BD Rhapsody™ System

BD® AbSeq and Sample Tag

Library Preparation Protocol
(for AbSeq-based cell calling)

23-24228(01)
2022-12

For Research Use Only

Regulatory information

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

History

Revision	Date	Change made
23-24228(01)	2022-12	New protein-only protocol.

Contents

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*.

Introduction

This protocol enables users to use cell surface proteins to profile cells independently of mRNA. Included here are instructions for how to generate BD® AbSeq single-cell libraries and Sample Tag libraries using the BD Rhapsody™ Single-Cell Analysis system, or the BD Rhapsody™ Express Single-Cell Analysis system. To create the libraries, BD® AbSeq Targets and Sample Tags are encoded on the BD Rhapsody™ Enhanced Cell Capture Beads then amplified in PCR1. After PCR1, the Sample Tag PCR1 products undergo a PCR2 reaction, followed by Index PCR. For BD® AbSeq libraries, PCR1 products are directly indexed. After index PCR, Sample Tag libraries and BD® AbSeq libraries can be combined for sequencing. Sequencing of libraries are completed on Illumina sequencers followed by data analysis that utilizes a BD protein cell calling pipeline which identifies cells without mRNA information.

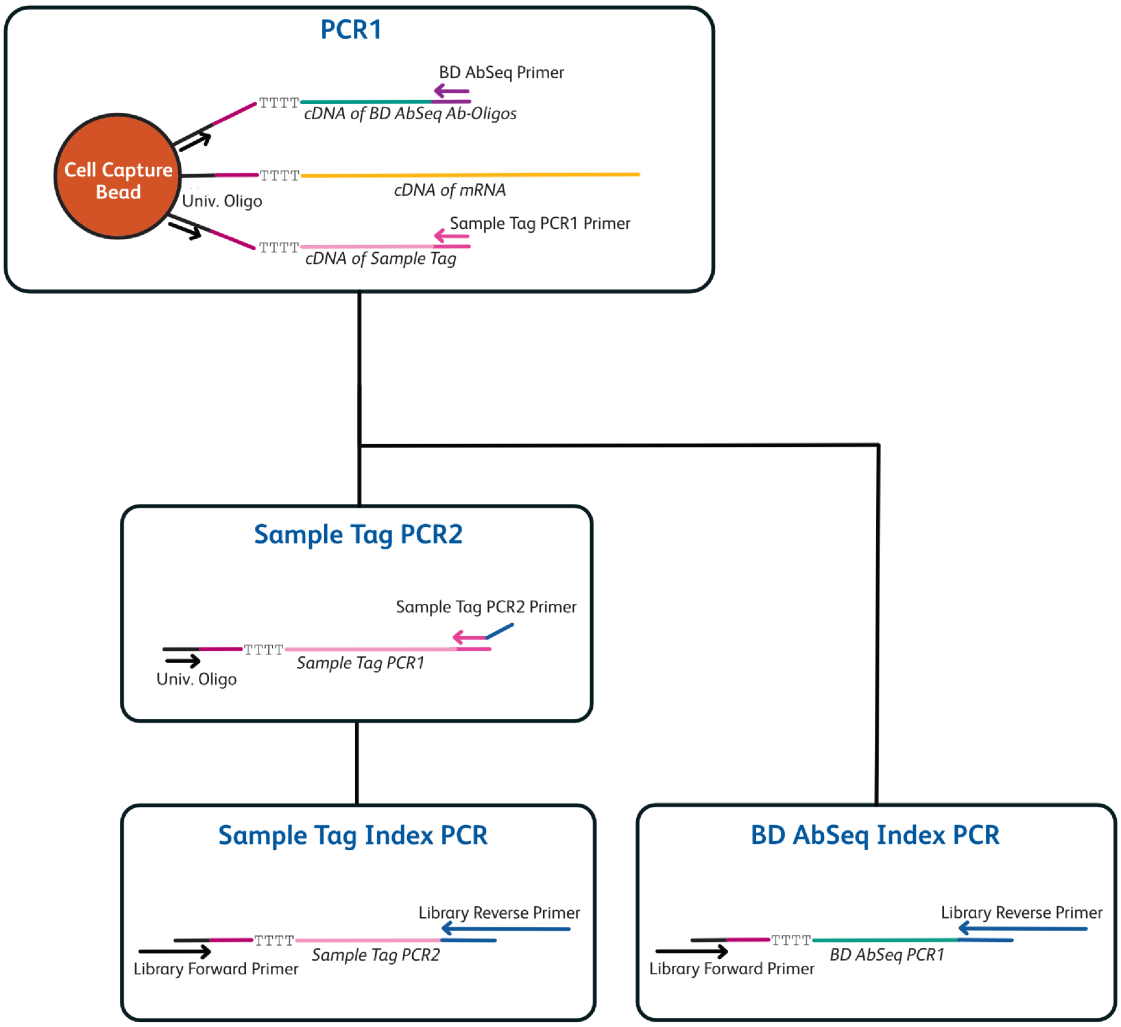
Considerations

BD® AbSeq Panel composition: The panel must include BD® AbSeq that can identify cell subtypes a user wishes to explore.

Cell Viability: For optimal results, this protocol should be used with cells that have greater than 80% viability. If using cells with lower viability, the accuracy of cell calling may be impacted.

This protocol only requires a subset of reagents from either the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit or the BD Rhapsody™ Whole Transcriptome Analysis (WTA) Amplification Kit. The remainder of the kit components may be saved and used for other mRNA workflows.

Workflow



Note: Univ. Oligo: Universal Oligo; region (dark purple) between universal oligo and poly(dT): cell label and Unique Molecular Identifier.

Required materials

- Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads
- Agencourt® AMPure® XP magnetic beads (Beckman Coulter Cat. no. A63880)
- Absolute ethyl alcohol, molecular biology grade (major supplier)
- Nuclease-free water (major supplier)

  Note: The kit provides enough to prepare the PCR MasterMixes. You will need to purchase additional nuclease-free water for the AMPure purification steps.

- 6-Tube Magnetic Separation Rack for 1.5 mL tubes (New England Biolabs Cat. no. S1506S)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Cat. no. Q32851)
- BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Cat. no. 633774)

Kit component	Part number	Cap color	Required
Nuclease-free water	650000076	Neutral	Yes
Bead RT/PCR Enhancer	91-1082	Black	Yes
PCR MasterMix	91-1083	White	Yes
Elution Buffer	91-1084	Pink	Yes
Universal Oligo	650000074	White	Yes
Library Forward Primer	91-1085	Red	Yes
Library Reverse Primer 1	650000080	Red	Yes
Library Reverse Primer 2	650000091	Red	Yes
Library Reverse Primer 3	650000092	Red	Yes
Library Reverse Primer 4	650000093	Red	Yes
Bead Resuspension Buffer	650000066	Black	Yes
Sample Tag PCR1 Primer	91-1088	Purple	Yes
Sample Tag PCR2 Primer	91-1089	Purple	Yes
BD® AbSeq Primer	91-1086	Green	Yes

- BD Rhapsody™ WTA Amplification Kit (Cat. no. 633801)

  Note: You will only need to thaw the components marked Yes in the following table.

Kit component	Part number	Cap color	Required
Nuclease-free water	650000076	Clear	Yes
WTA Extension Buffer	91-1114	Blue	No
WTA Extension Primers	91-1115	Blue	No
10 mM dNTP	650000077	Orange	No
Bead RT/PCR Enhancer	91-1082	Black	Yes
WTA Extension Enzyme	91-1117	Blue	No
PCR MasterMix	91-1118	White	Yes
Universal Oligo	650000074	White	Yes
BD® AbSeq Primer	91-1086	Green	Yes
WTA Amplification Primer	91-1116	White	No
Elution Buffer	91-1084	Pink	Yes
Bead Resuspension Buffer	650000066	Black	Yes
Library Forward Primer	91-1085	Red	Yes
Library Reverse Primer 1	650000080	Red	Yes
Library Reverse Primer 2	650000091	Red	Yes
Library Reverse Primer 3	650000092	Red	Yes
Library Reverse Primer 4	650000093	Red	Yes
Sample Tag PCR1 Primer	91-1088	Purple	Yes
Sample Tag PCR2 Primer	91-1089	Purple	Yes

For a complete list of materials, see the appropriate instrument user guide.

Best practices

- Use low-retention filtered pipette tips.
- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes. **Never vortex the beads. Pipet-mix only.**
- Remove supernatants without disturbing AMPure XP magnetic beads.

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents highlighted in any of the library kits mentioned above at room temperature (15–25 °C), and then place on ice.

Performing BD® AbSeq and Sample Tag PCR1

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

PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100.0	120.0
Universal Oligo	20.0	24.0
Bead RT/PCR Enhancer	12.0	14.4
Sample Tag PCR1 Primer	1.2	1.4
BD® AbSeq Primer	12.0	14.4
Nuclease-free water	54.8	65.8
Total	**200.0**	**240.0**

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Proceed as follows:

- Entire sample: Skip to **step 5**.
- Sub-sample: Proceed to **step 4**.

4 Sub-sample the Exonuclease I-treated beads:

a Based on the number of wells with viable cells and a bead detected by the BD Rhapsody™ scanner or the number of cells targeted for capture in the cartridge, determine the volume of beads to sub-sample for targeted sequencing.

b Pipet-mix to completely resuspend the beads, and pipet the calculated volume of bead suspension into a new 1.5-mL LoBind® tube.

Note: The remaining beads can be stored at 2–8 °C for up to 3 months.

5 Place the tube of Exonuclease I-treated beads in Bead Resuspension Buffer on a 1.5-mL magnet for <2 minutes. Remove and discard the supernatant.

6 Remove the tube from the magnet, and resuspend the beads in 200 µL of the PCR1 reaction mix. Do not vortex.

7 Ensuring that the beads are fully resuspended, pipet 50 µL of the PCR1 reaction mix with beads into each of four 0.2-mL PCR tubes.

Transfer any residual mix to one of the tubes.

8 Bring the reaction mix to the post-amplification workspace.

9 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	10-14[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 the 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, number of antibodies in BD® AbSeq panel, and cell number.

Suggested number of PCR cycles

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
1,000	14
2,500	13
5,000	12
10,000	11
20,000	10

10 Ramp the heated lid and heat block of the 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.

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

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

12 After PCR, briefly centrifuge the tubes.

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

Note: Retain the supernatant in the next step.

14 Place the 1.5-mL tube on the magnet for 2 minutes, and carefully pipet the supernatant (BD® AbSeq and 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 BD® AbSeq and Sample Tag PCR1 products

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

1 In a new 5.0-mL LoBind® tube, prepare 5 mL 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. Vortex the tube for 10 seconds to mix.

   Make fresh 80% ethyl alcohol and use it within 24 hours.

2 Bring the AMPure XP magnetic beads to room temperature (15–25 °C). Vortex at high speed for 1 minute until the beads are fully resuspended.

3 Pipet 280 µL AMPure XP beads into a 1.5 mL LoBind® tube with 200 µL BD® AbSeq and Sample Tag PCR1 products from Performing BD® AbSeq and Sample Tag PCR1 on page 8. Pipet-mix 10 times.

4 Incubate at room temperature (15–25 °C) for 5 minutes.

5 Place the tube on the magnet for 5 minutes. Remove the supernatant.

6 Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol, and incubate for 30 seconds. Remove and discard the supernatant.

7 Repeat **step 6** once for a total of two washes.

8 Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual ethyl alcohol supernatant from the tube.

9 Air-dry beads at room temperature (15–25 °C) for 5 minutes.

10 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 the performance.

11 Incubate at room temperature (15–25 °C) for 2 minutes, and briefly centrifuge.

12 Place the tube on the magnet until the solution is clear, usually within 30 seconds.

13 Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified BD® AbSeq and 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.

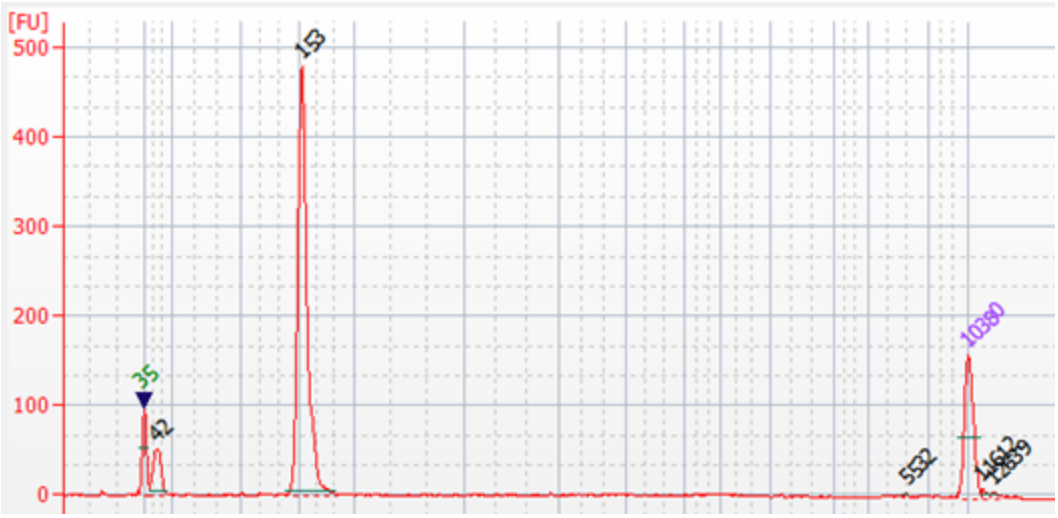
Quantifying BD® AbSeq and Sample Tag PCR1 products

1 Measure the yield of the BD® AbSeq and Sample Tag PCR1 products (~153 bp)—size varies on different instruments—by using the Agilent Bioanalyzer with the High Sensitivity Kit or the Agilent TapeStation system using the Agilient High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions.

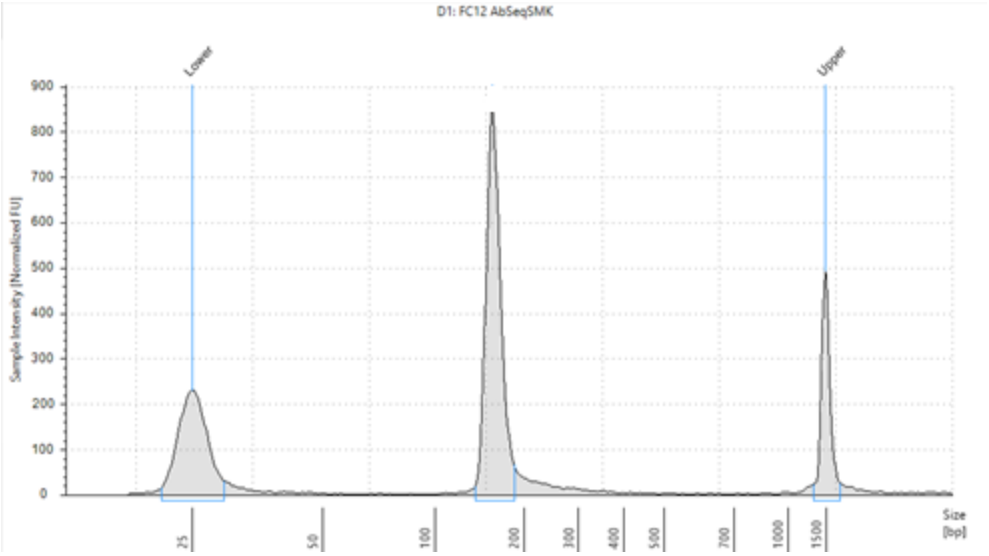
2 Dilute an aliquot of BD® AbSeq or Sample Tag PCR1products to 0.1–1.1 ng/µL with Elution Buffer before index PCR of BD® AbSeq PCR1 products. Use undiluted PCR1 products for Sample Tag PCR2 amplification.

Figure 1 BD® AbSeq and Sample Tag PCR1

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



Performing Sample Tag PCR2 on the BD® AbSeq and Sample Tag PCR1 products

This section describes how to amplify Sample Tag products through PCR. The PCR primers include partial Illumina sequencing adapters that enable the additions of full-length Illumina sequencing indices in the next PCR.

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

Sample Tag PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25.0	30.0
Universal Oligo	2.0	2.4
Sample Tag PCR2 Primer	3.0	3.6
Nuclease-free water	15.0	18.0
Total	**45.0**	**54.0**

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Bring the PCR2 reaction mixes into the post-amplification workspace.

4 Pipet 5.0 µL of PCR1 products into 45.0 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	10[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. Suggested PCR cycles might need to be optimized for different cell types and cell number.

STOPPING POINT: The PCR can run overnight.

Purifying Sample Tag PCR2 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the Sample Tag PCR2 products. The final product is purified double-stranded DNA.

Note: Perform purification in the post-amplification workspace.

1. Bring AMPure XP beads to room temperature (15–25 °C), and vortex at high speed for 1 minute until beads are fully resuspended.
2. To 50.0 µL of PCR2 products, pipet 90 µL AMPure beads.
3. Pipet-mix 10 times, and incubate at room temperature (15–25 °C) for 5 minutes.
4. Place the tube on the strip tube magnet for 3 minutes. Remove and discard the supernatant.
5. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol to 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 (15–25 °C) for 3 minutes.
9. Remove the 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 (15–25 °C) 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)to a new 1.5-mL LoBind® tube (purified Sample Tag PCR2 product).

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 with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.
14. Dilute an aliquot of the products with Elution Buffer to 0.1–1.1 ng/µL.

Performing index PCR to prepare final libraries

1 In the pre-amplification workspace, prepare the 1 library + 20% overage of the final amplification mix for each of the two products. Pipet reagents into a new 1.5-mL LoBind® tube on ice.

Note: For a single cartridge or sample, consider using the same index for all libraries for that cartridge or sample. If libraries are to be indexed differently, make separate index PCR mixes containing different library reverse primers for each library type.

Index PCR mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25.0	30.0
Library Forward Primer	2.0	2.4
Library Reverse Primer 1-4*	2.0	2.4
Nuclease-free water	18.0	21.6
Total	**47.0**	**56.4**
*For more than one cartridge, use different Library Reverse Primers for each Sample Tag library.		

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Bring index PCR mixes into the post-amplification workspace.

4 In two separate and new 0.2-mL PCR tubes:

- Sample Tag PCR2 products: Pipet 3.0 µL of 0.1–1.1 ng/µL products into 47.0 µL index PCR mix.
- BD® AbSeq PCR1 products: Pipet 3.0 µL of 0.1–1.1 ng/µL products into 47.0 µL index PCR 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	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 PCR cycles

Concentration Index PCR input for Sample Tag and BD® AbSeq 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 index PCR products

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

1. Bring AMPure XP beads to room temperature (15–25 °C), and vortex at high speed for 1 minute until the beads are fully resuspended.
2. Briefly centrifuge all of the index PCR products.
3. To 50.0 µL of the index PCR products, pipet:
   - BD® AbSeq library: 40 µL AMPure beads.
   - Sample Tag libraries: 50 µL AMPure beads.
4. Pipet-mix 10 times, and incubate at room temperature (15–25 °C) for 5 minutes.
5. Place each tube on strip tube magnet for 3 minutes. Remove and discard the supernatant.
6. Keeping the tubes 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 (15–25 °C) for 3 minutes.
10. Remove the tubes from the magnet and resuspend each bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
11. Incubate at room temperature (15–25 °C) for 2 minutes, and briefly centrifuge.
12. Place the tubes on the magnet until the solution is clear, usually within 30 seconds.
13. For each tube, pipet the entire eluate (~30 µL) into three separate new 1.5-mL LoBind® tubes (final sequencing libraries).
14. Perform quality control before freezing samples. See Performing quality control on the final sequencing libraries on page 16.

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

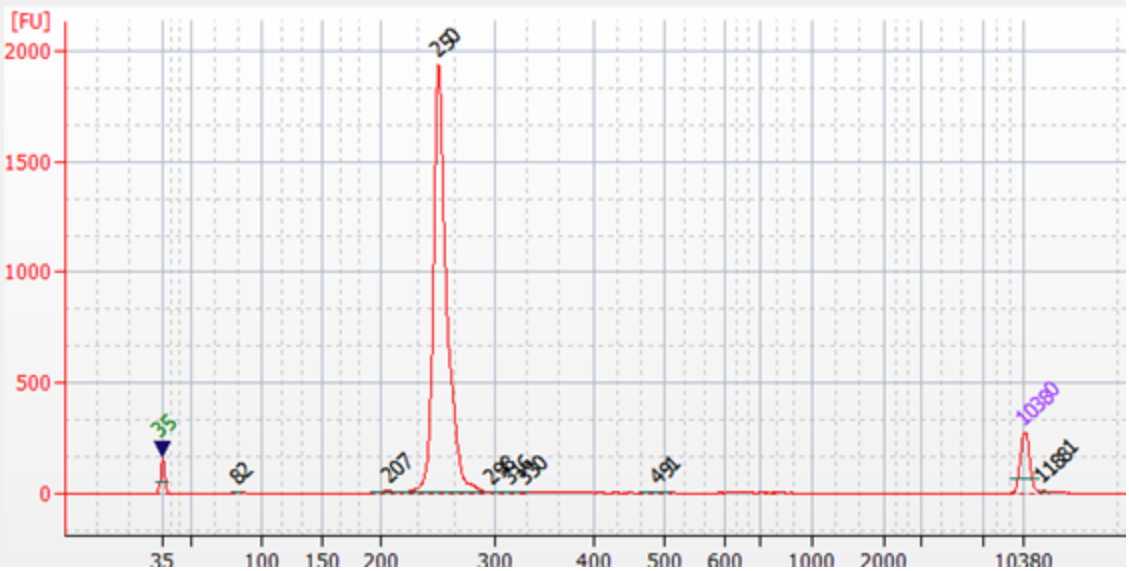
Performing quality control on the final sequencing libraries

Estimate the library 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 an Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions. The expected concentration of the libraries is greater than 1.5 ng/µL.

Figure 2 BD® AbSeq index PCR product

A. Sample Bioanalyzer high-sensitivity DNA trace

The expected size of BD® AbSeq index PCR products is ~250 bp.



B. BD® AbSeq TapeStation high-sensitivity D5000 trace

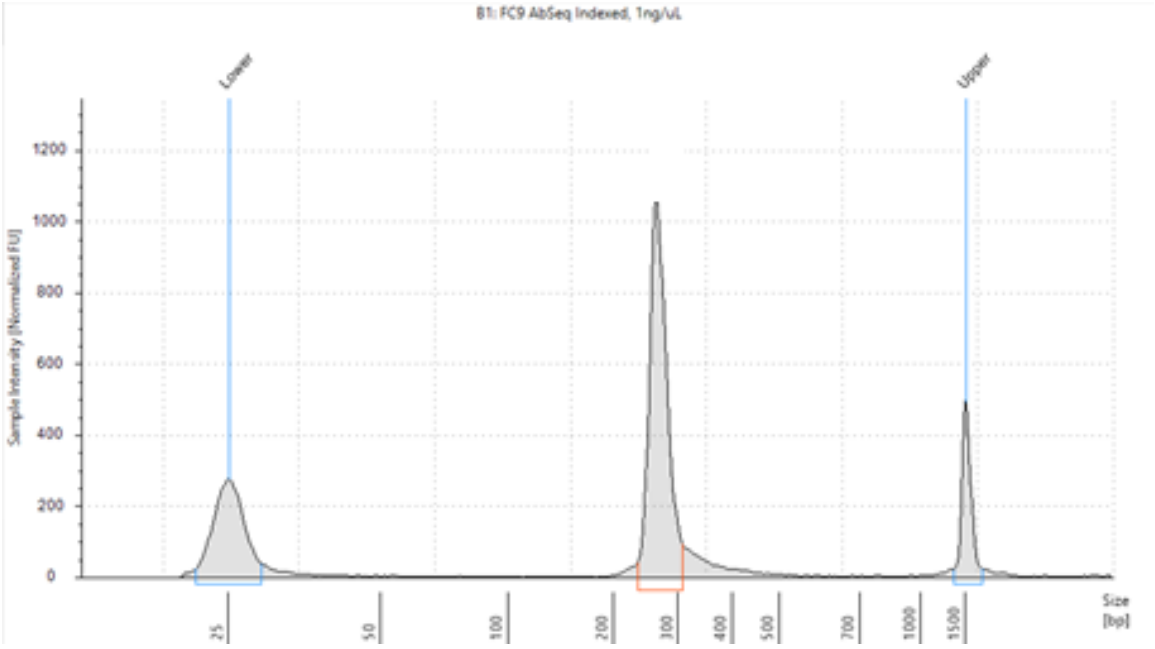
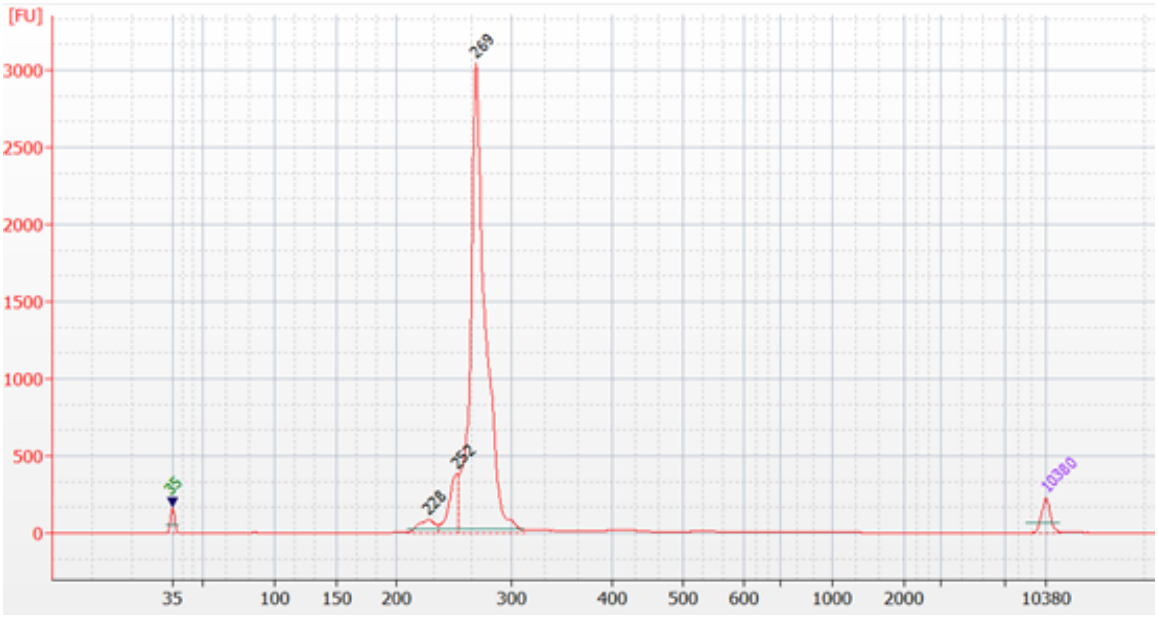


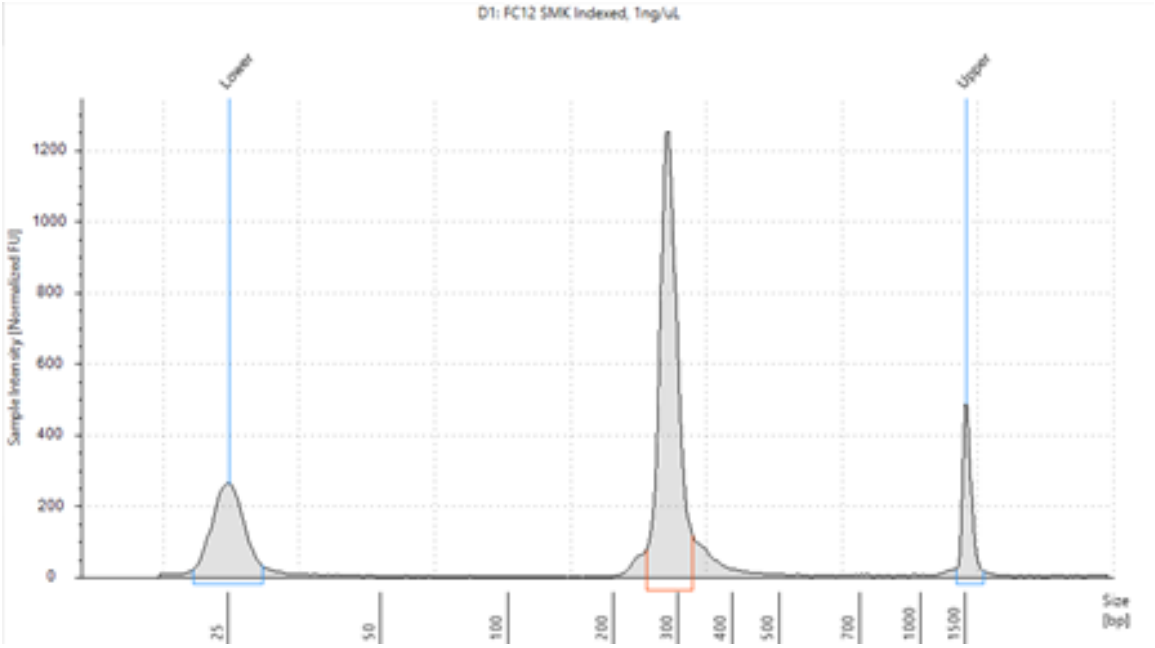
Figure 3 Sample Tag index PCR product

A. Sample Bioanalyzer high-sensitivity DNA trace

The expected size of Sample Tag index PCR product is ~270 bp. You might observe a smaller peak of ~250 bp, which corresponds to BD® AbSeq products (as shown).



B. Sample TapeStation high-sensitivity D1000 trace



Note: If the concentration or size of the library is outside of the expected range, see Troubleshooting on page 21 or contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Sequencing

Requirements

Run 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	Illumina[a]
Read Length	Recommend Read 1: 51 cycles; Read 2: 71 cycles
PhiX	1% recommended
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook*
a. To review Illumina Index 1 (i7) sequences, see Appendix on page 24.	

Sequencing recommendations

- Sequencing amount for BD® AbSeq libraries:
  - The amount of sequencing needed for BD® AbSeq libraries will vary depending on application, BD® AbSeq panel plexy, and cell type. We have observed that using 40,000 sequencing reads per cell for 40-plex BD® AbSeq libraries prepared from resting PBMCs achieves an RSEC sequencing depth of ~2.
- Sequencing amount for Sample Tag libraries:
  - Pooling samples of the same type: 120 reads/cell; for example, combining different donor PBMCs.
  - Pooling different sample types: 600 reads/cell; for example, combining Jurkat cells with PBMCs.

Sequencing flowcell loading and PhiX concentrations

Illumina system	Sequencing flowcell loading concentration	PhiX concentration
MiSeq V2[a]	6–10 pM	1%
MiSeq V3[a]	6–10 pM	1%
MiniSeq High or Mid Output	1–1.5 pM	1%
MiniSeq High or Mid Output	1–1.5 pM	1%
HiSeq 2500[a]	7–15 pM	1%
HiSeq 3000/4000[a]	3 nM	1%
a. Sample Tag and BD® AbSeq libraries have not been tested on these sequencing platforms.		

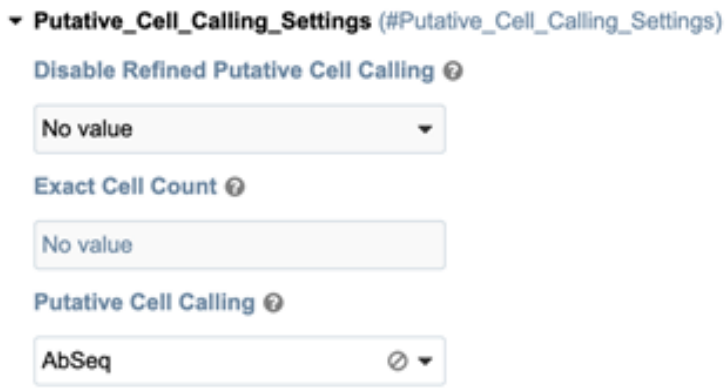
- First-time users are encouraged to start at the low end of the loading concentration recommendation to avoid over-clustering.
- Dilute PhiX to the same concentration as your library before combining samples to achieve the desired final concentration of PhiX. See Illumina instructions for detailed information on preparation and storage of PhiX and optimal cluster density ranges.

- Quantify sequencing libraries as recommended or according to Illumina or service provider instructions.

BD Rhapsody™ sequence analysis pipeline

Refer to the *BD®Single-Cell Multiomics Analysis Setup User Guide* to setup the BD Rhapsody™ Sequence Analysis Pipeline. To identify cells using the protein data from the BD® AbSeq libraries, select “AbSeq” from the “Putative Cell Calling” input options as shown. mRNA is set as the default. See example in Figure 4 below. Refer to the putative cell calling section of the *BD®Single-Cell Multiomics Bioinformatics Handbook* for more details.

Figure 4 Example of Putative Cell Calling Setting with BD® AbSeq data selected



Troubleshooting

Library preparation

Observation	Possible causes	Recommended solutions
BD® AbSeq PCR1 product size too low.	• BD® AbSeq Primer not added to PCR1. • Too few PCR1 cycles. • Incorrect volumes of AMPure XP beads used during purifying PCR product step.	• Contact BD Biosciences technical support at scomix@bdscomix.bd.com.
Yield of BD® AbSeq library too low after index PCR, but yield of BD® AbSeq PCR1 products is sufficient.	Too few index PCR cycles.	• Increase the number of cycles for index PCR.
	Only one primer (Library Forward or Library Reverse primer) added to index PCR mix.	• Ensure that both the Library Forward Primer and Library Reverse Primer are added to the index PCR mix, and repeat index PCR.

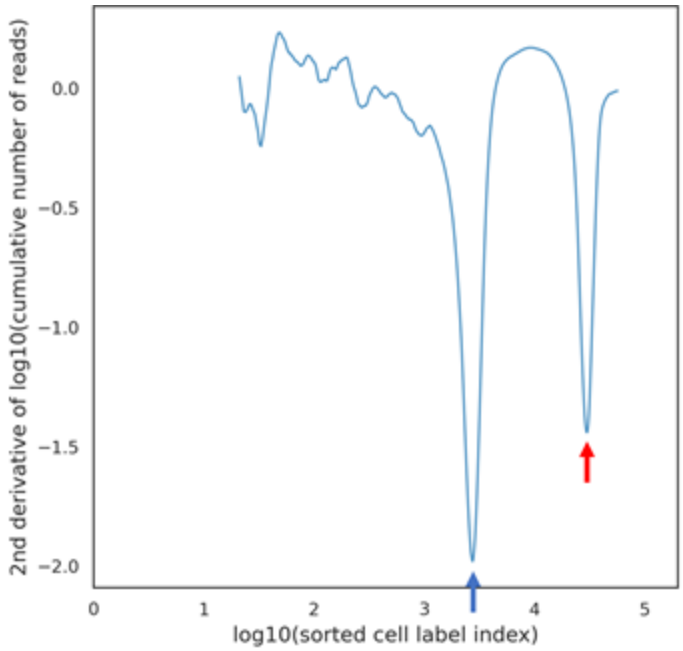
Sequencing

Observation	Possible causes	Recommended solutions
Over-clustering on the Illumina flow cell due to under-estimation of the library.	Inaccurate measurement of the library concentration.	• Quantitate library according to instructions in protocol.
Low sequencing quality.	Insufficient PhiX.	• Use the recommended concentration of PhiX with the library to be sequenced. See Sequencing flowcell loading and PhiX concentrations on page 19.
	Suboptimal cluster density, and/or library denaturation.	• See troubleshooting in Illumina documentation.
	Higher than 50 cycles for R2 reads that reads through polyA of BD® AbSeq oligo.	• Lower read 2 cycles to 50 or trim additional reads from read2 of BD® AbSeq.

Pipeline

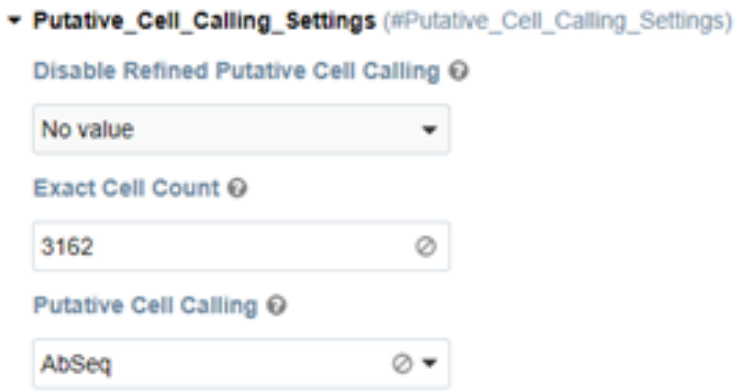
Observation	Possible causes	Recommended solutions
High number of putative cells and low number of RSEC molecules/cell.	BD® AbSeq noise is too high in the cartridge.	Wash 3 times after BD® AbSeq staining.
		Check “Cell_Label_Second_Derivative_Curve.png” file to determine the accurate cell number and use “exact cell count” tool to run pipeline. See the example in Figure 5 Second derivative of the cell label filter output plot on page 23. Red arrow indicates noise included in the cell calling and blue arrow indicates correct cell calling. Use the number on the Y-axis that aligns with the blue arrow to set as the exact cell count in the pipeline options and re-run the pipeline with this value.
Too few putative cells	BD® AbSeq panel does not include markers that are expressed in all cell types in the sample	Include at least one BD® AbSeq marker/cell types in all cell types in the sample.
Cells expressing two different cell markers.	Potential antibody aggregates called as cells.	Potential protein aggregates are identified by pipeline and annotated in “Protein Aggregates.” Filter out before analysis.
	Dead cells absorb antibodies non-specifically.	Use high viability sample.
		Filter potential dead cells according to BD® AbSeq profile.

Figure 5 Second derivative of the cell label filter output plot



The second derivative of the cell label filter output plot can be found on the seven bridges output page as described in the *BD® Single-Cell Multiomics Bioinformatics Handbook*.

Figure 6 Example to set up exact cell count



An estimated value of cells as indicated from the blue arrow in **Figure 5** above ($10^{3.5}$ = 3162) can be used as the exact cell count in the Putative Cell Calling Settings shown.

Appendix

Illumina index 1 (i7) sequences

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

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