BD Rhapsody™ System

BD® AbSeq and Sample Tag

Library Preparation Protocol

(for AbSeq-based cell calling)

23-25004(01)
2025-10

Regulatory information

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

History

Revision	Date	Change made
23-25004(01)	2025-10	Initial release

Contents

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™ HT Single-Cell Analysis system and BD Rhapsody™ HT Xpress System. For complete instrument procedures and safety information, refer to the *BD Rhapsody™ HT Single-Cell Analysis System Instrument User Guide* or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-Free Workflow*. 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 can be completed on various 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 uses the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit. If the BD OMICS-One™ WTA Next Amplification Kit is intended for use, contact BD Biosciences technical support at scomix@bd.com for further information. The remainder of the kit components may be saved and used for other mRNA workflows.

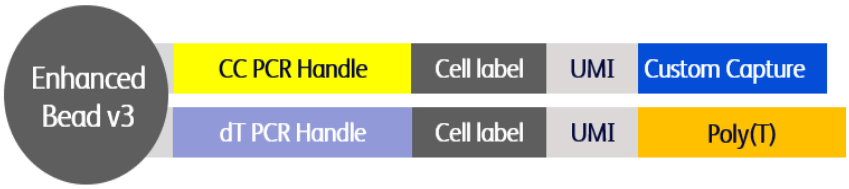
Symbols

The following symbols are used in this guide.

Symbols	Description
	Important information for maintaining measurement accuracy or data integrity.
	Noteworthy information.
	Procedural stopping point.

Workflow

BD Rhapsody™ Enhanced Cell Capture Bead V3

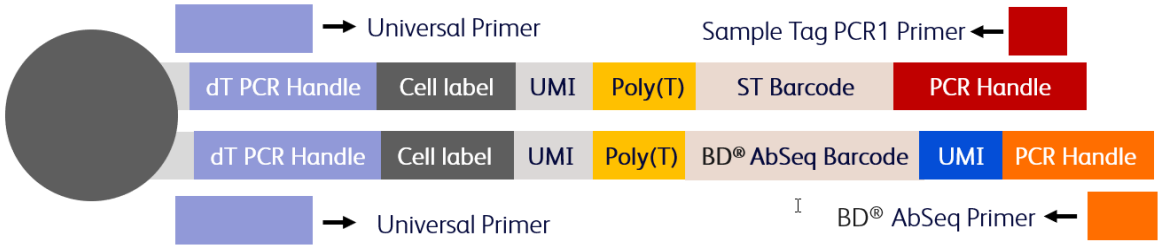


BD® AbSeq antibody-oligonucleotide

BD® Sample Tag oligo

1. BD® AbSeq and Sample Tag PCR1 (page 11)

BD® Abseq and Sample Tag products are amplified together

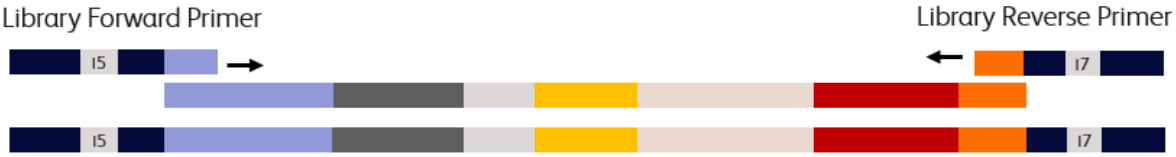


3. Sample Tag PCR2 (page 18)

Sample Tag product further amplified in PCR2

5. BD® AbSeq/Sample Tag index PCR (page 23)

Add Illumina adapters and indices



BD® AbSeq and Sample Tag index PCR are done separately.

Required and recommended materials

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

The kit provides enough nuclease-free water to prepare the PCR master mixes. You will need to purchase additional water for the AMPure® XP beads cleanup steps.

- 6-Tube Magnetic Separation Rack for 0.2 mL and 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)
- BD OMICS-One™ Dual Index Kit (Cat. no. 571899)
- BD OMICS-One™ XT Dual Index Kit A (Cat. no. 571973)
- BD OMICS-One™ XT Dual Index Kit B (Cat. no. 572304)

The BD Rhapsody™ Targeted panel usage is dependent on intended use.

BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (–20 ˚C)

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

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

Best practices

Bead handling

- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes.

Never vortex the beads. Pipet-mix only.

- Store BD Rhapsody™ Enhanced Cell Capture Beads at 4 °C.

Do not freeze.

- Bring AMPure® XP magnetic beads to room temperature (15–25 °C) before use. See manufacturer's instructions for more information.

Libraries

- BD® AbSeq and Sample Tag libraries can be sequenced separately or together with WTA Targeted mRNA libraries.

Master mix preparation

- Thaw reagents (except for enzymes) at room temperature.
- Keep enzymes at –25 °C to –15 °C until ready for use.
- Return reagents to correct storage temperature as soon as possible after preparing the master mix.
- Use only nuclease-free water throughout the protocol.

Supernatant handling

- Read this protocol carefully before beginning each section. Note which steps require you to keep the supernatant to avoid accidentally discarding required products.
- Remove supernatants without disturbing AMPure® XP magnetic beads.
- Make and use fresh 80% ethyl alcohol within 24 hours. Adjust the volume of 80% ethyl alcohol depending on the number of libraries.

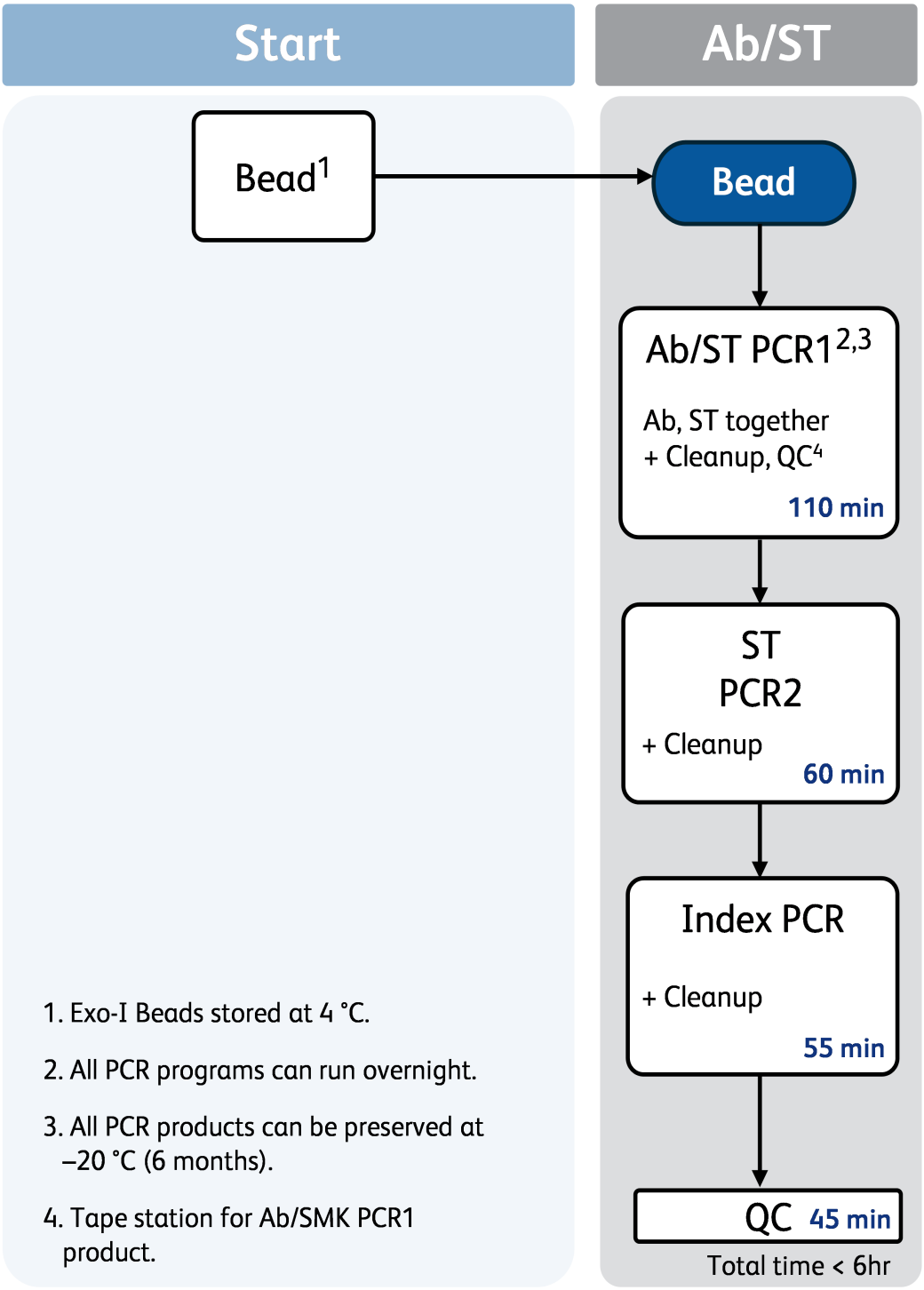
Additional documentation

- *BD Rhapsody™ HT Single-Cell Analysis System Extended-Lysis Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24984)
- *BD Rhapsody™ HT Xpress System Extended-Lysis Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24983)
- *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol* (doc ID 23-21339)
- *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol* (doc ID 23-22354)
- *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos for Intracellular CITE-seq Protocol* (doc ID 23-24464)
- *BD Rhapsody™ Sequence Analysis Pipeline User's Guide* (doc ID 23-24580)

Safety information

- For safety information, refer to the *BD Rhapsody™ HT Single-Cell Analysis System Instrument User Guide* (doc ID 23-24989) or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-Free Workflow* (doc ID 23-24988).

Time Considerations



Procedure

Continue this procedure after staining the antibodies as described in the *BD Rhapsody™ System Single-Cell Labeling with the BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol* (doc ID 23-2133) or the *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kit and BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol* (doc ID 23-22354).

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system using either of the following guides for cell capture, reverse transcription, and exonuclease treatment:

- *BD Rhapsody™ HT Single-Cell Analysis System Extended-Lysis Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24984)
- *BD Rhapsody™ HT Xpress System Extended-Lysis Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24983)

Ensure that the lysis step is performed for **5 minutes**. Do not extend the incubation to 10 minutes.

Ensure that the intended total cell load is between 1,000 and 100,000 single cells for this protocol. Cell load below or above this recommendation might not be suitable for the current protocol configuration. Then proceed as described in the following procedure.

BD® AbSeq/Sample Tag library amplification

This procedure comprises the following tasks:

1. BD® AbSeq and Sample Tag PCR1 (page 11)

2. BD® AbSeq/Sample Tag PCR1 cleanup and quality check (page 15)

3. Sample Tag PCR2 (page 18)

4. Sample Tag PCR2 cleanup and quantification (page 20)

5. BD® AbSeq/Sample Tag index PCR (page 23)

6. BD® AbSeq/Sample Tag index PCR cleanup and quality check (page 26)

1. BD® AbSeq and Sample Tag PCR1

Summary:

- Prepare BD® AbSeq/Sample Tag PCR1 mix
- Amplify using BD® AbSeq /Sample Tag PCR1 program

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
○	Universal oligo	650000074	Equilibrate to room temperature 30 minutes before setting up BD® AbSeq/Sample Tag PCR1. Centrifuge briefly.	–20 °C
●	Bead RT/PCR enhancer	91-1082		
●	Sample tag PCR1 primer	91-1088		
●	BD® AbSeq primer	91-1086		
○	Nuclease-free water	650000076		
Thaw and leave on ice until ready to use				
○	PCR master mix	91-1083	Centrifuge briefly before adding to mix.	–20 °C
Obtain				
Exonuclease I-treated cell capture beads			Centrifuge briefly before adding to mix.	4°C
Ice bucket				
1.5-mL DNA LoBind® tubes				
1.5-mL tube magnetic rack				
0.2-mL PCR tubes				
0.2-mL tube magnetic rack				
Set up				
Thermocycler with BD® AbSeq/Sample Tag PCR1 program				

Procedure steps:

This section describes how to amplify BD® AbSeq/Sample Tag products through PCR.

Perform this procedure in the pre-amplification workspace.

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

BD® AbSeq and Sample Tag PCR1 mix

Cap	Component	1 library (µL)	1 library with 20% overage (µL)	4 libraries with 20% overage (µL)	8 libraries with 20% overage (µL)
○	PCR master mix	100.0	120.0	480.0	960.0
○	Universal oligo	20.0	24.0	96.0	192.0
●	Bead RT/PCR enhancer	12.0	14.4	57.6	115.2
●	BD®AbSeq primer	1.2	1.4	5.8	11.5
●	Sample tag PCR1 primer	12.0	14.4	57.6	115.2
○	Nuclease-free water	54.8	65.8	263.0	526.1
	Total	**200.0**	**240.0**	**960.0**	**1920.0**

2. Pipet-mix the BD® AbSeq/Sample Tag PCR1 mix.
3. Place on **ice** until ready to use.
4. Proceed as follows:
   - Entire sample: Skip to **step 6**.
   - Sub-sample: Proceed to **step 5**.
5. 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 tube.

   The remaining Exonuclease I-treated beads can be stored in bead resuspension buffer at 4 °C for up to 1 year.

6. Place the tube of Exonuclease I-treated beads in bead resuspension buffer on a 1.5-mL magnet for **<2 minutes**.
7. Remove and discard the supernatant.

8. Remove the tube from the magnet and resuspend the beads in **200 µL** of the BD® AbSeq/Sample Tag PCR1 reaction mix. Do not vortex.
9. Pipet-mix thoroughly.
10. Split the reaction mix into **four** 0.2-mL PCR tubes with **50 µL** mix per tube.
11. Transfer any residual mix to one of the tubes.

Bring the tubes to the post-amplification workspace.

12. Run the following PCR program.

Do not use fast cycling mode.

AbSeq/Sample Tag PCR1 program

Step	Cycles	Temperature	Time
Hot start	1	95 °C[a]	3 minutes
Denaturation	Recommended PCR cycles[b] 1,000 cells: 14 cycles 2,500 cells: 13 cycles 5,000 cells: 12 cycles 10,000 cells: 11 cycles 20,000 cells: 10 cycles 40,000 cells: 9 cycles >80,000 cells: 8 cycles	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	1 minute
Final extension	1	72 °C	5 minutes
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. Recommended number of PCR cycles might require optimization for different cell types.

a. 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.

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

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

The PCR can run overnight but proceed with cleanup within 24 hours after PCR.

13. When the BD® AbSeq PCR1 program is complete, briefly centrifuge the tubes.
14. Put the tubes on a magnet for >**30 seconds**.

Retain the supernatant in the next step.

15. For each sample, collect and combine the supernatant from the four 0.2-mL PCR tubes into one new 1.5-mL LoBind® tube without disturbing the beads.

Discard the BD Rhapsody™ Enhanced Cell Capture Beads after use.

2. BD® AbSeq/Sample Tag PCR1 cleanup and quality check

Summary:

- BD® AbSeq/Sample Tag PCR1 Cleanup
- Quality check using Qubit Fluorometer and BioAnalyzer/TapeStation

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
	Elution buffer	91-1084	Centrifuge briefly.	–20 °C
AMPure® XP magnetic beads			Manufacturer's recommendations	
Qubit dsDNA HS Assay Kit				
Agilent BioAnalyzer High Sensitivity Kit **OR**				
Agilent TapeStation ScreenTape & Reagents				
Obtain				
BD® AbSeq/Sample Tag PCR1 product				4 °C
1.5-mL DNA LoBind® tubes				
0.2-mL PCR tubes				
1.5-mL PCR tube magnetic rack				
Set up				
Prepare fresh 80% ethyl alcohol				

Procedure steps:

This section describes how to perform a single-sided AMPure® XP beads cleanup for sequencing.

Perform the purification in the post-amplification workspace.

1. Bring AMPure® XP beads to room temperature.
2. Make fresh 80% (v/v) ethyl alcohol and use it within 24 hours.

   Adjust the volume of 80% ethyl alcohol depending on the number of samples. One sample requires 1 mL 80% ethyl alcohol.
3. Vortex the AMPure® XP beads until the beads are fully resuspended.
4. Briefly centrifuge the tubes with the BD® AbSeq/Sample Tag PCR1 product

   The volume must be exactly 200 µL. If the volume is less than 200 µL, use nuclease-free water to achieve the final volume.
5. Pipet **280 µL** of AMPure® XP beads (1.4x) into the tube with BD® AbSeq/Sample Tag PCR1 product.
6. Pipet-mix 10 times.
7. Briefly centrifuge the tube.

   Avoid getting AMPure® XP beads on the lid of the tube. Residual beads and PCR mix buffer can negatively impact downstream results.
8. Incubate at room temperature for **5 minutes**.
9. Place the tube on a magnet until the supernatant is clear **(<5 minutes)**.
10. Remove and discard the supernatant.
11. Keeping the tube on the magnet, gently pipet **500 µL** of fresh 80% ethyl alcohol into the tube.
12. Incubate for **30 seconds**.
13. Remove and discard the supernatant without disturbing the beads.
14. Repeat steps 11–13 once for a total of **two ethyl alcohol washes**.
15. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
16. Air-dry the beads at room temperature until the beads no longer look glossy **(~5 minutes)**.

    Do not overdry the AMPure® XP beads after the ethyl alcohol washes. Overdried beads appear cracked.
17. Remove the tube from the magnet.
18. Pipet **30 µL** of elution buffer into the tube.
19. Pipet-mix 10 times until the beads are fully resuspended.
20. Incubate at room temperature for **2 minutes**.

21. Briefly centrifuge the tube.
22. Place the tube on the magnet until the solution is clear **(~30 seconds)**.
23. Pipet the eluate **(30 µL)** into a new 1.5-mL tube.

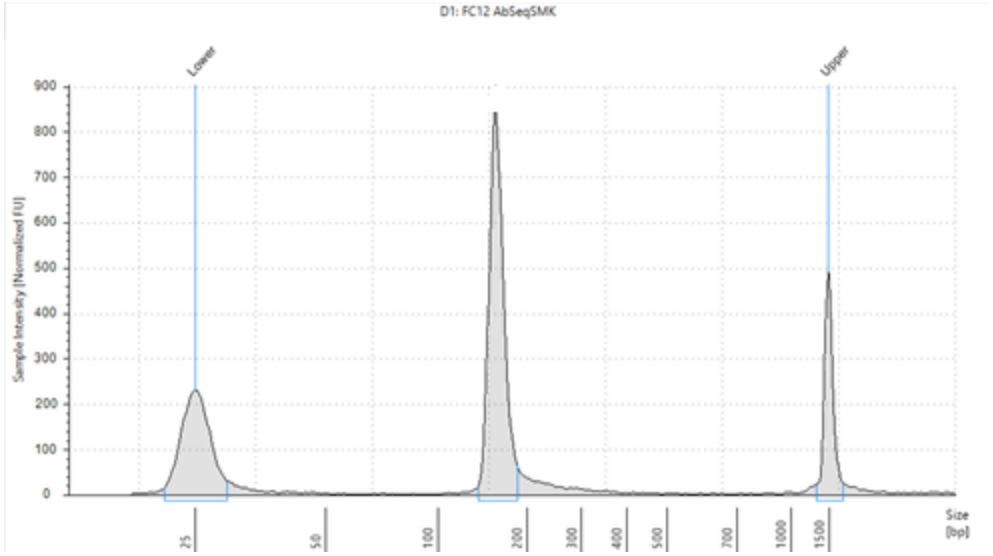
STOP The BD® AbSeq/Sample Tag PCR1 libraries can be stored at –20 °C for up to 6 months.

24. Perform quality control of the BD® AbSeq/Sample Tag PCR1 product with one 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

    The expected size of BD® AbSeq/Sample Tag PCR1 products is **~160 bp**.
25. Quantify the BD® AbSeq/Sample Tag PCR1 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay.
26. Dilute an aliquot of BD® AbSeq/Sample Tag PCR1 products to 0.1–1.1 ng/µL with Elution Buffer before5. BD® AbSeq/Sample Tag index PCR (page 23) . Use undiluted PCR1 products for Sample Tag PCR2 amplification.

    Refer to the representative trace in the following figure.

Figure 1 Representative TapeStation High-Sensitivity D1000 trace- BD® AbSeq and Sample Tag PCR1



If the concentration or size of the library is outside of the expected range, see Troubleshooting (page 32) or contact BD Biosciences technical support at scomix@bd.com.

3. Sample Tag PCR2

Summary:

- Prepare Sample Tag PCR2 mix
- Amplify using Sample Tag PCR2 program

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
○	Universal oligo	650000074	Equilibrate to room temperature 30 minutes before setting up Sample Tag PCR2. Centrifuge briefly.	–20 °C
●	Sample tag PCR2 primer	91-1089		
○	Nuclease-free water	650000076		
Thaw and leave on ice until ready to use				
○	PCR master mix	91-1083	Centrifuge briefly before adding to mix.	–20 °C
Obtain				
BD® AbSeq/Sample Tag PCR1 products				4 °C
Ice bucket				
0.2-mL PCR tubes				
Set up				
Thermocycler with Sample Tag PCR2 program				

Procedure steps:

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 a new 1.5-mL tube, pipet the following components to create the Sample Tag PCR2 reaction mix.

Sample Tag PCR2 reaction mix

Cap	Component	1 library (µL)	1 library with 20% overage (µL)	4 libraries with 20% overage (µL)	8 libraries 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. Pipet-mix the Sample Tag PCR2 mix.

Bring the Sample Tag PCR2 mix to the post-amplification workspace.

3. Pipet **5 µL** of BD® AbSeq/Sample Tag PCR1 products into **45 µL** Sample Tag PCR2 reaction mix.
4. Pipet-mix 10 times.
5. Run the following PCR program.

Do not use fast cycling mode.

Sample Tag PCR2 program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 minutes
Denaturation	10*	95 °C	30 seconds
Annealing		66 °C	30 seconds
Extension		72 °C	1 minute
Final extension	1	72 °C	5 minutes
Hold	1	4 °C	∞
**Recommended number of PCR cycles might require optimization for different cell types.*			

The PCR can run overnight.

6. When the Sample Tag PCR2 program is complete, briefly centrifuge the tubes.

4. Sample Tag PCR2 cleanup and quantification

Summary:

- Sample Tag PCR2 Cleanup
- Quantify using Qubit Fluorometer

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
	Elution buffer	91-1084	Centrifuge briefly.	–20 °C
AMPure® XP magnetic beads			Manufacturer's recommendations	
Qubit dsDNA HS Assay Kit				
Obtain				
Sample Tag PCR2 product				4 °C
1.5-mL DNA LoBind® tubes				
0.2-mL PCR tubes				
1.5-mL PCR tube magnetic rack				
Set up				
Prepare fresh 80% ethyl alcohol				

Procedure steps:

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

Perform purification in the post-amplification workspace.

1. Bring AMPure® XP beads to room temperature.
2. Make fresh 80% (v/v) ethyl alcohol and use it within 24 hours.

   Adjust the volume of 80% ethyl alcohol depending on the number of samples. One sample requires 0.5 mL 80% ethyl alcohol.
3. Vortex the AMPure® XP beads until the beads are fully resuspended.
4. Briefly centrifuge the tubes with the Sample Tag PCR2 product.
5. Transfer **50 µL** Sample Tag PCR2 product to a new 1.5-mL tube.

   The volume must be exactly 50 µL.
6. Pipet **60 µL** of AMPure® XP beads (1.2x) into the same tube.
7. Pipet-mix 10 times.
8. Briefly centrifuge the tube.
9. Incubate at room temperature for **5 minutes**.

   Avoid getting AMPure® XP beads on the lid of the tube. Residual beads and PCR mix buffer can negatively impact downstream results.
10. Place the tube on a magnet until the supernatant is clear **(<3 minutes)**.
11. Remove and discard the supernatant.
12. Keeping the tube on the magnet, gently pipet **200 µL** of fresh 80% ethyl alcohol into the tube.
13. Incubate for **30 seconds**.
14. Remove and discard the supernatant without disturbing the beads.
15. Repeat steps 12–14 once for a total of **two ethyl alcohol washes**.
16. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
17. Air-dry the beads at room temperature until the beads no longer look glossy **(~3 minutes)**

    Do not overdry the AMPure® XP beads after the ethyl alcohol washes. Overdried beads appear cracked.
18. Remove the tube from the magnet.
19. Pipet **30 µL** of elution buffer into the tube.

20. Pipet-mix 10 times until the beads are fully resuspended.
21. Incubate at room temperature for **2 minutes**.
22. Briefly centrifuge the tube.
23. Place the tube on the magnet until the solution is clear **(~30 seconds)**.
24. Pipet the eluate (**30 µL**) into a new 1.5-mL tube.

The Sample Tag PCR2 libraries can be stored at –20 °C for up to 6 months.

25. Quantify the Sample Tag PCR2 product with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.
26. Dilute an aliquot of the products with elution buffer to 0.1–1.1 ng/µL.

5. BD® AbSeq/Sample Tag index PCR

Summary:

- Prepare BD® AbSeq/Sample Tag index PCR mix
- Amplify using BD® AbSeq/Sample Tag index PCR program

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
🔴	Library forward primer	Various	Equilibrate to room temperature 30 minutes before setting up BD® AbSeq/Sample Tag index PCR. Centrifuge briefly.	–20 °C
🔴	Library reverse primer 1–4	Various		
⚪	Nuclease-free water	650000076		
Thaw and leave on ice until ready to use				
⚪	PCR master mix	91-1083	Centrifuge briefly before adding to mix.	–20 °C
Obtain				
BD® AbSeq PCR1/Sample Tag PCR2 products				4 °C
1.5-mL DNA LoBind® tubes				
0.2-mL PCR tubes				
Set up				
Thermocycler with BD® AbSeq/Sample Tag index PCR program				

Procedure steps:

This section describes how to generate BD® AbSeq/Sample Tag libraries compatible with various sequencing platforms, by adding full-length sequencing adapters and indices through PCR.

Perform this procedure in the post-amplification workspace.

1. In a new 1.5-mL tube, pipet the following components to create the BD AbSeq/Sample Tag index PCR mix.

   Prepare the BD® AbSeq and Sample Tag index PCR mix separately.

   BD® AbSeq/Sample Tag index PCR mix

Cap	Component	1 library (µL)	1 library with 20% overage (µL)	4 libraries with 20% overage (µL)	8 libraries 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*	2.0	2.4	N/A	N/A
○	Nuclease-free water	18.0	21.6	86.4	172.8
	Total	**47.0**	**56.4**	**216**	**432**
*For more than one lane cartridge, use different library reverse primers for each Sample Tag library.					

2. Pipet-mix the BD® AbSeq/Sample Tag index PCR mix.
3. Pipet **45 µL** of index PCR mix into a separate 0.2-mL PCR tube for each sample.
4. Add **2 µL** of reverse primer to each sample.
5. Place on **ice** until ready to use.
6. Bring the BD® AbSeq/Sample Tag index PCR mix to the post-amplification workspace.
   Add **3 µL** of the appropriate diluted template:
   - BD® AbSeq/Sample Tag PCR1 product from 2. BD® AbSeq/Sample Tag PCR1 cleanup and quality check (page 15) for AbSeq libraries
   - Sample Tag PCR2 product from 4. Sample Tag PCR2 cleanup and quantification (page 20) for Sample Tag libraries

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

Accurate primer assignment is essential to maintain sample identity during multiplexed sequencing.

7. Pipet-mix 10 times.
8. Run the following PCR program.

Do not use fast cycling mode.

BD® AbSeq/Sample Tag index PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 minutes
Denaturation	Index PCR input concentration* 0.5–1.1 ng/µL: 6 cycles 0.25–0.5 ng/µL: 7 cycles 0.1–0.25 ng/µL: 8 cycles	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	30 seconds
Final extension	1	72 °C	1 minute
Hold	1	4 °C	∞
*Recommended number of PCR cycles might require optimization for different cell types.			

The PCR can run overnight.

9. When the AbSeq/Sample Tag index PCR program is complete, briefly centrifuge the tubes.

6. BD® AbSeq/Sample Tag index PCR cleanup and quality check

Summary:

- BD® AbSeq/Sample Tag index PCR cleanup
- Quality check using Qubit Fluorometer and Bioanalyzer/TapeStation

Preparation list:

Item		BD Part Number	Preparation and Handling	Storage
Equilibrate to Room Temperature				
●	Elution buffer	91-1084	Centrifuge briefly.	–20 °C
○	Nuclease-free water	650000076		
AMPure® XP magnetic beads			Manufacturer's recommendations	
Qubit dsDNA HS Assay Kit				
Agilent BioAnalyzer High Sensitivity Kit **OR** Agilent TapeStation ScreenTape & Reagents				
Obtain				
BD® AbSeq/Sample Tag index PCR product				4 °C
1.5-mL DNA LoBind® tubes				
0.2-mL PCR tubes				
0.2-mL PCR tube magnetic rack				
Set up				
Prepare fresh 80% ethyl alcohol				

Procedure steps:

This section describes how to perform a single-sided AMPure® XP beads cleanup for sequencing. The final product is purified double-stranded DNA with full-length adapter sequences.

Perform the purification in the post-amplification workspace.

1. Bring AMPure® XP beads to room temperature.
2. Make fresh 80% (v/v) ethyl alcohol and use it within 24 hours.

   Adjust the volume of 80% ethyl alcohol depending on the number of samples. One sample requires 0.5 mL 80% ethyl alcohol.

3. Vortex the AMPure® XP beads until the beads are fully resuspended.
4. Transfer **50 µL** of the index PCR products to a 1.5 mL tube.

   The volume must be exactly 50 µL.

5. Pipet:
   - **50 µL** AMPure® XP beads (1.0x) for Sample Tag library.
   - **40 µL** AMPure® XP beads (0.8x) for BD® AbSeq library.
6. Pipet-mix 10 times.
7. Briefly centrifuge the tube.
8. Incubate at room temperature for **5 minutes**.

   Avoid getting AMPure® XP beads on the lid of the tube. Residual beads and PCR mix buffer can negatively impact downstream results.

9. Place the tube on a magnet until the supernatant is clear **(<3 minutes)**.
10. Remove and discard the supernatant.
11. Keeping the tube on the magnet, gently pipet **200 µL** of fresh 80% ethyl alcohol into the tube.
12. Incubate for **30 seconds**.
13. Remove and discard the supernatant without disturbing the beads.
14. Repeat steps 11–13 once for a total of **two ethyl alcohol washes**.
15. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
16. Air-dry the beads at room temperature until the beads no longer look glossy **(~3 minutes)**.

    Do not overdry the AMPure® XP beads after the ethyl alcohol washes. Overdried beads appear cracked.

17. Remove the tube from the magnet.

18. Pipet **30 µL** of elution buffer into the tube.
19. Pipet-mix 10 times until the beads are fully resuspended.
20. Incubate at room temperature for **2 minutes**.
21. Briefly centrifuge the tube.
22. Place the tube on the magnet until the solution is clear **(~30 seconds)**.
23. Pipet the eluate **(30 µL)** into a new 1.5-mL tube.
24. The purified eluate is the final sequencing library.

STOP The Index PCR libraries can be stored at –20 °C for up to 6 months.

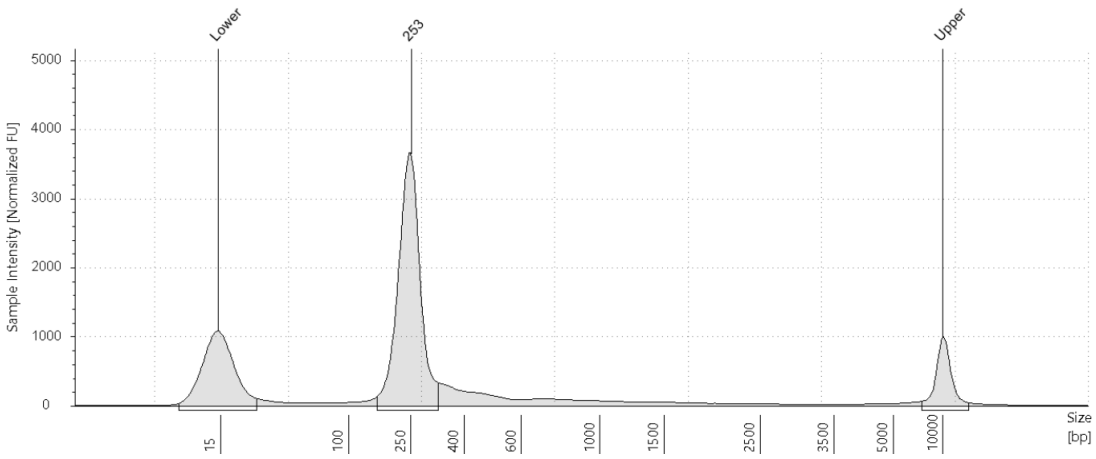
25. Quantify and perform quality control of the Index PCR library products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay and one of the following systems:
    - Agilent 2100 BioAnalyzer using the Agilent High Sensitivity DNA Kit
    - Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay

The expected concentration of the libraries from the Qubit™ Fluorometer is **>1.5 ng/µL**.

Refer to the representative traces in the following figures.

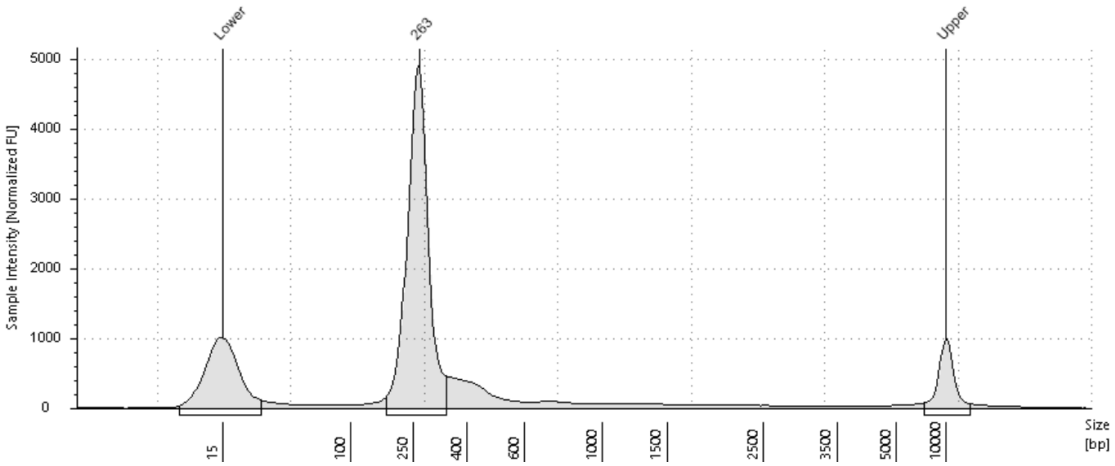
The expected size of BD® AbSeq index PCR products is ~250 bp.

Figure 2 Representative TapeStation High-Sensitivity D5000 trace–BD® AbSeq index product



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).

Figure 3 Representative TapeStation High-Sensitivity D1000 trace–Sample Tag index PCR product



If the concentration or size of the library is outside of the expected range, see Troubleshooting (page 32) or contact BD Biosciences technical support at scomix@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 and Element[a]
Read Length	Recommend Read 1: 51 Biosciences 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 (page 35).	

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.

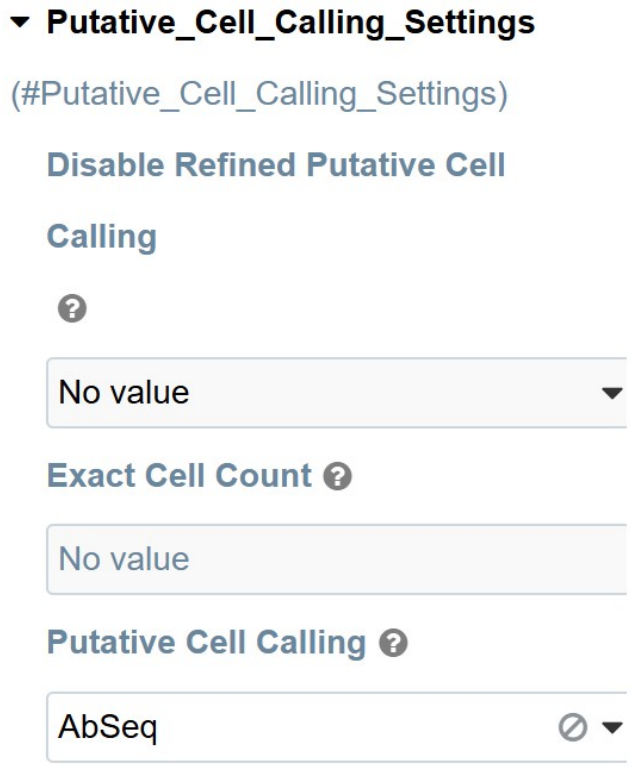
- Quantify sequencing libraries as recommended or according to Illumina or service provider instructions.

For other sequencing platforms (e.g. Element AVITI System), follow the manufacturer's sequencing recommendations. Loading concentration might need to be titrated to optimize yield.

BD Rhapsody™ sequence analysis pipeline

Refer to the *BD®Single-Cell Multiomics Analysis Setup User Guide* to set up 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. • PCR1 product yield too low. • Incorrect volumes of AMPure® XP beads used during purifying PCR product step.	• Contact BD Biosciences technical support at scomix@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.

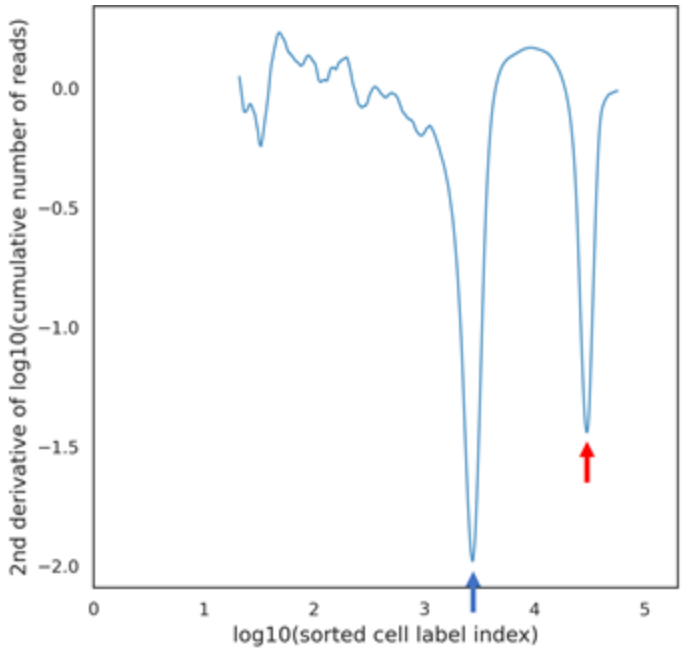
Sequencing

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 (page 30).
	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

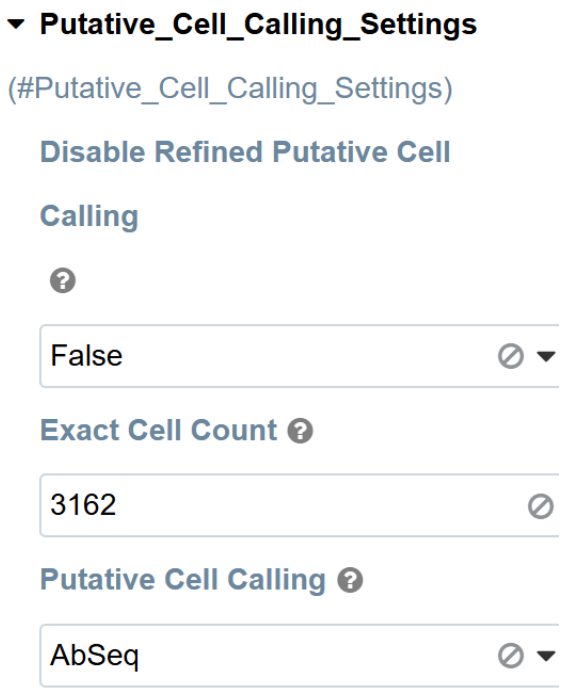
Observation	Possible causes	Recommended solutions
High number of putative cells and low number of RSEC molecules/cell.	BD® AbSeq noise is too high in the cartridge.	Wash 3 times after BD® AbSeq staining.
		Check “Cell_Label_Second_ Derivative_Curve.png” file to determine the accurate cell number and use “exact cell count” tool to run pipeline. See the example in Figure 5 Second derivative of the cell label filter output plot (page 34). 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 the preceding **Figure 5** ($10^{3.5}$= 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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