

 **BD Rhapsody™ System**
BD® AbSeq

Library Preparation Protocol
(for AbSeq-based cell calling)

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Regulatory information

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History

Revision	Date	Change made
23-24227(01)	2022-12	New protein-only protocol.

Contents

Introduction	4
Considerations	4
Workflow	5
Required materials	6
Best practices	7
Before you begin	7
Performing BD® AbSeq PCR1	8
Purifying BD® AbSeq PCR1 products	10
Quantifying BD® AbSeq PCR1 products	11
Performing BD® AbSeq Index PCR to prepare final BD® AbSeq library	12
Purifying index PCR products	13
Performing quality control on the final sequencing libraries	13
Sequencing	15
Requirements	15
Sequencing recommendations	15
Sequencing amount for BD® AbSeq libraries	15
BD Rhapsody™ sequence analysis pipeline	16
Troubleshooting	17
Library preparation	17
Sequencing	17
Pipeline	18
Appendix	20
Illumina index 1 (i7) sequences	20

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 using the BD Rhapsody™ Single-Cell Analysis system, or the BD Rhapsody™ Express Single-Cell Analysis system. 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 are completed on Illumina sequencers followed by data analysis that utilizes BD[®] AbSeq-based cell calling pipeline which identifies cells without mRNA information.

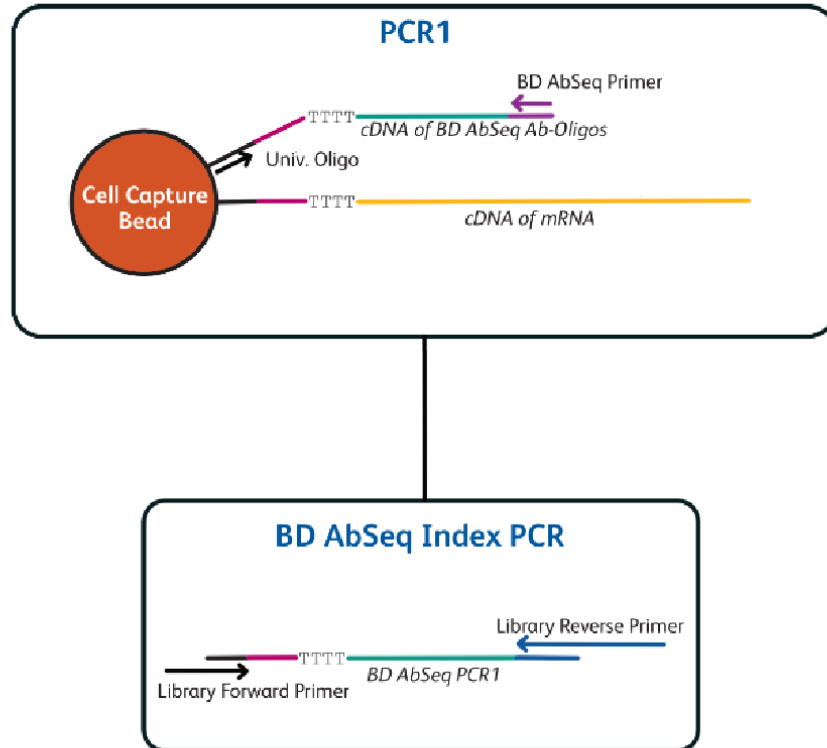
Considerations

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 either BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit or 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)

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	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	No
Sample Tag PCR2 Primer	91-1089	Purple	No
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	No
Sample Tag PCR2 Primer	91-1089	Purple	No

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 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
BD [®] AbSeq Primer	12.0	14.4
Nuclease-Free Water	Up to 40	Up to 49.2
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:

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

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 up to 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 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 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 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 240 µL AMPure XP beads into a 1.5-mL LoBind[®] tube with 200 µL AbSeq PCR1 from [Performing BD[®] AbSeq 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 supernatant from the tube.
- 9 Air-dry the 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 Elution Buffer. Vigorously pipet-mix until the 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 PCR1 products).

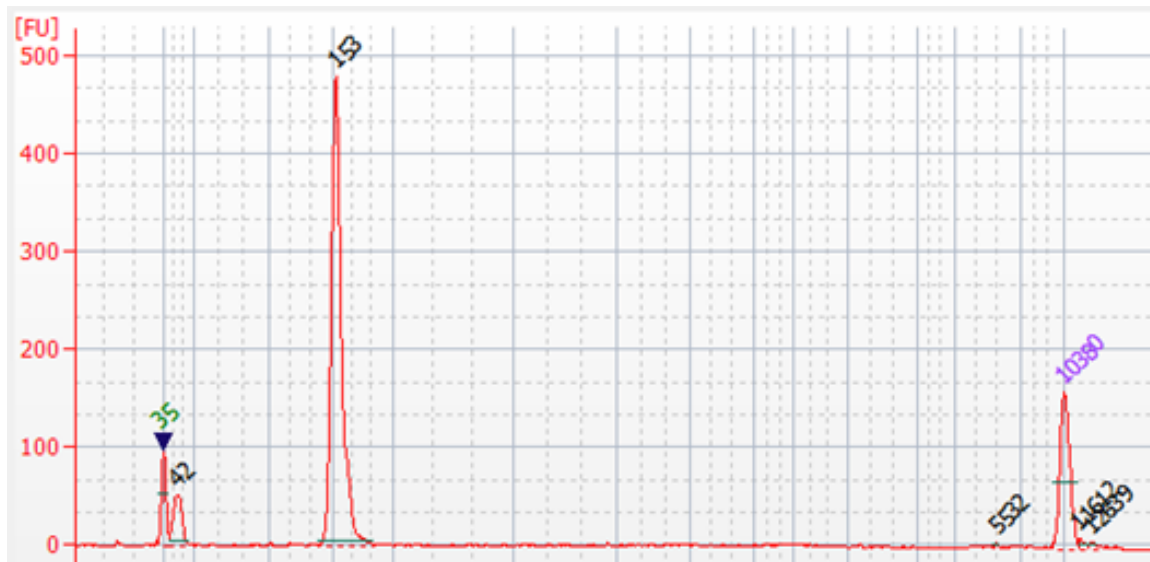
STOPPING POINT: Store at 2–8 °C before proceeding within 24 hours or at –25 °C to –15 °C for up to 6 months.

Quantifying BD® AbSeq PCR1 products

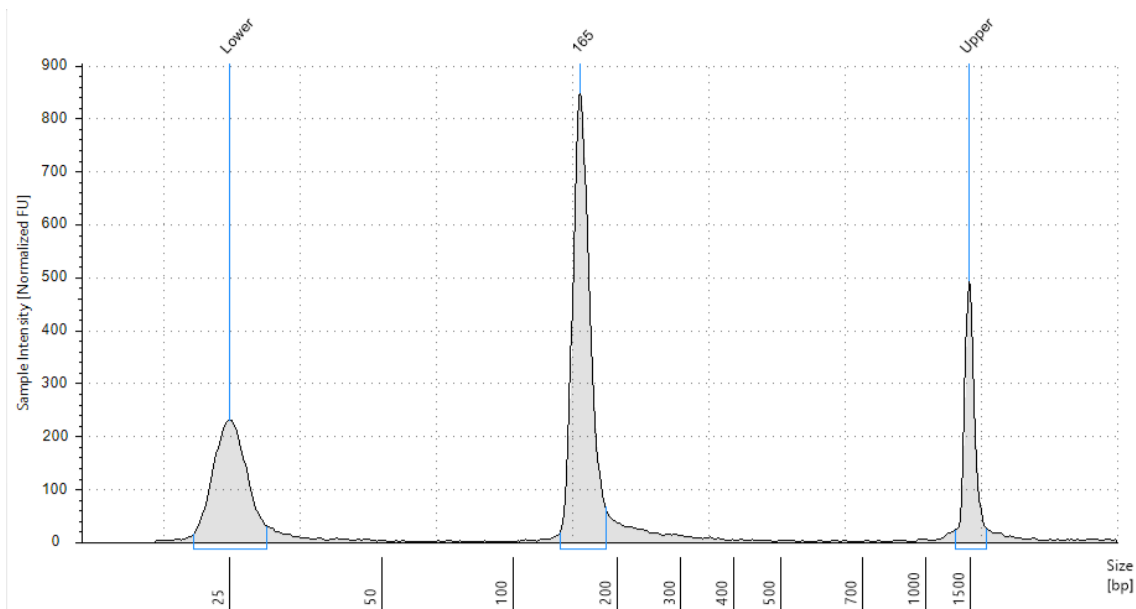
1. Measure the yield of the BD® AbSeq PCR1 products (~153 bp)—size varies on different instruments—by using the Agilent Bioanalyzer with the High Sensitivity Kit or the Agilent TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions.
2. Dilute an aliquot of BD® AbSeq PCR1 products to 0.1–1.1 ng/μL with Elution Buffer before index PCR of BD® AbSeq PCR1 products. See [Performing BD® AbSeq Index PCR to prepare final BD® AbSeq library on page 12](#).

Figure 1 BD® AbSeq PCR1 products

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



Performing BD[®] AbSeq Index PCR to prepare final BD[®] AbSeq library

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

Note: If libraries are to be indexed differently, make separate index PCR mixes containing different library reverse primers for each library.

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

- 2 Gently vortex mix, briefly centrifuge, and place back on ice.
- 3 Bring index PCR mixes into the post-amplification workspace. Pipet 3.0 μL of 0.1–1.1 ng/μL products into 47.0 μL index PCR mix.
- 4 Gently vortex, and briefly centrifuge.
- 5 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 BD [®] AbSeq libraries (ng/μL)	Suggested number of 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 Pipet 40 µL of AMPure beads into 50.0 µL of the index PCR products.
- 4 Pipet-mix 10 times, and incubate at room temperature (15–25 °C) for 5 minutes.
- 5 Place the tube on the strip tube magnet for 3 minutes. Remove and discard the supernatant.
- 6 Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube, and incubate for 30 seconds. Remove 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 supernatant from the tube.
- 9 Air-dry the beads at room temperature (15–25 °C) for 3 minutes.
- 10 Remove the tube from the magnet, and resuspend the bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
- 11 Incubate at room temperature (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 entire eluate (~30 µL) into two separate and 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 13](#).

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

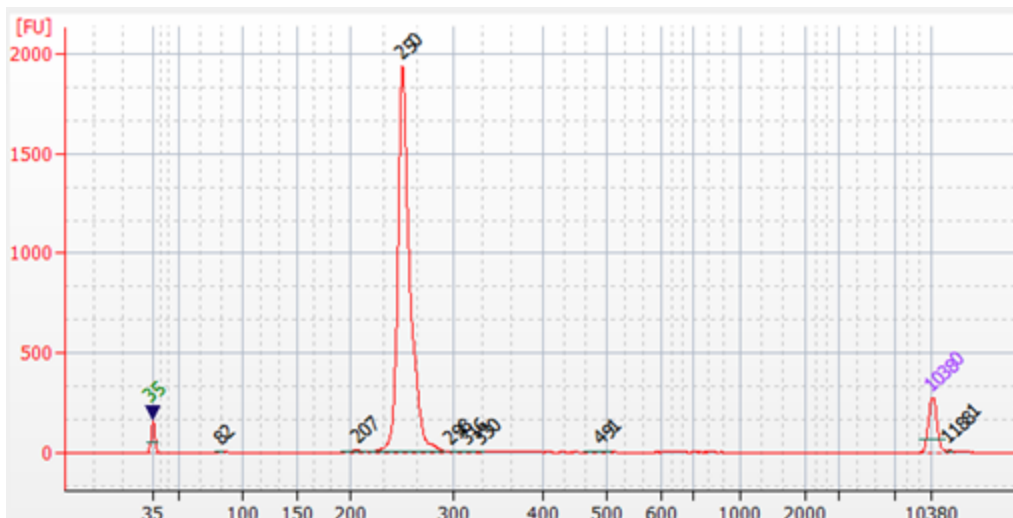
Performing quality control on the final sequencing libraries

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.

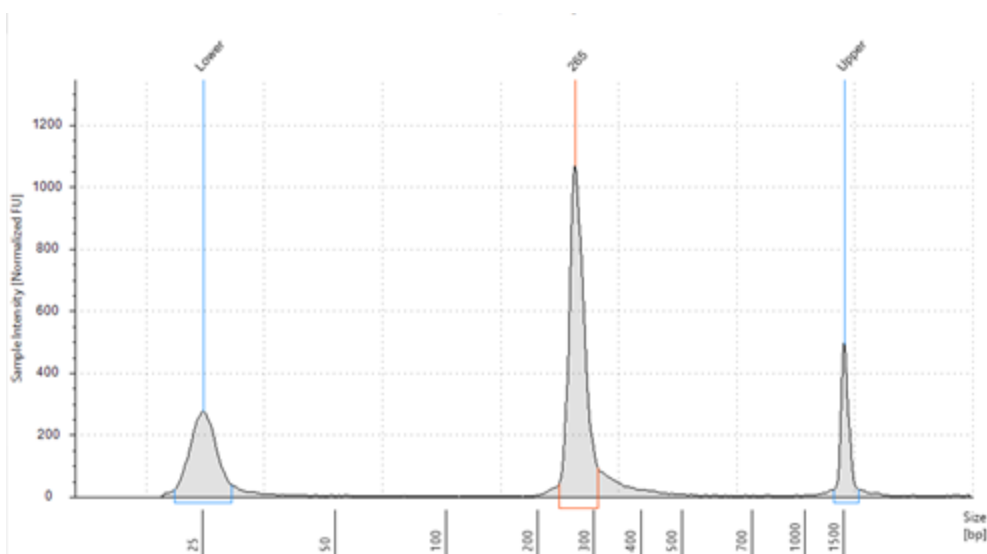
Figure 2 Final BD[®] AbSeq library

A. Sample Bioanalyzer high-sensitivity DNA trace

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



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 17](#), or contact BD Biosciences technical support at scomix@bdscomix.bd.com.

Sequencing

Requirements

Run setup for Illumina® BaseSpace and sample sheet sequencing.

Required parameters

Parameter	Requirement
Platform	Illumina ^a
Read Length	Recommend Read 1: 51 cycles; Read 2: 50 cycles
PhiX	1% recommended
Analysis	See the <i>BD® Single-Cell Multiomics Bioinformatics Handbook</i>
a. To review Illumina Index 1 (i7) sequences, see Appendix on page 20 .	

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.

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 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

▼ **Putative_Cell_Calling_Settings** (#Putative_Cell_Calling_Settings)

Disable Refined Putative Cell Calling ⓘ

No value ▼

Exact Cell Count ⓘ

No value

Putative Cell Calling ⓘ

AbSeq ⓘ ▼

Troubleshooting

Library preparation

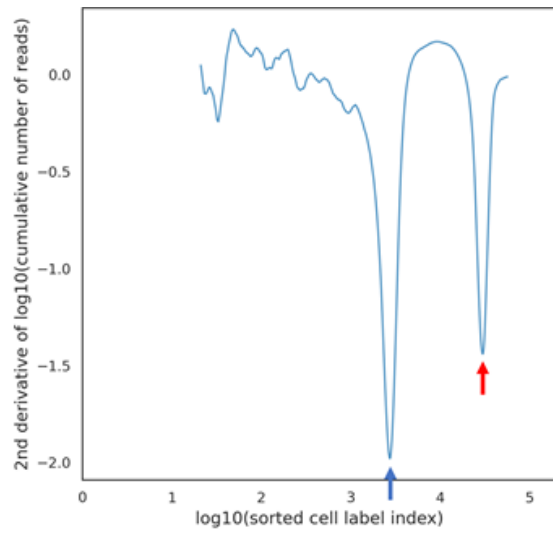
Observation	Possible causes	Recommended solutions
BD® AbSeq PCR1 product size too low.	<ul style="list-style-type: none"> BD® AbSeq Primer not added to PCR1. Too few PCR1 cycles. Incorrect volumes of AMPure XP beads used during purifying PCR product step. 	<ul style="list-style-type: none"> Contact BD 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.	<ul style="list-style-type: none"> Increase the number of cycles for index PCR.
	Only one primer (Library Forward or Library Reverse primer) added to index PCR mix.	<ul style="list-style-type: none"> 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.	<ul style="list-style-type: none"> Quantitate library according to instructions in protocol.
Low sequencing quality.	Insufficient PhiX.	<ul style="list-style-type: none"> Use the recommended concentration of PhiX with the library to be sequenced. See Sequencing flowcell loading and PhiX concentrations on page 15.
	Suboptimal cluster density, and/or library denaturation.	<ul style="list-style-type: none"> See troubleshooting in Illumina documentation.
	Higher than 50 cycles for R2 reads that reads through polyA of AbSeq oligo.	<ul style="list-style-type: none"> Lower read 2 cycles to 50 or trim additional reads from read2 of 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 on page 19 . 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

▼ **Putative_Cell_Calling_Settings** (#Putative_Cell_Calling_Settings)

Disable Refined Putative Cell Calling ⓘ

False

Exact Cell Count ⓘ

3162

Putative Cell Calling ⓘ

AbSeq

Appendix

Illumina index 1 (i7) sequences

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

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