BD Rhapsody™ System

Single-Cell ATAC-Seq and Sample Tag

Library Preparation Protocol

23-24798(01)
2024-08

Regulatory information

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

History

Revision	Date	Change made
23-24798(01)	2024-08	Initial release.

Contents

Introduction

This protocol provides instructions on sample multiplexing and generating a single-cell ATAC library using the BD Rhapsody™ Single-Cell Analysis System.

The BD® Nuclear Sample Multiplexing Kit utilizes an innovative antibody-oligo technology to provide higher sample throughput for single-nucleus library preparation. Every antibody-oligo in the BD® Nuclear Sample Multiplexing Kit, referred to as a a Sample tag, has a unique sample oligo barcode conjugated to an antibody that can universally target nuclei. Up to six samples can be labeled and pooled prior to single-cell capture with the BD Rhapsody Single-Cell Analysis System.

To generate a single-cell ATAC library with the BD Rhapsody™ Single Cell Analysis System, a specific variant of Tn5 transposase is used to fragment and simultaneously insert Next Generation Sequencing (NGS) primers into the accessible chromatin regions of individual cells or nuclei for sequencing on compatible Illumina® sequencers. It enables uncovering insights into the chromatin structure and factors that affect gene expression of cells at single-cell level.

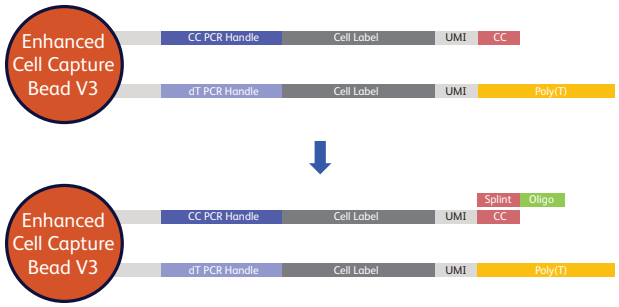
For complete instrument procedures and safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*.

Workflow overview

The *BD Rhapsody™ System Single-Cell ATAC-Seq Library Preparation Protocol* enables profiling of the epigenomic landscape at a single nuclei level.

ATAC workflow

Splint bead: Starting from the BD Rhapsody™ Enhanced Bead V3 layout, add splint oligonucleotide to the beads to assist capturing of genomic DNA.

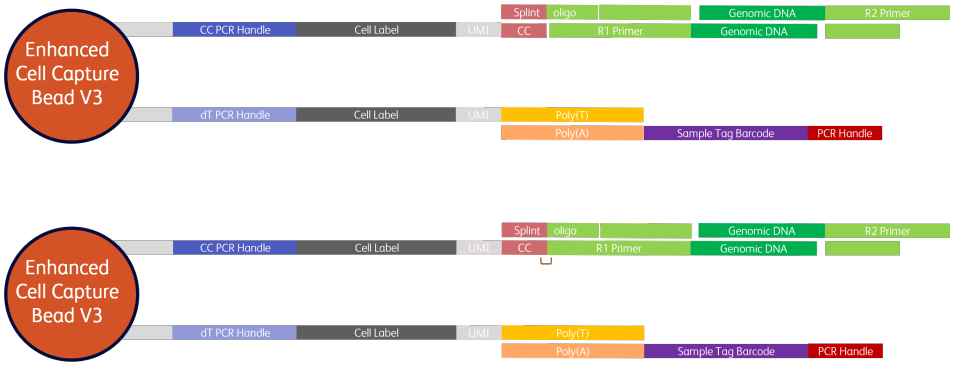


Nuclei isolation: Nuclei isolation protocol depends on the sample type. For details, see Nuclei isolation (page 16).

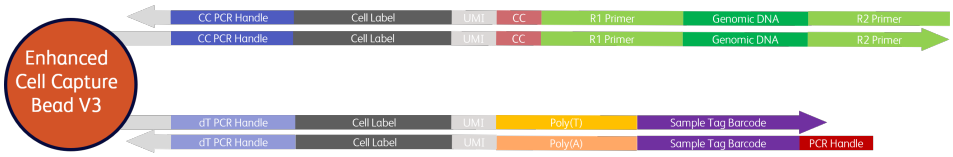
Tagmentation: During the bulk in-situ tagmentation process, expose the nuclei to a tagmentation mix, containing Tagmentase. This enzyme targets accessible genomic regions (open chromatin areas), cutting the DNA and simultaneously attaching preloaded adapter sequences to the ends of each DNA fragment. For substep details, see Tagmentation (page 18).

Single-cell capture: Perform cell lysis in a microwell. The genomic DNA sequences are captured by the splint-oligo-bonded CC strands. Sample Tags are captured by poly(T).

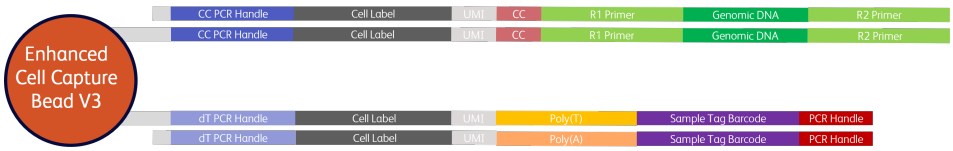
Ligation: Ligate BD Rhapsody™ bead oligo and tagmented DNA using DNA ligase.



Gap filling ATAC fragments: This process performs ATAC fragment gap filling and extension to beads oligo. Complementary DNA of Sample Tag is synthesized from captured Sample Tag.



Splint oligo removal and Exonuclease I treatment: To remove unused oligos from the beads.



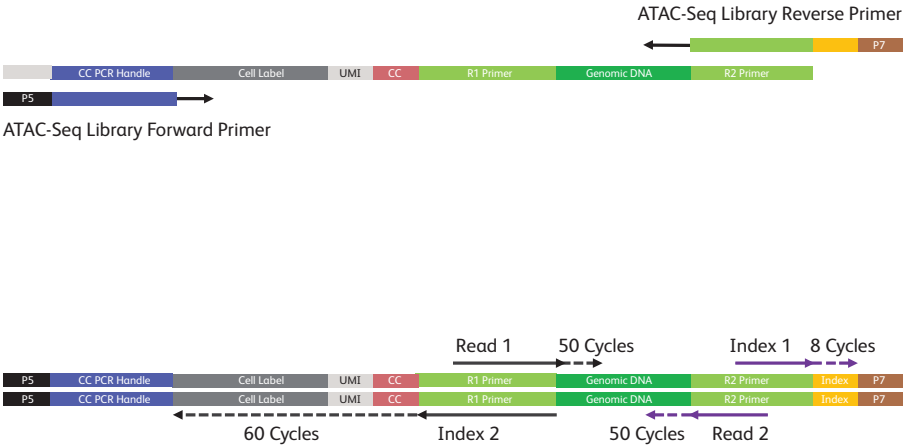
ATAC fragment denaturation and PCR amplification:

Supernatant: Denature the genomic DNA template off the bead. Illumina® adapters and indices are added during the ATAC product amplification.

Bead: Proceed through the Sample Tag library workflow—see Sample Tag library amplification workflow (page 6).

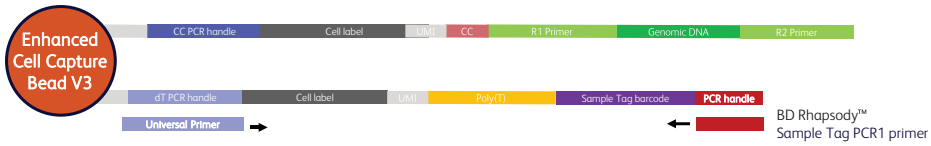
Sequencing:

Read 1: 50 cycles
Read 2: 50 cycles
Index 1: 8 cycles
Index 2: 60 cycles



Sample Tag library amplification workflow

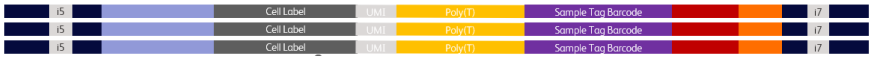
Sample Tag PCR1: Sample Tag product is amplified in PCR1.



Sample Tag PCR2: Sample Tag product is further amplified in PCR2.

Sample Tag Index PCR: Add Illumina adapters and indices.

Final Sample Tag library product:



Note: In the preceding diagrams, CC is an abbreviation for Custom Capture.

Required and recommended materials

Required reagents

Store the reagents at the storage temperature specified on the label.

Material	Supplier	Catalog No.
BD Rhapsody™ Tagmentation and Supplemental Reagents Kit	BD Biosciences	571201
BD Rhapsody™ ATAC-Seq Amplification Kit	BD Biosciences	571356
BD Rhapsody™ Enhanced Cartridge Reagent Kit V3[a]	BD Biosciences	667052
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit	BD Biosciences	633774
BD Rhapsody™ 8-Lane Cartridge	BD Biosciences	666262
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
Nuclease-free water	Major supplier	–
N,N-Dimethylformamide	MilliporeSigma	D4551-250ML
DyeCycle™ Green[b]	Thermo Fisher Scientific	V35004
Dimethylsulfoxide (DMSO)	Major supplier	–
70% ethyl alcohol or 70% isopropyl alcohol[c]	Major supplier	–
Exonuclease I (E. coli)	New England Biolabs	M0293S
Ethylenediaminetetraacetic acid (EDTA)	Major supplier	–
MS Nucleoporin P62 sample tag 1	BD Biosciences	460291
MS Nucleoporin P62 sample tag 5	BD Biosciences	460293
MS Nucleoporin P62 sample tag 7	BD Biosciences	460294
MS Nucleoporin P62 sample tag 8	BD Biosciences	460295
MS Nucleoporin P62 sample tag 9	BD Biosciences	460296
MS Nucleoporin P62 sample tag 10	BD Biosciences	460297

[a] The Enhanced Cartridge Reagent Kit V3 must be used to perform this protocol.

[b] Protect DyeCycle™ Green from light. See manufacturer's storage recommendations.

[c] To clean the BD Rhapsody™ Xpress System and the BD Rhapsody™ Scanner, see the *BD Rhapsody™ Single-Cell Analysis System Installation and Maintenance Guide*. Instead of 70% alcohol, 10% (w/v) bleach can be used.

Recommended consumables

Supplies	Supplier	Catalog No.
Gilson™ PIPETMAN™ DIAMOND Tipack™ filter tips, 100–1200 µL for BD Rhapsody™ P8xP1200 µL pipette (or BD Rhapsody™ P1200 µL pipette) (Recommended) Or ZAP™ SLIK 1000 µL low-retention aerosol filter pipet tips for BD Rhapsody™ P8xP1200 µL pipette (or BD Rhapsody™ P1200 µL pipette) (Alternative)	Thermo Fisher Scientific Labcon	F171803G 1177-965-008-9
Low retention, filtered pipette tips (20 µL, 200 µL, 1000 µL)	Major supplier	–
Falcon® tube with cell strainer cap	Corning	352235
INCYTO disposable hemocytometer	INCYTO	CN DHC-N01-5
60-mL reagent reservoir self-standing[a]	BD Biosciences	666626
Corning® 96-well polypropylene cluster tube, 8-tube strip format, sterile[b]	Corning	4413
0.2-mL PCR 8-strip tubes	Major supplier	–
15-mL conical tube	Major supplier	–
50-mL conical tube	Major supplier	–
DNA LoBind® tubes, 1.5 mL	Eppendorf	022431021
DNA LoBind® tubes, 2.0 mL	Eppendorf	022431048
DNA LoBind® tubes, 5.0 mL	Eppendorf	0030108310
Reagent reservoir (sterile, non-pyrogenic, RNase/DNase free), 10 mL	VistaLab	3054-1012 3054-1013
Reagent reservoir (sterile, non-pyrogenic, RNase/DNase free), 25 mL	VistaLab	3054-1002 3054-1003
Deep 96-well 2-mL polypropylene plate	Major supplier	–
Lint-free cloth (Kim-Wipes)	Major supplier	–
Qubit™ assay tubes	Thermo Fisher Scientific	Q32856
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent High Sensitivity DNA Kit	Agilent	5067-4626

[a] Waste collection container for the BD Rhapsody™ HT Xpress System.

[b] These are the bead retrieval tubes to be used with the BD Rhapsody™ HT Xpress System.

Required equipment

Equipment	Supplier	Catalog No.
Single-channel pipettes (P20, P200, P1000)	Major supplier	–
BD Rhapsody™ HT Xpress Package	BD Biosciences	666730
BD Rhapsody™ Scanner	BD Biosciences	633701
Hemocytometer adapter[a]	BD Biosciences	633703
BD Rhapsody™ P8xP1200 µL pipette-HTX[b]	BD Biosciences	666718
BD Rhapsody™ P1200 µL Pipette – HTX[c]	BD Biosciences	666719
Temperature-controlled centrifuge	Major supplier	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
Microcentrifuge for 1.5–2.0-mL tubes	Major supplier	–
Microcentrifuge for 0.2-mL tubes	Major supplier	–
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Agilent® 2100 Bioanalyzer	Agilent Technologies	G2940CAG
Invitrogen™ DynaMag™-2 magnet	Thermo Fisher Scientific	12321D
Low-profile magnetic separation stand for 0.2-mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
Ice bucket	Major supplier	–
Vortexer	Major supplier	–
Digital timer	Major supplier	–

[a] Included with the BD Rhapsody™ Scanner.

[b] Part of the BD Rhapsody™ Xpresss Package. Items can be ordered separately.

[c] Only required if not using the BD Rhapsody™ P8xP1200µL Pipette – HTX.

Before you begin

- Ensure that you have the correct kits for this protocol. Matching cap colors indicate that you have the correct kit.

BD Rhapsody™ Enhanced Cartridge Reagent Kit V3

Cap Color	Name	Quantity
	BD Rhapsody™ HT Enhanced Cell Capture Beads V3	4
	Sample buffer	1
	Cartridge wash buffer 1	1
	Cartridge wash buffer 2	1
	Lysis buffer	4
	Bead wash buffer	1
	Waste collection container	4
	1M DTT	1

ATAC-Seq Amplification Kit

Cap Color	Reagent Name	Quantity
	Ligation buffer	1
	Ligase	1
	Nuclease-free water	2
	Gap-filling enzyme	1
	Gap-filling buffer	1
	Gap-filling enhancer	1
	dNTP	1
	ATAC-Seq library forward primer	1
	ATAC-Seq library reverse primer 1	1
	ATAC-Seq library reverse primer 2	1
	ATAC-Seq library reverse primer 3	1
	ATAC-Seq library reverse primer 4	1
	ATAC-Seq library reverse primer 5	1
	ATAC-Seq library reverse primer 6	1
	ATAC-Seq library reverse primer 7	1
	ATAC-Seq library reverse primer 8	1
	Elution buffer	1
	Bead resuspension buffer	1
	PCR master mix	1

ATAC-Seq Tagmentation and Supplemental Reagents Kit

Cap Color	Name	Quantity
	Tagmentase	1
	Tagmentation buffer	1
	10X PBS	1
	Digitonin 2%	1
	Tween 20 10%	1
	Nuclease-free water	1
	Universal ATAC-Seq splint oligo	1
	Splint oligo annealing buffer	1
	Splint-bead wash buffer	1
	Nuclei buffer	2
	Proteinase K, molecular biology grade	1

BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit

Cap Color	Name	Quantity
	Nuclease-free water	1
	Bead RT/PCR enhancer	1
	PCR master mix	1
	Universal oligo	1
	Sample Tag PCR1 primer	1
	Sample Tag PCR2 primer	1
	BD® AbSeq PCR1 primer	1
	Library reverse primer 1–4	1 each
	Library forward primer	1
	Bead resuspension buffer	1
	Elution buffer	1

- Thaw reagents (not enzymes) in the BD Rhapsody™ Tagmentation and Supplemental Reagents Kit (Cat. No. 571201) and BD Rhapsody™ ATAC-Seq Amplification Kit (Cat. No. 571356) at room temperature (15–25 °C), and then place on ice. Keep enzymes at –25 °C to –15 °C.

  Note: Only thaw the reagents needed for the day.
- Dilute 2% Digitonin to 1% Digitonin with nuclease-free water.
- Prepare tagmentation buffer with dimethylformamide (DMF): Thaw and transfer 200 µL of tagmentation buffer into a new 1.5-mL LoBind® tube, add 50 µL of 100% DMF into the tube, and mix by vortexing. Tagmentation buffer with DMF can be stored at –25 °C to –15 °C for later use.
- Place on ice the following components of the BD Rhapsody™ Enhanced Cartridge Reagent Kit V3 (Cat. No. 667052):

- Sample buffer
- 1M DTT
- Bead wash buffer
- BD Rhapsody™ Enhanced Cell Capture Beads V3

- Visually inspect the lysis buffer for any precipitation. If precipitation is not present, leave the lysis buffer at room temperature (15–25 °C) until ready to use. If precipitation is present, incubate the lysis buffer at room temperature for 1 hour. Invert to mix, but do not vortex. Once the solution is clear, continue leaving the lysis buffer at room temperature until ready to use.
- Open the tube while holding the DTT tube vertically. The solution is overlain with an inert/non-oxygen-containing gas. A non-vertical tube will allow the inert gas to pour off. After opening the DTT tube once, seal and store the tube at –25 °C to –15 °C.
- Thaw DyeCycle™ Green at room temperature (15–25 °C). Follow the manufacturer's instructions and protect it from light.
- Aliquot 100% ethyl alcohol and cartridge reagent buffers in 10-mL or 25-mL reagent reservoirs based on the number of lanes used, as instructed by the following table. Do not aliquot for single lane.

Component	For 1 lane (mL)	For 2 lanes (mL)	For 3 lanes (mL)	For 4 lanes (mL)	For 5 lanes (mL)	For 6 lanes (mL)	For 7 lanes (mL)	For 8 lanes (mL)
100% ethyl alcohol	0.05	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Cartridge wash buffer 1	0.76	3.50	5.25	7.00	8.75	10.50	12.25	14.00
Cartridge wash buffer 2	0.38	2.00	3.00	4.00	5.00	6.00	7.00	8.00

- For a single cartridge workflow, adhere to the specified buffer volume as recommended in the *BD Rhapsody™ Protocol for Single Cell Capture and cDNA Synthesis*.
- When conducting the experiment in accordance with the *BD Rhapsody™ HT Xpress System Instrument User Guide* for a scanner-free workflow, it is essential to utilize a thermomixer for the **Bead Agitation** step. It is important to keep the cartridge leveled.

Best practices

- Use low-retention filtered pipette tips.
- Use wide-bore tips when handling nuclei.
- It is important to keep the nuclei isolation on ice at all times.
- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes. Never vortex the beads. Pipet-mix only.
- Bring AMPure XP magnetic beads to room temperature before use.
- Remove supernatants without disturbing AMPure XP magnetic beads.
- It is recommended to use a swinging-bucket centrifuge for pelleting cells and nuclei.
- For a complete list of materials for the BD Rhapsody™ system, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 23-24257).

Important: The BD Rhapsody™ Enhanced Cartridge Reagent Kit V3 (Cat. No. 667052) must be used for this protocol. The BD Rhapsody™ Tagmentation and Supplemental Reagents Kit (Cat. No. 571201) is not compatible with the BD Rhapsody™ Enhanced Cartridge Reagent Kit (Cat. No. 664887).

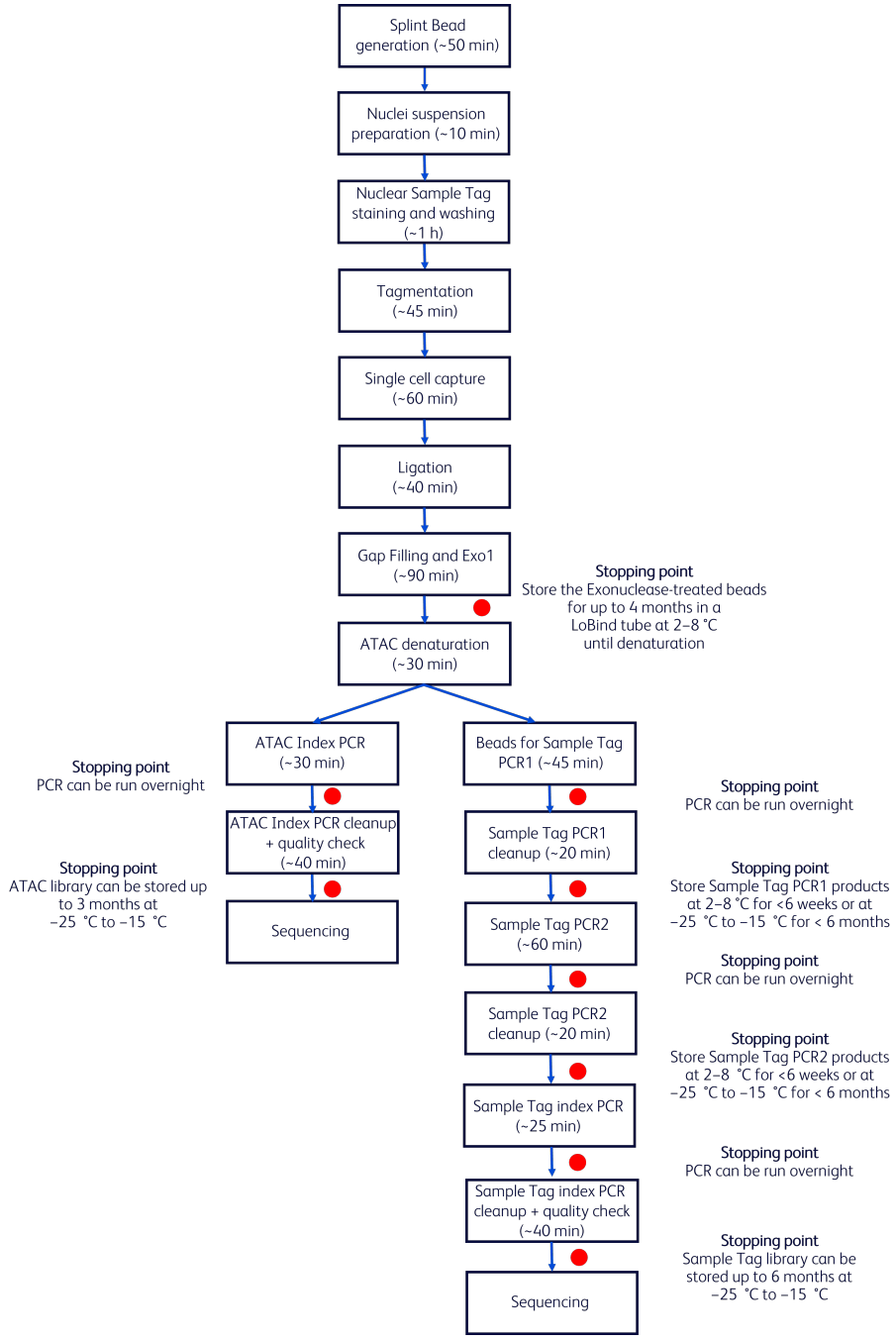
Additional documentation

- *BD Rhapsody™ Preparing Single-Cell Suspensions Protocol* (Doc ID: 23-24126)
- *BD Rhapsody™ HT Single-Cell Capture and Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (Doc ID: 23-24252)
- *BD® Rhapsody Sequence Analysis Pipeline User's Guide* (Doc ID: 23-24580)

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID: 23-24257).

Time considerations



Procedure

The range of intended total nuclei load on a cartridge is between 1,000 to 50,000 nuclei for this protocol. Nuclei load below or above this recommended range may not be suitable for current protocol configuration. Follow the procedures listed in this section.

Important: The BD Rhapsody™ Enhanced Cartridge Reagent Kit V3 (Cat. No. 667052) must be used for this protocol.

Splint bead generation

1. Set the thermomixers to 25 °C and 70 °C.
2. Obtain BD Rhapsody™ Enhanced Cell Capture Beads V3.
3. Place the tube on a magnet rack to magnetize beads down until the solution is clear.
4. Remove and discard the supernatant.
5. Remove the tube from the magnet rack and pipet 1 mL of splint-bead wash buffer to the tube.
6. Resuspend beads by slowly pipetting up and down 10 times.
7. Transfer the resuspended beads into a new 1.5-mL LoBind® tube.
8. Place the tube on a magnet rack to magnetize beads down until the solution is clear.
9. Remove and discard the supernatant.
10. Remove the tube from the magnet rack and add the following into the tube.

Color	ATAC-Seq Tagmentation and Supplemental Kit Component	Volume (µL)
	Splint-bead wash buffer	160
	Splint oligo annealing buffer	20
	Universal ATAC-Seq splint oligo	20

11. Briefly centrifuge, then resuspend the beads by slowly pipetting up and down 10 times.
12. Place the tube in the thermomixer at 70 °C and incubate for 5 minutes with 1200 rpm mixing.
13. Transfer the tube to the thermomixer at 25 °C and incubate for 30 minutes with 1200 rpm mixing.
14. Remove the tube from the thermomixer, briefly centrifuge, and place the tube on a magnet rack to magnetize the beads down until the solution is clear.
15. Remove and discard the supernatant.
16. Remove the tube from the magnet rack and add 1 mL of splint-bead wash buffer.
17. Resuspend the beads by slowly pipetting up and down 10 times.
18. Place the tube on the magnet rack to magnetize the beads down until the solution is clear.
19. Remove and discard the supernatant.
20. Repeat wash with 1 mL of splint-bead wash buffer 2 more times for a total of three washes.
21. After the final wash, resuspend the beads in 380 µL of cold sample buffer by slowly pipetting up and down 10 times.
22. Store on ice if proceeding on the same day, or at 2–8 °C for up to 1 week.

Nuclei isolation

Note: Reagents required for nuclei isolation are not included in the kits.

For optimal results with each specific sample type, refer to established nuclei isolation protocols and identify the method best suited for the cells or tissues of interest.

Labeling nuclei with Sample Tags

1. Prepare ATAC-SMK buffer (for buffer composition, contact scomix@bdscomix.bd.com). Each sample needs 5 mL of ATAC-SMK buffer for Sample Tag staining and washing.
2. Resuspend up to 1 million nuclei pellet in 200 µL ATAC-SMK buffer.
3. Briefly centrifuge the Sample Tag tubes to collect the contents at the bottom.
4. For each sample, add 2 µL of nuclear Sample Tag reagent to the nuclei suspension tube. Gently pipet-mix with a wide bore tip for 10 times.
5. Incubate the samples on ice for 30 minutes.

Washing labeled nuclei

Note: Sufficient post-labeling washing is important for reducing noise that comes from residual unbound antibodies being captured onto 3' capture beads during single-nuclei capture. However, some nuclei loss occurs with each additional wash. Users can choose to perform more or fewer washes depending on the abundance of their sample.

1. Transfer each labeled nuclei suspension to a 5-mL polystyrene Falcon® tube.
2. Pipet 2 mL of ATAC-SMK buffer to labeled nuclei and pipet-mix for 10 times.
3. Centrifuge each tube at 500 × g for 5 minutes at 4 °C to pellet the nuclei.
4. Uncap each tube and invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from tube rim.
5. Pipet 2 mL of ATAC-SMK buffer to each tube and resuspend by pipet-mixing for 10 times.
6. Centrifuge at 500 × g for 5 minutes at 4 °C to pellet the nuclei.
7. Uncap each tube and invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-wiper to remove residual supernatant from tube rim.
8. (Optional) Repeat steps 5-7 once more for a total of 3 washes.
9. Resuspend the Sample Tag labeled nuclei pellet in 25 µL nuclei buffer with a wide bore tip by gentle pipetting 10 times, targeting for 10,000 nuclei/µL and keep on ice.

Prepare nuclei suspension for tagmentation

1. Dilute an aliquot of nuclei 20-fold in cold sample buffer from the BD Rhapsody™ Enhanced Cartridge Reagent Kit for nuclei counting.
   a. Pipet 95 µL of cold sample buffer into a new 1.5-mL LoBind® tube.
   b. Ensure the nuclei are well suspended by gently pipet-mixing.
   c. Pipet 5 µL of the nuclei suspension into the tube with 95 µL cold sample buffer. Keep the remaining nuclei on ice.
   d. Pipet 0.5 µL of 5 mM DyeCycle Green into the tube.
   e. Gently pipet-mix with a wide-bore tip 10 times and incubate on ice for 5 minutes to stain the nuclei, protected from light.
2. Count the stained nuclei immediately using the BD Rhapsody™ Scanner.
   a. Ensure the stained nuclei are well suspended by gently pipet-mixing.
   b. Pipet 10 µL into INCYTO disposable hemocytometer and count using the scanner.
   c. Multiply the reading by 20 to calculate the concentration of unstained nuclei.
      - If unstained nuclei concentration is >10,000 nuclei/µL, dilute the nuclei to 10,000 nuclei/µL with nuclei buffer and keep on ice.
      - If the unstained nuclei concentration is <10,000 nuclei/µL, keep on ice. Adjust the nuclei volume and nuclease-free water in the tagmentation reaction.
3. Pool Sample Tag labeled nuclei at a desired ratio. Multiplexed nuclei are ready for tagmentation.

Tagmentation

1. Set a thermomixer to 37 °C.
2. In a new 1.5-mL LoBind® tube, add the following reagents in order. Pipet-mix the buffer and Tn5 tagmentase 10 times before adding nuclei. Ensure the nuclei are well suspended by gently pipet-mixing with a wide bore tip. Add 50,000 Sample Tag labeled nuclei and gently pipet-mix 5–10 times with a wide-bore tip.

   Tagmentation mix

Color	Kit Component	Volume (µL)
●	Tagmentation buffer with DMF	25
○	Nuclease-free water	13[a]
●	10X PBS	2
●	Digitonin 1%[b]	0.5
●	Tween20, 10%	0.5
●	Tagmentase	4
	Nuclei	5[a]
	[a] If the nuclei concentration is less than 10,000 nuclei/µL, adjust the volume of nuclei and nuclease-free water. [b] 1% Digitonin is diluted from 2% stock with nuclease-free water.	

   Note: Successful tagmentation has been performed with 50,000 nuclei in 50 µL reaction. When using less than 50,000 nuclei, proportionally scale down the reaction.
3. Incubate the reaction at 37 °C for 30 minutes in a thermomixer without shaking.

   Note: During the incubation time, start priming the cartridge by following step 1 in section BD Rhapsody™ cartridge workflow (no scan other than indicated) (page 19).
4. After incubation, add 400 µL of cold sample buffer into the Tagmentation mix.
5. Gently pipet-mix 5 times and keep on ice.
6. Wet the cell strainer with 50 µL of sample buffer. Filter the tagmented nuclei through a Falcon tube with cell strainer cap. Place the tube on ice.

7. Stain an aliquot of tagmented nuclei and count the stained nuclei immediately using the BD Rhapsody™ Scanner.

   Note: DO NOT STAIN THE ENTIRE SAMPLE.

   Nuclei staining dye can impact ATAC-seq data. Consequently, no cartridge scanner metrics will be collected.

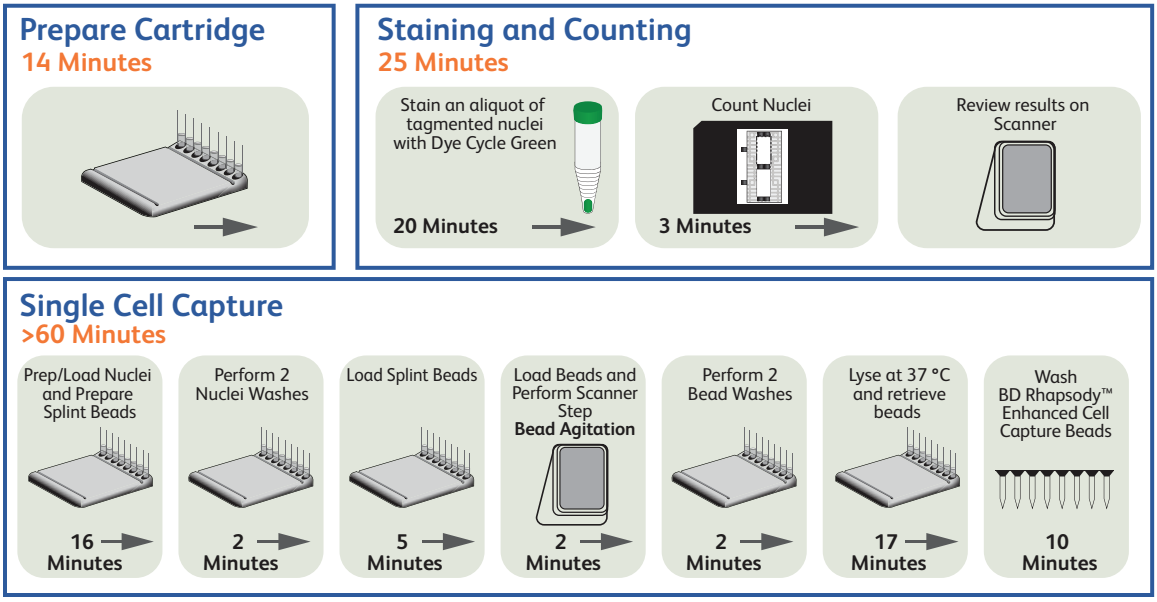
   a. Ensure the nuclei are well suspended by gently pipet-mixing.
   b. Pipet 50 µL of the nuclei suspension into a new 1.5-mL LoBind® tube. Keep the remaining nuclei suspension on ice.
   c. Pipet 1.25 µL of 5-times diluted DyeCycle™ Green* (1 mM) into the tube containing the 50 µL nuclei aliquot.

      Note: *Dilute 5 mM DyeCycle™ Green 5 fold to 1 mM with DMSO.

   d. Pipet-mix 5 times with wide-bore tip and incubate on ice for 5 minutes to stain the nuclei, protected from light.

8. Count the stained nuclei immediately using the BD Rhapsody™ Scanner.
   a. Ensure the stained nuclei are well suspended by gently pipet-mixing.
   b. Pipet 10 µL into INCYTO disposable hemocytometer and count using the scanner.
   c. Viability information is not applicable. Use the concentration to calculate loading dilution.

BD Rhapsody™ cartridge workflow (no scan other than indicated)



1. Prime the cartridge.
   a. Prior to priming the cartridge, scan at least one lane of the empty cartridge for Cell Load scan. For detailed instructions, see *BD Rhapsody™ HT Single-Cell Capture and Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (Doc ID: 23-24252).
   b. Place waste collection container and cluster tube in the BD Rhapsody™ HT Xpress System.

c. Carefully peel off the seal on the cartridge inlet of the lanes to be used.

d. Prime the cartridge using the following table steps with BD Rhapsody™ P8xP1200µL pipette:

Step number	Material to load	Volume (µL/lane)	Pipette mode	Incubation at room temperature
1	100% ethyl alcohol	50	EtOH Prime	N/A
2	Air	380	Prime/Wash	N/A
3	Room temp. Cartridge wash buffer 1	380	Prime/Wash	1 min
4	Air	380	Prime/Wash	N/A
5	Room temp. Cartridge wash buffer 1	380	Prime/Wash	3 min
6	Air	380	Prime/Wash	N/A
7	Room temp. Cartridge wash buffer 2	380	Prime/Wash	≤4 hr

2. Prepare single-nuclei suspension for cartridge loading:

   Use the BD Rhapsody™ Scanner to calculate the number of nuclei for cartridge loading.

   a. Select Sample Calculator.

   b. Select the correct cartridge type. For the BD Rhapsody™ 8-Lane Cartridge, use 0120.

   c. Calculate the volumes of tagmented nuclei and sample buffer needed to prepare a nuclei suspension of 380 µL (this volume is for one lane).

   d. Prepare 380 µL nuclei suspension for cartridge loading by mixing unstained tagmented nuclei with cold sample buffer according to the displayed volumes on the scanner. Ensure the stock solution of each sample is well suspended by gently pipet-mixing with a wide-bore tip before pooling. Keep the nuclei suspension on ice.

   e. If working with multiple samples, transfer each tube of prepared nuclei suspension into a 96-deep-well plate for multiple lane loading. Keep the 96-deep-well plate on ice.

3. Load tagmented nuclei in the cartridge:

   a. Load the cartridge with materials listed in the following table using the BD Rhapsody™ P8xP1200µL pipette:

Material to load	Volume (µL) 1 lane	Pipette mode
Air	380	Prime/Wash
• Gently pipet mix with a multi-channel pipette to completely resuspend the nuclei. • Set the BD Rhapsody™ P8xP1200µL pipette (or BD Rhapsody™ P1200µL pipette) to **Load** mode. • Immediately load.		
Nuclei suspension	320	Load

   Note: Air bubbles that might appear at the inlet or outlet of the cartridge do not affect cartridge performance.

   b. Incubate at room temperature (15–25 °C) for 8 minutes.

4. Wash the loaded nuclei with cold sample buffer:

   Note: Do not omit this step. It is necessary to obtain good ATAC data.

   a. Place the cartridge on the BD Rhapsody™ HT Xpress System.
   b. Set the BD Rhapsody™ P8x1200 µL pipette to **Prime/Wash** mode.
   c. Load the cartridge with materials listed in the following table using the BD Rhapsody™ P8x1200 µL pipette:

Material to load	Volume (µL) 1 lane	Pipette mode
Air	380	Prime/Wash
Cold sample buffer	380	Prime/Wash
Air	380	Prime/Wash
Cold sample buffer	380	Prime/Wash

5. Load and wash cell-capture beads:

   a. Place the cartridge on the BD Rhapsody™ HT Xpress System.
   b. Set the BD Rhapsody™ P8xP1200 µL pipette to **Prime/Wash** mode.
   c. Bring the splint beads generated from the Splint bead generation (page 15) steps.
   d. Load the cartridge with materials listed below using the BD Rhapsody™ P8xP1200 µL pipette:

Material to load	Volume (µL) 1 lane	Pipette mode
Air	380	Prime/Wash
• Gently pipet mix with a multi-channel pipette to completely resuspend the beads. • Set the BD Rhapsody™ P8xP1200µL pipette (or BD Rhapsody™ P1200µL pipette) to Load mode. • With a new set of pipette tips, immediately load the beads. Check the pipette tips to make sure that there are no air bubbles inside the tips before loading. Otherwise, dispense the beads into the 96-deep well plate and aspirate with a new set of pipette tips.		
Splint beads	320	Load

   e. Incubate the cartridge at room temperature (15–25 °C) for 3 minutes.
   f. Perform scanner step: **Bead Agitation**.
   g. After bead agitation is complete, tap **OK**, then **Eject**. Remove the cartridge from the scanner.
   h. Place the cartridge on the BD Rhapsody™ HT Xpress System.
   i. Set the BD Rhapsody™ P8xP1200µL pipette to Prime/Wash mode.
   j. Load the cartridge with materials listed below using the BD Rhapsody™ P8xP1200µL pipette:

Material to load	Volume (µL) 1 lane	Pipette mode
Air	380	Prime/Wash
Cold sample buffer	380	Prime/Wash
Air	380	Prime/Wash
Cold sample buffer	380	Prime/Wash

6. Lyse nuclei:
   a. Add 75.0 µL of 1 M DTT to one room-temperature 15-mL lysis buffer bottle and briefly vortex mix. Use the lysis buffer with DTT within 24 hours, and then discard.
   b. Pipette 0.5 mL of lysis buffer with DTT into a new 1.5-mL LoBind® tube. Add 25 µL of Proteinase K to the tube immediately before the lysis step, and gently pipet-mix 5 times.

      Note: 0.5 mL is enough for one lane. Scale up proportionally if multiple lanes are used.
   c. Set the BD Rhapsody™ P8xP1200µL pipette to Lysis mode.
   d. Load the cartridge with materials listed using the BD Rhapsody™ P8xP1200µL pipette:

Material to load	Volume (µL) 1 lane	Pipette mode
Lysis buffer with DTT and Proteinase K	280	Lysis

   e. Carefully remove the cartridge from the BD Rhapsody™ HT Xpress System. Slowly transfer the cartridge into an incubator at 37 °C and incubate for 10 minutes. Maintain the recommended lysis time for best performance.

      Note: It is important to keep the cartridge leveled.

7. Retrieve cell-capture beads:
   a. Ensure the cluster tube 8-tube strip is placed into the BD Rhapsody™ HT Xpress System drawer. Label the tubes appropriately.
   b. Ensure that the BD Rhapsody™ P8xP1200µL pipette is set to **Retrieval** mode.
   c. Move the front slider to BEADS on the BD Rhapsody™ HT Xpress System.
   d. Carefully bring the cartridge from the 37 °C incubator to the BD Rhapsody™ HT Xpress System and allow the cartridge to cool down for 5 minutes.
   e. Gently pull the top RETRIEVAL slider toward and on top of the cartridge.
   f. Leave the retrieval magnet in the down position for 1 minute.
   g. Aspirate 1,000 µL lysis buffer with DTT using the BD Rhapsody™ P8xP1200µL pipette.
   h. Press down on the BD Rhapsody™ P8xP1200µL pipette to seal against the gasket.
   i. Push back the top RETRIEVAL magnet, and immediately load 1,000 µL lysis buffer with DTT.
   j. Remove the pipette from the gasket and purge the tips.
   k. Move the front slider to OPEN and remove the cluster tube with the bottom adapter to a flat, secure surface.
   l. Remove the cluster tube from the bottom adapter. Gently pipet-mix the beads and transfer into a new 1.5-mL LoBind® tube. Keep on ice.
   m. If beads are still left in the cluster tube, add 100 µL of lysis buffer with DTT, rinse the cluster tube, and transfer into the 1.5-mL LoBind® tube from the previous substep.
   n. Immediately proceed to the next step (Wash cell-capture beads).

8. Wash cell-capture beads:
   a. Place the tube on a magnet rack for 2 minutes.
   b. Remove and discard the supernatant. Avoid leaving lysis buffer or bubbles in the tube.
   c. Remove the tube from the magnet, and pipet 1.0 mL cold bead wash buffer into the tube. Pipet-mix.
   d. Place the tube on the magnet rack for 2 minutes. Remove and discard the supernatant.
   e. Remove the tube from magnet, and pipet 1.0 mL cold bead wash buffer into the tube. Pipet-mix, and place on ice.

   Note: Start Ligation ≤30 minutes after washing retrieved cell-capture beads with bead wash buffer.

Ligation

1. Set a thermomixer to 25 °C.
2. In a new 1.5-mL or 2.0-mL LoBind® tube, add the following components:

   Ligation mix

Color	Kit component	For 1 library (µL)	For 1 library with 10% overage (µL)	For 4 libraries with 10% overage (µL)	For 8 libraries with 10% overage (µL)
●	Ligation buffer	20	22	88	176
●	Ligase	10	11	44	88
○	Nuclease-free water	170	187	748	1496
	Total	**200**	**220**	**880**	**1760**

3. Gently vortex Ligation mix, briefly centrifuge and place on ice.
4. Place the tube of washed cell-capture beads on a magnet rack for 2 minutes. Remove and discard the supernatant.
5. Remove the tube from the magnet and pipet 200 µL of Ligation mix into the tube.
6. Resuspend the beads by pipet-mixing 10 times.
7. Transfer the whole reaction into a new 1.5-mL LoBind® tube.
8. Incubate the tube in the thermomixer at 25 °C for 30 minutes with 1,200 rpm mixing.

Gap filling

1. Program the thermomixer.
   a. 1,200 rpm and at 25 °C for 10 minutes.
   b. 1,200 rpm and at 37 °C for 15 minutes.
   c. 1,200 rpm and at 45 °C for 10 minutes.
   d. 1,200 rpm and at 55 °C for 10 minutes.

   IMPORTANT: Confirm "Time Mode" is set to **Time Control** before the program begins.
2. In a new 1.5-mL or 2.0-mL LoBind® tube, add the following components and mix.

   Gap filling mix

Color	Kit component	For 1 library (µL)	For 1 library with 10% overage (µL)	For 4 libraries with 10% overage (µL)	For 8 libraries with 10% overage (µL)
○	Nuclease-free Water	138	151.8	607.2	1214.4
●	dNTP	20	22	88	176
●	Gap-filling enhancer	12	13.2	52.8	105.6
●	Gap-filling buffer	20	22	88	176
●	Gap-filling enzyme	10	11	44	88
	Total	**200**	**220**	**880**	**1760**

3. Place the tube with ligase-treated beads on a magnet rack for 2 minutes. Remove and discard the supernatant.
4. Resuspend the beads in 200 µL of gap filling mix.
5. Place the tube in the thermomixer. Start the program set in step 1.

Splint oligo removal

1. Set the thermomixers to 60 °C and 37 °C.
2. Upon completion of gap filling, remove the tube from the thermomixer and place on the magnet for 2 minutes. Remove and discard the supernatant.
3. Remove the tube from the magnet and pipet 200 µL of splint oligo removal buffer into the tube. For buffer composition, contact scomix@bdscomix.bd.com.
4. Resuspend the beads by pipet-mixing 10 times.
5. Incubate the tube in the thermomixer at 60 °C for 5 minutes with 1,200 rpm mixing.
6. Remove the tube from the thermomixer and immediately place it on ice for 5 minutes.

Exonuclease I treatment

1. In a new 1.5-mL or 2.0-mL LoBind® tube, add the following components and gently vortex mix. Briefly centrifuge then place on ice.

   Exonuclease I mix

Kit component	For 1 library (µL)	For 1 library with 10% overage (µL)	For 4 libraries with 10% overage (µL)	For 8 libraries with 10% overage (µL)
10X Exonuclease I buffer	20	22	88	176
Exonuclease I	10	11	44	88
Nuclease-free water	170	187	748	1496
Total	**200**	**220**	**880**	**1760**

2. Remove the tube from ice, quick spin and place on the magnet for 2 minutes. Remove and discard the supernatant.
3. Remove the tube from the magnet and pipet 200 µL of Exonuclease I mix into the tube.
4. Resuspend the beads by pipet-mixing 10 times.
5. Incubate the tube in the thermomixer at 37 °C for 30 minutes with 1,200 rpm mixing.
6. Remove the tube from the thermomixer and add 4 µL of 0.5M EDTA to the Exonuclease I-treated beads. Pipet-mix 10 times.
7. Briefly centrifuge and then place the tube on the magnet for 2 minutes. Remove and discard the supernatant.
8. Remove the tube from the magnet and pipet 200 µL of bead resuspension buffer into the tube. Resuspend the beads by pipet-mixing 10 times.

   STOPPING POINT: Exonuclease I - treated beads can be stored at 2–8 °C for up to 4 months.

Performing single-cell ATAC library index PCR

1. Set a thermomixer to 95 °C.
2. Choose between using the entire sample or a sub-sample of the gap filling-treated beads. If using the entire sample, skip to step 4. If using a subsample, proceed to step 3.
3. (Optional) Subsample the gap filling-treated beads:
   - Determine the volume of beads to subsample for sequencing, based on the expected number of nuclei captured on beads in the final bead-resuspension volume.
   - Completely resuspend the beads by pipet-mixing, then pipet the calculated volume of bead suspension into a new 1.5-mL LoBind® tube. If needed, bring the total volume up to 200 µL with bead resuspension buffer.

   Note: The remaining beads can be stored in bead resuspension buffer at 4 °C for up to 4 months.
4. Place the tube with gap filling-treated beads on a magnet rack for 2 minutes. Remove and discard the supernatant.
5. Pipet 40 µL of elution buffer to the beads. Pipet-mix.
6. Incubate the tube in the thermomixer at 95 °C for 5 minutes (no shaking).
7. Remove the tube from the thermomixer and immediately place the tube on ice for 5 minutes.
8. Remove the tube from ice, quick spin, and then place the tube on a magnet rack until the solution is clear.
9. Transfer the entire supernatant to a new 0.2-mL PCR tube. Keep on ice.
10. Pipet 40 µL of elution buffer to the beads. Pipet-mix.
11. Incubate the tube in the thermomixer at 95 °C for 5 minutes (no shaking).
12. Remove the tube from the thermomixer and immediately place the tube on ice for 5 minutes.
13. Remove the tube from ice, quick spin and then place the tube on the magnet rack until the solution is clear.
14. Transfer the entire supernatant into the PCR tube with the previously collected 40 µL eluted supernatant. Total 80 µL of ATAC products.
15. Resuspend the beads with 200 µL bead resuspension buffer. Store the beads at 2–8 °C until ready for Sample Tag library generation as described in Sample Tag library amplification (page 30).
16. In a new 1.5-mL tube, add the following components. Gently vortex mix, briefly centrifuge, then place on ice.

ATAC index PCR mix

Color	Kit component	For 1 library (µL)	For 1 library with 10% overage (µL)	For 4 libraries with 10% overage (µL)	For 8 libraries with 10% overage (µL)
○	PCR master mix	30	33	132	264
○	ATAC-Seq library forward primer	6	6.6	26.4	52.8
●	ATAC-Seq library reverse primer(1–8)[a]	6	6.6	–	–
	Total	**42**	**46.2**	**158.4**	**316.8**

a. For more than one ATAC library, use a different ATAC-Seq library reverse primer for each library.

17. Combine the ATAC index PCR mix with ATAC products as follows:
    - For one sample, the ATAC index PCR mix includes an ATAC-Seq library reverse primer. Combine 42 µL of the mix with 80 µL of ATAC product. Pipet-mix 10 times, and then split the reaction volume (122 µL) into two 0.2-mL PCR tubes.
    - If working with multiple samples, the ATAC index PCR mix does not include ATAC-Seq library reverse primer because the reverse primer must be sample-specific. In separate tubes for each sample, combine 36 µL of the ATAC index PCR mix with 80 µL of ATAC product and 6 µL of the ATAC-Seq library reverse primer that is specifically assigned to the sample. Pipet-mix 10 times, and then split the reaction volume (122 µL) into two 0.2-mL PCR tubes.
18. Gently vortex mix and briefly centrifuge.
19. In post-amplification workspace. Run the following PCR program. (Volume = 60 µL)

Step	Cycles	Temperature	Time
Hot start	1	98 °C	45 s
Denaturation	12–16 cycles[a]	98 °C	10 s
Annealing		66 °C	30 s
Extension		72 °C	30 s
Final extension	1	72 °C	1 min
Hold	1	10 °C	∞

a. Suggested PCR cycles might need to be optimized for different cell types and number of cells.

STOPPING POINT: The PCR can run overnight.

Recommended number of PCR cycles

Number of cells in ATAC PCR	Suggested number of PCR cycles
≥10,000	12
10,000–5,000	13
4,999–1,000	14
<1,000	16

Purifying ATAC index PCR product

1. Perform the purification in post-amplification workspace.
2. Bring AMPure XP beads to room temperature (15–25 °C) and vortex at high speed for 1 minute until beads are fully resuspended.
3. In a new 5.0-mL LoBind® tube, prepare 2 mL (per sample) fresh 80% (v/v) ethyl alcohol by combining 1.6 mL absolute ethyl alcohol, molecular biology grade, with 0.4 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol (1 mL/sample) and use it within 24 hours.
4. When the ATAC Index PCR is complete, briefly centrifuge to collect the contents at the bottom of the tubes.
5. In a new 1.5-mL LoBind® tube, combine the two reactions of each sample for a total volume of 120 µL.

6. Pipet 144 µL of AMPure XP beads into the tube containing 120 µL of ATAC Index PCR products. Pipet-mix 10 times.
7. Incubate at room temperature (15–25 °C) for 5 minutes.
8. Place the tube on the 1.5-mL tube magnet for 3 minutes. Remove and discard the supernatant.
9. Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant.
10. Repeat step 9 once for a total of two washes.
11. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
12. Air-dry the beads at room temperature (15–25 °C) for 5 minutes.
13. Remove the tube from the magnet and resuspend the beads in 40 µL of elution buffer. Pipet-mix until the beads are fully resuspended.
14. Incubate at room temperature (15–25 °C) for 2 minutes.
15. Briefly centrifuge and place the tube on the magnet until the solution is clear, usually ≤30 seconds.
16. Transfer the supernatant (~40 µL) containing the purified ATAC library into a new 1.5-mL LoBind® tube.

    STOPPING POINT: Store at 2–8 °C if proceeding on the same day, or at –25 °C to –15 °C for up to 3 months.

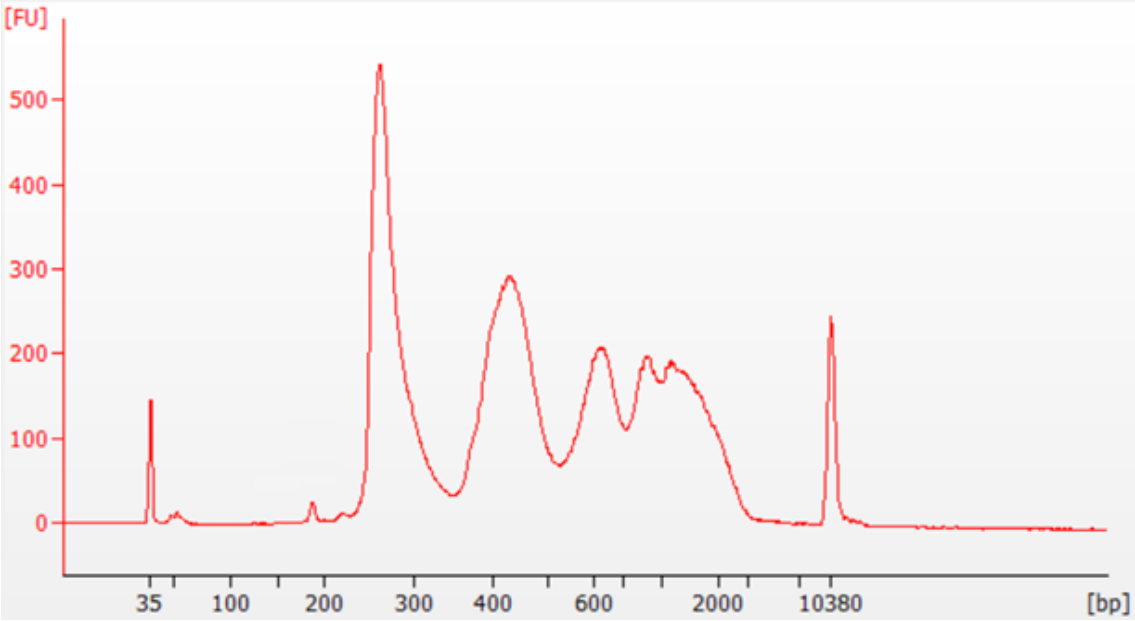
Performing quality control on the final sequencing libraries

1. Measure the concentration of each ATAC library by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer and Qubit dsDNA HS assay, and perform quality control of the ATAC library using either of the following systems:
   a. Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit
   b. Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape Assay
2. If needed, dilute the library to the quantitative range of the Agilent 2100 Bioanalyzer. Measure the fragment size of the library following the manufacturer's instructions.
   a. If the concentration is >5 ng/µL, dilute the library to ≤5 ng/µL with elution buffer.
   b. Measure the average fragment size of the ATAC libraries within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the Agilent High Sensitivity DNA Kit (Agilent Cat. No. 5067-4626). Follow the manufacturer's instructions.

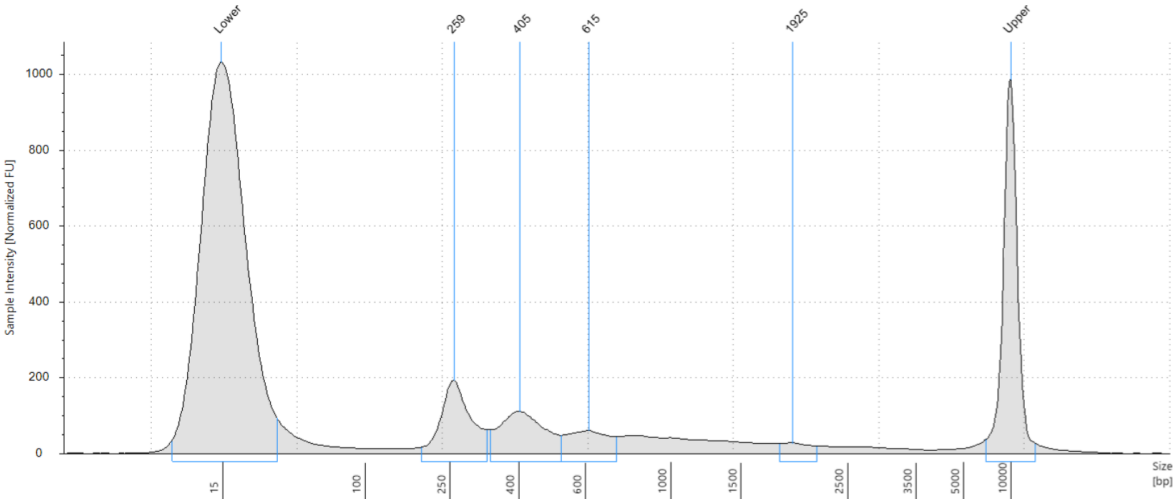
      The following diagram shows a representative ATAC library trace from the Bioanalyzer and TapeStation, where the majority of the fragments are distributed between ~200–2000 bp.

Figure 1 BD Rhapsody™ ATAC Library

A. Bioanalyzer High Sensitivity DNA trace



B. TapeStation high-sensitivity D5000 trace



Sample Tag library amplification

Before you begin

- Obtain beads from step 15 of Performing single-cell ATAC Library Index PCR (page 26).
- Thaw reagents in the BD Rhapsody™ targeted mRNA and AbSeq Amplification Kit at room temperature (15–20 °C), then immediately place on ice.

Performing Sample Tag PCR1

1. In the pre-amplification workspace, pipet reagents into a new 1.5 mL LoBind® tube on ice.

Sample Tag PCR1 reaction mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% coverage (µL)	For 8 libaries with 20% overage (µL)
○	PCR master mix	100.0	120.0	480.0	960.0
○	Universal oligo	1.2	1.4	5.8	11.5
●	Bead RT/PCR enhancer	12.0	14.4	57.6	115.2
●	Sample Tag PCR1 primer	1.2	1.4	5.8	11.5
○	Nuclease-free water	85.6	102.7	410.9	821.8
	Total	**200**	**240**	**960**	**1920**

2. Gently vortex the mix, briefly centrifuge, and place back on ice.
3. Place the tube of ATAC-denatured beads from step 15 of Performing single-cell ATAC Library Index PCR (page 26) on 1.5 mL magnet for <2 minutes. Remove and discard the supernatant.
4. Remove the tube from the magnet, and resuspend the beads in a 200 µL Sample Tag PCR1 reaction mix. Do not vortex.
5. Ensuring that the beads are fully resuspended, pipet 50 µL PCR1 reaction mix with the beads into each of the four 0.2-mL PCR tubes. Transfer any residual mix to one of the tubes.
6. Bring the reaction mix to the post-amplification workspace.

7. Program the thermal cycler. **Do not use fast cycling mode**.

Program thermal cycler

Step	Cycles	Temperature	Time
Hot start	1	95 °C[a]	3 min
Denaturation	9–13[b]	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

a. To avoid beads settling due to prolonged incubation time on thermal cycler before the denaturation step, it is critical to pause the instrument at 95 °C before loading the samples. Different thermal cyclers might have different pause time settings. In certain brands of thermal cyclers, however, we have observed a step-skipping error with the pause/unpause functions. To ensure that the full three-minute denaturation is not skipped, verify that the pause/unpause functions are working correctly on your thermal cycler. To avoid the step- skipping problem, a one-minute 95 °C pause step can be added immediately before the three-minute 95 °C denaturation step.

b. Suggested PCR cycles might need to be optimized for different cell types and cell number.

Suggested number of PCR cycles

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
1,000	13
2,500	12
5,000	11
10,000	10
20,000	9

8. Ramp heated lid and heat block of post-amplification thermal cycler to 95 °C by starting the thermal cycler program and then pausing it.

   Note: Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension.

9. For each 0.2-mL PCR tube, gently pipet-mix, immediately place tube in thermal cycler, and unpause the thermal cycler program.

   STOPPING POINT: The PCR can run overnight but proceed with purification up to 24 hours after PCR.

10. After PCR, briefly centrifuge tubes.

11. Pipet-mix and combine the four reactions into a new 1.5-mL LoBind® tube.

    Note: Retain the supernatant in the next step.

12. Place the 1.5-mL tube on magnet for 2 minutes, and carefully pipet the supernatant (Sample Tag PCR1 products) into the new 1.5 mL LoBind® tube without disturbing the beads.

    Note: (Optional) Remove the tube with the BD Rhapsody™ Enhanced Cell Capture Beads from the magnet, and pipet 200 µL cold Bead Resuspension Buffer into the tube. Pipet-mix. Do not vortex. Store beads at 2–8 °C in the post-amplification workspace.

Purifying Sample Tag PCR1 products by single-sided size selection

1. In a new 5.0-mL LoBind® tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by combining 4.0 mL absolute ethyl alcohol, molecular biology grade (major supplier) with 1.0 mL nuclease-free water (major supplier). Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries. Volumes provided in the following table are enough to cover all PCR clean ups throughout the protocol.

   80% Ethyl Alcohol

Component	For 1 library (mL)	For 4 libraries (mL)	For 8 libraries (mL)
100% ethyl alcohol	4	16	32
Nuclease -free water	1	4	8
Total	**5**	**20**	**40**

2. Bring AMPure XP beads to room temperature. Vortex at high speed for 1 minute until the beads are fully resuspended.
3. Pipet 280 µL AMPure XP beads into a tube with 200 µL Sample Tag PCR1 products—see step 12 of Performing Sample Tag PCR1 (page 31). Pipet-mix 10 times.
4. Incubate at room temperature for 5 minutes.
5. Place 1.5-mL LoBind® tube on magnet for 5 minutes.
6. Keeping the tube on the magnet, remove and discard the supernatant.
7. Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol, and incubate 30 seconds. Remove and discard the supernatant.
8. Repeat step 7 once for a total of two washes.
9. Keeping the tube on the magnet, use a small-volume pipette to remove and discard residual supernatant from the tube.
10. Air-dry beads at room temperature for 5 minutes.
11. Remove the tube from the magnet, and resuspend the bead pellet in 30 µL of elution buffer. Vigorously pipet-mix until beads are uniformly dispersed. Small clumps do not affect performance.
12. Incubate at room temperature for 2 minutes, and briefly centrifuge.
13. Place the tube on the magnet until the solution is clear, usually within 30 seconds.
14. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified Sample Tag PCR1 products).

    STOPPING POINT: Store at 2–8 °C before proceeding within 24 hours or at –25 °C to –15 °C for up to 6 months

Performing PCR2 on Sample Tag PCR1 products

1. In the pre-amplification workspace, pipet reagents into a new 1.5-mL LoBind® tube on ice.

Sample Tag PCR2 reaction mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% coverage (µL)	For 8 libaries with 20% overage (µL)
◯	PCR master mix	25.0	30.0	120.0	240.0
◯	Universal oligo	2.0	2.4	9.6	19.2
●	Sample Tag PCR2 primer	3.0	3.6	14.4	28.8
◯	Nuclease-free water	15.0	18.0	72.0	144.0
	Total	**45.0**	**54.0**	**216.0**	**432.0**

2. Gently vortex the mix, briefly centrifuge, and place back on ice.
3. Bring the PCR2 reaction mixes into the post-amplification workspace.
4. In a new 0.2-mL PCR tubes: Pipet 5.0 µL Sample Tag PCR1 products into 45 µL Sample Tag PCR2 reaction mix.
5. Gently vortex, and briefly centrifuge.
6. Program the thermal cycler. Do not use fast cycling mode.

Program thermal cycler

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	9[a]	95 °C	30 s
Annealing		66 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

a. Cycle .number might require optimization according to cell number and type.

STOPPING POINT: PCR program can run overnight.

Purifying Sample Tag PCR2 products

Note: Perform purification in the post-amplification workspace.

1. Bring AMPure XP beads to room temperature, and vortex at high speed for 1 minute until the beads are fully resuspended.
2. To 50.0 µL Sample Tag PCR2 products, pipet 60 µL AMPure beads.
3. Pipet-mix 10 times, and incubate at room temperature for 5 minutes.
4. Place each tube on the strip tube magnet for 3 minutes. Remove and discard the supernatant.
5. Keeping each tube on the magnet, for each tube, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant.
6. Repeat step 5 once for a total of two washes.
7. Keeping each tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
8. Air-dry the beads at room temperature for 3 minutes.
9. Remove each tube from the magnet, and resuspend each bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
10. Incubate at room temperature for 2 minutes, and briefly centrifuge.
11. Place the tube on the magnet until the solution is clear, usually within 30 seconds.
12. Pipet the entire eluate (~30 µL) of sample into a new 1.5-mL LoBind® tubes (purified Sample Tag PCR2 products).

    STOPPING POINT: Store at 2–8 °C before proceeding on the same day or at –25 °C to –15 °C for up to 6 months.
13. Estimate the concentration of sample by quantifying 2 µL of Sample Tag PCR2 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.
14. Dilute an aliquot of the products to 0.1–1.1 ng/µL with elution buffer.

Performing index PCR to prepare final Sample Tag libraries

1. In the pre-amplification workspace, pipet the following reagents into a new 1.5-mL LoBind® tube on ice.

Sample Tag Index PCR mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% coverage (µL)	For 8 libaries with 20% overage (µL)
○	PCR master mix	25.0	30.0	120.0	240.0
●	Library forward primer	2.0	2.4	9.6	19.2
●	Library reverse primer 1–4[a]	2.0	2.4	–	–
○	Nuclease-free water	18.0	21.6	86.4	172.8
	Total	**47.0**	**56.4**	**216.0**	**432.0**

a. For more than one library, use different library reverse primers for each library.

2. Gently vortex mix, briefly centrifuge, and place back on ice.
3. In a new 0.2-mL PCR tube, combine Sample Tag index PCR mix with diluted Sample Tag PCR2 products as follows:
   - For one sample, add 47 µL of Sample Tag index PCR mix to the PCR tube in which 3 µL of 0.1–1.1 ng/uL Sample Tag PCR2 products will be added in post-amplification workspace.
   - If working with multiple samples, combine 45 µL of Sample Tag index PCR mix with 2 µL of corresponding Library reverse primer to the PCR tube in which 3 µL of 0.1–1.1 ng/uL Sample Tag PCR2 products will be added in post-amplifcation workspace.
4. Bring index PCR mixes into the post-amplification workspace.
5. Pipet 3.0 µL of 0.1–1.1 ng/µL Sample Tag PCR2 products into 47.0 µL index PCR mix.
6. Gently vortex, and briefly centrifuge.

7. Program the thermal cycler. Do not use fast cycling mode.

Program thermal cycler

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	6–8[a]	95 °C	30 s
Annealing		60°C	30 s
Extension		72 °C	30 s
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

a. Suggested PCR cycles.

Suggested number of PCR cycles

Concentration index PCR input for Sample Tag libraries (ng/µL)	Suggested PCR cycles
0.5–1.1	6
0.25–0.5	7
0.1–0.25	8

STOPPING POINT: The PCR can run overnight.

Purifying Sample Tag index PCR products

Note: Perform the purification in the post-amplification workspace.

1. Bring AMPure XP beads to room temperature, and vortex at high speed for 1 minute until the beads are fully resuspended.
2. Briefly centrifuge the index PCR products.
3. To 50.0 µL of the Sample Tag index PCR products, pipet 40 µL AMPure beads.
4. Pipet-mix 10 times, and incubate at room temperature for 5 minutes.
5. Place the tubes on the strip tube magnet for 3 minutes. Remove and discard the supernatant.
6. Keeping the tube on the magnet, for each tube, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant.
7. Repeat step 6 once for a total of two washes.
8. Keeping the tubes on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
9. Air-dry the beads at room temperature for 3 minutes.
10. Remove the tubes from the magnet and resuspend the bead pellet in 30 µL of elution buffer. Pipet-mix until the beads are fully resuspended.
11. Incubate at room temperature for 2 minutes, and briefly centrifuge.
12. Place the tubes on the magnet until the solution is clear, usually within 30 seconds.

13. Pipet the entire eluate (~30 µL) into to a new 1.5-mL LoBind® tubes (final Sample Tag sequencing libraries).
14. Perform quality control.

    STOPPING POINT: Store at –25 °C to –15 °C for up to 6 months until sequencing.

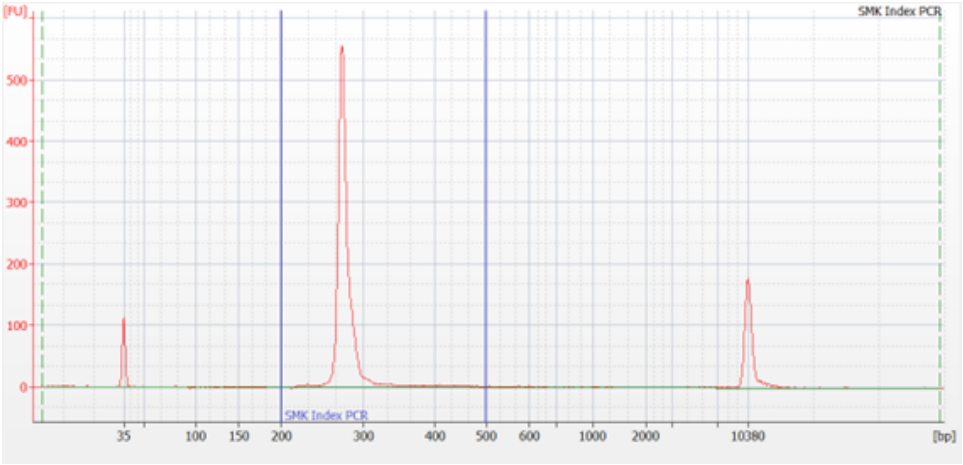
Performing quality control on final sequencing libraries

1. Estimate the concentration by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer or Agilent 4200 TapeStation. Follow the manufacturer's instructions. The expected concentration of the libraries is >1.5 ng/µL.
2. Measure the average fragment size of the Sample Tag library by using the Agilent Bioanalyzer with the High Sensitivity Kit for 50–7,000 bp, 5–1,000 pg/µL. Follow the manufacturer’s instructions.

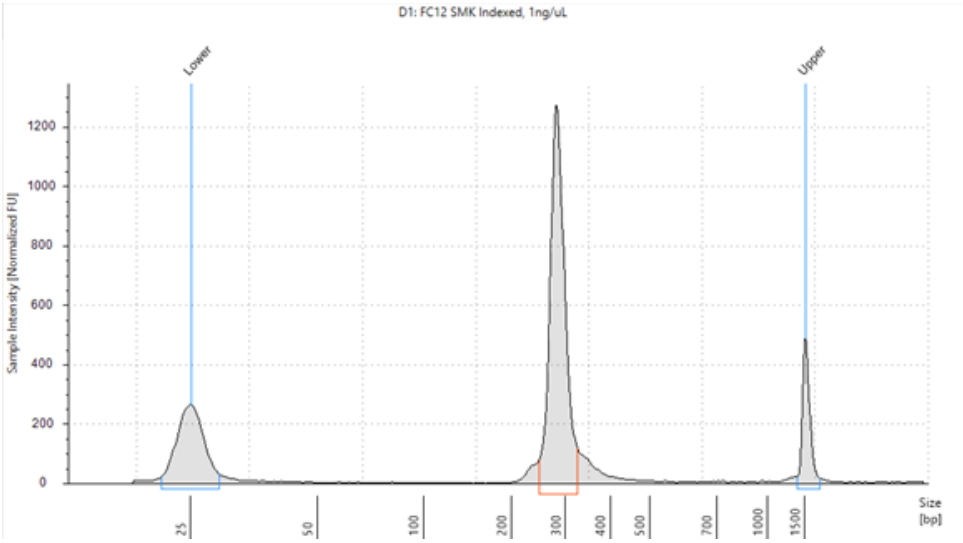
   The Sample Tag library should show a peak of ~270 bp. Peak sizes may vary depending on instrumentation or assay used for measurement.

Figure 2 Sample Tag indexed product

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



Sequencing

ATAC library requirements

- Recommended sequencing depth: 50,000 read pairs per cell.
- Required parameters:

Parameter	Requirement
Platform[a]	Illumina®[a]
Paired-end reads	Recommended: Read 1: 50 cycles; Read 2: 50 cycles Index 1: 8 cycles; Index 2: 60 cycles
PhiX	1% recommended
Analysis	See the *BD® Rhapsody Sequence Analysis Pipeline User's Guide* (Doc ID: 23-24580)
a. To review the Illumina® Index 1 (i7) sequences, see the following table.	

Illumina® index (i7) sequences

Library reverse primer	Sequences
1	TAAGGCGA
2	CGTACTAG
3	AGGCAGAA
4	TCCTGAGC
5	GGACTCCT
6	TAGGCATG
7	CTCTCTAC
8	CAGAGAGG

Sample Tag library requirements

- Recommended sequencing depth: 2,000 read pairs per cell.
- Required parameters:

Parameter	Requirement
Platform[a]	Illumina®[a]
Paired-end reads	Recommended: Read 1: 50 cycles; Read 2: 50 cycles; Index 1: 8 cycles
PhiX	1% recommended
Analysis	See the *BD® Rhapsody Sequence Analysis Pipeline User's Guide* (Doc ID: 23-24580)
a. To review the Illumina® Index 1 (i7) sequences, see the following table.	

ATAC and Sample Tag library sequencing recommendations

- For a NextSeq High or Mid Output run and MiniSeq High or Mid Output run, load the flow cell at a concentration in the range of 1.5–1.8 pM with 1% PhiX spike-in.
- Sample Tag libraries can be sequenced together with ATAC libraries generated from the same experiment.

  For Sample Tag, we recommend 2,000 reads per cell.

Illumina® index for Sample Tag (i7) sequences

Library reverse primer	Sequence
1	GCTACGCT
2	CGAGGCTG
3	AAGAGGCA
4	GTAGAGGA

Single-cell ATAC and Sample Tag library sequencing analysis pipeline

Contact your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com for access to the latest BD Rhapsody™ sequence analysis pipeline.

Appendix

Note: Our ATAC kits are configured for use with the BD Rhapsody™ HT Xpress System. For BD Rhapsody™ Express System users, contact your local Field Application Specialist (FAS) or us at scomix@bdscomix.bd.com, if Proteinase K quantity proves insufficient.

Rhapsody single-lane cartridge workflow (no scan other than indicated)

1. Prime the cartridge.
   a. Prior to priming the Rhapsody™ cartridge, scan the empty cartridge for Cell Load scan. For detailed instructions, see *BD Rhapsody™ System - Single-Cell Capture and cDNA Synthesis with BD Rhapsody Single-Cell Analysis System* (Doc ID: 23-22951(02).
   b. Place waste collection container in the BD Rhapsody™ Express System.
   c. Prime the cartridge using the following table steps with a BD Rhapsody™ P1200M pipette.

Step number	Material to load	Volume (µL)	Pipette mode	Incubation at room temperature
1	100% ethyl alcohol	700	Prime/Treat	N/A
2	Air	700	Prime/Treat	N/A

Step number	Material to load	Volume (µL)	Pipette mode	Incubation at room temperature
3	Room temp. Cartridge Wash Buffer 1	700	Prime/Treat	1 min
4	Air	700	Prime/Treat	N/A
5	Room temp. Cartridge Wash Buffer 1	700	Prime/Treat	10 min
6	Air	700	Prime/Treat	N/A
7	Room temp. Cartridge Wash Buffer 2	700	Prime/Treat	≤4 hr

2. Prepare single-nuclei suspension for cartridge loading:

   Use the BD Rhapsody™ Scanner to calculate the number of nuclei for cartridge loading.

   a. Use the Samples Calculator on the scanner to calculate the volumes of tagmented nuclei and sample buffer needed to prepare a nuclei suspension of 650 µL (this volume is for one cartridge).

   b. Select the correct cartridge type. For the BD Rhapsody™ single-lane cartridge, use 0119.

   c. Prepare 650 µL nuclei suspension for cartridge loading by mixing unstained tagmented nuclei with cold sample buffer according to the displayed volumes on the scanner. Ensure the stock solution of each sample is well suspended by gently pipet-mixing with a wide-bore tip before pooling. Keep the nuclei suspension on ice.

3. Load tagmented nuclei in the cartridge:

   a. Load the cartridge with materials listed in the following table using the BD Rhapsody™ P1200M pipette:

Material to load	Volume (µL)	Pipette mode
Air	700	Prime/Treat
• Set the BD Rhapsody™ P1200M pipette to **Cell Load** mode. • Pipet-mix the cell suspension using a manual P1000 pipette		
Nuclei suspension	575	Cell Load

   Note: Press button to aspirate 40 µL air, and then immerse tip in cell suspension. Press button again to aspirate 575 µL of cold nuclei suspension. Dispense 615 µL of air and cell suspension. Air bubbles that might appear at the inlet or outlet of the cartridge do not affect cartridge performance.

   b. Incubate at room temperature (15–25 °C) for 15 minutes.

4. Wash the loaded nuclei with cold sample buffer:

   Note: Do not omit this step. It is necessary to obtain good ATAC data.

   a. Place the cartridge on the BD Rhapsody™ Express System.

   b. Set the BD Rhapsody™ P1200M pipette to **Prime/Treat** mode.

   c. Load the cartridge with materials listed in the following table using the BD Rhapsody™ P1200M pipette:

Material to load	Volume (µL)	Pipette mode
Air	700	Prime/Treat
Cold sample buffer	700	Prime/Treat
Air	700	Prime/Treat
Cold sample buffer	700	Prime/Treat

5. Load and wash BD Rhapsody™ Enhanced Cell Capture Beads:

   a. Place the cartridge on the BD Rhapsody™ Express System.

   b. Set the BD Rhapsody™ P1200M pipette to **Prime/Treat** mode.

   c. Bring the splint beads generated from the Splint bead generation (page 15) step. Add another 370 µL sample buffer to increase the bead suspension volume to 750 µL.

   d. Load the cartridge with materials listed below using the BD Rhapsody™ P1200M pipette:

Material to load	Volume (µL)	Pipette mode
Air	700	Prime/Treat
• Gently pipet-mix to completely resuspend the beads. • Set the BD Rhapsody™ P1200M pipette to **Bead Load** mode. • Immediately load the beads. Check the pipette tips to make sure that there are no air bubbles inside the tips before loading.		
Splint Beads	630	Bead Load

   e. Incubate the cartridge at room temperature (15–25 °C) for 3 minutes.

   f. Perform scanner step: **Bead Load.**

   g. Once Bead Load is completed, tap **OK**, then **Eject**. Remove the cartridge from the scanner.

   h. Place the cartridge on the BD Rhapsody™ Express System.

   i. Set the BD Rhapsody™ P1200M pipette to **Wash mode**.

   j. Load the cartridge with materials listed below using the BD Rhapsody™ P1200M pipette:

Material to load	Volume (µL)	Pipette mode
Air	700	Wash
Cold sample buffer	700	Wash
Air	700	Wash
Cold sample buffer	700	Wash
Note: Press the button once to aspirate 720 µL air or reagent. Insert the tip into the cartridge, and press the button once to dispense 700 µL air or liquid. Remove pipette tip, and press the button once to dispense remaining 20 µL of air or liquid.		

6. Lyse nuclei:
   a. Add 75.0 µL of 1 M DTT to one room temperature 15-mL lysis buffer bottle and briefly vortex mix. Use the lysis buffer with DTT within 24 hours, and then discard.
   b. Pipette 1 mL of lysis buffer with DTT into a new 1.5-mL LoBind® tube. Add 50 µL of Proteinase K to the tube immediately before the lysis step, and gently pipet-mix 5 times.
   c. Set the BD Rhapsody™ P1200M pipette to **Lysis** mode.
   d. Load the cartridge with materials listed using the BD Rhapsody™ P1200M pipette:

Material to load	Volume (µL)	Pipette mode
Lysis buffer with DTT and Proteinase K	550	Lysis

   e. Carefully remove the cartridge from the BD Rhapsody™ Express System. Slowly transfer the cartridge into an incubator at 37 °C and incubate for 10 minutes. Maintain the recommended lysis time for best performance.

      Note: It is important to keep the cartidge leveled.

7. Retrieve cell-capture beads:
   a. Place the 5-mL LoBind tube into the BD Rhapsody™ Express System drawer. Label the tubes appropriately.
   b. Ensure that the BD Rhapsody™ P5000M pipette is set to **RETRIEVAL** mode.
   c. Move the front slider to **BEADS** on the BD Rhapsody™ Express System.
   d. Carefully bring the cartridge from the 37 °C incubator to the BD Rhapsody™ Express System and allow the cartridge to cool down for 5 minutes.
   e. Move the left slider to **RETRIEVAL.**
   f. Leave the retrieval magnet in the down position for 30 seconds.
   g. Aspirate 5,000 µL lysis buffer with DTT using the BD Rhapsody™ P5000M pipette.
   h. Press down on the BD Rhapsody™ P5000M pipette to seal against the gasket.

i. Move the left slider to the middle position (**0**), and immediately load 4,950 µL of Lysis Buffer with DTT.

j. Remove the pipette from the gasket and purge the tips.

k. Move the front slider to **OPEN**, and place the 5-mL LoBind tube on the large magnet with the 15-mL tube adapter for 1 minute.

l. Immediately proceed to Washing BD Rhapsody™ Enhanced Cell Capture Beads in the following section.

m. Appropriately dispose of the cartridge, Waste Collection Container, and Lysis Buffer with DTT.

8. Washing BD Rhapsody™ Enhanced Cell Capture Beads:

a. After the 1-minute incubation, leaving the 5-mL tube containing retrieved BD Rhapsody™ Enhanced Cell Capture Beads on the large magnet, remove all but ~1 mL of supernatant without disturbing the beads.

b. Remove the tube from the magnet. Gently pipet-mix the beads and transfer them to a new 1.5-mL LoBind tube.

c. If there are still beads left in the 5-mL tube, add 0.5 mL of Lysis Buffer with DTT, rinse the 5-mL tube, and transfer to the 1.5-mL LoBind tube from the previous step.

d. Place the tube on a magnet for 2 minutes.

e. Remove and discard the supernatant. Avoid leaving lysis buffer or bubbles in the tube. Otherwise, the lysis buffer might cause the ligation reaction to fail.

f. Remove the tube from the magnet, and pipet 1.0 mL cold bead wash buffer into the tube. Pipet-mix.

g. Place the tube on the magnet rack for 2 minutes. Remove and discard the supernatant.

h. Remove the tube from the magnet, and pipet 1.0 mL cold bead wash buffer into the tube. Pipet-mix, and place on ice.

Note: Start ligation ≤30 minutes after washing retrieved cell-capture beads with bead wash buffer.

i. Proceed to Ligation (page 23)

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