Single Cell

RNA Sequencing Kit v1.1

Protocol

For Research Use Only.

Legal Notices

Document 1020796, Rev C, Jul 2024
© 2024 Scale Biosciences, Inc.

3210 Merryfield Row
San Diego, CA 92121, United States
https://scale.bio/
support@scale.bio

Scale Biosciences, Inc ("ScaleBio"). All rights reserved. No part of this document may be reproduced, distributed, or transmitted in any form or by any means, electronic or mechanical, including photocopying, recording, or any information storage and retrieval system, without the prior written permission of ScaleBio. This document is provided for information purposes only and is subject to change or withdrawal by ScaleBio at any time.

Disclaimer of Warranty:
TO THE EXTENT PERMITTED BY APPLICABLE LAW, SCALEBIO PROVIDES THIS DOCUMENT "AS IS" WITHOUT WARRANTY OF ANY KIND, INCLUDING WITHOUT LIMITATION, ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, OR NONINFRINGEMENT. IN NO EVENT WILL SCALEBIO BE LIABLE TO YOU OR ANY THIRD PARTY FOR ANY LOSS OR DAMAGE, DIRECT OR INDIRECT, FROM THE USE OF THIS DOCUMENT, INCLUDING WITHOUT LIMITATION, LOST PROFITS, LOST INVESTMENT, BUSINESS INTERRUPTION, GOODWILL, OR LOST DATA, EVEN IF SCALEBIO IS EXPRESSLY ADVISED IN ADVANCE OF THE POSSIBILITY OF SUCH LOSS OR DAMAGE. Any warranties applicable to the ScaleBio products are set forth in the Terms and Conditions accompanying such product and such Terms and Conditions are not modified in any way by the terms of this notice.

Trademark Information:
ScaleBio may make reference to products or services provided by other companies using their brand names or company names solely for the purpose of clarity, and does not assert any ownership rights over those third-party marks or names. Images were created with BioRender.com

Patent Information:
ScaleBio products may be covered by one or more patents as indicated at: https://scale.bio/legal-notice/

Terms and Conditions:
The use of the ScaleBio products described herein is subject to ScaleBio's Terms and Conditions that accompany the product, or such other terms as have been agreed to in writing between ScaleBio and the user.

Intended Use:
All products and services described herein are intended FOR RESEARCH USE ONLY and NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Table of Contents

Required Materials

ScaleBio Single Cell RNA Sequencing Kit v1.1 (PN 950884) Consumables and Reagents:

Kit Module	Consumable	Part Number	Qty	Cap Color	Storage Temp
RNA Kit RT Module (PN 2020004)	Wash Buffer	202100002	1	-	-20°C
	RT Barcode Plate	202110002	1	-	-20°C
	RT Enzyme Mix	202110003	1	Green	-20°C
	RT Buffer Conc.	202110004	1	Green	-20°C
	RT Additive	202110005	1	Green	-20°C
RNA Kit Ligation Module (PN 2020005)	Ligation Barcode Plate	202110006	1	-	-20°C
	Ligation Enzyme Mix	202110007	1	Blue	-20°C
	Ligation Buffer Conc.	202110008	1	Blue	-20°C
RNA Kit Tagment and i5 Index PCR Module (PN 935932)	Second Strand Buffer Conc.	202110009	1	Purple	-20°C
	Second Strand Enzyme Mix	202110010	1	Purple	-20°C
	Cleanup Enzyme v1.1	935999	1	Brown	-20°C
	Tagment Buffer Conc.	202100003	1	Red	-20°C
	Tagment Enzyme Mix	202100004	1	Red	-20°C
	Index PCR Enzyme Mix	202110012	2	Orange	-20°C
	Adaptor Primer i7-1 Tube	936006	1	Orange	-20°C
	Adaptor Primer i5 Barcode Plate	936013	1	-	-20°C
	Elution Buffer	202110014	1	Clear	-20°C
RNA Kit Workflow Consumables Module (PN 2020007)	Index PCR Additive	202110016	1	Orange	RT
	Tagment Stop Solution	202110017	1	Black	RT
	Final Distribution Plate	202110018	1	-	RT
	ScaleBio Sample Collection Funnel	202100005	2	-	RT

Consumables and reagents manufactured by other vendors:

Consumable or reagent	Supplier	Part Number
Nuclease-free water	Various	Various
Ethanol (pure)	Various	Various
SPRIselect	Beckman Coulter	B23317
Cell counting dye	Various	Various
Pipette tips (nuclease-free, filtered, low retention for P1000, P200, P20) *	Various	Various
0.2-mL PCR tube strips (nuclease-free)	Various	Various
1.5-mL DNA LoBind tubes *	Eppendorf	0030108418
5-mL DNA LoBind tubes *	Eppendorf	0030108310
Microseal 'B' PCR Plate Sealing Film	Bio-Rad	MSB1001
Agilent High Sensitivity DNA Kit for Bioanalyzer 2100 (or equivalent kit for alternative fragment analyzer) **	Agilent	5067-4626
NEBNext Library Quant Kit for Illumina **	NEB	E7630

* Required for best assay performance.

** Required for quality control of library.

Equipment List:

Item	Supplier	Part Number
Pipettes (P1000, P200, P20, P10, P2)	Various	Various
Multi-channel pipettes (P100 or P200, P10)	Various	Various
Magnetic stand for 1.5-mL tubes	Various	Various
Magnetic stand for 0.2-mL strip tubes	Various	Various
96-well aluminum cooler blocks	Various	Various
Vortex mixer	Various	Various
Shaker for semi-skirted 96-well plates (2000 rpm, e.g. ThermoMixer) *	Various	Various
Cell counter	Various	Various
Centrifuge with temperature control and swing-bucket rotor (1.5-mL tubes, 96-well deep well plates)	Various	Various
Thermocycler with lid temperature control (0-105°C) for semi-skirted 96-well plates	Various	Various
2100 Bioanalyzer Instrument (or equivalent system) **	Agilent	G2939BA
qPCR machine **	Various	Various

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

** Required for quality control of library.

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.
- Dedicate separate laboratory workspaces to pre-amplification process and post-amplification process.
- Routinely wipe work surfaces with a 10% bleach solution to remove DNA amplicon contaminants.
- We strongly recommend against the use of plate mixers with foam attachments. Foam particulates can break off, attach to well plates, and potentially interfere with the assay.

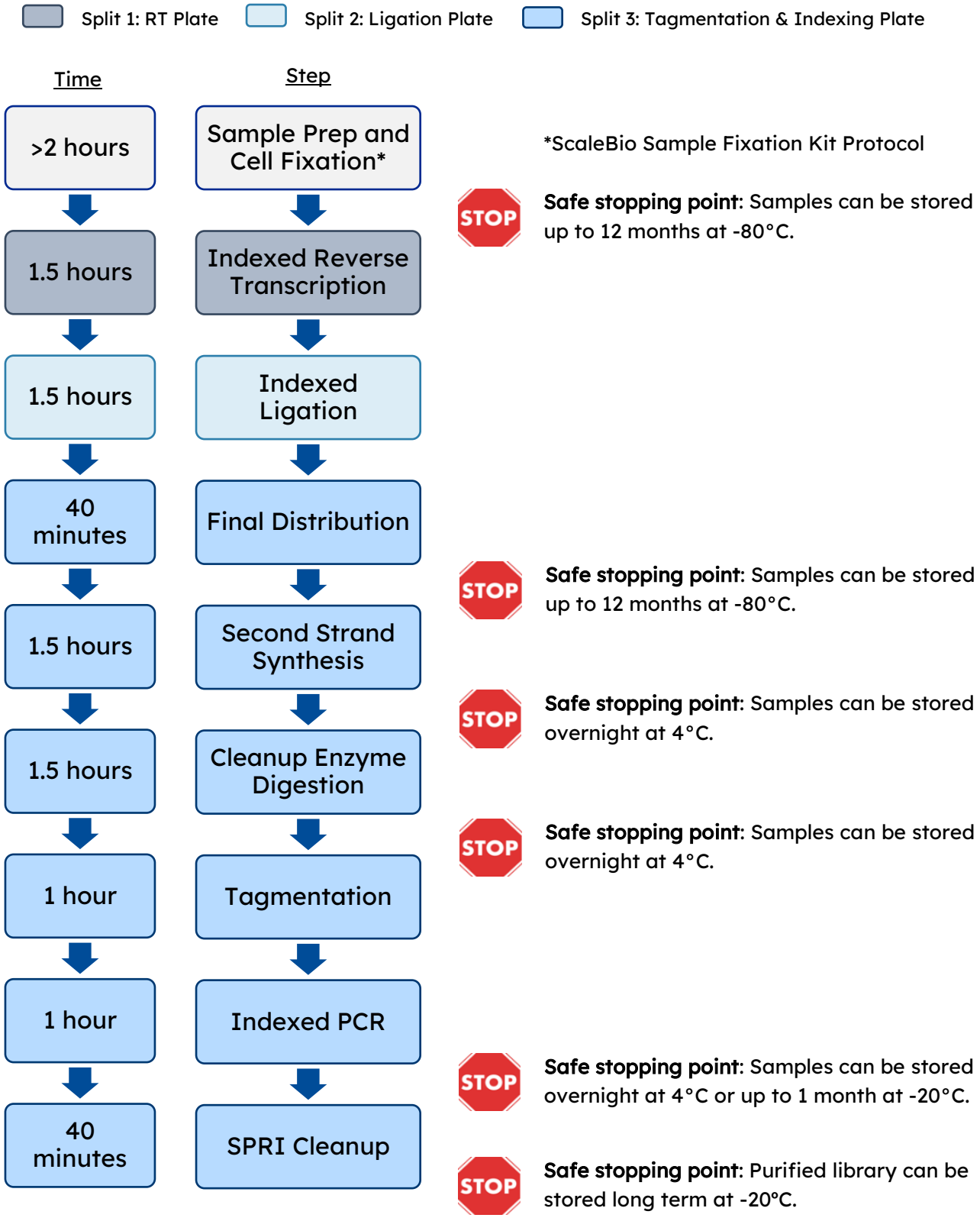
For RNase-free sample processing:

- 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.
- Wear disposable gloves and change them frequently.

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.

Workflow Diagram



Assay Introduction

The ScaleBio™ Single Cell RNA Sequencing Kit v1.1 provides an instrument-free workflow for the analysis of gene expression at the single cell level. The 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%. It enables multiplexing of up to 96 samples from multiple sources and can be performed from start to finish in just two days.

Additional kits and protocols are needed for customizing the workflow (i.e. using custom primers), or for increasing the number of recovered cells (i.e. up to 500,000 cells) as shown in Figure 1. For assistance with these additional workflows, please contact your local Field Application Scientist or support@scale.bio.

Figure 1: Overview of Protocols

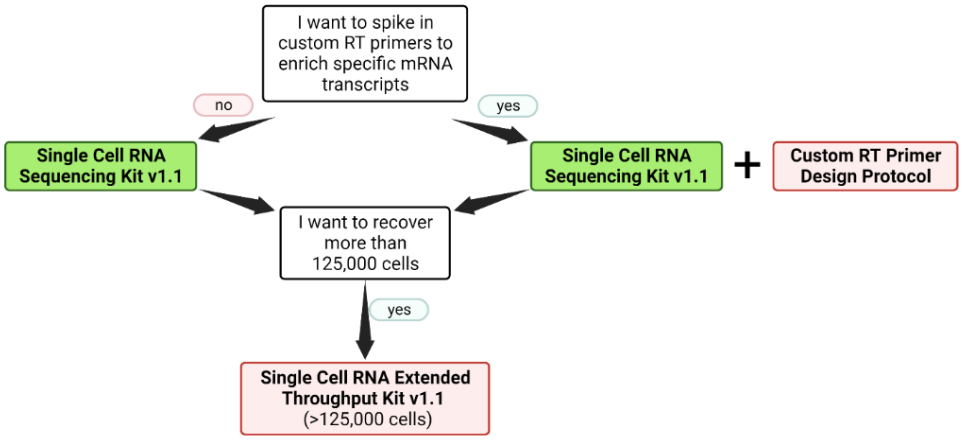
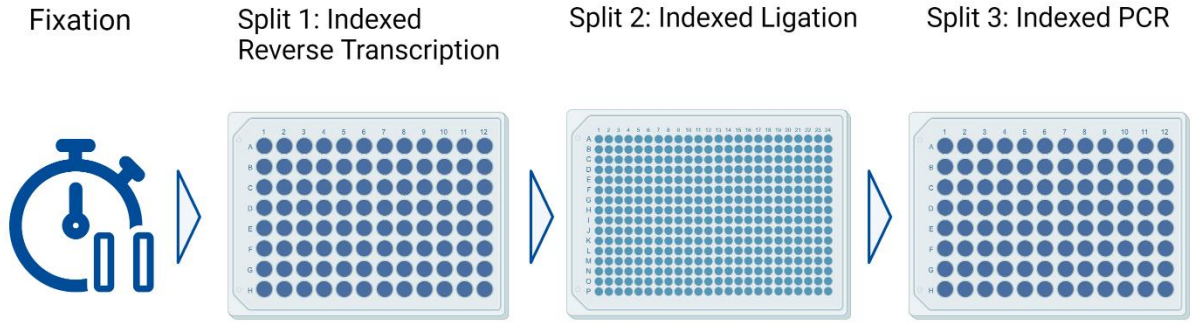


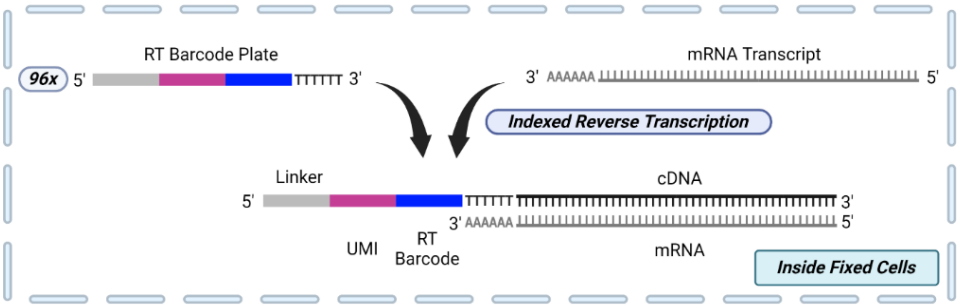
Figure 2: Overall procedure



Upstream fixation of single cell suspensions with the ScaleBio Sample Fixation Kit accommodates an input of 400,000 to 2.5 million cells per sample. Fixed samples can be processed directly into the RNA assay or stored at -80°C until ready for use.

In the ScaleBio Single Cell RNA Sequencing Kit v1.1 protocol, fixed cells are distributed across the 96-well RT Barcode Plate, using 10,000 cells per well for cDNA synthesis and the addition of 96 unique RT Barcodes (one unique barcode per well).

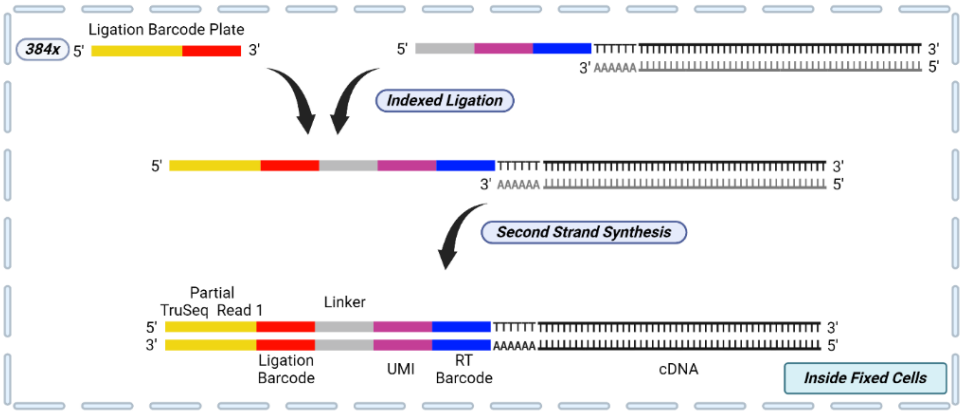
Figure 3: Split 1 – Indexed Reverse Transcription



Cells are pooled by centrifugation using the provided ScaleBio Sample Collection Funnel. Pooled cells are then distributed across the 384-well Ligation Barcode Plate for addition of ligation adaptors containing the TruSeq Read 1 sequence as well as 384 unique Ligation Barcodes (one unique barcode per well).

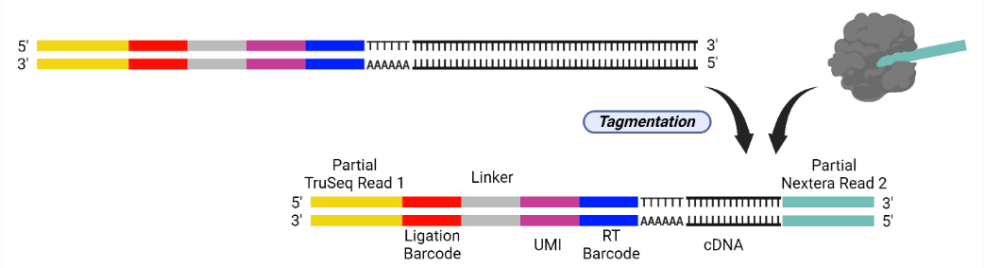
Cells are pooled again with a ScaleBio Sample Collection Funnel and distributed across the 96-well Final Distribution Plate. Second strand synthesis is then performed, followed by a cleanup enzyme digestion step to break down cells.

Figure 4: Split 2 – Indexed Ligation and Second Strand Synthesis



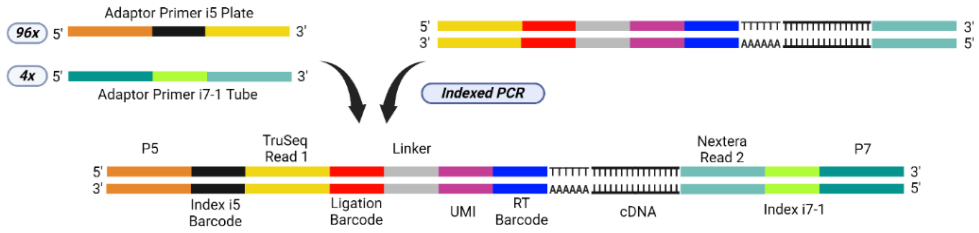
This is followed by tagmentation to attach the Nextera Read 2 sequence for subsequent amplification of the mRNA transcripts.

Figure 5: Split 3 – Tagmentation



An indexed PCR is performed to add 96 unique Index i5 Barcodes (one unique barcode per well). The i7 Index contains a pool of four different indices for increased nucleotide diversity and color balancing during sequencing.

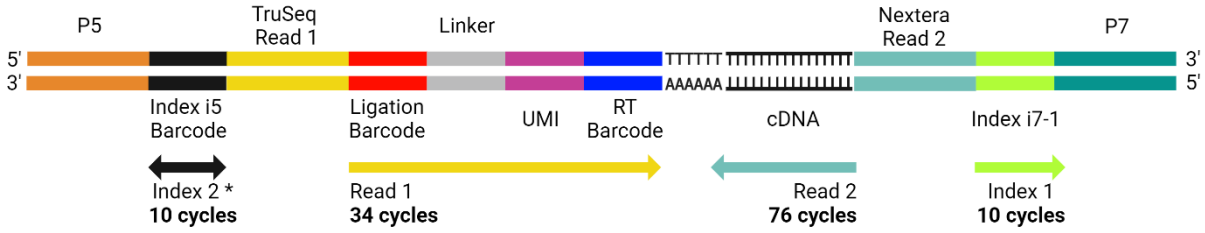
Figure 6: Indexed PCR



ScaleBio single cell libraries are compatible with standard Illumina sequencing. The ScaleBio bioinformatics pipeline (ScaleBio Seq Suite) can be used to both demultiplex and assign reads to individual cells. The algorithm output includes basic single-cell RNA sequencing QC metrics and files required for further secondary and tertiary downstream analysis.

Please note that this protocol is to be performed with the ScaleBio Single Cell RNA Sequencing Kit v1.1 and must use the ScaleBio Seq Suite RNA Data Analysis Pipeline **version 1.4** or later.

Figure 7: Final RNA Library Structure



* orientation depends on sequencer and sequencing chemistry

Step 1: Initial Distribution and Reverse Transcription

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
ScaleBio Sample Fixation Kit	Fixed cell samples	-	-80°C	On ice	✗	✗
RT Module	RT Barcode Plate	-	-20°C	On ice	✗	✓
	RT Enzyme Mix	Green	-20°C	On ice	✗	✓
	RT Buffer Conc.	Green	-20°C	On ice	✓	✓
	RT Additive	Green	-20°C	On ice	✓	✓
	Wash Buffer *	-	-20°C	RT, then ice	-	-
Workflow Consumables Module	ScaleBio Sample Collection Funnel	-	RT	RT	-	-
Other Vendors	Cell counting dye 5-mL DNA LoBind Eppendorf tubes Microseal 'B' PCR Plate Sealing Film					

* Can be thawed at 4°C overnight prior to starting the workflow.

Before you begin:

- If frozen, thaw cells on ice. Do not vortex.
- Once thawed, invert the Wash Buffer to ensure it is fully mixed.
- Fully chill a 96-well metal block on ice.
- Bring centrifuges that accommodate 96-well plates and 5-mL Eppendorf tubes to 4°C.
- Start Program 1 on a thermocycler and hold at 55°C with a lid temperature of 65°C.

Procedure:

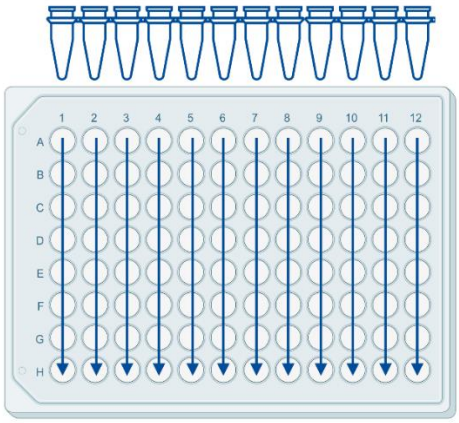
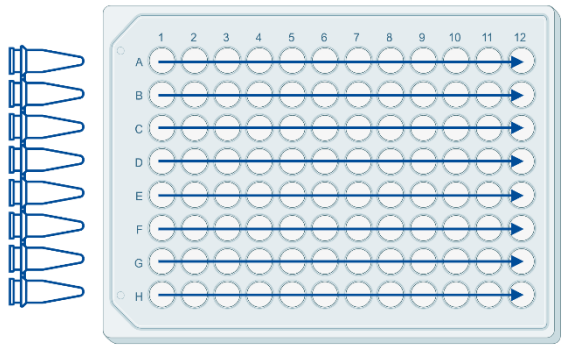
1. Briefly spin down the thawed **RT Barcode Plate** and place on a 96-well metal block on ice.
2. Determine the concentration of the cell suspension prepared with the ScaleBio Sample Fixation Kit using cell counting equipment. Keep the cell suspension **on ice**.

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. 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.

3. **On ice**, flick the cells gently and dilute with Wash Buffer to achieve a concentration of **2000 cells per µL**. Gently pipette mix the cells and Wash Buffer.

Note: 5 µL of diluted cell suspension (total of ~10,000 cells) will be distributed into each well of the **RT Barcode Plate** in the following step. Calculate the total volume of cell suspension to be distributed with ~20% overage. To prepare a full plate distribution, use Figure 8 as guidance for cell calculation and distribution.

Figure 8: Dispensing guidelines for distribution into RT Barcode Plate

For distribution into columns, using a 12-channel multichannel pipette and a 12-tube strip	
• Prepare cell suspension in a 12-tube strip by adding **50 µL** of the diluted cell suspension to each tube (includes 25% overage). Distribute **5 µL** of cells to each well from this stock by using a 12-channel pipette.	
For distribution into rows using an 8-channel multichannel pipette and an 8-tube strip	
• Prepare cell suspension in an 8-tube strip by adding **75 µL** of the diluted cell suspension to each tube (includes 25% overage). Distribute **5 µL** of cells to each well from this stock by using an 8-channel pipette.	

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.
6. Shake the plate at **2000 rpm** for **30 seconds**.
7. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.

8. Incubate the RT Barcode Plate in a **pre-heated** thermocycler according to Program 1.

Program 1: Annealing of RT Barcode

Lid Temperature	Reaction Volume
65°C	7 µL
Temperature	**Time**
55°C	∞
Skip the HOLD step after placing the plate in the thermocycler.	
55°C	5 min

Note: Annealing time is critical for this step. Pre-heat the thermocycler to 55°C **in advance** and set up a timer for 5 minutes. Place the plate into the pre-heated thermocycler, then start the timer immediately.

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. Start Program 2 on a thermocycler and **hold at 4°C** with a lid temperature of 65°C.
12. **On ice**, prepare the Reverse Transcription Master Mix according to Table 1.

Table 1: Reverse Transcription Master Mix

Reagent	Volume (µL)
RT Buffer Conc.	240
RT Additive	60
RT Enzyme Mix	60
Total volume	**360**

13. Gently pipette mix the RT Master Mix until the solution is homogeneous and briefly spin down.
14. **On ice**, aliquot the RT Master Mix into each tube of an 8- or 12-tube strip:
    a. For an **8-tube strip**, aliquot **42 µL** into each tube.
    b. For a **12-tube strip**, aliquot **28 µL** into each tube.
15. 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.

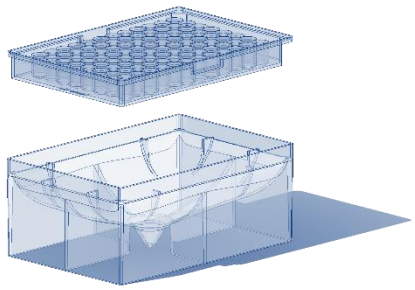
16. Seal the RT Barcode Plate and place on a plate shaker.
17. Shake the plate at **2000 rpm** for **30 seconds**.
18. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C** and place **on ice**.
19. Incubate the RT Barcode Plate in a **pre-cooled** thermocycler according to Program 2.

Program 2: Reverse Transcription

Lid Temperature	Reaction Volume
65°C	10 µL
Temperature	**Time**
4°C	∞
Skip the HOLD step after placing the plate in the thermocycler.	
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	∞

20. Once the thermocycler program is completed, 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. Shake the plate at **2000 rpm** for **30 seconds.**
22. Unpack the ScaleBio Sample Collection Funnel and place **on ice**. **Do not touch the inside of the collection funnel**.
23. Unseal the RT Barcode Plate, place the collection funnel on top, and quickly invert the assembly according to Figure 9.

Figure 9: ScaleBio Sample Collection Funnel and Plate Assembly



24. Centrifuge the collection funnel with an appropriately weighted balance at **400 x g** for **3 minutes** at **4°C**.

Caution: After centrifugation, a cell pellet may or may not be visible in the bottom of the funnel well. Resuspend the pooled material gently with a P1000 pipette before transfering the material.

25. Transfer pooled material **(~1 mL)** from the collection funnel into a **5-mL DNA LoBind** Eppendorf tube and place **on ice**.

26. Add **5 mL** of Wash Buffer to the now empty collection funnel, rinsing the sides of the collection funnel 2-3 times to collect the residual liquid on the collection funnel into the center. **Leave the Wash Buffer** in the collection funnel on ice; this volume will be used in step 30.
27. Centrifuge the 5-mL tube containing the pooled material from the RT Barcode Plate at **500 x g** for **8 minutes** at **4°C**.
28. 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.

29. Gently flick the tube several times to resuspend the pellet in the residual volume.
30. Collect the Wash Buffer from the collection funnel and add it to the loosened cell pellet.
31. Place the inverted RT Barcode Plate over the collection funnel.
32. Centrifuge the collection funnel with an appropriately weighted balance at **400 x g** for **1 minute** at **4°C.**
33. Collect any remaining Wash Buffer from the collection funnel and add it to the 5 mL tube.
34. Centrifuge the tube at **500 x g** for **8 minutes** at **4°C**.
35. Carefully remove supernatant without disturbing the pellet, leaving **~50 µL** of residual volume as shown above.
36. Gently flick the tube several times to resuspend the pellet in the residual volume.
37. Add **3000 µL** of ice-cold Wash Buffer to the tube with pooled cells.
38. Proceed directly to *Step 2: Ligation*.

Step 2: Ligation

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
Step 1: Initial Distribution and Reverse Transcription	Wash Buffer	-	On ice	On ice	-	-
Ligation Module	Ligation Barcode Plate	-	-20°C	On ice	✗	✓
	Ligation Buffer Conc.	Blue	-20°C	On ice	✓	✓
	Ligation Enzyme Mix	Blue	-20°C	On ice	✗	✓
Workflow Consumables Module	ScaleBio Sample Collection Funnel	-	RT	RT	-	-
Other Vendors	Microseal 'B' PCR Plate Sealing Film					

Procedure:

1. Briefly spin down the thawed **Ligation Barcode Plate** and place on ice.
2. **On ice**, prepare the Ligation Master Mix by adding the components to the pooled and washed cells in the specified order according to Table 2.

   Table 2: Ligation Master Mix

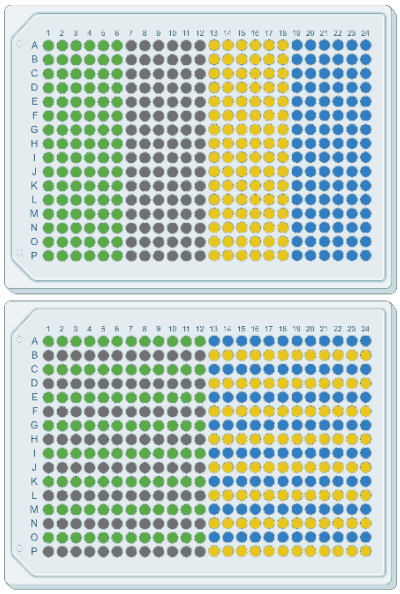
Reagent	Volume (µL)
Pooled cells	3050
Ligation Buffer Conc.	450
Ligation Enzyme Mix	135
Total volume	3635

3. Using a P1000 pipette tip, **gently** pipette mix the Ligation Master Mix until the solution is homogeneous and quickly proceed to the next step.
4. 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 **215 µL** into each tube.
   b. For a **12-tube strip**, aliquot **145 µL** into each tube.

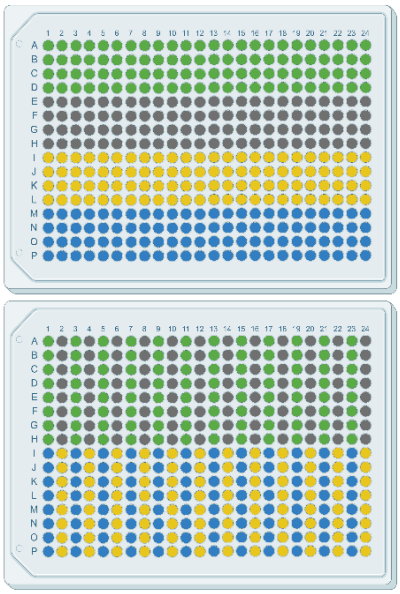
   This is enough volume for distribution for half of the Ligation Barcode Plate.

Figure 10: Schematic examples for loading 384-well plate. Each color corresponds to one full tip box, for a total of four tip boxes needed to load the full plate.

8-channel multichannel pipette



12-channel multichannel pipette



5. 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.

6. Repeat steps 2-4, 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.
7. Seal the Ligation Barcode Plate and place on a plate shaker.
8. Shake the plate at **2000 rpm** for **30 seconds**.
9. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
10. Incubate the Ligation Barcode Plate on the bench according to Program 3.

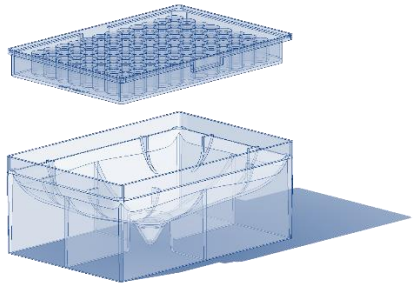
Program 3: Ligation

Lid Temperature	Reaction Volume
OFF	10 µL
Temperature	**Time**
Room temperature (18-25°C)	30 min

Note: The ligation time can be extended from 30 minutes up to 2 hours with no negative impact on the assay performance.

11. Place the Ligation Barcode Plate on ice and incubate until top of the plate is fully chilled. This may take **up to 5 minutes**.
12. 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.
13. Shake the plate at **2000 rpm** for **30 seconds**.
14. Unpack a ScaleBio Sample Collection Funnel and **place on ice**. **Do not touch the inside of the collection funnel**.
15. Unseal the Ligation Barcode Plate, place the collection funnel on top, and quickly invert the assembly according to Figure 11.

Figure 11: ScaleBio Sample Collection Funnel and Plate Assembly



16. Centrifuge the collection funnel with an appropriately weighted balance at **400 x g** for **3 minutes** at **4°C**.

Caution: After centrifugation, a cell pellet may or may not be visible in the bottom of the funnel well. Resuspend the pooled material gently with a P1000 pipette before transfering the material.

17. Transfer pooled material **(~4 mL)** from collection funnel into a **5-mL DNA LoBind** Eppendorf tube and place on ice.
18. Add **5 mL** of cold Wash Buffer to the now empty collection funnel, rinsing the sides of the collection funnel 2-3 times to collect the residual liquid on the collection funnel into the center. **Leave the Wash Buffer** in the collection funnel on ice; this volume will be used in step 22.
19. Centrifuge the 5-mL tube containing the pooled material from the Ligation Barcode Plate at **500 x g** for **8 minutes** at **4°C**.
20. Carefully remove supernatant without disturbing the pellet, leaving **~50 µL** of residual volume.

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

21. Gently flick the tube several times to resuspend the pellet in the residual volume.
22. Collect the Wash Buffer from the collection funnel and add it to the loosened cell pellet.

23. Place the inverted Ligation Barcode Plate over the collection funnel.
24. Centrifuge the collection funnel with an appropriately weighted balance at **400 x g** for **1 minute** at **4°C.**
25. Collect any remaining Wash Buffer from the collection funnel and add it to the 5 mL tube.
26. Centrifuge the tube at **500 x g** for **8 minutes** at **4°C**.
27. Carefully remove supernatant without disturbing the pellet, leaving **~50 µL** of volume.
28. Gently flick the tube several times to resuspend the pellet in the residual volume.
29. Resuspend pellet in **100 µL** of Wash Buffer for a total volume of **~150 µL**.
30. Proceed directly to *Step 3: Final Distribution*.

Step 3: Final Distribution

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
Step 2: Ligation	Wash Buffer	-	On ice	On ice	-	-
Workflow Consumables Module	Final Distribution Plate	-	RT	On ice	-	-
Other Vendors	Cell counting dye Microseal 'B' PCR Plate Sealing Film					

Before you begin:

- Fully chill a 96-well metal block on ice.

Procedure:

1. Determine the concentration of the cell suspension using a 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 all cells in Wash Buffer to achieve a final concentration of **400 cells per µL**.
3. Gently pipette mix the tube of cells and aliquot the suspension into each tube of an 8- or 12-tube strip:
    a. For an **8-tube strip**, aliquot **58 µL** into each tube.
    b. For a **12-tube strip**, aliquot **39 µL** into each tube.
4. Use a multichannel pipette to distribute **4 µL** of the suspension into the bottom of each well of the 96-well Final Distribution Plate, for a total of 1600 cells loaded per well.
5. Seal the Final Distribution Plate.
6. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**, and place on ice.
7. Store the Final Distribution Plate, or proceed to *Step 4: Second Strand Synthesis and Cleanup Enzyme Digestion*.

Note: For the remaining cells that are not distributed into the 96-well plate at this time, we recommend storing the cell suspension in Wash Buffer at 400 cells per µL at -80°C up to 12 months.

Safe stopping point. The Final Distribution Plate can be stored at -80°C overnight, or for up to 12 months, before proceeding with Second Strand Synthesis.

Step 4: Second Strand Synthesis and Cleanup Enzyme Digestion

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
Tagment and i5 Index PCR Module	Second Strand Buffer Conc.	Purple	-20°C	On ice	✓	✓
	Second Strand Enzyme Mix	Purple	-20°C	On ice	✗	✓
	Cleanup Enzyme v1.1	Brown	-20°C	On ice	✓	✓
Other Vendors	Nuclease-free water Microseal 'B' PCR Plate Sealing Film					

Do not re-freeze Cleanup Enzyme v1.1 once it has been thawed. Reagent is stable at 4C up to 24 hours.

Before you begin:

- If frozen, thaw Final Distribution Plate from *Step 3: Final Distribution* on ice and briefly spin down.
- 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 according to Table 3.

Table 3: Second Strand Synthesis Master Mix

Reagent	Volume (µL)
Nuclease-free water	161
Second Strand Buffer Conc.	84
Second Strand Enzyme Mix	35
Total volume	**280**

2. Thoroughly and gently pipette mix the Second Strand Synthesis Master Mix until it is homogeneous, briefly spin down, and place **on ice**.
3. On a pre-chilled metal block **on ice** distribute prepared Second Strand Synthesis Master Mix into each tube of an 8- or 12-strip tube, using the volumes below:
    a. For an **8-tube strip**, aliquot **32 µL** into each tube.
    b. For a **12-tube strip**, aliquot **22 µL** into each tube.
4. Using a multichannel pipette add **2 µL** of the Second Strand Synthesis 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 at **2000 rpm** for **30 seconds**.
7. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
8. Incubate the Final Distribution Plate in a thermocycler according to Program 4.

Program 4: Second Strand Synthesis

Lid Temperature	Reaction Volume
OFF	6 µL
Temperature	**Time**
16°C	60 min
4°C	∞

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 v1.1 into each tube of an 8- or 12-strip tube, using the volumes below:
    a. For an **8-tube strip**, aliquot **32 µL** into each tube.
    b. For a **12-tube strip**, aliquot **22 µL** into each tube.
11. Using a multichannel pipette add **2 µL** of Cleanup Enzyme v1.1 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 at **2000 rpm** for **30 seconds**.
14. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
15. Incubate the Final Distribution Plate in a thermocycler according to Program 5.

Program 5: Cleanup Enzyme Digestion

Lid Temperature	Reaction Volume
85°C	8 µL
Temperature	**Time**
37°C	60 min
75°C	20 min
4°C	∞

16. Briefly centrifuge the plate and place on a pre-chilled metal block **on ice**.
17. Store the plate, or proceed to *Step 5: Tagmentation and Index PCR*.

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

Step 5: Tagmentation and Index PCR

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
Tagment and i5 Index PCR Module	Adaptor Primer i7-1 Tube	Orange	-20°C	On ice	✓	✓
	Index PCR Enzyme Mix	Orange	-20°C	On ice	✗	✓
	Adaptor Primer i5 Barcode Plate	-	-20°C	On ice	✗	✓
	Tagment Buffer Conc.	Red	-20°C	On ice	10 sec, max speed *	✓
	Tagment Enzyme Mix	Red	-20°C	On ice		✓
Workflow Consumables Module	Tagment Stop Solution	Black	RT	RT		✓
	Index PCR Additive	Orange	RT	On ice	✗	✓
Other Vendors	Nuclease-free water Microseal 'B' PCR Plate Sealing Film					

* 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. **On ice**, prepare the Tagmentation Master Mix by combining the components in the order specified according to Table 4.

   Table 4: Tagmentation Master Mix

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. **On ice**, 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 at **2000 rpm** for **30 seconds**.
7. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
8. Incubate the Final Distribution Plate in a thermocycler according to Program 6.

Program 6: Tagmentation

Lid Temperature	Reaction Volume
65°C	13 µL
Temperature	**Time**
55°C	10 min

9. Place the plate on the benchtop and cool down to reach **room temperature**.
10. Vortex the Tagment Stop Solution for **10 seconds** at maximum speed and briefly spin down.
11. **At room temperature**, 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. **At room temperature** and 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. Seal Final Distribution Plate and place on plate shaker.
14. Shake the plate at **2000 rpm** for **30 seconds**.
15. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
16. Incubate the Final Distribution Plate in a thermocycler according to Program 7.

Program 7: Tagmentation Stop

Lid Temperature	Reaction Volume
65°C	15.6 µL
Temperature	**Time**
55°C	15 min

17. Place the plate **on ice**.
18. **On ice**, prepare the Index PCR Master Mix in a **5-mL DNA LoBind** tube by combining the components in the order specified according to Table 5.

Table 5: Index PCR Master Mix

Reagent	Volume (µL)
Index PCR Additive	240
Adaptor Primer i7-1	48
Index PCR Enzyme Mix	2400
Total Volume	**2688**

19. Using a P1000, pipette mix the Index PCR Master Mix until the solution is homogeneous.
20. 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 **162 µL** into each tube for distribution to half of the Final Distribution Plate. After the first distribution, add another **162 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 **215 µL** into each tube.
21. Using a multichannel pipette add **22.4 µL** of Index PCR Master Mix to each well of the Final Distribution Plate.

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

22. Briefly spin down the thawed **Adaptor Primer i5 Barcode Plate** and place on ice.
23. Using a multichannel pipette, transfer **2 µL** from the 96-well **Adaptor Primer i5 Barcode Plate** to the corresponding well of the **Final Distribution Plate**.

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

24. Seal the Final Distribution Plate and place on plate shaker.
25. Shake the plate at **2000 rpm** for **30 seconds**.
26. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C**.
27. Incubate the Final Distribution Plate in a thermocycler according to Program 8.

Program 8: Index PCR

Lid Temperature		Reaction Volume	
105°C		40 µL	
Step	**Temperature**	**Time**	**Cycles**
1	70°C	5 min	1
2	98°C	30 sec	1
3	98°C	10 sec	14 cycles (steps 3-5)
4	63°C	30 sec	
5	72°C	1 min	
6	72°C	3 min	1
7	4°C	∞	

27. Store the plate or proceed to *Step 6: Index PCR Purification.*

Safe stopping point. The plate can be stored overnight at 4°C or up to 1 month at -20°C prior to purification.

Step 6: Index PCR Purification

Instructions below are for purification of a single library pool from **all wells** from the Final Distribution Plate.

Users may wish to sequence a subset of wells of the 96-well Final Distribution plate (representing a proportion of the total recovered cells from the workflow) before proceeding to sequencing the entire plate. For purification of a subset of wells, follow the instructions in *Appendix A: RNA Purification of a Single Column from Final Distribution Plate*.

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

Source	Material	Cap Color	Take from:	Thaw at:	Brief Vortex	Brief Spin
Tagment and i5 Index PCR Module	Elution Buffer	-	-20°C	RT	✓	✓
Other Vendors	Nuclease-free water SPRIselect beads Ethanol (pure) 0.2-mL PCR tube strips (nuclease-free) 1.5-mL DNA LoBind tubes Agilent HS DNA Kit for Bioanalyzer (or similar fragment analyzer) NEBNext Library Quant Kit for Illumina					

Before you begin:

- Prepare 3 mL fresh 80% ethanol.
- Perform the SPRIselect cleanup at room temperature.

Procedure:

First SPRIselect Cleanup

Caution: Change pipette tips between wells to avoid cross-contamination.

1. Pool **5 µL** from each well of the Final Distribution Plate into a **1.5-mL DNA LoBind** tube (**480 µL** total volume) following the guidance below:
   a. For an **8-tube strip**, transfer **5 µL** with a multi-channel pipette from each plate column into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **60 µL** from each strip tube to the 1.5-mL tube.
   b. For a **12-tube strip**, transfer **5 µL** with a multi-channel pipette from each plate row into the tube strip. Using a single channel pipette, pipette mix the pool and transfer **40 µL** from each strip tube to the 1.5-mL tube.
2. Vortex the SPRIselect 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 1.5-mL tube containing Indexed PCR products.
4. Vortex to mix.
5. Incubate at **room temperature** for **5 minutes**.
6. Briefly spin the tube and place on the magnetic stand for **5 minutes**.
7. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
8. Keep the tube on the magnetic stand and add **1 mL** of 80% ethanol to the beads.
9. Incubate on the magnetic stand for **30 seconds**.
10. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
11. Repeat steps 8-10 for a total of two washes.
12. Briefly spin the tube and place on the magnetic stand.
13. Remove residual 80% ethanol without disturbing the beads.
14. Air dry the beads for **2 minutes**.
15. Remove the tube from the magnetic stand and add **50 µL** Elution Buffer.
16. Vortex to mix.
17. Incubate the tube **off** the magnetic stand for **5 minutes.**
18. Briefly spin the tube and place on the magnetic stand until the solution is clear.
19. Transfer the supernatant to a new **0.2-mL** PCR tube and discard the beads.

Second SPRIselect Cleanup

1. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
2. Transfer **40 µL** of SPRIselect beads (0.8X) to the 0.2-mL tube.
3. Vortex to mix.
4. Incubate at **room temperature** for **5 minutes**.
5. Briefly spin the tube and place on the magnetic stand for **5 minutes**.
6. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
8. Incubate on the magnetic stand for **30 seconds**.
9. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
10. Repeat steps 7-9 for a total of two washes.
11. Briefly spin the tube and place on the magnetic stand.
12. Remove residual 80% ethanol without disturbing the beads.
13. Air dry the beads for **2 minutes**.
14. Remove the tube from the magnetic stand and add **30 µL** Elution Buffer.
15. Vortex to mix.
16. Incubate the tube **off** the magnetic stand for **5 minutes**.
17. Briefly spin the tube and place on the magnetic stand until the solution is clear.
18. Transfer the supernatant to a new tube and discard the beads.
19. Proceed directly to *Step 7: Library Quality Control*.

Step 7: Library Quality Control

1. Determine the average fragment size of the RNA Library using Agilent High Sensitivity DNA Kit for the 2100 Bioanalyzer instrument (or equivalent reagent kit for high sensitivity DNA fragment analysis for an alternative system). Example RNA Library traces are shown in Figure 12 (Bioanalyzer) and Figure 13 (Tapestation). The RNA Library was diluted to 1 ng/µL and loaded with 1 µL on each system; it represents a mixed experiment consisting of both PBMC and Barnyard samples.

Figure 12: Representative RNA Library Traces on Bioanalyzer

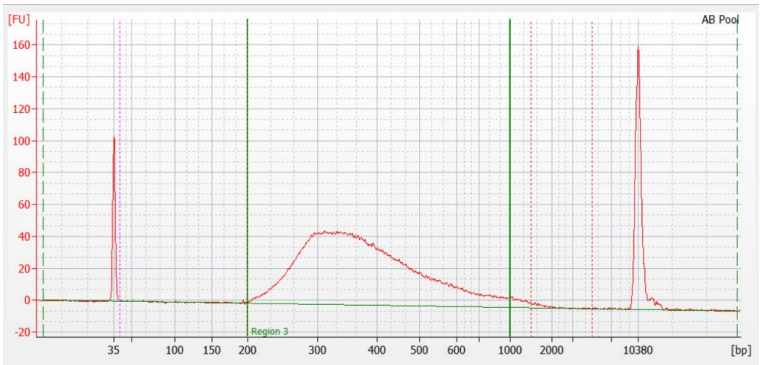
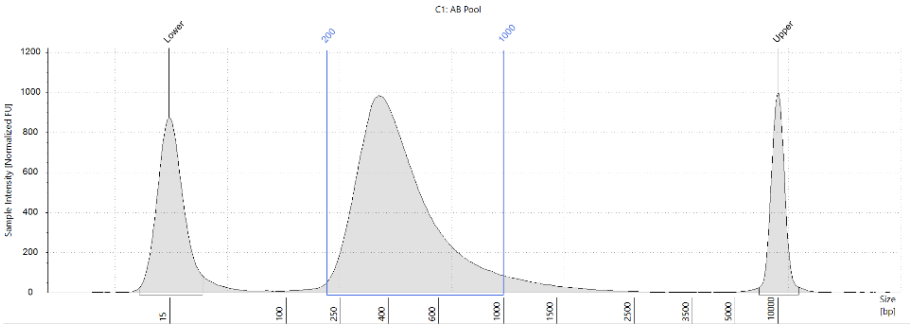


Figure 13: Representative RNA Library Traces on Tapestation



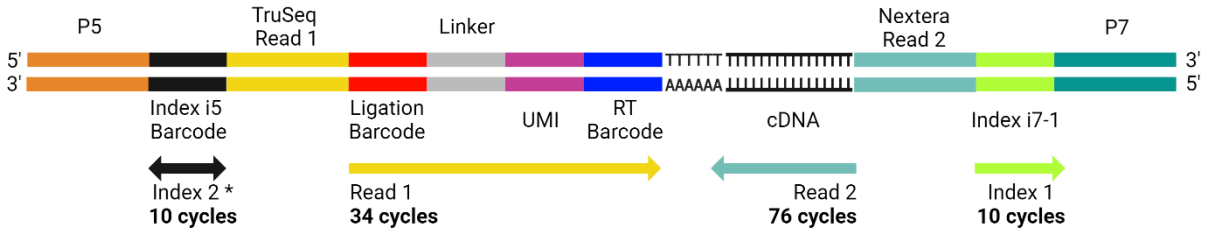
2. Determine RNA Library concentration for sequencing with a commercial qPCR kit for Illumina libraries according to manufacturer's protocol (for example, NEBNext Library Quant Kit for Illumina or equivalent kit). Perform RNA Library dilution and clustering according to sequencing manufacturers parameters.

Caution: It is strongly recommended to quantify the RNA Library by qPCR. Failing to do so may lead to under-clustering on sequencing flow cell.

3. Store the RNA Library or proceed to *Step 8*: *Sequencing Parameters*.

Safe Stopping Point. Purified library can be stored long term at -20°C.

Step 8: Sequencing Parameters



* orientation depends on sequencer and sequencing chemistry

Read	Length	Purpose
Read 1	34 cycles	RT Barcode (96 different 10 bp barcodes) UMI (Unique Molecular Identifier, 8 bp) Ligation Barcode (384 different 9 bp barcodes)
Read 2	76 cycles	Transcript
Index 1	10 cycles	Index i7 (pool of 4 different indices for base balancing)
Index 2	10 cycles	Index i5 Barcode (96 different 10 bp barcodes)

Sequencing Depth
We recommend a minimum sequencing depth of **20,000 read pairs per cell**. Expected cell recovery is ~125,000 cells for a full RT Barcode plate loaded with 10,000 cells per well.

Recommended Final Loading Concentrations	
NovaSeq 6000	200 pM
NextSeq 2000	0.9 nM (on-board denaturation)
NextSeq 550	1.4 pM
PhiX	1% (optional)

Caution: To ensure correct sample demultiplexing, libraries processed by multiple ScaleBio Single Cell RNA Sequencing Kits cannot be pooled nor sequenced together on the same lane of a flow cell.

Please note that the ScaleBio Single Cell RNA Sequencing Kit v1.1 must use the ScaleBio Seq Suite RNA Data Analysis Pipeline **version 1.4** or later for data analysis.

For implementation of the ScaleBio RNA Bioinformatics Pipeline (ScaleBio Seq Suite), please contact your local Field Application Scientist for more information.

Appendix A: RNA Purification of a Single Column from Final Distribution Plate

Instructions below are for purification of a single library from a **single column of wells** from the Final Distribution Plate.

Users may wish to sequence a single column of the 96-well Final Distribution plate (representing ~10,000 recovered cells) before proceeding to sequencing the entire plate.

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

Source	Material
Other Vendors	Nuclease-free water SPRIselect beads Ethanol (pure) Qiagen EB Buffer (10 mM Tris-Cl, pH 8.5) 0.2-mL PCR tube strips (nuclease-free) 1.5-mL DNA LoBind tubes Agilent HS DNA Kit for Bioanalyzer (or similar fragment analyzer) NEBNext Library Quant Kit for Illumina

Before you begin:

- Prepare 1 mL of fresh 80% ethanol for each column that is purified.
- Perform the SPRIselect cleanup at room temperature.

Procedure:

First SPRIselect Cleanup

1. Transfer **5 µL** from each well of a single column of the Final Distribution Plate into a new **0.2-mL PCR tube** for cleanup (**40 µL** total volume), and record the column used.
2. Pipette mix the pool.
3. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
4. Transfer **32 µL** of SPRIselect beads (0.8X) to the 0.2-mL tube containing Index PCR products.
5. Vortex to mix.
6. Incubate at **room temperature** for **5 minutes**.
7. Briefly spin the tube and place on the magnetic stand for **5 minutes**.
8. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
9. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
10. Incubate on the magnetic stand for **30 seconds**.
11. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
12. Repeat steps 9-11 for a total of two washes.
13. Briefly spin the tube and place on the magnetic stand.

14. Remove residual 80% ethanol without disturbing the beads.
15. Air dry the beads for **2 minutes**.
16. Remove the tube from the magnetic stand and add **50 µL** nuclease-free water.
17. Vortex to mix.
18. Incubate the tube **off** the magnetic stand for **5 minutes**.
19. Briefly spin the tube and place on the magnetic stand until the solution is clear.
20. Transfer the supernatant to a **new 0.2-mL** PCR tube and discard the beads.

Second SPRIselect Cleanup

1. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
2. Transfer **40 µL** of SPRIselect beads (0.8X) to the 0.2-mL tube.
3. Vortex to mix.
4. Incubate at **room temperature** for **5 minutes**.
5. Briefly spin the tube and place on the magnetic stand for **5 minutes**.
6. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
7. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
8. Incubate on the magnetic stand for **30 seconds**.
9. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
10. Repeat steps 7-9 for a total of two washes.
11. Briefly spin the tube and place on the magnetic stand.
12. Remove residual 80% ethanol without disturbing the beads.
13. Air dry the beads for **2 minutes**.
14. Remove the tube from the magnetic stand and add **15 µL** Elution Buffer.
15. Vortex to mix.
16. Incubate the tube **off** the magnetic stand for **5 minutes**.
17. Briefly spin the tube and place on the magnetic stand until the solution is clear.
18. Transfer the supernatant to a new tube and discard the beads.
19. Proceed to *Step 7: Library Quality Control*.

Document Revision History

Revision	Revision Date	Document ID	Changes
Rev A	Nov 2023	1020796	Initial release.
Rev B	Jun 2024	1020796	Sample handling optimized for improved cell recovery. Clarified reaction and storage temperature conditions.
Rev C	Jul 2024	1020796	Increased overages.