BD Rhapsody™ System

BD® AbSeq

Library Preparation Protocol

(for AbSeq-based cell calling)

23-25003(01)
2025-10

Regulatory information

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

History

Revision	Date	Change made
23-25003(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 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 BD® AbSeq library, AbSeq targets are encoded on the BD Rhapsody™ Enhanced Cell Capture Beads then amplified in PCR1. PCR1 products are then directly indexed. Sequencing of libraries can be completed on various sequencers followed by data analysis that utilizes BD® AbSeq-based 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 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.

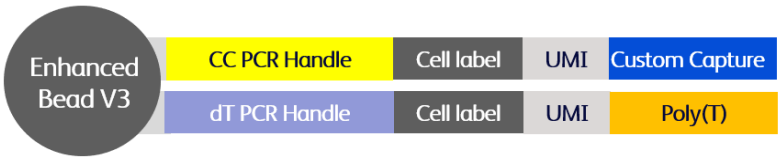
Symbols

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

1. BD® AbSeq PCR1 (page 11)

BD® AbSeq products are amplified in PCR1

3. BD® AbSeq index PCR (page 18)

Add Illumina adapters and indices

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 MasterMixes. You will need to purchase additional nuclease-free 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)

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

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	No
●	Sample Tag PCR2 primer	1	No
●	BD® AbSeq 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 Agencourt® AMPure® XP magnetic beads to room temperature (15–25 °C) before use. See manufacturer's instructions for more information.

Libraries

- BD® AbSeq libraries can be sequenced separately or together with 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.

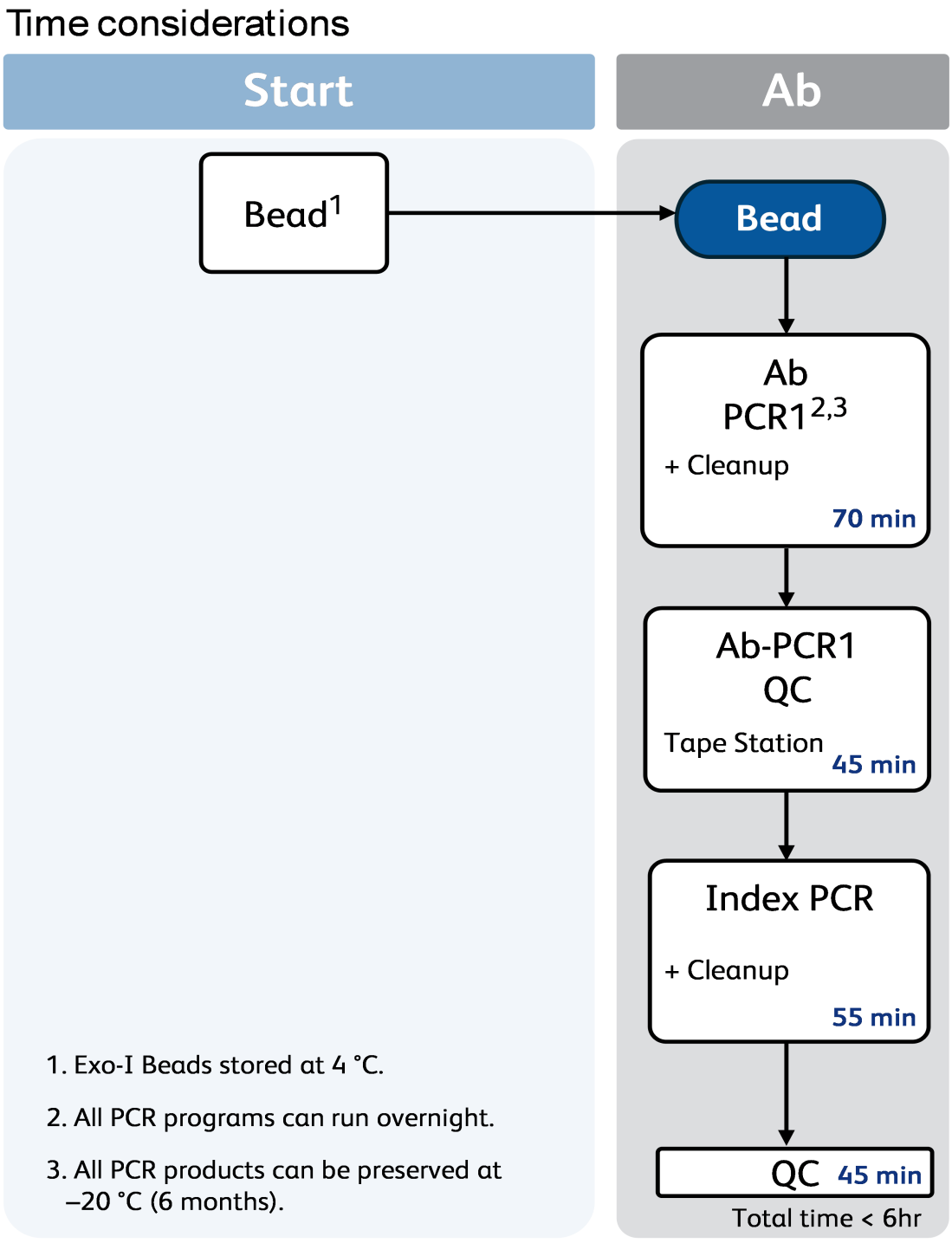
Additional documentation

- *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® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol* (doc ID 23-24262)
- *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol* (doc ID 23-22314)
- *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 BD® AbSeq Ab-Oligos (1 plex to 40 plex) Protocol* (doc ID 23-24262) or the *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos (41 plex to 100 plex) Protocol* (doc ID 23-22314).

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

This procedure comprises the following tasks:

- 1. BD® AbSeq PCR1 (page 11)
- 2. BD® AbSeq PCR1 cleanup and quality check (page 15)
- 3. BD® AbSeq index PCR (page 18)
- 4. BD® AbSeq index PCR cleanup and quality check (page 21)

1. BD® AbSeq PCR1

Summary:

- Prepare BD® AbSeq PCR1 mix.
- Amplify using BD® AbSeq 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 PCR1. Centrifuge briefly.	–20 °C
●	Bead RT/PCR enhancer	91-1082		
●	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 and keep on ice until ready.	4 °C
Ice bucket				
0.2-mL PCR tubes				
1.5-mL DNA LoBind® tubes				
0.2-mL tube magnetic rack				
1.5-mL tube magnetic rack				
Set up				
Thermocycler with BD® AbSeq PCR1 program				

Procedure steps:

This section describes how to amplify BD® AbSeq 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 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	12.0	14.4	57.6	115.2
○	Nuclease-free water	56.0	67.2	268.8	537.6
	Total	**200.0**	**240.0**	**960.0**	**1920.0**

2. Pipet-mix the BD® AbSeq 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:
   1. 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.
   2. 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 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.

BD® AbSeq 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 PCR1 cleanup and quality check

Summary:

- BD® AbSeq 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 PCR1 product				4 °C
1.5-mL DNA LoBind® 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 requires1 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 PCR1 product.
5. Transfer **200 µL** BD® AbSeq PCR1 product to a new 1.5-mL tube.

   The volume must be exactly 200 µL. If the volume is <200 µL, use nuclease-free water to achieve the final volume.
6. Pipet **280 µL** of AMPure® XP beads (1.4x) 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 **(<5 minutes).**
11. Remove and discard the supernatant.
12. Keeping the tube on the magnet, gently pipet **500 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 **(~5 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.

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

25. Perform quality control of the BD® AbSeq PCR1 product with one of the following systems:

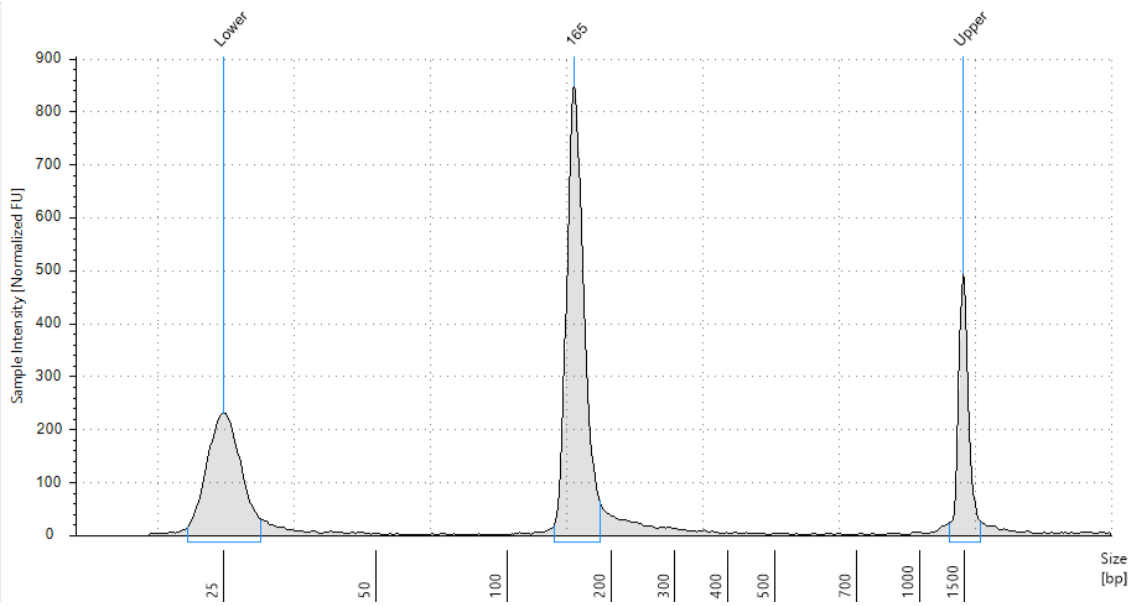
    • Agilent 2100 BioAnalyzer using the Agilent High Sensitivity DNA Kit

    • Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay

    The expected size of BD® AbSeq PCR1 products is **~160 bp**. Refer to the representative traces in the following figures.
26. Quantify the BD® AbSeq PCR1 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay.
27. Dilute an aliquot of BD® AbSeq PCR1 products to **0.1–1.1 ng/µL** with elution buffer before 3. BD® AbSeq index PCR (page 18).

    Refer to the representative trace in the following figure.

Figure 1 Representative TapeStation High-Sensitivity D1000 trace–BD® AbSeq PCR1 product



3. BD® AbSeq index PCR

Summary:

- Prepare BD® AbSeq index PCR mix
- Amplify using BD® AbSeq 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 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 product			Centrifuge briefly before adding to mix.	4 °C
Ice bucket				
0.2-mL PCR tubes				
Set up				
Thermocycler with BD® AbSeq index PCR program				

Procedure steps:

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

Perform this procedure in the pre-amplification workspace.

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

BD® AbSeq index PCR mix

Cap	Component	For 1 library (µL)	For 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 BD® AbSeq Sample Tag library.					

2. Pipet-mix the BD® AbSeq 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.

Bring the BD® AbSeq index PCR mix to the post-amplification workspace.

6. Add **3 µL** of diluted BD® AbSeq PCR1 from 2. BD® AbSeq PCR1 cleanup and quality check (page 15) product to the reaction mix.

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 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 BD® AbSeq index PCR program is complete, briefly centrifuge the tubes.

4. BD® AbSeq index PCR cleanup and quality check

Summary:

- BD® AbSeq 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 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% ethyl alcohol and use 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 BD® AbSeq index PCR product.
5. Transfer **50 µL** BD® AbSeq index PCR product to a new 1.5-mL tube.

   The volume must be exactly 50 µL. If the volume is <50 µL, use nuclease-free water to achieve the final volume.
6. Pipet **40 µL** of AMPure® XP beads (0.8x) 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 purified eluate is the final sequencing library.

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

25. Quantify and perform quality control of the BD® AbSeq index PCR product 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

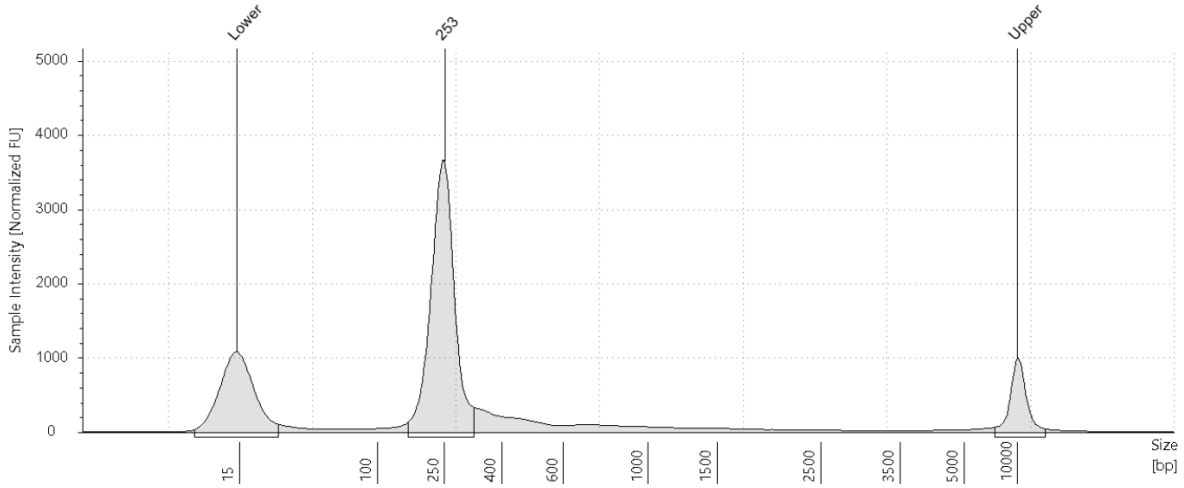
    • Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay

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

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

    Refer to the representative trace in the following figure.

Figure 2 Representative TapeStation High-Sensitivity D1000 trace–BD® AbSeq index product



If the concentration or size of the library is outside of the expected range, see Troubleshooting (page 26), or contact BD Biosciences technical support at scomix@bd.com.

Sequencing

Requirements

Run setup for Illumina® BaseSpace and sample sheet sequencing.

Required parameters

Parameter	Requirement
Platform	Illumina and Element Biosciences[a]
Read Length	Recommend Read 1: 51 cycles; Read 2: 50 cycles
PhiX	1% recommended
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook*
a. To review Index 1 (i7) sequences, see Appendix (page 29).	

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 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. BD® AbSeq libraries have not been tested on these sequencing platforms.		

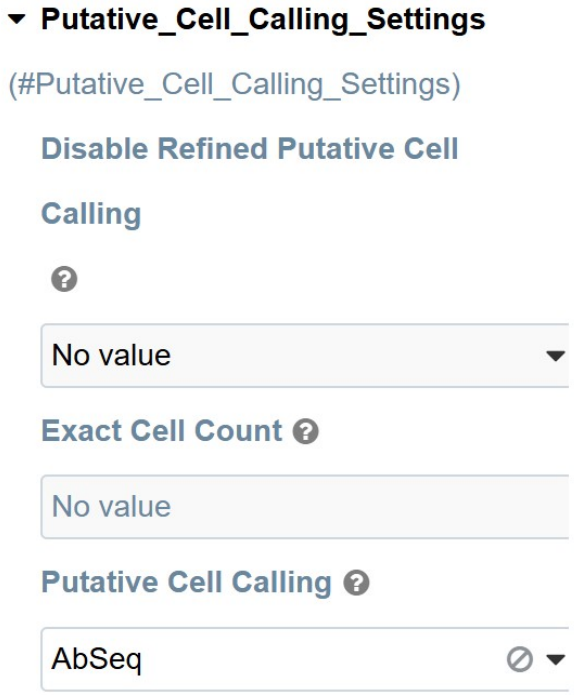
- First-time users are encouraged to start at the low end of the loading concentration recommendation to avoid over-clustering.
- Dilute PhiX to the same concentration as your library before combining samples to achieve the desired final concentration of PhiX. See Illumina instructions for detailed information 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 3 below. Refer to the putative cell calling section of the *BD® Single-Cell Multiomics Bioinformatics Handbook* for more details.

Figure 3 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.

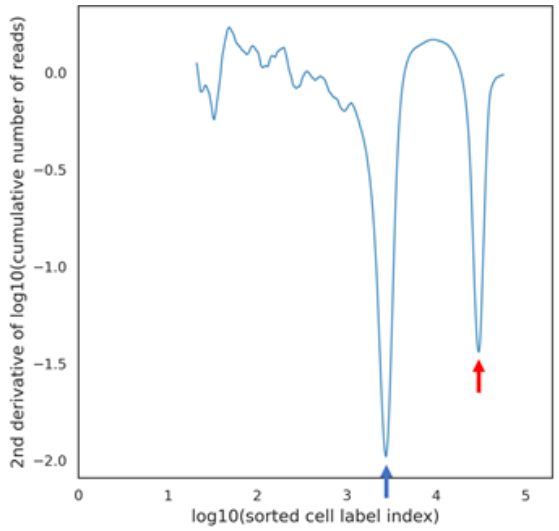
Sequencing

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 24).
	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

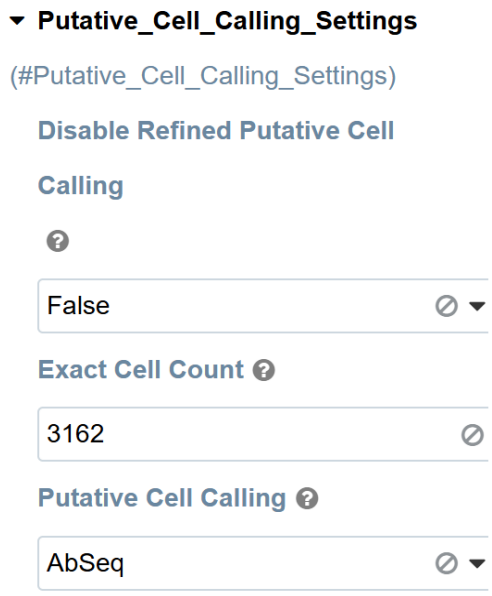
Observation	Possible causes	Recommended solutions
High number of putative cells and low number of RSEC molecules/cell.	BD® AbSeq noise is too high in the cartridge.	Wash 3 times after BD® AbSeq staining.
		Check “Cell_Label_Second_ Derivative_Curve.png” file to determine the accurate cell number and use “exact cell count” tool to run pipeline. See the example in Figure 4 Second derivative of the cell label filter output plot (page 28). 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 4 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 5 Example to set up exact cell count



An estimated value of cells, as indicated from the blue arrow in the preceding Figure 4 (103.5 = 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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