BD Rhapsody™ System

Single-Cell ATAC-Seq, mRNA Whole Transcriptome Analysis, and Sample Tag

Library Preparation Protocol

23-24799(01)
2024-08

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Regulatory information

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History

Revision	Date	Change made
23-24799(01)	2024-08	Initial release.

Contents

Introduction

This protocol provides instructions on sample multiplexing and generating a single-cell ATAC library and whole transcriptome analysis (WTA) mRNA library with cell-capture beads 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. In combination with the BD Rhapsody™ WTA Amplification Kit, a 3' WTA approach is used to simultaneously profile mRNA expression of the tagmented single nuclei/cells. It enables uncovering insights into the chromatin structure and factors that affect gene expression of cells at single-cell level.

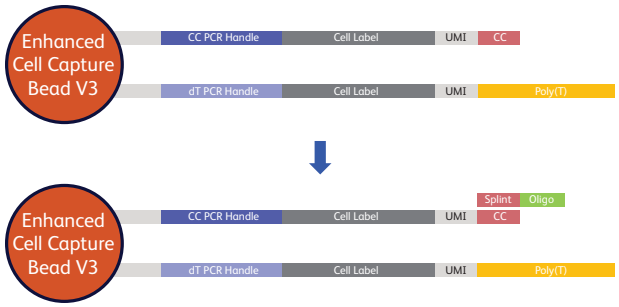
For complete instrument procedures and safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*.

Workflow overview

The *BD Rhapsody™ System Single-Cell ATAC-Seq and mRNA Whole Transcriptome Analysis Library Preparation Protocol* offers a comprehensive multiomic solution, enabling simultaneous profiling of the epigenomic landscape and gene expression within the same single nuclei.

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.

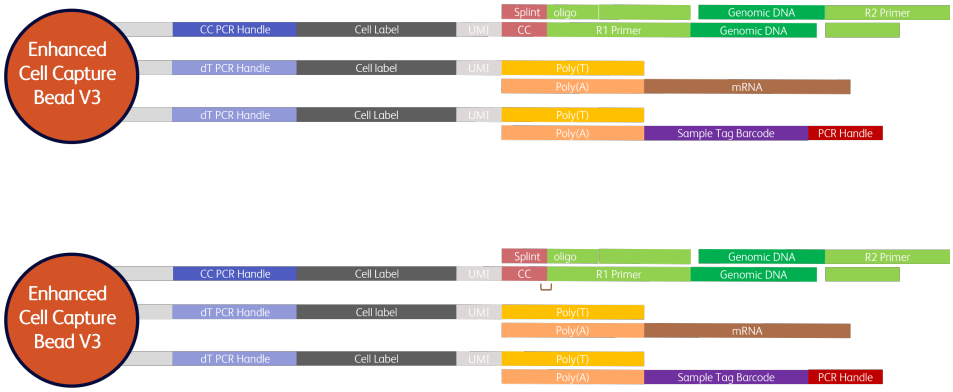


Nuclei isolation: Nuclei isolation protocol depends on the sample type. For details, see Nuclei isolation (page 18).

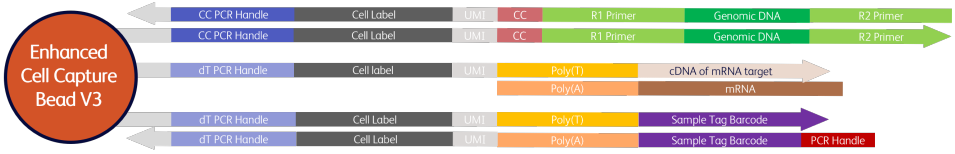
Tagmentation: During the bulk in-situ tagmentation process, expose the nuclei to a tagmentation mix, containing Tagmentase. This enzyme targets accessible genomic regions (open chromatin areas), cutting the DNA and simultaneously attaching preloaded adapter sequences to the ends of each DNA fragment. For substep details, see Tagmentation (page 20).

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

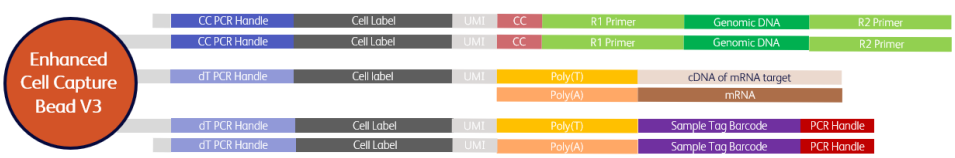
Ligation: Ligate BD Rhapsody™ bead oligo and tagmented DNA using DNA ligase.



Reverse transcription: This process performs ATAC fragment gap filling and extension to beads oligo. Complementary DNA and second strand of Sample Tag are synthesized from captured mRNA and sample tags.

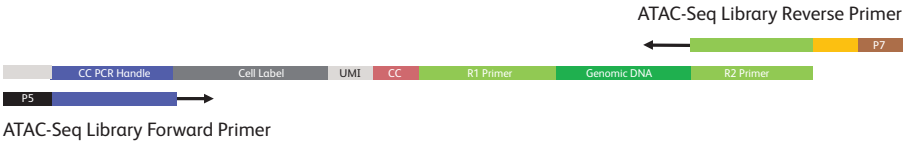


Splint oligo removal and Exonuclease I treatment: To remove unused oligos from the beads.



ATAC fragment denaturation and PCR amplification:

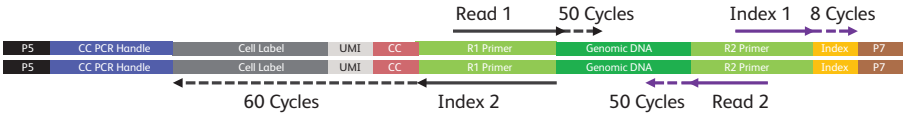
Supernatant: Denature the genomic DNA template off the bead. Illumina® adapters and indices are added during the ATAC product amplification.



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

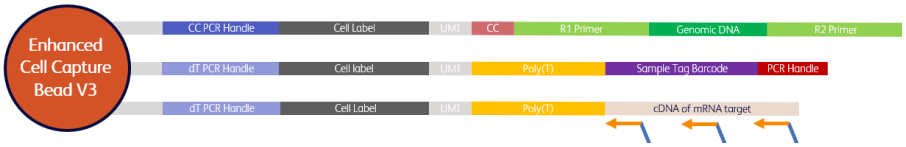
Sequencing:

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

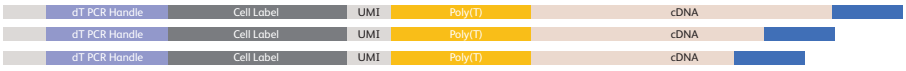


WTA library amplification workflow

WTA Random Priming and Extension: Random priming on the bead.

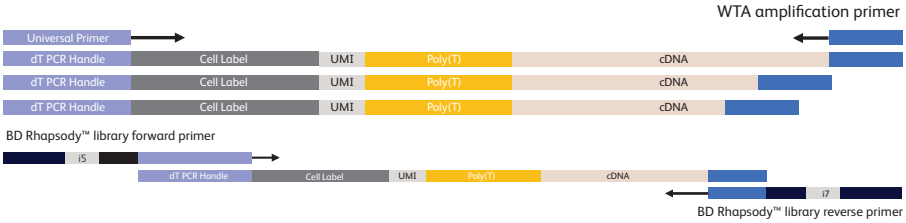


Purify RPE product: Denature off the RPE product.

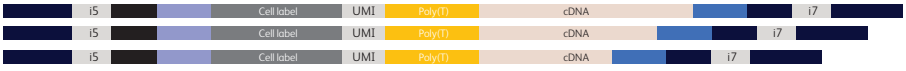


WTA RPE PCR: Amplify the RPE product.

WTA Index PCR: Add Illumina® adapters and indices.

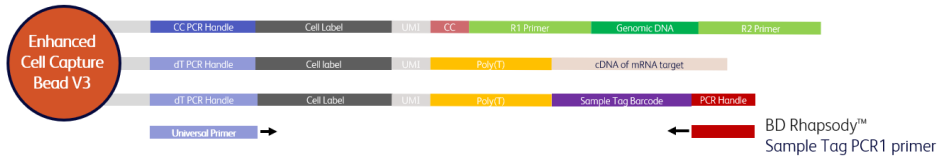


Final WTA Product



Sample Tag library amplification workflow

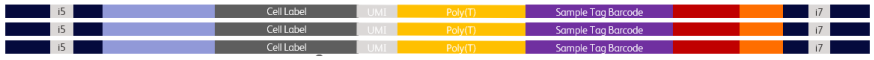
Sample Tag PCR1:
Sample Tag product is amplified in PCR1.



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™ Multiomic ATAC-Seq Amplification Kit	BD Biosciences	571361
BD Rhapsody™ Enhanced Cartridge Reagent Kit V3[a]	BD Biosciences	667052
BD Rhapsody™ cDNA Kit	BD Biosciences	633773
BD Rhapsody™ WTA Amplification Kit	BD Biosciences	633801
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	–
BD® RNase Inhibitor (sold separately)	BD Biosciences	570751
70% ethyl alcohol or 70% isopropyl alcohol[c]	Major supplier	–
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

BD Rhapsody™ cDNA Kit

Cap Color	Name	Quantity
	RT buffer	1
	RT 0.1M DTT	1
	Reverse transcriptase	1
	dNTP	1
	RNase Inhibitor	1
	Bead RT/PCR enhancer	1
	10X Exonuclease I buffer	1
	Exonuclease I	1
	Nuclease-free water	2
	Bead resuspension buffer	1

BD Rhapsody™ WTA Amplification Kit

Cap Color	Name	Quantity
	WTA extension primers	1
	WTA extension buffer	1
	WTA extension enzyme	1
	10 mM dNTP	1
	Nuclease-free water	3
	Bead RT/PCR enhancer	3
	WTA amplification primer	1
	PCR master mix	1
	Universal oligo	2
	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	2
	Bead resuspension buffer	3
	Elution buffer	2

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

Multiomic ATAC-Seq Amplification Kit

Cap Color	Name	Quantity
	Ligation buffer	1
	Ligase	1
	Nuclease-free water	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
	RNase Inhibitor	1
	0.1M DTT, molecular biology grade	1
	Bead resuspension buffer	1
	Elution buffer	1
	Splint oligo removal buffer	2
	PCR master mix	1

- Thaw reagents (not enzymes) in the BD Rhapsody™ Tagmentation and Supplemental Reagents Kit (Cat. No. 571201) and BD Rhapsody™ Multiomic ATAC-Seq Amplification Kit (Cat. No. 571361) 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 and use RNase Inhibitor to keep RNA intact.
- 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

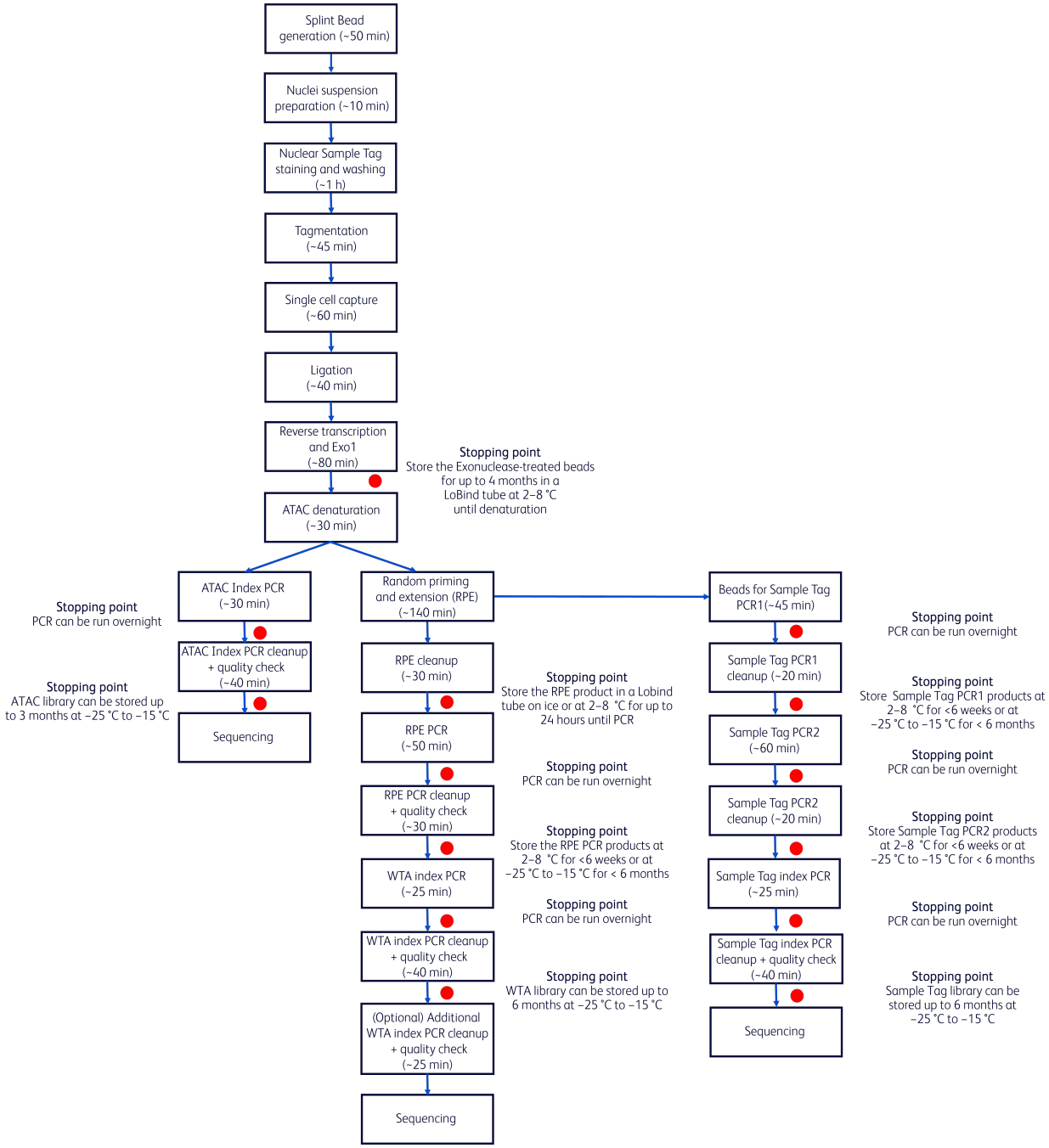
- *BD Rhapsody™ Preparing Single-Cell Suspensions Protocol* (Doc ID: 23-24126)
- *BD Rhapsody™ HT Single-Cell Capture and Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (Doc ID: 23-24252)

- *BD Rhapsody™ mRNA Whole Transcriptome Analysis (WTA) Library Preparation Protocol* (Doc ID: 23-24117 (02))
- *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. Prepare *modified* ATAC-SMK buffer by combining the following reagents in a new 1.5-mL LoBind® tube. Pipet-mix for 10 times and keep it on ice.

Kit component	For 1 sample (µL)	For 1 sample with 10% overage (µL)	For 4 samples with 10% overage (µL)	For 6 samples with 10% overage (µL)
ATAC-SMK buffer	193	212.3	849.2	1273.8
BD RNase Inhibitor	5	5.5	22	33
0.1 M DTT	2	2.2	8.8	13.2
Total	**200**	**220**	**880**	**1320**

3. Resuspend up to 1 million nuclei pellet in 200 µL *modified* ATAC-SMK buffer.
4. Briefly centrifuge the nuclear Sample Tag tubes to collect the contents at the bottom.
5. 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.
6. 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.

Prepare modified nuclei buffer by combining the following reagents in a new 1.5-mL LoBind® tube. Pipet-mix 10 times and keep it on ice.

Color	Component	Catalog No.	Volume (µL)
●	Nuclei buffer	51-9023091	193
●	RNase Inhibitor	51-9024039	5
○	0.1M DTT	51-9022688	2
	Total		200

1. Transfer each labeled nuclei suspension to a 5-mL polystyrene Falcon® tube.
2. Pipet 2 mL of cold 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 cold 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 *modified* 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 modified 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. Prepare modified cold sample buffer with RNase Inhibitor as in the following table. Pipet-mix 10 times and keep on ice. If multiple samples are processed, scale it up (1 mL/sample).

   Modified sample buffer with RNase Inhibitor

Color	Material	Volume
○	Cold sample buffer	1 mL
●	RNase Inhibitor	25 µL

3. 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	11.75[a]
●	10X PBS	2
●	RNase Inhibitor	1.25
●	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.

4. 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 21).

5. After incubation, add 400 µL of modified cold sample buffer with RNase Inhibitor into the Tagmentation mix.
6. Gently pipet-mix 5 times and keep on ice.
7. 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.

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

   Note: DO NOT STAIN THE ENTIRE SAMPLE.

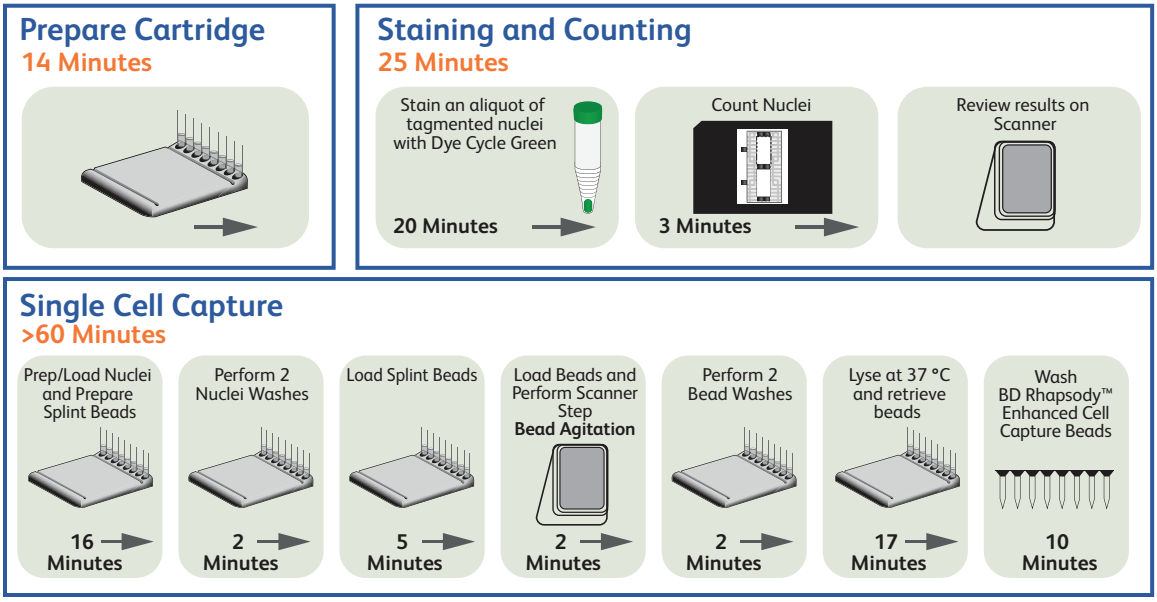
   Nuclei staining dye can impact ATAC-seq data. Consequently, no cartridge scanner metrics will be collected.

   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.
9. 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 modified sample buffer with RNase Inhibitor 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 modified cold sample buffer with RNase Inhibitor 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 17) 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. Otherwise, the lysis buffer might cause the reverse transcription reaction to fail.
   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

Before you begin, thaw reagents (except for the enzymes) in the BD Rhapsody™ cDNA Kit along with necessary reagents in the BD Rhapsody™ Multiomic ATAC-Seq Amplification Kit to room temperature and then place on ice. Keep enzymes at –25 °C to –15 °C. Ensure you have 0.5 M EDTA readily available for the Exonuclease I Treatment steps.

1. Set the thermomixers to 25 °C, 42 °C, and 60 °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
●	RNase Inhibitor	5	5.5	22	44
○	Nuclease-free water	165	181.5	726	1452
	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.
9. Proceed to the Reverse transcription (RT) (page 26) steps immediately.

Reverse transcription (RT)

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

   RT 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)
	RT buffer	40	44	176	352
	dNTP	20	22	88	176
	RT 0.1M DTT	10	11	44	88
	Bead RT/PCR enhancer	12	13.2	52.8	105.6
	RNase Inhibitor	10	11	44	88
	Reverse transcriptase	10	11	44	88
	Nuclease-free water	98	107.8	431.2	862.4
	Total	**200**	**220**	**880**	**1760**

2. Upon completion of the Ligation (page 25) steps, 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 RT Mix into the tube.
4. Resuspend the beads by pipet-mixing 10 times.
5. Incubate the tube in the thermomixer at 42 °C for 30 minutes with 1,200 rpm mixing.

Splint oligo removal

1. Upon completion of reverse transcription, remove the tube from the thermomixer and place on the magnet for 2 minutes. Remove and discard the supernatant.
2. Remove the tube from the magnet and pipet 200 µL of splint oligo removal buffer into the tube.
3. Resuspend the beads by pipet-mixing 10 times.
4. Incubate the tube in the thermomixer at 60 °C for 5 minutes with 1,200 rpm mixing.
5. 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

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)
●	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 Exonuclease I - treated beads. If using the entire sample, skip to step 4. If using a subsample, proceed to step 3.
3. (Optional) Subsample the Exonuclease I - 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 Exonuclease I - 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 WTA library generation as described in WTA library amplification (page 32).
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 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 magnet rack 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.

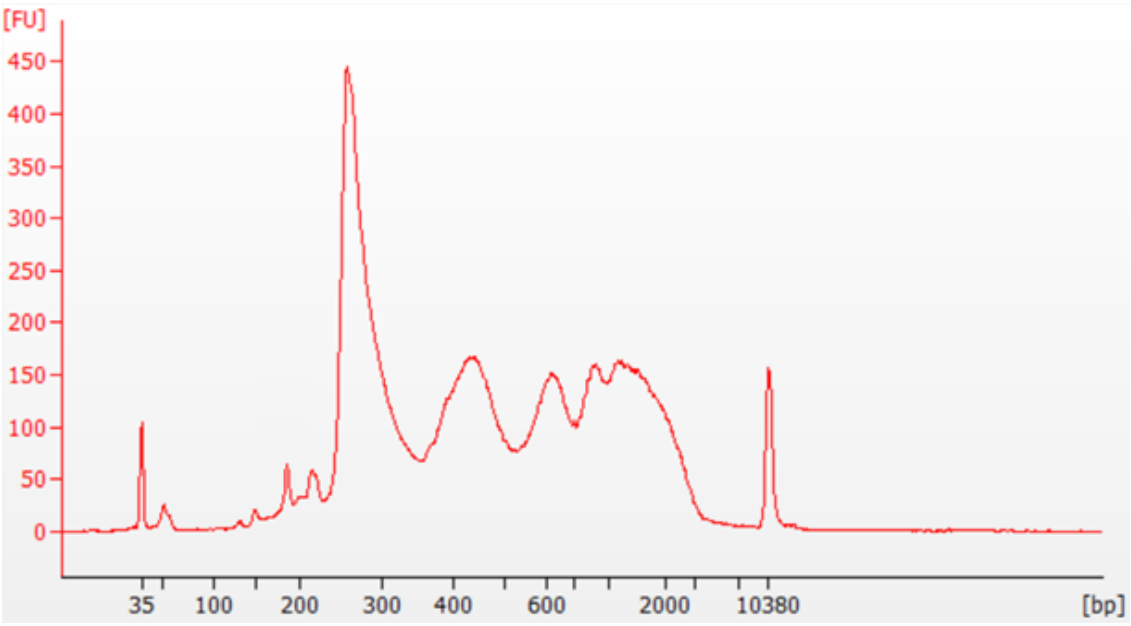
Performing quality control on the final sequencing libraries

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.

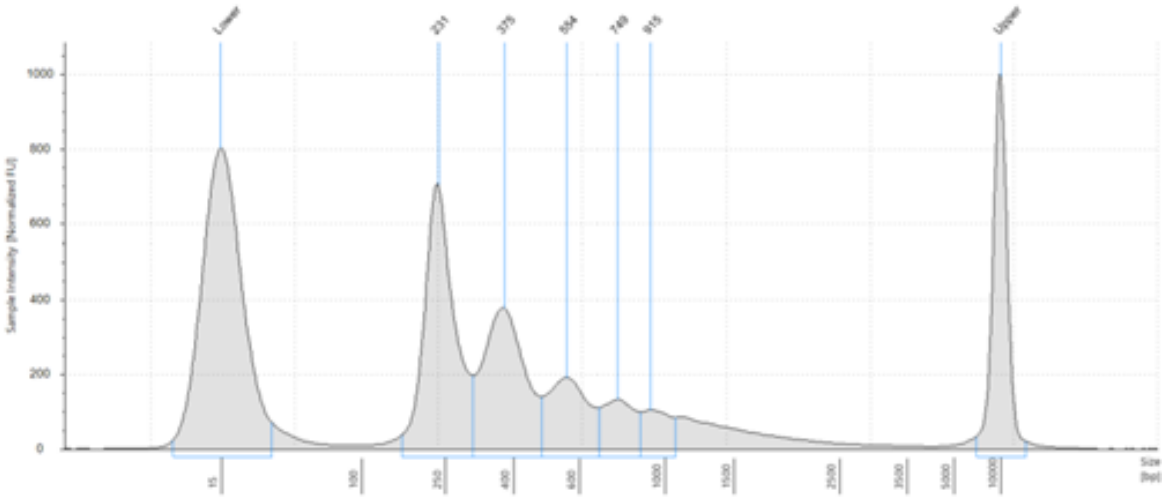
      The following diagram shows a representative ATAC library trace from the Bioanalyzer and TapeStation, where the majority of the fragments are distributed between ~200–2000 bp.

Figure 1 BD Rhapsody™ ATAC Library

A. Bioanalyzer High Sensitivity DNA trace



B. TapeStation high-sensitivity D5000 trace



WTA library amplification

Before you begin:

- Obtain beads from step 15 of Performing single-cell ATAC Library Index PCR (page 28).
- Thaw reagents (except for the enzymes) in the BD Rhapsody™ WTA Amplification Kit at room temperature (15–25 °C), then immediately place on ice. Keep enzymes at –25 °C to –15 °C.

Performing random priming and extension (RPE) on BD Rhapsody™ Enhanced Cell Capture Beads with cDNA

This section describes how to generate random priming products. First, random primers are hybridized to the cDNA on the BD Rhapsody™ Enhanced Cell Capture Beads, then extended with an enzyme. This hybridization and extension is repeated a second time to increase assay sensitivity.

Note: Perform this procedure in the pre-amplification workspace.

1. Set a heat block to 95 °C, one thermomixer to 37 °C, and another thermomixer to 25 °C.
2. In a new 1.5-mL LoBind® tube, pipet the following reagents:

   Random primer mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
●	WTA extension buffer	20	24	96	192
●	WTA extension primers	20	24	96	192
○	Nuclease-free water	134	160.8	643.2	1,286.4
	Total	**174**	**208.8**	**835.2**	**1,670.4**

3. Pipet-mix the random primer mix and keep at room temperature.
4. Resuspend the beads with a pipette.
5. Place the tube with beads in a 95 °C heat block for 5 minutes (no shaking).
6. Briefly centrifuge the tube, then immediately place the tube in the 1.5-mL magnetic separation rack. Remove and discard the supernatant. Avoid drying out the BD Rhapsody™ Enhanced Cell Capture Beads.
7. Remove the tube from the magnet and use a low-retention tip to pipet 87 µL of random primer mix into the tube. Pipet-mix 10 times to resuspend the beads. Save the remaining volume of random primer mix for a second RPE. Keep random primer mix at room temperature.
8. Incubate the tube in the following order:
   a. At 95 °C in a heat block (no shaking) for 5 minutes.
   b. Thermomixer at 1,200 rpm and at 37 °C for 5 minutes.

c. Thermomixer at 1,200 rpm and at 25 °C for 5 minutes.

9. Briefly centrifuge the tube and keep it at room temperature.
10. In a new 1.5-mL LoBind® tube, pipet the following reagents:

    Primer extension enzyme mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
	10 mM dNTP	8	9.6	38.4	76.8
	Bead RT/PCR enhancer	12	14.4	57.6	115.2
	WTA extension enzyme	6	7.2	28.8	57.6
	Total	**26**	**31.2**	**124.8**	**249.6**

11. Pipet-mix the primer extension enzyme mix.
12. Pipet 13 µL of the primer extension enzyme mix into the sample tube containing the beads (for a total volume of 100 µL) and keep at room temperature until ready. Save the remaining volume of primer extension enzyme mix for a second RPE. Keep primer extension enzyme mix on ice.
13. Program the thermomixer as follows:
    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 the Time Mode is set to **Time Control** before the program begins.
14. Place the sample tube containing the beads and primer extension enzyme mix in the thermomixer. Start the program set up in the preceding step.
15. Place the tube in a 1.5-mL tube magnet and remove and discard the supernatant.
16. Remove the tube from the magnet and resuspend the beads in 205 µL of elution buffer using a P200 pipette.
17. To denature the random priming products off the beads, pipet to resuspend the beads. Then:
    a. Incubate the sample at 95 °C in a heat block for 5 minutes (no shaking).
    b. Place the tube in a thermomixer at any temperature for 10 seconds at 1,200 rpm to resuspend the beads.
18. Place the tube in a 1.5-mL tube magnet. Immediately transfer 200 µL of the supernatant containing the random primer extension (RPE) product to a new 1.5-mL LoBind® tube.
19. Repeat **steps 7 to 18** for a second RPE product. Store supernatant containing RPE product on ice.

20. Pipet 200 µL of cold bead resuspension buffer to the tube with leftover beads. Gently resuspend the beads by pipet-mixing only. Do not vortex. Store the beads at 2–8 °C until ready for Sample Tag library generation as described in Sample Tag library amplification (page 44) for up to 3 months in the pre-amplification workspace. Immediately proceed to Purifying RPE product.

Purifying RPE product

This section describes how to perform a single-sided AMPure cleanup, which removes primer dimers and other small molecular weight byproducts. The final product is purified single-stranded DNA. An additional cleanup is recommended for low cell input (<5,000 cells) before the next PCR to ensure maximum removal of the unwanted small molecular weight byproducts.

Note: Perform this procedure in the pre-amplification workspace.

1. In a new 15-mL conical tube, prepare 10 mL of fresh 80% (v/v) ethyl alcohol by pipetting 8.0 mL of absolute ethyl alcohol to 2.0 mL of nuclease-free water (from major supplier). Vortex the tube for 10 seconds.

   Note: Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2. Bring the AMPure XP magnetic beads to room temperature. Vortex the beads at high speed for 1 minute until they are fully resuspended.
3. If the RPE sample volume is <400 µL, increase the volume to 400 µL with elution buffer. Pipet 720 µL of AMPure XP magnetic beads into the tube containing the 400 µL of RPE product supernatant. Pipet-mix at least 10 times, then briefly centrifuge.
4. Incubate the suspension at room temperature for 10 minutes.
5. Place the suspension on the 1.5-mL tube magnet for 5 minutes. Remove and discard the supernatant.
6. While keeping the tube on the magnet, gently add 1 mL of fresh 80% ethyl alcohol into the tube.
7. Incubate the sample on the magnet for 30 seconds. Remove and discard the supernatant.
8. Repeat the 80% ethyl alcohol wash for a total of two washes.
9. While keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
10. For best results, briefly centrifuge the AMPure beads while still wet and place the tube back on the magnet. Remove and discard any excess ethanol that may collect at the bottom. Air dry the beads at room temperature until no longer glossy (~15–20 minutes).
11. Remove the tube from the magnet and pipet 40 µL of elution buffer into the tube. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
12. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
13. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
14. Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product.

Note: For samples with low cell input starting with fewer than 5,000 PBMCs, perform the additional RPE purification steps detailed in the following subsection.

Additional RPE purification steps for cell input <5,000 PBMC cells

1. Use nuclease-free water to bring the volume in the PCR tube containing purified RPE product to 100 µL and transfer to a 1.5-mL LoBind® tube.

   Note: It is critical for the final volume to be exactly 100 µL to achieve the desired size selection of the purified RPE product.

2. Pipet-mix 10 times, then briefly centrifuge.
3. Pipet 180 µL of AMPure XP magnetic beads into the tube containing 100 µL of eluted RPE product from the first round of purification.
4. Pipet-mix 10 times, then briefly centrifuge.
5. Repeat **step 4** through **step 14** in the preceding subsection once more, resulting in a total of two rounds of purification.
6. Elute into a new PCR tube (~40 µL).

STOPPING POINT: Store the RPE product in a LoBind® tube on ice or at 4 °C for up to 24 hours until PCR.

Performing RPE PCR

This subsection describes how to generate more RPE product through PCR amplification, so that there are multiple copies of each random-primed molecule.

1. In the pre-amplification workspace, in a new 1.5-mL LoBind® tube, pipet the following components.

   RPE PCR mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
◯	PCR master mix	60	72	288	576
◯	Universal oligo	10	12	48	96
◯	WTA amplification primer	10	12	48	96
	Total	**80**	**96**	**384**	**768**

2. Add 80 µL of the RPE PCR mix to the tube with the 40 µL of purified RPE product. Pipet-mix 10 times.
3. Split the RPE PCR reaction mix into two PCR tubes with 60 µL of reaction mix per tube.

4. Bring the reaction to the post-amplification workspace and run the following PCR program:

PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 minutes
Denaturation	Refer to the following table, Recommended number of PCR cycles.[a,b]	95 °C	30 s
Annealing		60 °C	1 minute
Extension		72 °C	1 minute
Final extension	1	72 °C	2 minutes
Hold	1	4 °C	∞

a. Suggested PCR cycles might need to be optimized for different cell types and number of cells.
b. Recommended number of PCR cycles is based on resting PBMCs only.

Recommended number of PCR cycles

Number of cells in RPE PCR	Suggested PCR cycles for resting PBMCs
1,000–9,999	13
10,000	12
20,000	11

5. When the RPE PCR reaction is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

STOPPING POINT: The PCR can run overnight.

Purifying RPE PCR amplification product (single-sided cleanup)

This section describes how to perform a single-sided AMPure cleanup to remove unwanted small molecular weight products. The final product is purified double-stranded DNA.

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

1. Combine the two RPE PCR reactions into a new 1.5-mL tube.
2. Briefly centrifuge the tube with the RPE PCR product.

IMPORTANT: It is critical for the final volume to be exactly 120 µL to achieve the appropriate size selection of the purified RPE PCR product. If the volume is <120 µL, bring the volume to 120 µL with elution buffer.

3. Bring AMPure XP magnetic beads to room temperature (15–25 °C). Vortex the AMPure XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
4. Pipet 96 µL of AMPure XP magnetic beads into the tube containing 120 µL of RPE PCR product. Pipet-mix at least 10 times, then briefly centrifuge the samples. Use care to avoid getting AMPure on the lid of the tube, as residual AMPure and PCR mix buffer can negatively impact downstream results.
5. Incubate the suspension at room temperature for 5 minutes.
6. Place the suspension on the tube magnet for 3 minutes. Discard the supernatant.
7. While keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol to the tube.

8. Incubate the samples for 30 seconds on the magnet. Remove and discard the supernatant.
9. Repeat the 80% ethyl alcohol wash for a total of two washes.
10. While keeping the tubes on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.
12. Remove the tube from the magnet and pipet 40 µL of elution buffer into the tube. Pipet-mix the suspension at least 10 times until beads are fully suspended.
13. Incubate the samples at room temperature for 2 minutes. Briefly centrifuge the tubes to collect the contents at the bottom.
14. Place the tubes on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 µL) into new 1.5-mL LoBind® tubes. The RPE PCR product is ready for index PCR.

    STOPPING POINT: The RPE PCR libraries can be stored at –20 °C for up to 6 months or 4 °C for up to 6 weeks.

16. Quantify and perform quality control of the RPE PCR products with a Qubit Fluorometer using the Qubit dsDNA HS Assay and either of the following systems:

    - Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit
    - Agilent 4200 TapeStation system using the Agilent High Sensitivity D5000 ScreenTape Assay

    a. Check that the concentration from the Qubit Fluorometer is ~0.5 to 10 ng/µL.

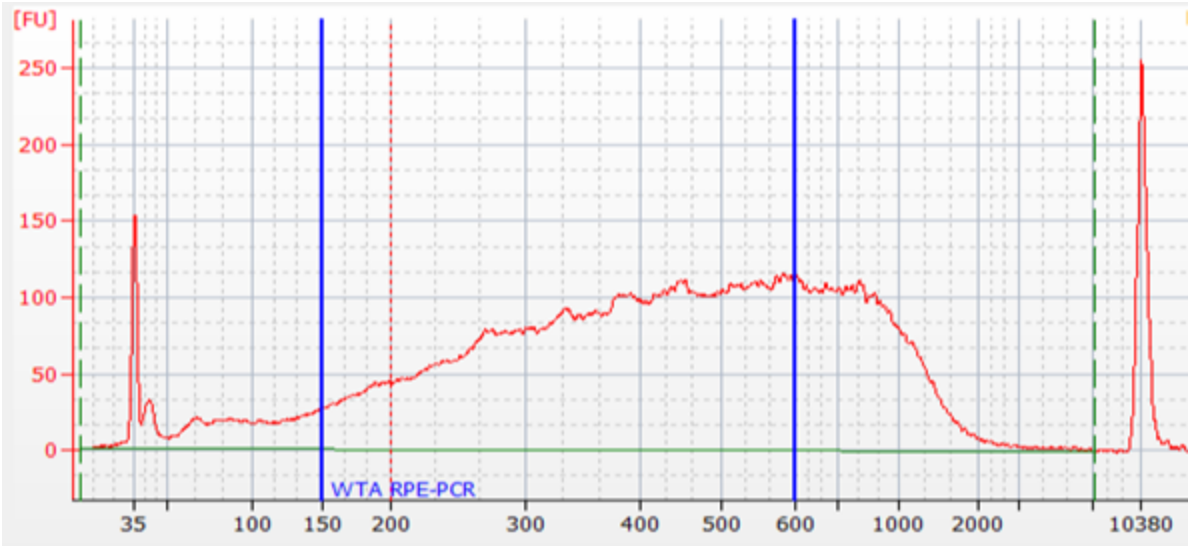
    b. Check that the Bioanalyzer or TapeStation trace shows a broad peak from ~150 to 2,000 bp. Use the concentration from 150 to 600 bp to calculate how much template to add into Index PCR. Refer to the blue-boxed regions in the sample trace images.

    The Bioanalyzer or TapeStation is used to calculate molarity for the WTA library because of the distribution in fragment sizes for this library type.

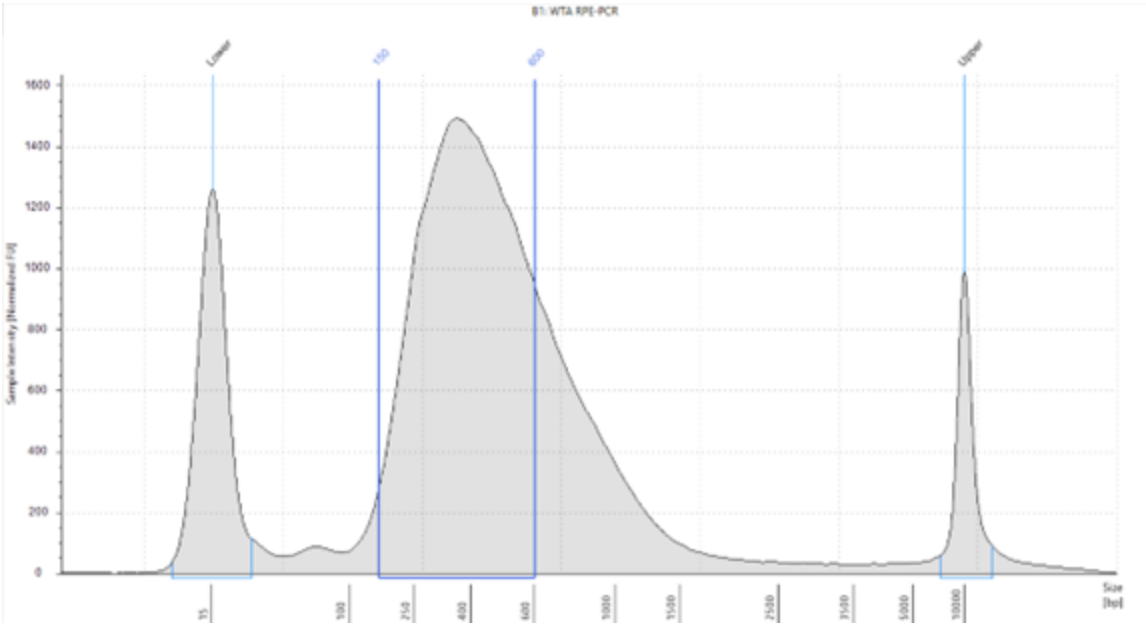
    Note: Although there are products <150 bp and >600 bp, these products will be removed in the double-sided cleanup after the index PCR.

RPE PCR product traces

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D5000 trace



Performing WTA index PCR

This section describes how to generate libraries compatible with the Illumina® sequencing platform, by adding full-length Illumina® sequencing adapters and indices through PCR.

Note: Perform this procedure in the post-amplification workspace.

1. Dilute the RPE PCR products from Purifying RPE PCR amplification product (single-sided cleanup) (page 36) with Elution Buffer such that the concentration of the 150–600 bp peak is 2 nM. If the product concentration is <2 nM, do not dilute and continue.

   For example: If the Bioanalyzer measurement of the 150–600 bp peak is 6 nM, then dilute the sample threefold with Elution Buffer to 2 nM.

2. In a new 1.5-mL tube, pipet the following components:

   WTA index PCR mix

Color	Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	PCR master mix	25	30	120	240
●	Library forward primer	5	6	24	48
●	Library reverse primer (1–4)[a]	5	6	–	–
○	Nuclease-free water	5	6	24	48
	Total	**40**	**48**	**168**	**336**

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

3. Gently vortex mix, briefly centrifuge, and place back on ice.
4. In a new 0.2-mL PCR tube, combine WTA index PCR mix with diluted RPE PCR products as follows:
   a. For one sample, combine 40 µL of WTA index PCR mix with 10 µL of 2 nM of RPE PCR products.
   b. If working with multiple libraries, in separate tubes for each sample, combine 35 µL of WTA index PCR mix with 5 µL of the corresponding library reverse primer and 10 µL of 2 nM RPE PCR products.
5. Pipet-mix 10 times.

6. Run the following PCR program:

PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 minutes
Denaturation	Refer to the following table, Recommended number of PCR cycles.	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	30 s
Final extension	1	72 °C	1 minute
Hold	1	4 °C	∞

Recommended number of PCR cycles

Concentration of diluted RPE PCR products	Recommended number of PCR cycles
1 to <2 nM	9
2 nM	8

If the concentrations of diluted RPE PCR products are <1 nM, additional PCR cycles might be needed.

STOPPING POINT: The PCR can run overnight.

7. When the WTA index PCR is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

Purifying WTA index PCR product (dual-sided cleanup)

This section describes how to perform a double-sided AMPure cleanup to ensure that the library is at a proper size (~250–1,000 bp) for Illumina® sequencing. The final product is purified double-stranded DNA with full-length Illumina® adapter sequences.

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

1. Add 60 µL of nuclease-free water to the WTA index PCR product for a final volume of 110 µL.
2. Transfer 100 µL of WTA index PCR product into a new 0.2-mL PCR tube.
3. Bring AMPure XP magnetic beads to room temperature. Vortex the AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
4. Add 60 µL of AMPure XP magnetic beads to the 0.2-mL PCR tube from **step 2**.
5. Pipet-mix at least 10 times, then briefly centrifuge the samples.
6. Incubate the suspensions at room temperature for 5 minutes, then place on the 0.2-mL strip tube magnet for 2 minutes.
7. Pipet 15 µL of AMPure XP magnetic beads into a different strip tube.
8. While the strip tube in **step 6** is still on the magnet, carefully, without disturbing the beads, remove and transfer the 160 µL of supernatant into the 0.2-mL strip tube with AMPure XP magnetic beads (from step 7) and pipet-mix 10 times.

9. Incubate the suspension at room temperature for 5 minutes, then place the new tube on a 0.2-mL tube magnet for 1 minute.
10. While on the magnet, carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP magnetic beads.
11. While keeping the tubes on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol into the tubes.
12. Incubate the samples for 30 seconds on the magnet.
13. While on the magnet, carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP magnetic beads.
14. Repeat the 200 µL of fresh 80% ethyl alcohol wash for a total of two washes.
15. While keeping the tubes on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
16. Leave the tubes open on the magnet to dry the AMPure XP magnetic beads at room temperature for ~1 minute. Do not over-dry the AMPure XP magnetic beads.
17. Remove tube from magnet and pipette 30 µL of elution buffer into the tubes and pipet-mix to completely resuspend the AMPure XP magnetic beads.
18. Incubate the samples at room temperature for 2 minutes.
19. Briefly centrifuge the tubes to collect the contents at the bottom.
20. Place the tubes on the magnet until the solution is clear, usually ~30 seconds.
21. Pipet the eluate (~30 µL) into new 1.5-mL LoBind® tubes. The WTA index PCR eluate is the final sequencing libraries.

    STOPPING POINT: The index PCR libraries can be stored at –20 °C for up to 6 months until sequencing.
22. Quantify and perform quality control of the index PCR libraries with a Qubit Fluorometer using the Qubit dsDNA HS Assay and either of the following systems:

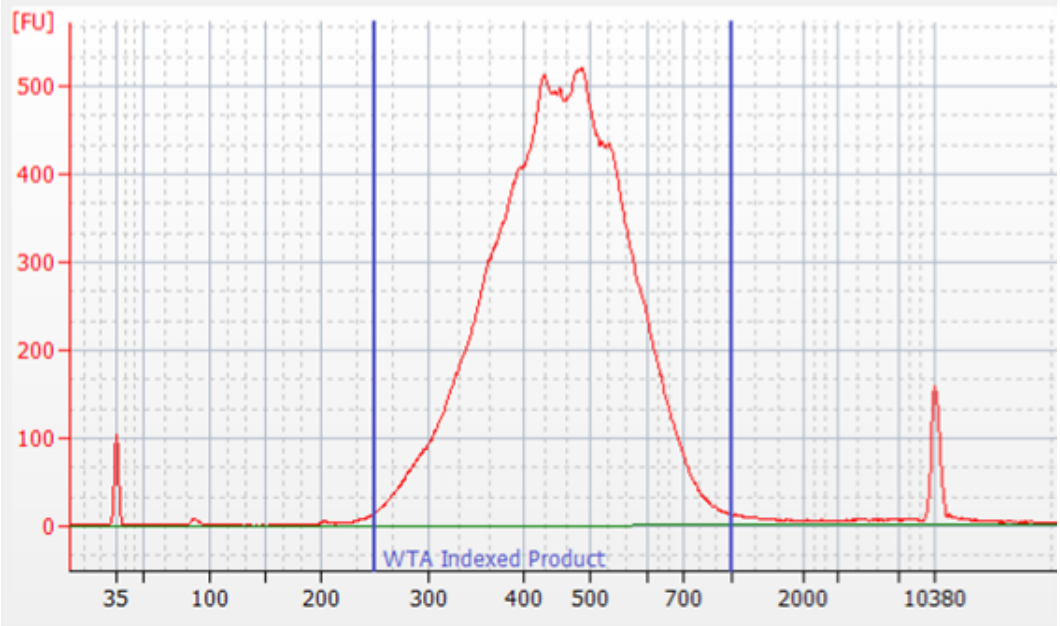
    - Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit
    - Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape Assay

    a. Check that the concentration from the Qubit Fluorometer is >1.0 ng/µL.

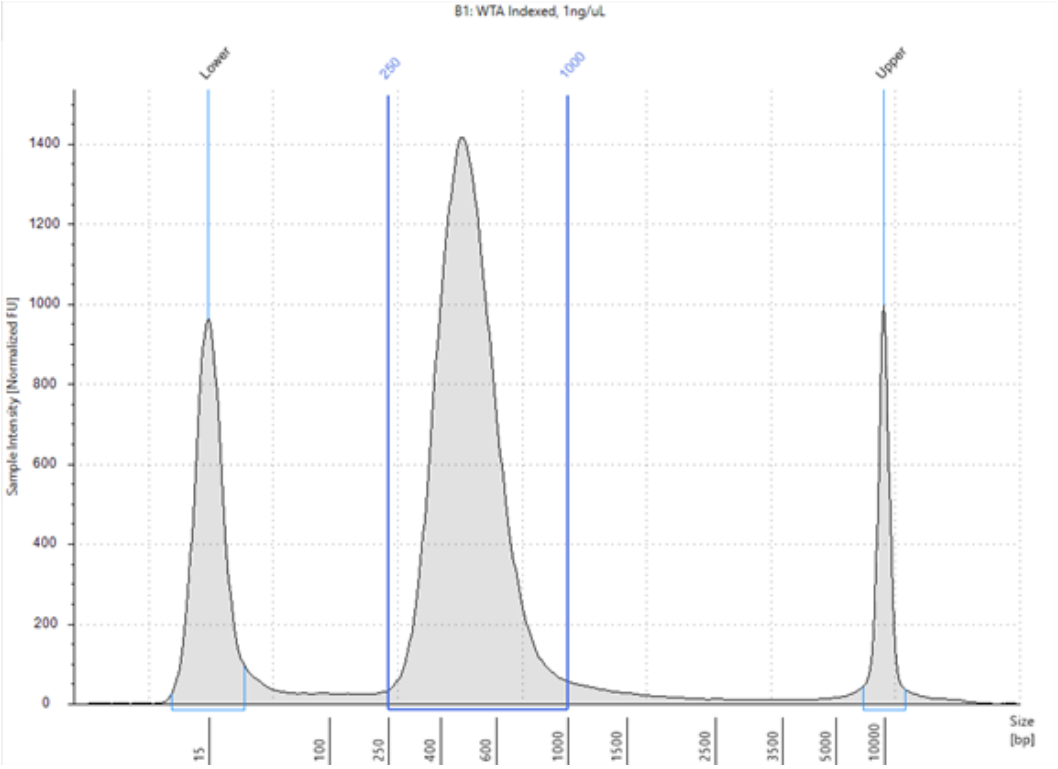
    b. Check that the Bioanalyzer or TapeStation trace shows a peak from ~250 to 1,000 bp. See the following example.

WTA index PCR product

A. Sample Bioanalyzer high-sensitivity DNA trace

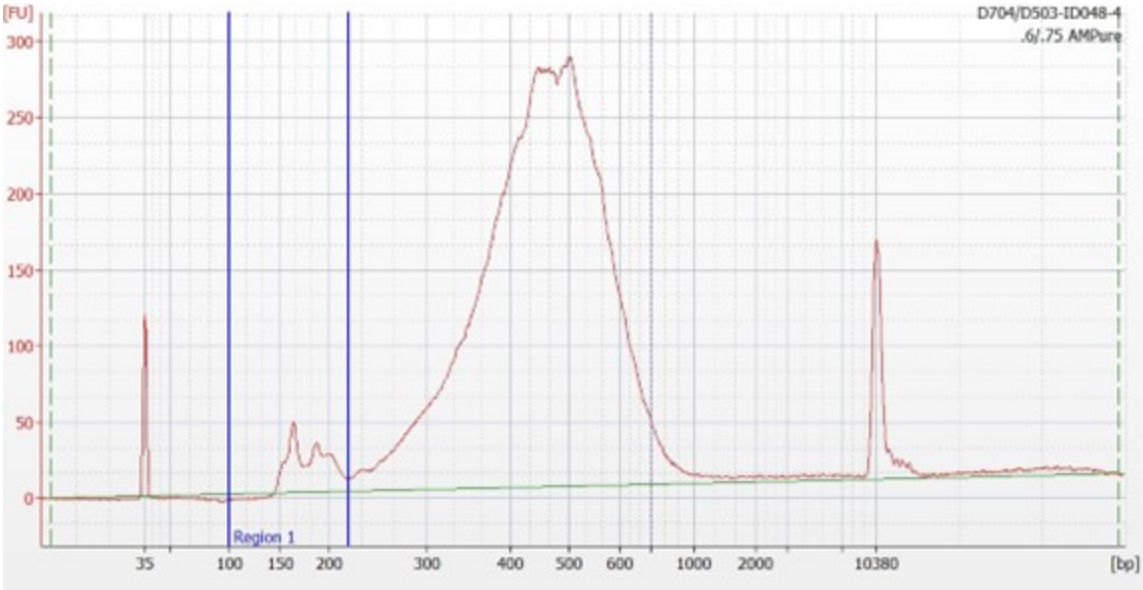


B. Sample TapeStation high-sensitivity D5000 trace



Note: If a <270 bp peak is observed as shown in the following example, a second round of AMPure XP magnetic purification is recommended. See Additional WTA index PCR purification steps (page 43).

Sample Bioanalyzer high-sensitivity DNA trace for an index PCR product with observable peaks of <270 bp



Additional WTA index PCR purification steps

If a <270 bp peak is observed from the Bioanalyzer or TapeStation traces, perform a second round of AMPure XP magnetic purification:

1. To the tube from **step 21** in the preceding subsection, bring the total purified WTA index PCR elute volume up to 100 µL with nuclease-free water.

   Note: It is critical for the final volume to be exactly 100 µL to achieve the desired size selection of the purified WTA index PCR library.

2. Pipet-mix 10 times, then briefly centrifuge.
3. Pipet 75 µL of AMPure XP magnetic beads into the tube containing 100 µL of eluted WTA index PCR product from the first round of purification.
4. Pipet-mix 10 times, then briefly centrifuge.
5. Repeat steps 9–21 in Purifying WTA index PCR product (dual-sided cleanup) (page 41) once more, resulting in a total of two rounds of purification.
6. Collect the elute (~30 µL) to a new PCR tube.
7. Repeat the quality control step, step 22 in Purifying WTA index PCR product (dual-sided cleanup) (page 41).

STOPPING POINT: The index PCR libraries can be stored at –20 °C for up to 6 months until sequencing.

Sample Tag library amplification

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 RPE-treated beads from step 20 of Performing random priming and extension (RPE) on BD Rhapsody™ Enhanced Cell Capture Beads with cDNA (page 34) 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 45). 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.

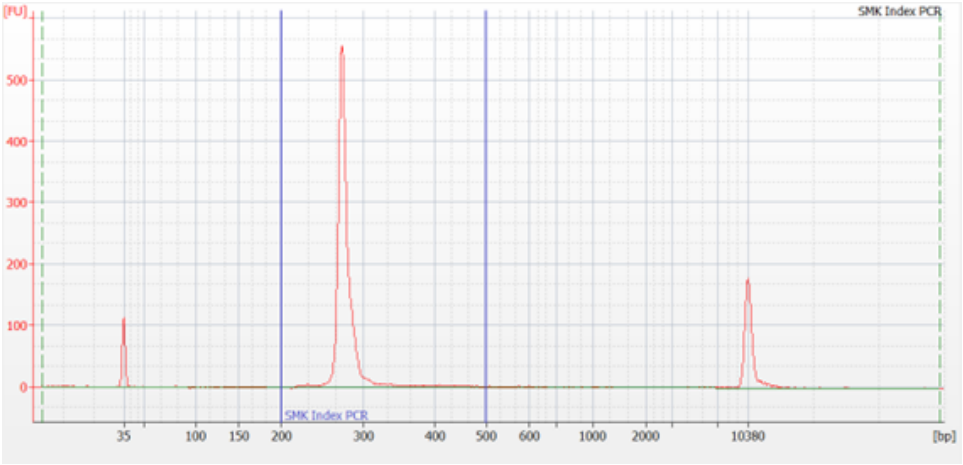
Performing quality control on final sequencing libraries

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.

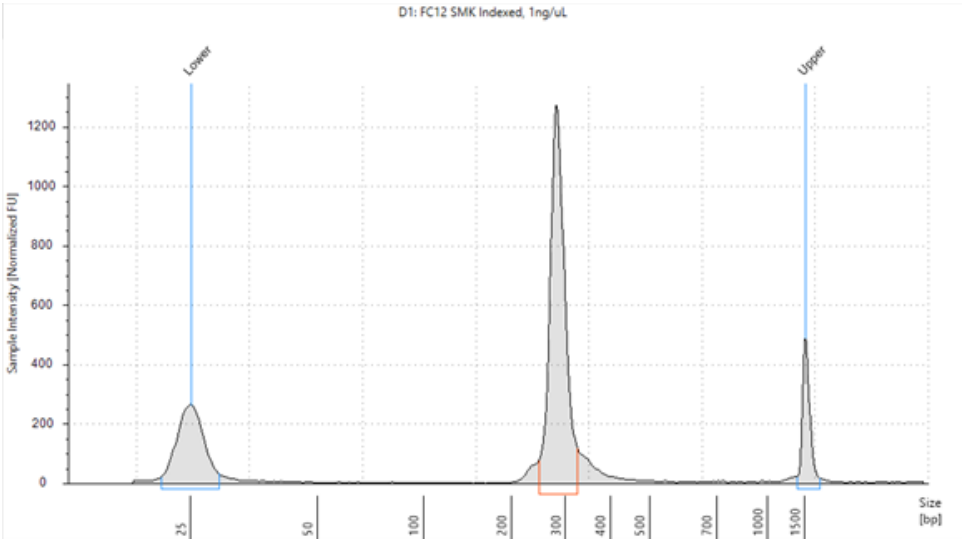
   The Sample Tag library should show a peak of ~270 bp. Peak sizes may vary depending on instrumentation or assay used for measurement.

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

ATAC 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.

WTA and Sample Tag libary requirements

- Run the setup for Illumina® BaseSpace and sample sheet sequencing. Enter the pooled libraries as one sample if both libraries were made with the same library reverse primer or if both libraries share the same i7 index.
- Required parameters:

Parameter	Requirement
Platform[a]	Illumina® [a]
Paired-end reads	Recommend: Read 1: 51 cycles; Read 2: 71 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 table following the WTA library sequencing recommendations.	

Illumina®index 1 (i7) sequences

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

WTA 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 1.5–1.8 pM with 1% PhiX for a sequencing run.
- Sequencing depth for WTA can vary depending on whether the sample contains high- or low-content RNA cells. For resting PBMCs, the reads-per-cell recommendations are as follows:
  - 10,000 reads per cell for shallow sequencing. Genes per cell and UMI per cell detected is generally lower but can be useful for cell type identification.
  - 50,000 reads per cell for moderate sequencing.
  - 100,000 reads per cell for deep sequencing to harvest the majority of UMIs in the library.
- For Sample tag, we recommend 2000 reads per cell.

Single-cell ATAC-Seq WTA 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 RNase inhibitor or Proteinase K quantities prove 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
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 modified sample buffer with RNase inhibitor 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 modified sample buffer with RNase inhibitor 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 17) 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 25)

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