the bead pellet appears matte instead of glossy but not cracked.
33. Remove the tube from the magnetic stand and add 15 µL nuclease-free water.
34. Vortex to mix and briefly spin down to collect liquid at the bottom of the tube.
35. Incubate the tube off the magnetic stand for 5 minutes and then briefly spin down.
36. Place the tube on a magnetic stand until the solution is clear.
37. Transfer the supernatant to a fresh tube.
38. 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.
39. Determine library concentration for sequencing with *NEBNext Library Quant Kit for Illumina* according to manufacturer's protocol. Perform library dilution and clustering according to sequencing manufacturers parameters.