Single Cell CRISPR Guide Enrichment Kit v1.1

Protocol

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Legal Notices

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Table of Contents

Required Materials

Cas9 CRISPR Guide Enrichment Kit v1.1 (PN 955097) Consumables and Reagents:

Kit Module	Consumable	Part Number	Qty	Cap Color	Storage Temp
RNA Kit Cas9 CRISPR RT Module (PN 945061)	Wash Buffer	202100002	1	-	-20°C
	Cas9 CRISPR RT Barcode Plate	202110019	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
RNA Kit CRISPR Guide Enrichment Module (PN 2020035)	CRISPR PCR Enzyme Mix	202110021	1	Orange	-20°C
	CRISPR Amp Forward Primer	202110022	1	Blue	-20°C
	CRISPR Amp Reverse Primer 1	202110023	1	Blue	-20°C
	Elution Buffer	202110014	2	Clear	-20°C

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
Qubit dsDNA HS Assay Kit	Thermo Fisher	Q33230
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 final 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
Qubit 4 Fluorometer	Thermo Fisher	Q33238
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.

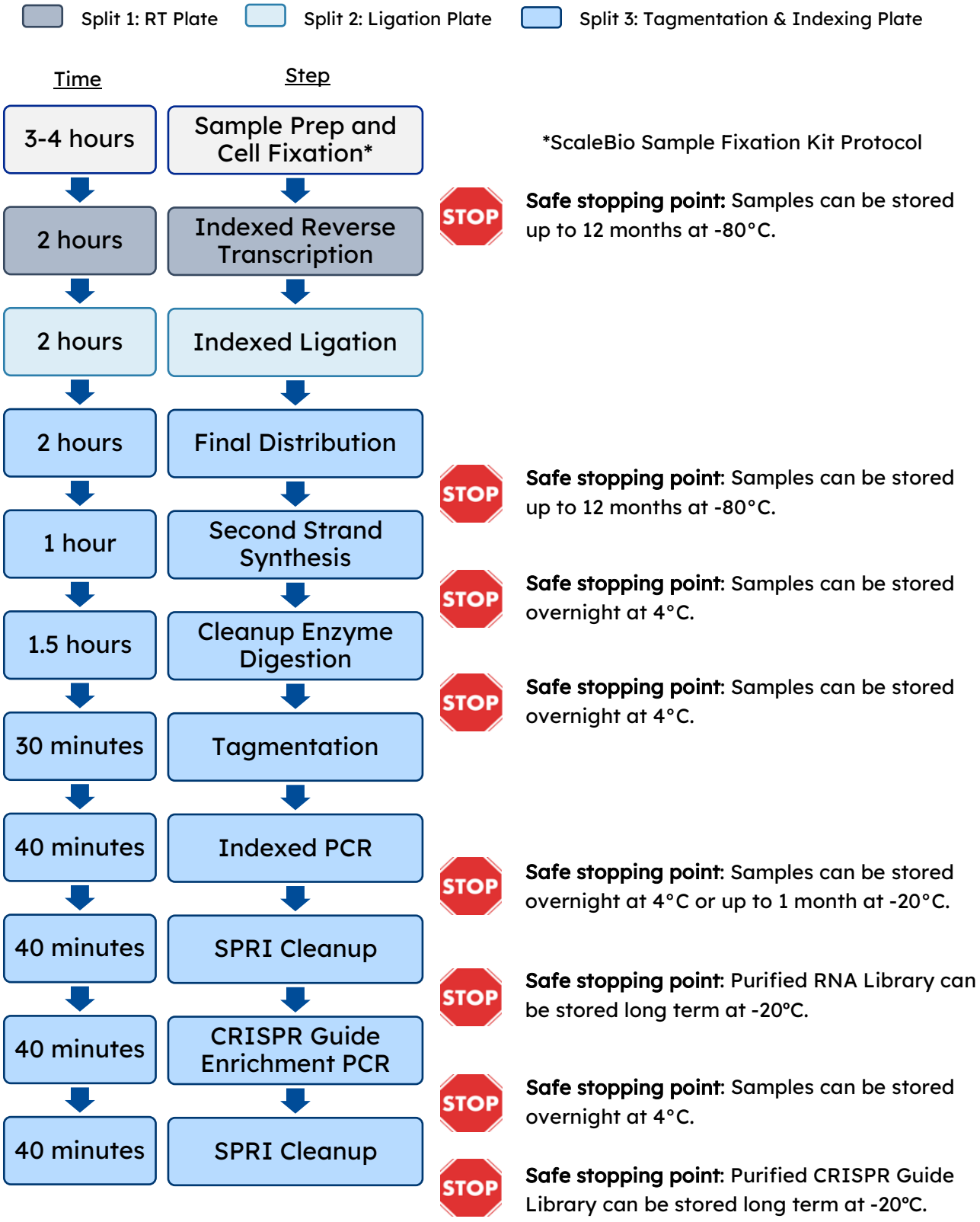
For RNase-free sample processing:

- 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™ CRISPR Guide Enrichment Kit v1.1 provides an instrument-free workflow that increases sample and cell throughput, while reducing cost and bench time. Upstream fixation of single-cell suspensions with the ScaleBio Sample Fixation Kit allows for storage for up to six months 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 two days and includes multiple safe stopping points.

This kit enables the capture of CRISPR guide sequences derived from a CROP-seq vector through specific RT primers on the Cas9 CRISPR RT Barcode Plate. The RT primers are compatible with the scaffold structure required by the Cas9 enzyme. This kit also includes a targeted enrichment PCR step after pooling, cleanup, and quantification of the final RNA library, using a human U6 primer to specifically amplify the CRISPR guides. Figure 1 outlines which add-on protocols might be needed for customizing the workflow.

Caution: If you are not using a CROP-seq vector and the Cas9 enzyme, please refer to the customization instructions in *Appendix D: Customized Protocol Without Cas9 and CROP-seq Vector.*

Please reach out to support@scale.bio or your local Field Application Scientist to verify your CROP-seq vector and CRISPR guide design are compatible with the assay.

Figure 1: Overview of Protocols

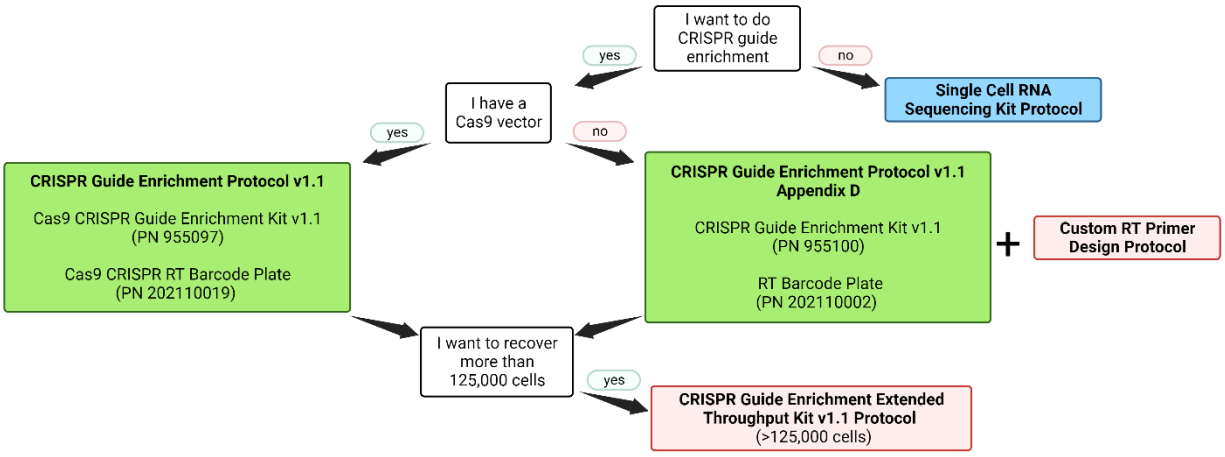
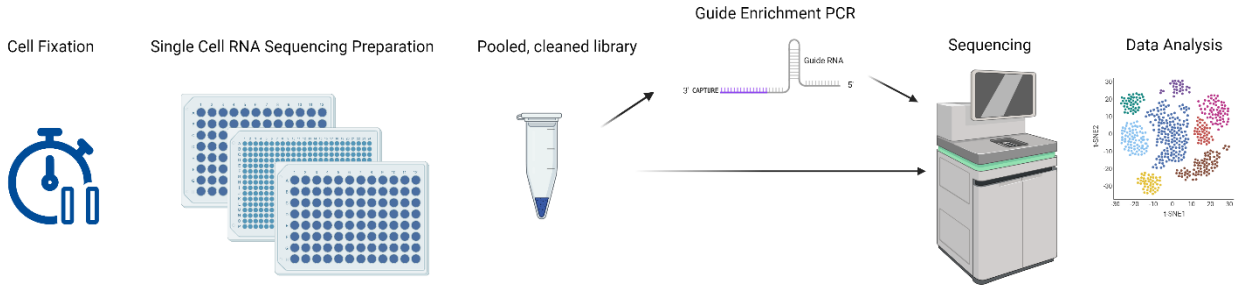
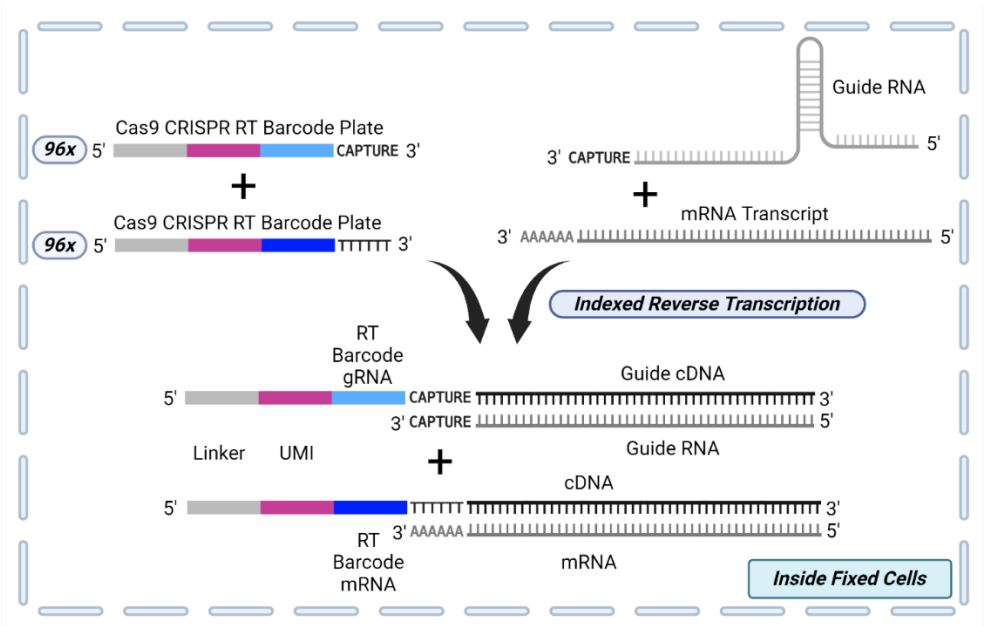


Figure 2: Overall procedure



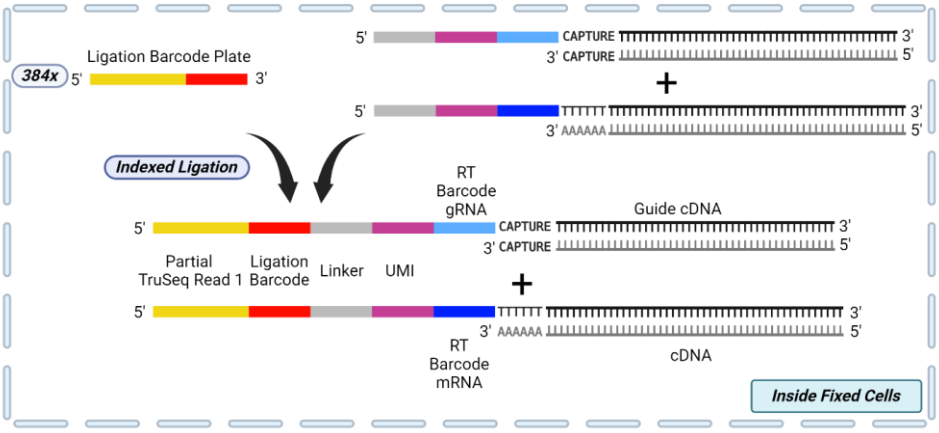
Cell fixation accommodates an input of 400,000 to 2.5 million cells and multiple samples can be fixed simultaneously in 2 hours or less. These samples can be processed directly into the assay or stored at -80°C until ready for use. Fixed cells are distributed across the 96-well Cas9 CRISPR RT Barcode Plate, using 10,000 cells per well for cDNA synthesis and addition of 96 unique RT Barcodes to mRNA transcripts as well as 96 unique Cas9 RT Barcodes to guide RNAs (one unique barcode per target per well).

Figure 3: Split 1 – Indexed Reverse Transcription



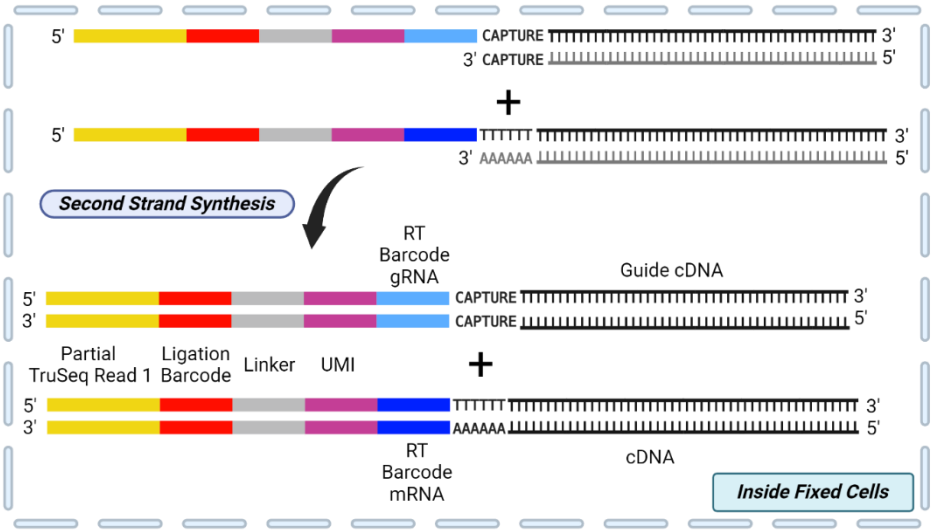
Cells are pooled by centrifugation using the provided ScaleBio Sample Collection Funnel, which significantly decreases bench time and cell loss. Pooled cells are then distributed across the 384-well Ligation Barcode Plate for addition of ligation adaptors containing the TruSeq Read 1 sequence as well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

Figure 4: Split 2 – Indexed Ligation



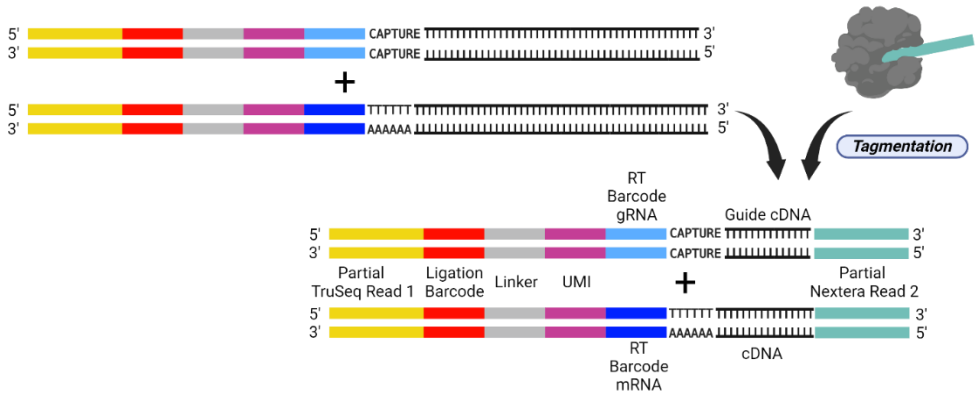
Cells are pooled again and distributed across the 96-well Final Distribution Plate. Second strand synthesis is then performed, followed by a cleanup enzyme digestion step to break down cells.

Figure 5: Split 3 – Final Distribution and Second Strand Synthesis



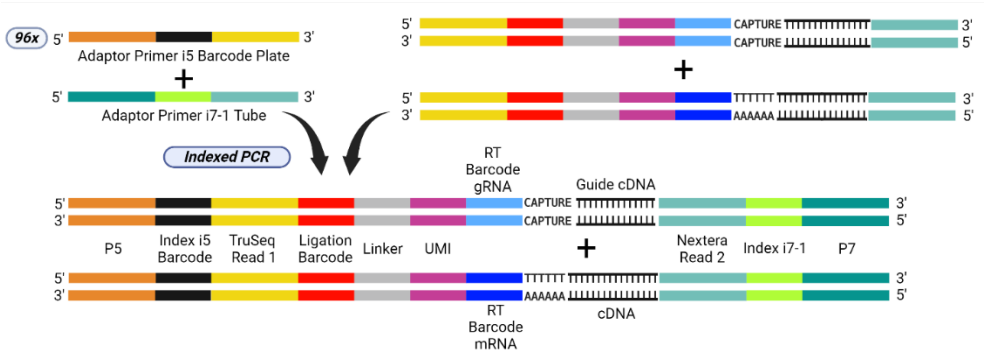
This is followed by tagmentation to attach the Nextera Read 2 sequence for subsequent amplification of both the mRNA transcripts as well as the guide RNA.

Figure 6: Tagmentation



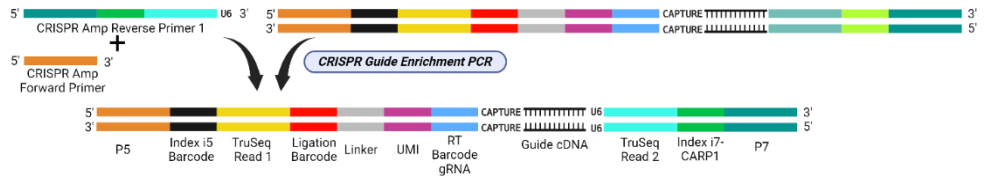
An indexed PCR is performed to add 96 unique Index i5 Barcodes (one unique barcode per well). The i7 Index contains a pool of four different indices for better nucleotide diversity and color balancing during sequencing. This library pool is called the RNA Library as it predominantly consists of mRNA targets.

Figure 7: Indexed PCR



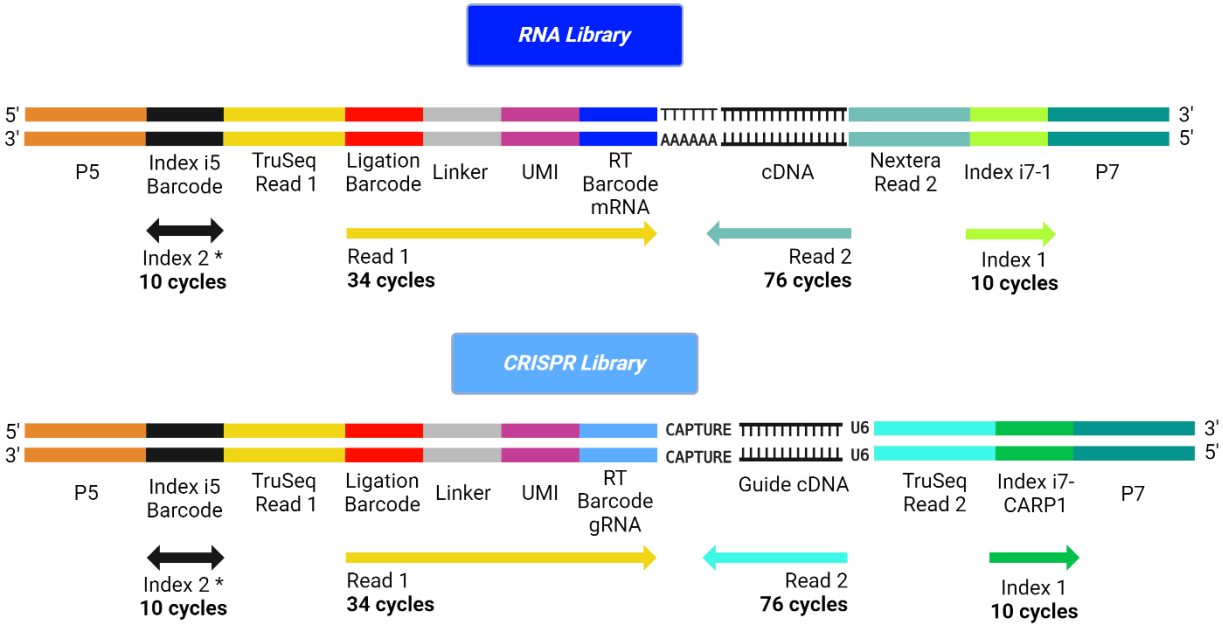
An aliquot of the RNA Library pool is then further amplified in a subsequent PCR to enrich the guide RNA constructs from the library pool in order to create the CRISPR Library.

Figure 8: CRISPR Guide Enrichment PCR



ScaleBio Single Cell libraries are compatible with standard Illumina sequencing. ScaleBio Seq Suite, the ScaleBio bioinformatics pipeline, can be used to both demultiplex and assign reads to individual cells. Output of this pipeline includes basic single-cell sequencing QC metrics and files required for further downstream analysis.

Figure 9: Final Library Structures



* 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	✗	✗
Cas9 CRISPR RT Module	Cas9 CRISPR 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:

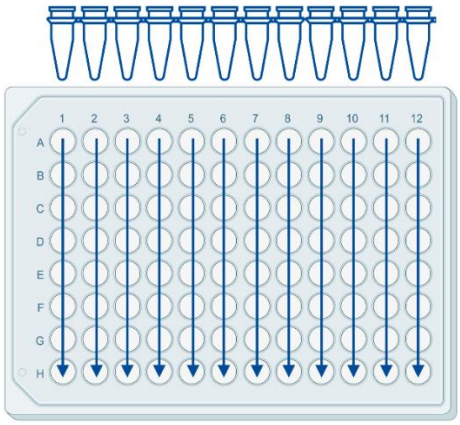
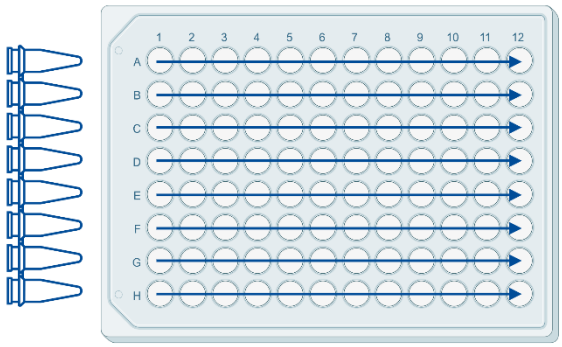
1. Briefly spin down the thawed **Cas9 CRISPR RT Barcode Plate** and place on a 96-well metal block on ice.
2. Determine the concentration of the cell suspension prepared with the ScaleBio Sample Fixation Kit using cell counting equipment. Keep the cell suspension **on ice**.

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 **Cas9 CRISPR 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 10 as guidance for cell calculation and distribution.

Figure 10: Dispensing guidelines for distribution into Cas9 CRISPR 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 Cas9 CRISPR RT Barcode Plate **on ice**, immediately distribute **5 µL** of cells to each well of the Cas9 CRISPR RT Barcode Plate.

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

5. Seal the Cas9 CRISPR 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 Cas9 CRISPR 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 Cas9 CRISPR 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 Cas9 CRISPR 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 Cas9 CRISPR 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 Cas9 CRISPR RT Barcode Plate and place on a plate shaker.
17. Shake the plate at **2000 rpm** for **30 seconds**.

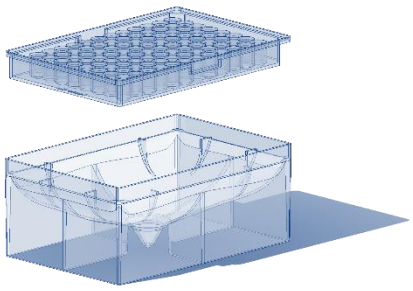
18. Briefly spin down the plate at **100 x g** for **30 seconds** at **4°C** and place **on ice**.
19. Incubate the Cas9 CRISPR RT Barcode Plate in a **pre-cooled** thermocycler according to Program 2.

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 Cas9 CRISPR 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 Cas9 CRISPR RT 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



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 Cas9 CRISPR 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 Cas9 CRISPR 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

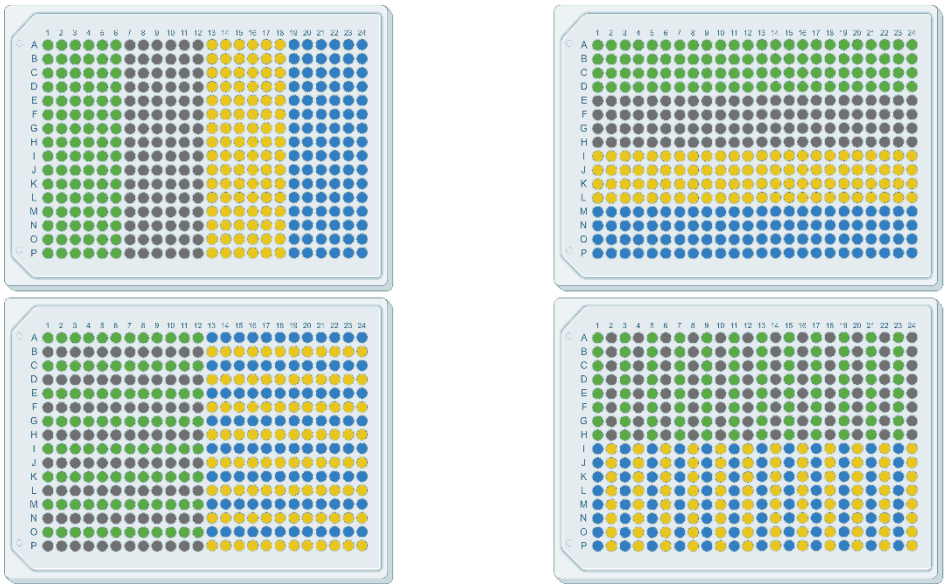
Reagent	Volume (µL)
Pooled cells	3050
Ligation Buffer Conc.	450
Ligation Enzyme Mix	135
Total volume	**3635**

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 12: 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**



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

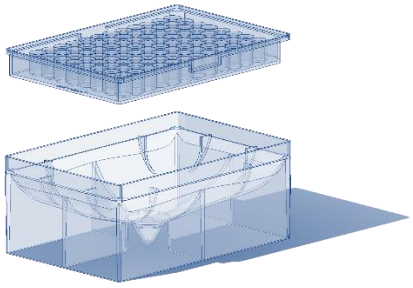
Program 3: Ligation

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.

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. Shake the plate at **2000 rpm** for **30 seconds**.
13. Unpack a ScaleBio Sample Collection Funnel and **place on ice**. **Do not touch the inside of the collection funnel**.
14. Unseal the Ligation Barcode Plate, place the collection funnel on top, and quickly invert the assembly according to Figure 13.

Figure 13: ScaleBio Sample Collection Funnel and Plate Assembly



15. 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.

16. Transfer pooled material **(~ 4 mL)** from collection funnel into a **5-mL DNA LoBind** Eppendorf tube and place on ice.
17. 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 21.
18. Centrifuge the 5-mL tube containing the pooled material from the Ligation Barcode Plate at **500 x g** for **8 minutes** at **4°C**.
19. 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.

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

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

Caution: 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	∞	

28. 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

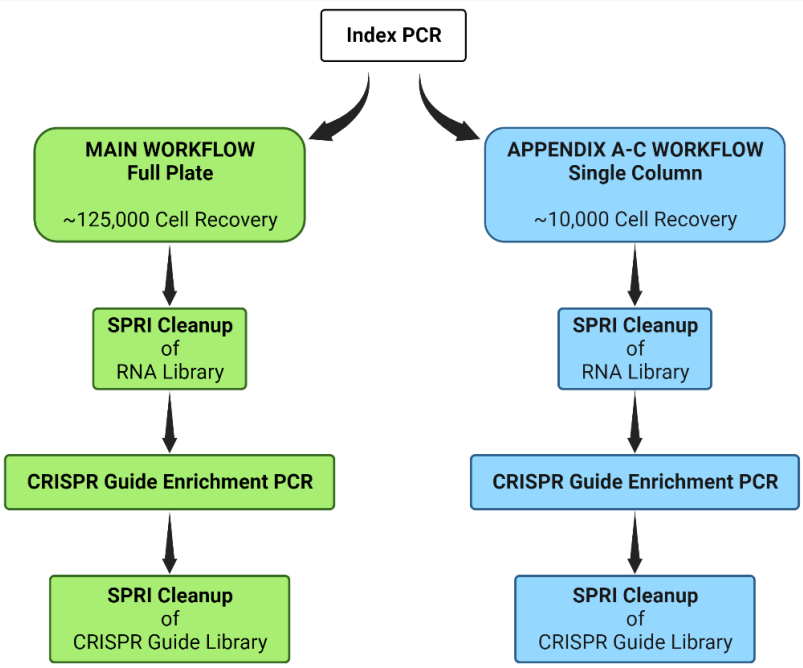
Instructions below are for purification of a single library pool from **all wells** from the Final Distribution Plate.

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

Note: CRISPR Guide Enrichment PCR should be performed on the **same** RNA library being sequenced. If purifying a single column of wells, please use the **same** purified library pool as starting material for CRISPR Guide Enrichment PCR (see Figure 14).

Note: Cleaning up a subset of wells is strongly recommended if running a new sample type with the CRISPR workflow.

Figure 14: Workflow Decision Tree



For purification of a **single column** of wells, please proceed directly to the instructions contained in the following chapters:

1. *Appendix A: RNA Purification of a Single Column from Final Distribution* Plate
2. *Appendix B: CRISPR Guide Enrichment PCR from a Single Column*
3. *Appendix C: CRISPR Guide Enrichment PCR Purification from a Single Column*

Please review the table below to prepare reagents for purification of **all wells** 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 Qubit dsDNA HS Assay Kit Agilent HS DNA Kit for Bioanalyzer (or similar fragment analyzer)					

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.

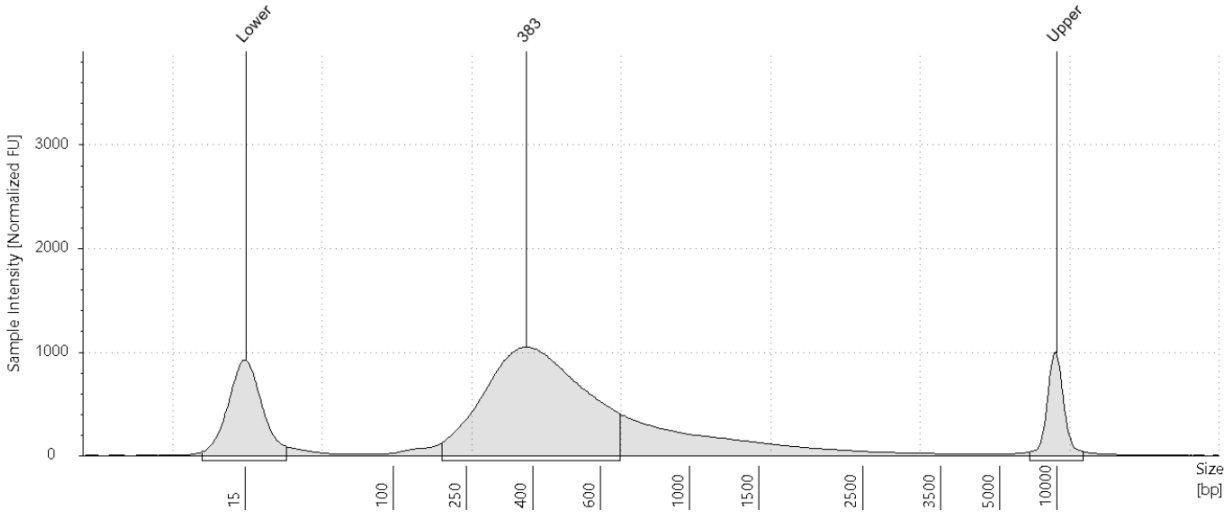
Second SPRIselect Cleanup

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**.
4. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
5. Keep the tube on the magnetic stand and add **200 µL** of 80% ethanol to the beads.
6. Incubate on the magnetic stand for **30 seconds**.
7. Once the solution is clear, carefully remove and discard the supernatant without disturbing the beads.
8. Repeat steps 5-7 for a total of two washes.
9. Briefly spin the tube and place on the magnetic stand.
10. Remove residual 80% ethanol without disturbing the beads.
11. Air dry the beads for **2 minutes**.
12. Remove the tube from the magnetic stand and add **30 µL** Elution Buffer.
13. Vortex to mix.
14. Incubate the tube **off** the magnetic stand for **5 minutes**.
15. Briefly spin the tube and place on the magnetic stand until the solution is clear.
16. Transfer the supernatant to a new tube and discard the beads.
17. Proceed directly to *Step 7: RNA Library Quality Control*.

Step 7: RNA 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 15 (Tapestation). The RNA Library was diluted to 1 ng/µL and loaded with 2 µL on Tapestation; it represents a human K562 cell line.

Figure 15: Representative RNA Library Trace on Tapestation



2. Quantify the RNA Library concentration using the Qubit dsDNA HS assay kit.
3. Dilute an aliquot of the RNA Library to **0.25 ng/µL** in **30 µL** with Elution Buffer and proceed to *Step 8: CRISPR Guide Enrichment PCR*.
4. 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.

5. Store the RNA Library, or proceed to *Step 11*: Sequencing Parameters.

> **Safe Stopping Point**. Cleaned RNA Library can be stored long term at -20°C. **For CRISPR Library**. Proceed to *Step 8: CRISPR Guide Enrichment PCR*.

Step 8: CRISPR Guide Enrichment PCR

Instructions are given below for performing guide enrichment on the RNA Library prepared from **all wells** of the Final Distribution Plate.

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
CRISPR Guide Enrichment Module	CRISPR PCR Enzyme Mix	Orange	-20°C	On ice	✗	✓
	CRISPR Amp Forward Primer	Blue	-20°C	On ice	✗	✓
	CRISPR Amp Reverse Primer 1	Blue	-20°C	On ice	✗	✓
Other Vendors	Nuclease-free water Microseal 'B' PCR Plate Sealing Film					

Procedure:

1. Take **30 µL** of SPRIselect cleaned RNA Library (diluted to **0.25 ng/µL)** from *Step 6: Index PCR Purification*.
2. **On ice**, prepare the CRISPR Enrichment PCR Master Mix by combining the components in the order specified in Table 6.

   Table 6: CRISPR Enrichment PCR Master Mix

Reagent	Volume (µL)
Nuclease-free water	90
CRISPR Amp Forward Primer	15
CRISPR Amp Reverse Primer 1	15
CRISPR PCR Enzyme Mix	150
Total volume	**270**

3. Pipette mix the CRISPR Guide Enrichment PCR Master Mix until the solution is homogeneous.
4. Distribute **18 µL** of CRISPR Guide Enrichment PCR Master Mix to 12 wells of a new strip-well tube.
5. Add **2 µL** of diluted library to each of the 12 wells containing the CRISPR Enrichment PCR Master Mix.
6. Shake the strip-well tubes for **30 seconds** at **2000 rpm**.
7. Briefly spin down the strip-well tubes.
8. Incubate the strip-well tube in a thermocycler according to Program 9.

Program 9: CRISPR Guide Enrichment PCR

Lid Temperature		Reaction Volume	
105°C		20 µL	
Step	**Temperature**	**Time**	**Cycles**
1	98°C	30 sec	1
2	98°C	10 sec	21 cycles (steps 2-4)
3	65°C	30 sec	
4	72°C	30 sec	
5	72°C	3 min	1
6	4°C	∞	

9. Store the strip-well tube, or proceed to *Step 9: CRISPR Guide Enrichment PCR Purification*.

Safe stopping point. The strip-well tube can be stored overnight at 4°C prior to purification.

Step 9: CRISPR Guide Enrichment PCR Purification

Instructions are given below for purification of a single library from **all wells** of the CRISPR Guide Enrichment PCR reaction.

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	Clear	-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)					

Before you begin:

- Prepare 1 mL fresh 80% ethanol.
- Perform the SPRIselect cleanup at room temperature.

Procedure:

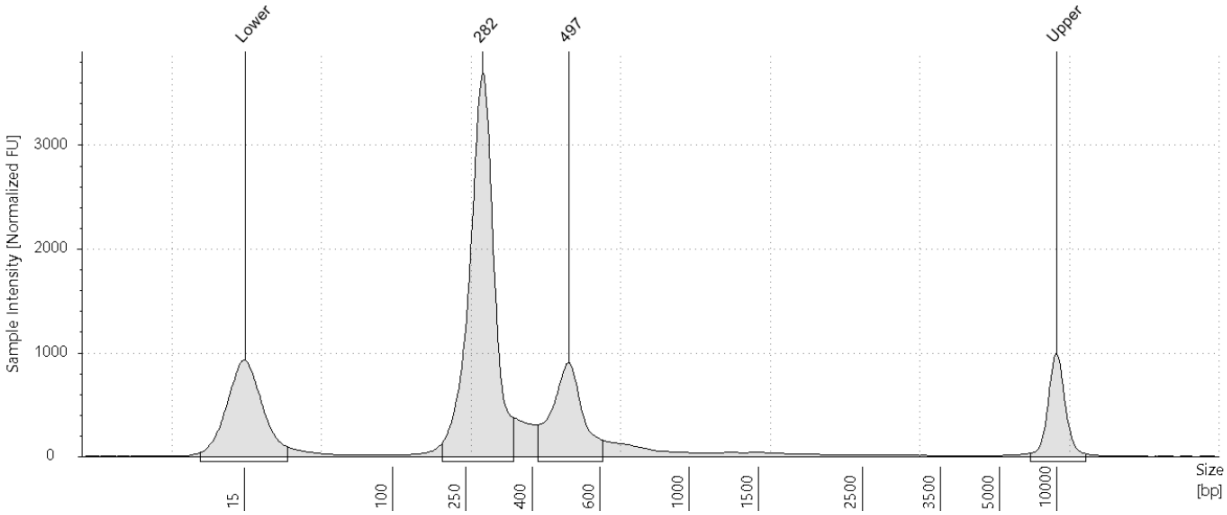
1. Pool **10 µL** from 12 wells of the CRISPR Guide Enrichment PCR strip-well tube into a new **1.5-mL DNA LoBind** tube (**120 µL** total volume).
2. Pipette mix the pool.
3. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
4. Transfer **120 µL** of SPRIselect beads (1X) to the 1.5-mL tube containing the CRISPR Guide Enrichment 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** 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 **15 µL** Elution Buffer.

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 tube and discard the beads.
21. Proceed directly to *Step 10: CRISPR Library Quality Control*.

Step 10: CRISPR Library Quality Control

1. Determine the average fragment size of the CRISPR 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 CRISPR Library traces are shown in Figure 16 (Tapestation). The CRISPR Library was diluted to 1 ng/µL and loaded with 2 µL on Tapestation; it represents a guide enrichment example from the same human K562 cell line shown in Figure 15.

Figure 16: Representative CRISPR Library Traces on Tapestation



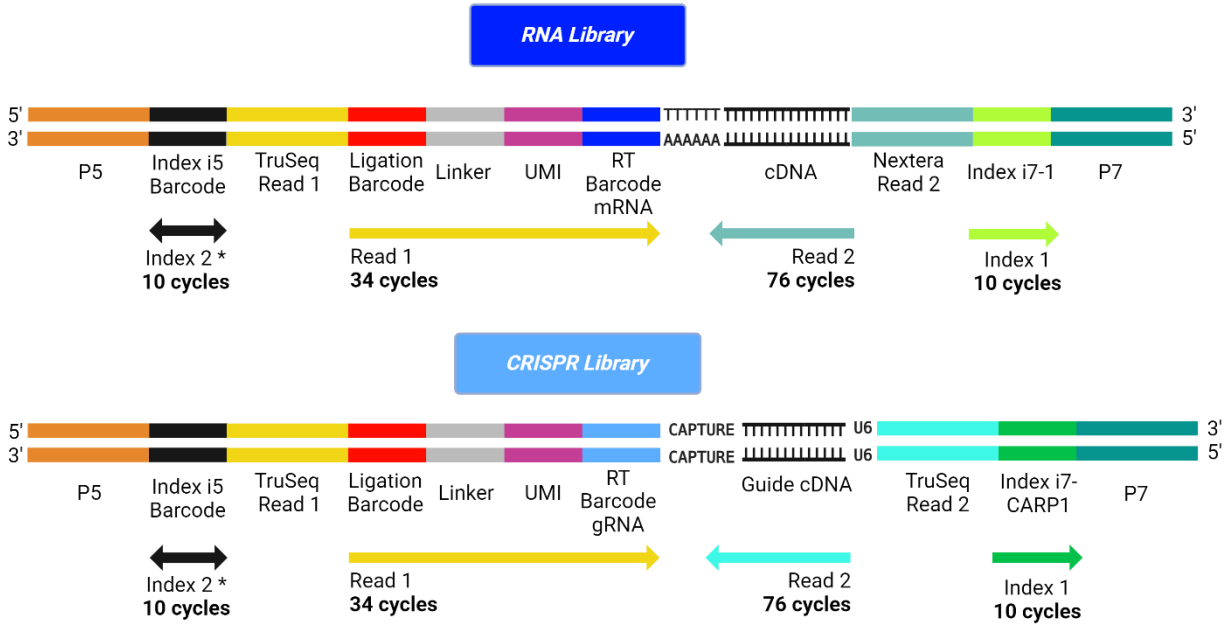
2. Determine CRISPR Library concentration for sequencing with a commercial qPCR kit for Illumina libraries according to manufacturer's protocol (for example, NEBNext Library Quant Kit for Illumina or equivalent kit). Perform CRISPR Library dilution and clustering according to sequencing manufacturers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

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

3. Store the CRISPR Library, or proceed to *Step 11*: Sequencing Parameters.

> **Safe Stopping Point**. Cleaned CRISPR Library can be stored long term at -20°C.

Step 11: 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 or CRISPR Guide
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
We recommend a minimum sequencing depth of **20,000 read pairs per cell for RNA Library and 2,000 read pairs per cell for CRISPR Library**. Expected cell recovery is ~125,000 cells for a full Cas9 CRISPR RT Barcode plate loaded with 10,000 cells per well. We recommend to pool and sequence both the RNA Library and CRISPR Library together.

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

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.

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 Qubit dsDNA HS Assay Kit Agilent HS DNA Kit for Bioanalyzer (or similar fragment analyzer)

Before you begin:

- Prepare 1 mL of fresh 80% ethanol for each column being 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** (**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 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** nuclease-free water.
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.
4. Proceed to *Step 7: RNA Library Quality Control* and then to *Appendix B: CRISPR Guide Enrichment PCR from a Single Column*.

Safe Stopping Point. Cleaned RNA Library can be stored long term at -20°C.
For CRISPR Library: Proceed to *Appendix B: CRISPR Guide Enrichment PCR from a Single Column*.

Appendix B: CRISPR Guide Enrichment PCR from a Single Column

Instructions are given below for performing guide enrichment on the RNA Library prepared from a **single column of wells** of the Final Distribution Plate.

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
CRISPR Guide Enrichment Module	CRISPR PCR Enzyme Mix	Red	-20°C	On ice	✗	✓
	CRISPR Amp Forward Primer	Blue	-20°C	On ice	✗	✓
	CRISPR Amp Reverse Primer 1	Blue	-20°C	On ice	✗	✓
Other Vendors	Nuclease-free water Microseal 'B' PCR Plate Sealing Film					

Procedure:

1. Take **10 µL** of SPRIselect cleaned RNA Library (diluted to **0.25 ng/µL**) from *Appendix A: RNA Purification of a Single Column from Final Distribution Plate*.
2. **On ice**, prepare the CRISPR Enrichment PCR Master Mix for a single column by combining the components in the order specified in Table 7.

Table 7: CRISPR Enrichment PCR Master Mix for a Single Column

Reagent	Volume (µL)
Nuclease-free water	7.5
CRISPR Amp Forward Primer	1.25
CRISPR Amp Reverse Primer 1	1.25
CRISPR PCR Enzyme Mix	12.5
Total Volume	**22.5**

3. Pipette mix the CRISPR Guide Enrichment PCR Master Mix until the solution is homogeneous.
4. Distribute **18 µL** of CRISPR Guide Enrichment PCR Master Mix to a single well of a new strip tube.
5. Add **2 µL** of diluted library to the well containing CRISPR Guide Enrichment PCR Master Mix.
6. Shake the strip-well tube for **30 seconds** at **2000 rpm**.
7. Briefly spin down the strip-well tube.
8. Incubate the strip-well tube in a thermocycler according Program 10.

Program 10: CRISPR Guide Enrichment PCR

Lid Temperature		Reaction Volume	
105°C		20 µL	
Step	**Temperature**	**Time**	**Cycles**
1	98°C	30 sec	1
2	98°C	10 sec	21 cycles (steps 2-4)
3	65°C	30 sec	
4	72°C	30 sec	
5	72°C	3 min	1
6	4°C	∞	

9. Store overnight at 4°C, or proceed to *Appendix C: CRISPR Guide Enrichment PCR Purification from a Single Column*.

Safe stopping point. The strip-well tube can be stored overnight at 4°C prior to purification.

Appendix C: CRISPR Guide Enrichment PCR Purification from a Single Column

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 Qubit dsDNA HS Assay Kit Agilent HS DNA Kit for Bioanalyzer (or similar fragment analyzer)

Before you begin:

- Prepare 1 mL fresh 80% ethanol.
- Perform the SPRIselect cleanup at room temperature.

Procedure:

1. Transfer **15 µL** of the CRISPR Guide Enrichment reaction from *Appendix B: CRISPR Guide Enrichment PCR from a Single Column* into a **new 0.2-mL PCR tube**.
2. Vortex the SPRIselect beads at high speed for **1 minute**. The beads should appear homogeneous and uniform in color.
3. Transfer **15 µL** of SPRIselect beads (1X) to the tube containing the CRISPR Guide Enrichment PCR products from a single column.
4. Vortex to mix.
5. Incubate at room temperature for **5 minutes**.
6. Briefly spin 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 **200 µL** 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 pellet for **2 minutes.**
15. Remove the tube from the magnetic stand and add **15 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 tube and discard the beads.

20. Proceed to *Step 10: CRISPR Library Quality Control*.

Safe Stopping Point. Cleaned CRISPR Library can be stored long term at -20°C.

Appendix D: Customized Protocol Without Cas9 and CROP-seq Vector

The CRISPR Guide Enrichment Protocol is used for experimental CRISPR designs including a CROP-seq vector and the Cas9 enzyme. RT Primers compatible with the scaffold structure required by the Cas9 enzyme are contained within the Cas9 CRISPR RT Barcode Plate.

If the planned CRISPR screen is not using the Cas9 enzyme nor a CROP-seq vector, both the ScaleBio CRISPR kit as well as the protocol need to be adapted. Custom RT Primers need to be designed up front and spiked manually into the RT Barcode Plate.

The table below outlines which part numbers and protocols need to be used depending on the:

	Experimental Design With Cas9		Experimental Design Without Cas9	
	Name	**Part Number**	**Name**	**Part Number**
ScaleBio Kit:	Cas9 CRISPR Guide Enrichment Kit v1.1	955097	CRISPR Guide Enrichment Kit v1.1	955100
Kit Modules:	Cas9 CRISPR RT Module*	945061	RT Module*	2020004
	Ligation Module	2020005	Ligation Module	2020005
	Tagment and i5 Index PCR Module	935932	Tagment and i5 Index PCR Module	935932
	Workflow Consumables Module	2020007	Workflow Consumables Module	2020007
	CRISPR Guide Enrichment Module	2020035	CRISPR Guide Enrichment Module	2020035
*** Included Barcode Plate:**	Cas9 CRISPR RT Barcode Plate	202110019	RT Barcode Plate	202110002
Protocol(s):	CRISPR Guide Enrichment Kit v1.1 Protocol	n/a	CRISPR Guide Enrichment Kit v1.1 Protocol Custom RT Primer Design Protocol	n/a

For assistance with these additional workflows, please contact support@scale.bio or your local Field Application Scientist.

Document Revision History

Revision	Revision Date	Document ID	Changes
Rev B	Feb 2024	1020765	Initial release.
Rev C	Jul 2024	1020765	Sample handling optimized for improved cell recovery. Clarified reaction and storage temperature conditions.