

 **BD Rhapsody™ System**
Targeted mRNA and BD® AbSeq
Library Preparation Protocol

Copyrights

No part of this publication may be reproduced, transmitted, transcribed, stored in retrieval systems, or translated into any language or computer language, in any form or by any means: electronic, mechanical, magnetic, optical, chemical, manual, or otherwise, without prior written permission from BD.

The information in this guide is subject to change without notice. BD reserves the right to change its products and services at any time. Although this guide has been prepared with every precaution to ensure accuracy, BD assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information. BD welcomes customer input on corrections and suggestions for improvement.

Patents and Trademarks

For US patents that may apply, see bd.com/patents.

BD, the BD Logo and BD Rhapsody are trademarks of Becton, Dickinson and Company or its affiliates. All other trademarks are the property of their respective owners. © 2024 BD. All rights reserved.

Regulatory information

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

History

Revision	Date	Change made
23-24123(01)	2021-12	Initial release.
23-24123(02)	2022-11	Updated for BD Rhapsody™ Enhanced Cell Capture Beads version 2.0.
23-24123(03)	2024-11	Added the BD® OMICS-One Dual Index Kit. Added Workflows chapter. Updated Time Considerations workflow. Updated sequencing section. Added sequencing recommendation. Added BD Rhapsody™ oligo sequences to appendix.

Contents

Introduction	4
Symbols	4
Protocol kits	5
Workflows	7
Targeted mRNA library amplification workflow	7
BD® AbSeq library amplification workflow	7
Required and recommended materials	8
Required reagents	8
Recommended consumables	9
Equipment	10
Best practices	10
Additional documentation	11
Safety information	11
Time considerations	12
Procedure	12
Targeted mRNA and BD® AbSeq library amplification	12
Before you begin	13
Step 1: Targeted mRNA and BD® AbSeq PCR1	13
Step 2: Targeted mRNA and BD® AbSeq PCR1 cleanup	15
Step 3: Quantifying BD® AbSeq PCR1 products	17
Step 4: Targeted mRNA PCR2	18
Step 5: Targeted mRNA PCR2 cleanup	19
Step 6: Targeted mRNA and BD® AbSeq index PCR	20
Step 7: Targeted mRNA and BD® AbSeq index PCR cleanup and quality check	22
Sequencing	25
Read requirements for libraries	25
Sequencing recommendations	26
Sequencing analysis pipeline	26
Troubleshooting	27
Library preparation	27
Sequencing	29
Appendix	30
Oligonucleotides in BD Rhapsody™ Targeted mRNA Amplification Kit	30
BD® AbSeq Ab-Oligo common motifs	32
Contact Information	33

Introduction

This protocol provides instructions for creating single-cell Targeted mRNA and BD® AbSeq libraries after cell capture on the BD Rhapsody™ HT Single-Cell Analysis system or the BD Rhapsody™ HT Xpress System for sequencing on various sequencers. For complete instrument procedures and safety information, see the *BD Rhapsody™ HT System Instrument User Guide* or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-free Workflow*.

To create the libraries, BD Rhapsody™ mRNA targets and BD® AbSeq are encoded on the BD Rhapsody™ Enhanced Cell Capture Beads and then amplified in PCR1. After PCR1, the BD® AbSeqPCR1 products are separated from the Targeted mRNA PCR1 products by double-sided size selection with Agencourt® AMPure® XP magnetic beads. Size selection of library molecules is achieved by specific and successive use of volume ratios between DNA samples and AMPure beads.

Successful preparation of Targeted mRNA, and BD® AbSeq libraries requires that:

- The BD® AbSeq PCR1 products undergo a separate index PCR from Targeted mRNA products with library index primers.
- After index PCR, the Targeted mRNA and BD® AbSeq libraries can be combined for sequencing.

Symbols

The following symbols are used in this guide.

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

Protocol kits

Before you begin, ensure that you have the correct kits for this protocol. Matching cap colors indicate you have the correct kit, along with the catalog numbers found in the [Required and recommended materials \(page 8\)](#) section.



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

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

Cap Color	Name	Quantity
○	PCR master mix	1
○	Universal oligo	1
●	Elution buffer	1
●	Bead RT/PCR enhancer	1
●	Library forward primer	1
●	Library reverse primer 1–4	1 each
○	Nuclease-free water	1
●	Bead resuspension buffer	1
●	Sample Tag PCR1 primer	2
●	Sample Tag PCR2 primer	1
●	BD® AbSeq PCR1 primer	1

BD Rhapsody™ Immune Response Panel Hs (–20 °C)

Cap Color	Name	Quantity
●	PCR1 primers immune response Hs	1
●	PCR2 primers immune response Hs	1

BD Rhapsody™ Immune Response Panel Mm (–20 °C)

Cap Color	Name	Quantity
●	PCR1 primers immune response Mm	1
●	PCR2 primers immune response Mm	1

BD Rhapsody™ Onco-BC Panel Hs (-20 °C)

Cap Color	Name	Quantity
●	PCR1 primers Onco-BC Hs	1
●	PCR2 primers Onco-BC Hs	1

BD Rhapsody™ T Cell Expression Panel Hs (-20 °C)

Cap Color	Name	Quantity
●	PCR1 primers T cell expression Hs	1
●	PCR2 primers T cell expression Hs	1

BD® OMICS-One Dual Index Kit (-20 °C)

Cap Color	Name	Quantity
●	Dual index forward primer 1–8	1 each
●	Dual index reverse primer 1–8	1 each

Workflows

Targeted mRNA library amplification workflow

Step 1: Targeted mRNA and BD® AbSeq PCR1 (page 13):
Universal primer and Targeted PCR1 primer panel copy target region from bead.

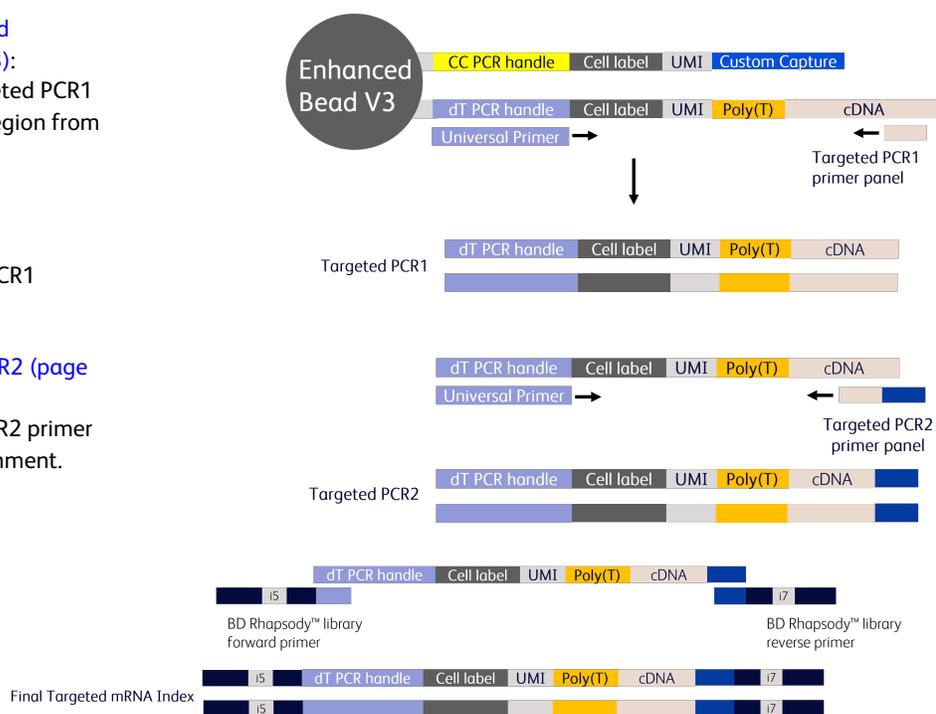
Amplify in solution. Collect supernatant as Targeted PCR1 product.

Step 4: Targeted mRNA PCR2 (page 18):

Amplify using Targeted PCR2 primer panel for nested PCR enrichment.

Step 6: Targeted mRNA and BD® AbSeq index PCR (page 20):

Add adapters and indices.



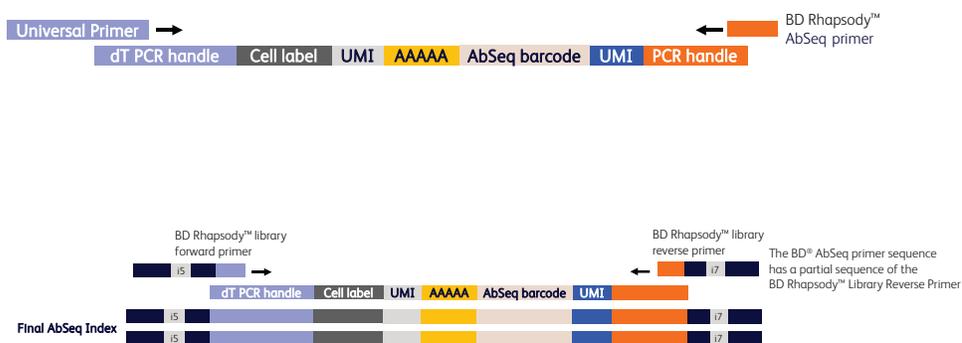
BD® AbSeq library amplification workflow

Step 1: Targeted mRNA and BD® AbSeq PCR1 (page 13):

BD® AbSeq product is amplified in PCR1.

Step 6: Targeted mRNA and BD® AbSeq index PCR (page 20):

Add BD® AbSeq adapters and indices.



Required and recommended materials

Required reagents

Store the reagents at the storage temperature specified on the label.

Material	Supplier	Catalog no.
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit*	BD Biosciences	633774
BD Rhapsody™ Immune Response Panel Hs* †	BD Biosciences	633750
BD Rhapsody™ Immune Response Panel Mm* †	BD Biosciences	633753
BD Rhapsody™ Onco-BC Panel HS* †	BD Biosciences	633752
BD Rhapsody™ T Cell Expression Panel Hs* †	BD Biosciences	633751
BD® OMICS-One Dual Index Kit‡	BD Biosciences	571899
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol, molecular biology grade	Major supplier	–
Nuclease-free water	Major supplier	–

* For processing more than four libraries, two orders of this catalog number are required.

† Dependent on targeted amplification samples used.

‡ Recommended for unique dual indexing with high-throughput (more than eight) library preparation workflows.

Recommended consumables

Material	Supplier	Part number/Catalog no.
Pipettes (P10, P20, P200, P1000)	Major supplier	–
Low-retention, filtered pipette tips	Major supplier	–
0.2-mL PCR 8-strip tubes	Major supplier	–
Axygen® 96-Well PCR Microplates*	Corning	PCR96HSC
Or, MicroAmp Optical 96-Well Reaction Plate*	Thermo Fisher Scientific	N8010560
MicroAmp Clear Adhesive Film*	Thermo Fisher Scientific	4306311
15-mL conical tube	Major supplier	–
DNA LoBind® tubes, 1.5 mL	Eppendorf	0030108051
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent High Sensitivity DNA Kit	Agilent	5067-4626
Or, Agilent High Sensitivity D1000 ScreenTape	Agilent	5067-5584
Agilent High Sensitivity D1000 Reagents	Agilent	5067-5585
Or, Agilent High Sensitivity D5000 ScreenTape	Agilent	5067-5592
Agilent High Sensitivity D5000 Reagents	Agilent	5067-5593

* Recommended for processing high-throughput (more than eight) library preparation workflows.

Equipment

Material	Supplier	Catalog no.
Microcentrifuge for 1.5–2.0-mL tubes	Major supplier	–
Microcentrifuge for 0.2-mL tubes	Major supplier	–
Vortexer	Major supplier	–
Digital timer	Major supplier	–
PCR thermal cycler	Major supplier	–
6-tube magnetic separation rack for 1.5-mL tubes Or, 12-tube magnetic separation rack [†] Or, Invitrogen™ DynaMag™-2 magnet [†]	New England Biolabs New England Biolabs Thermo Fisher Scientific	S1506S S1509S 12321D
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
Magnetic Stand–96 [‡]	Thermo Fisher Scientific	AM10027
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Agilent® 2100 Bioanalyzer Or, Agilent® 4200 TapeStation System	Agilent Technologies Agilent Technologies	G2940CAG G2991AA
Heat block	Major supplier	–

[†] Recommended for processing greater than six samples.

[‡] Recommended for processing high-throughput (more than eight) library preparation workflows.

Best practices

Cell capture

- For best results, ensure that cells have high viability before proceeding with cell capture.

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 the *AMPure® XP User's Guide* for information.

Libraries

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

Supernatant handling

- Read this protocol carefully before beginning each section. Note which steps require you to keep 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.

Bead amplification

- Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension. Start the thermocycler program immediately after mixing.

Additional documentation

- *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24252)
- *BD Rhapsody™ HT Xpress System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24253)
- *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™ 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 23-24607) or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-Free Workflow* (doc ID 23-24256).

Time considerations

Station	Workflow	Timing	Stopping Point & Storage
Targeted mRNA and BD® AbSeq Library Amplification			
Post-Amplification Workspace	Step 1: Targeted mRNA and BD® AbSeq PCR1 (page 13)	100 minutes 	PCR overnight
	Step 2: Targeted mRNA and BD® AbSeq PCR1 cleanup (page 15)		<24 hours at 4 °C or <6 months at –20 °C
	Step 3: Quantifying BD® AbSeq PCR1 products (page 17)BD® AbSeq PCR1 Quality Check		<24 hours at 4 °C or <6 months at –20 °C
	Step 4: Targeted mRNA PCR2 (page 18)	80 minutes 	PCR overnight
	Step 5: Targeted mRNA PCR2 cleanup (page 19)		<24 hours at 4 °C or <6 months at –20 °C
	Step 6: Targeted mRNA and BD® AbSeq index PCR (page 20)	70 minutes 	PCR overnight
	Step 7: Targeted mRNA and BD® AbSeq index PCR cleanup and quality check (page 22)		<6 months at –20 °C

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 treatment:

- *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24252)
- *BD Rhapsody™ HT Xpress System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24253)

Targeted mRNA and BD® AbSeq library amplification

This procedure comprises the following tasks:

- [Step 1: Targeted mRNA and BD® AbSeq PCR1 \(page 13\)](#)
- [Step 2: Targeted mRNA and BD® AbSeq PCR1 cleanup \(page 15\)](#)
- [Step 3: Quantifying BD® AbSeq PCR1 products \(page 17\)](#)
- [Step 4: Targeted mRNA PCR2 \(page 18\)](#)
- [Step 5: Targeted mRNA PCR2 cleanup \(page 19\)](#)
- [Step 6: Targeted mRNA and BD® AbSeq index PCR \(page 20\)](#)
- [Step 7: Targeted mRNA and BD® AbSeq index PCR cleanup and quality check \(page 22\)](#)

Before you begin

- Obtain Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads.
- Keep enzymes on ice or keep frozen before use.
- Thaw the reagents in the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit at room temperature and then place on ice.

Step 1: Targeted mRNA and BD[®] AbSeq PCR1



Before using BD Rhapsody™ 10X PCR1 custom primers or BD Rhapsody™ 10X PCR1 supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody™ Targeted (pre-designed) primer panels are provided at 1X concentration and should not be diluted.

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

PCR1 reaction mix

Cap	Component	For 1 library (μL)	For 1 library with 20% overage (μL)	For 4 libraries with 20% overage (μL)	For 8 libraries with 20% overage (μL)
○	PCR master mix	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
●	PCR1 Targeted mRNA primer panel* †	40.0	48.0	192.0	384.0
	(Optional) PCR1 panel supplement* †	(10.0)	(12.0)	(48.0)	(96.0)
●	BD [®] AbSeq primer	12.0	14.4	57.6	115.2
○	Nuclease-free water	16.0 (6.0)	19.2 (7.2)	76.8 (28.8)	153.6 (57.6)
	Total	200.0	240.0	960.0	1920.0

* Order from BD Biosciences.

† BD Rhapsody™ Targeted (pre-designed) primer panels are provided at 1X. Ensure custom and supplement panels are diluted to 1X before use.

2. Gently vortex mix, briefly centrifuge, and place back on ice.
3. Based on the sample size, complete one of the following actions:
 - If using a subsample, proceed to step 4.
 - If using the entire sample, skip to step 5.

4. To subsample 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.



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

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 PCR1 reaction mix. Do not vortex.
7. Ensure that the beads are fully resuspended, then pipet 50 µL of 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. Run the following PCR program on the thermal cycler.

PCR1 conditions for Targeted mRNA panel

Step	Cycles	Temperature	Time
Hot start	1	95 °C*	3 min
Denaturation	8–15 [†]	95 °C	30 seconds
Annealing		60 °C	3 min
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

* 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 3-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 1-minute 95 °C pause step can be added immediately before the 3-minute 95 °C denaturation step.

[†] Recommended PCR cycles might require optimization for different cell types and cell number.

Recommended number of PCR cycles

Number of cells in PCR1	Recommended PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12
10,000	11
20,000	10
40,000	9
80,000–100,000	8

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.



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 tubes 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 from the same sample into a new 1.5-mL LoBind[®] tube.



Retain the supernatant in the next step.

14. Place the 1.5-mL tube on magnet for 2 minutes, and carefully pipet the supernatant (mRNA Targeted PCR1 products) into the new 1.5-mL LoBind[®] tube without disturbing the beads. Discard the beads.

Step 2: Targeted mRNA and BD[®] AbSeq PCR1 cleanup

This section describes how to perform double-sided AMPure cleanup to separate the shorter BD[®] AbSeq PCR1 products (~160 bp) from the longer Targeted mRNA PCR1 products (350–800 bp).



Keep both the supernatant (BD[®] AbSeq products) and the AMPure beads (Targeted mRNA products) for purification.



Perform PCR1 purification in the post-amplification workspace.

1. Bring the AMPure® XP magnetic beads to room temperature.
2. In a new 15-mL conical tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL nuclease-free water (or refer to the following table for additional libraries). Vortex the tube for 10 seconds to mix.



Make fresh 80% ethyl alcohol and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries. Volumes provided in the following table are enough to cover all PCR clean ups throughout the protocol.

80% Ethyl Alcohol

Component	For 1 library (mL)	For 4 libraries (mL)	For 8 libraries (mL)
100% ethyl alcohol	4	16	32
Nuclease-free water	1	4	8
Total	5	20	40

3. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
4. Briefly centrifuge PCR1 products.

The final volume must be exactly 200 µL to achieve the appropriate size selection of the purified PCR1 product. If the volume is less than 200 µL, use elution buffer to achieve the final volume.
5. Pipet 140 µL of AMPure® beads into the 200 µL of Targeted mRNA and BD® AbSeq PCR1 products. Pipet-mix 10 times, then briefly centrifuge the samples.
6. Incubate at room temperature for 5 minutes.
7. Place the 1.5-mL LoBind® tube on the magnet for 5 minutes or until the supernatant is clear.
8. Keeping the tube on the magnet, transfer the 340 µL of supernatant (BD® AbSeq PCR1 products) to a new 1.5-mL tube without disturbing the beads (Targeted mRNA PCR1 products).
9. Store the supernatant (step 8) on ice while purifying and eluting the Targeted mRNA products. Purify the BD® AbSeq PCR1 products after purifying the Targeted mRNA PCR1 products.
10. Keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant without disturbing the beads.
11. Repeat step 10 once for a total of two ethyl alcohol washes.
12. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
13. Air-dry the beads at room temperature for 5 minutes.



Do not overdry the AMPure® beads after the ethanol washes. Overdried beads appear cracked.

14. Remove the tube from the magnet and pipet 30 µL of elution buffer to resuspend the bead pellet. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.
15. Incubate at room temperature for 2 minutes and briefly centrifuge.
16. Place the tube on the magnet until the solution is clear, usually ~30 seconds.

17. Pipet the eluate (~30 μL) into a new 1.5-mL LoBind[®] tube (purified Targeted mRNA PCR1 products).
18. To purify BD[®] AbSeq PCR1 products, pipet 100 μL of AMPure[®] beads into the tube with 340 μL supernatant from step 8. Pipet-mix 10 times, then briefly centrifuge the samples.
19. Incubate at room temperature for 5 minutes.
20. Place the 1.5-mL LoBind[®] tube on the magnet for 5 minutes or until the supernatant is clear. Remove and discard the supernatant without disturbing the beads.
21. Keeping the tube on the magnet, gently pipet 500 μL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant without disturbing the beads.
22. Repeat step 21 for a total of two ethyl alcohol washes.
23. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
24. Air-dry the beads at room temperature for 5 minutes.



Do not overdry the AMPure[®] beads after the ethanol washes. Overdried beads appear cracked.

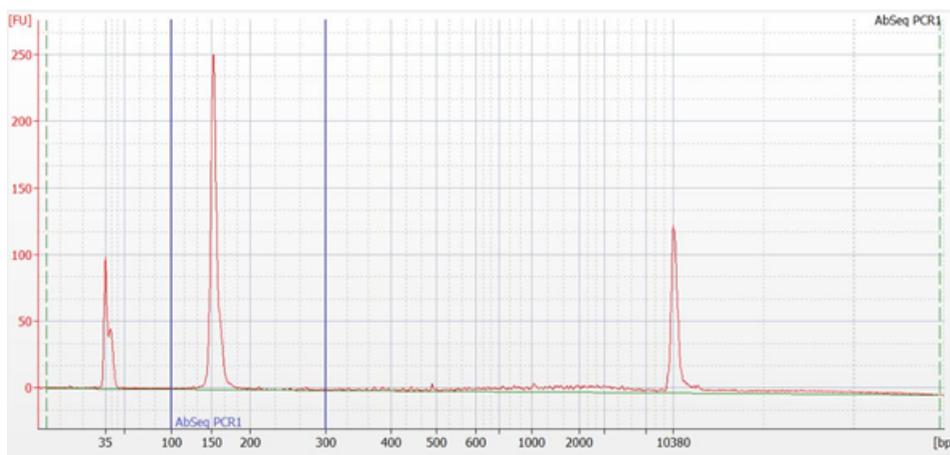
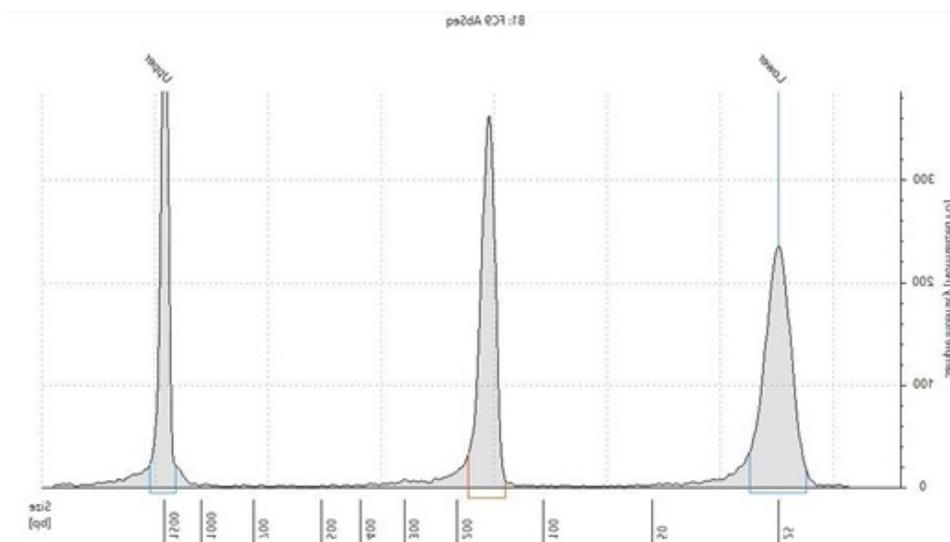
25. Remove the tube from the magnet and pipet 30 μL of eution buffer into the tube to resuspend the bead pellet. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.
26. Incubate at room temperature for 2 minutes and briefly centrifuge.
27. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
28. 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.

Step 3: Quantifying BD[®] AbSeq PCR1 products

1. Measure the yield of the largest peak of the BD[®] AbSeq PCR1 products (~160 bp) by using the Agilent Bioanalyzer with the High Sensitivity Kit or Agilent TapeStation. Follow the manufacturer's instructions. Peak sizes may vary depending on instrumentation or assay used for measurement.

Figure 1 BD® AbSeq PCR1 sample bioanalyzer high-sensitivity DNA trace**Figure 2** BD® AbSeq PCR1 sample TapeStation high-sensitivity D1000 trace

2. Dilute an aliquot of BD® AbSeq products to 0.1–1.1 ng/μL with elution buffer before index PCR of BD® AbSeq PCR1 products.

Step 4: Targeted mRNA PCR2



Before using BD Rhapsody™ 10X PCR1 Custom primers or BD Rhapsody™ 10X PCR1 Supplement primers, dilute 1 part of the 10X PCR primer stock to 9 parts of IDTE buffer to prepare a 1X primer solution. BD Rhapsody™ Targeted (pre-designed) primer panels are provided at 1X concentration and should not be diluted.



Only the Targeted mRNA PCR1 products require PCR2 amplification. The BD® AbSeq PCR1 products require only index PCR.

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

Targeted mRNA PCR2 reaction mix

Cap	Component	For 1 library (μL)	For 1 library with 20% overage (μL)	For 4 libraries with 20% overage (μL)	For 8 libraries with 20% overage (μL)
○	PCR master mix	25.0	30.0	120.0	240.0
○	Universal oligo	2.0	2.4	9.6	19.2
●	PCR2 primer panel* †	10.0	12.0	48.0	96.0
	(Optional) PCR2 panel supplement* †	(2.5)	(3.0)	(12.0)	(24.0)
○	Nuclease-free water	8.0 (5.5)	9.6 (6.6)	38.4 (26.4)	76.8 (52.4)
	Total	45.0	54.0	216.0	432.0

* Order from BD Biosciences.

† BD Rhapsody™ Targeted (pre-designed) primer panels are provided at 1X. Ensure custom panels are diluted to 1X before use.

2. Gently vortex mix, briefly centrifuge. Pipet 45 μL of PCR2 reaction mix into one 0.2-mL PCR tube for each library and place back on ice.
3. Bring the PCR2 reaction mixes to the post-amplification workspace.
4. Pipet 5.0 μL of purified Targeted mRNA PCR1 products into 45.0 μL of PCR2 reaction mix to create the Targeted mRNA PCR2 reaction mix.
5. Gently vortex and briefly centrifuge.
6. Run the following PCR program on the thermal cycler.

PCR2 conditions for Targeted mRNA

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10	95 °C	30 seconds
Annealing		60 °C	3 min
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞



STOPPING POINT: The PCR can run overnight.

Step 5: Targeted mRNA PCR2 cleanup



Perform PCR2 purification in the post-amplification workspace.

1. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.
2. Briefly centrifuge the PCR2 products.



The final volume must be exactly 50 µL to achieve the appropriate size selection of the purified PCR2 product. If the volume is less than 50 µL, use elution buffer to achieve the final volume.

3. To 50 µL of the PCR2 products, pipet AMPure® beads as follows:
 - Targeted mRNA library: 40 µL of AMPure® beads
4. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
5. Place the suspension on the 0.2-mL strip tube magnet for 3 minutes or until the supernatant is clear. Remove and discard the supernatant without disturbing the beads.
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 and discard the supernatant without disturbing the beads.
7. Repeat step 6 once for a total of two alcohol washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
9. Air-dry the beads at room temperature for 3 minutes.



Do not overdry the AMPure® beads after the ethanol washes. Overdried beads appear cracked.

10. Remove the tube from the magnet and pipet 30 µL of elution buffer into the tube. Pipet-mix at least 10 times to completely resuspend the AMPure® XP magnetic beads.
11. Incubate the sample at room temperature for 2 minutes.
12. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
13. Pipet the entire eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified Targeted mRNA PCR2 products).



STOPPING POINT: Store at 2–8 °C before proceeding on the same day, or at –25 °C to –15 °C for up to 6 months.

14. Quantify the concentration of the Targeted mRNA PCR2 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.

Step 6: Targeted mRNA and BD® AbSeq index PCR

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



If additional unique or combinatorial indexing is needed, use the BD® OMICS-One Dual Index Kit primers.

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

Targeted mRNA and BD[®] AbSeq index PCR mix

Cap	Component	For 1 library (μL)	For 1 library with 20% overage (μL)	For 4 libraries with 20% overage (μL)	For 8 libraries with 20% overage (μL)
○	PCR master mix	25.0	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	–	–
○	Nuclease-free water	18.0	21.6	86.4	172.8
	Total	47.0	56.4	216.0	432.0

* For more than one library, use different library reverse primers for each library. For recommendations about how to index libraries, contact your local Field Application Specialist or go to scomix@bdscomix.bd.com.

† For more than four libraries, use the BD[®] OMICS-One Dual Index Kit.

For sequencing on Illumina systems, refer to the Illumina guidelines for preparing libraries with balanced index combinations.

2. Gently vortex mix and briefly centrifuge. Pipet 47 μL of Targeted mRNA or BD[®] AbSeq index PCR mix into one 0.2-mL PCR tube for each library, and place back on ice.
3. Bring the Index PCR mixes to post-amplification workspace.
4. In separate and new 0.2-mL PCR tubes:
 - Targeted mRNA PCR2 products: Pipet 3.0 μL of 0.2–2.7 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. Run the following PCR program on the thermal cycler.

Index PCR conditions for Targeted mRNA

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	6–8*	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	30 seconds
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

* See suggested PCR cycles.

Suggested PCR cycles

Concentration index PCR input for mRNA Targeted libraries (ng/μL)	Concentration index PCR input for BD® AbSeq libraries (ng/μL)	Suggested PCR cycles
1.2–2.7	0.5–1.1	6
0.6–1.2	0.25–0.5	7
0.2–0.6	0.1–0.25	8



STOPPING POINT: The PCR can run overnight.

Step 7: Targeted mRNA and BD® AbSeq index PCR cleanup and quality check

Perform PCR purification in the post-amplification workspace.

1. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until the beads are fully resuspended.
2. Briefly centrifuge the index PCR products.



The final volume must be exactly 50 μL to achieve the appropriate size selection of the purified PCR1 product. If the volume is less than 50 μL, use elution buffer to achieve the final volume.

3. To 50 μL of the index PCR products, pipet AMPure® beads as follows:
 - Targeted mRNA library: 35 μL AMPure® beads
 - BD® AbSeq library: 40 μL AMPure® beads
4. Pipet-mix the PCR products 10 times, then briefly centrifuge the samples.
5. Incubate at room temperature for 5 minutes.
6. Place the suspension on the 0.2-mL strip tube magnet for 3 minutes or until the supernatant is clear. Remove and discard the supernatant without disturbing the beads.
7. Keeping the tube on the magnet, gently add 200 μL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant without disturbing the beads.
8. Repeat step 7 for a total of two ethyl alcohol washes.
9. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
10. Air-dry the beads at room temperature for 2 minutes.



Do not overdry the AMPure® beads after the ethanol washes. Overdried beads appear cracked.

11. Remove the tube from the magnet and pipet 30 μL of elution buffer into the tube. Pipet-mix at least 10 times to fully resuspend the AMPure® XP magnetic beads.
12. Incubate at room temperature for 2 minutes.

13. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
14. Pipet the eluate (~30 μL) into a new 1.5-mL LoBind® tube (final sequencing library).

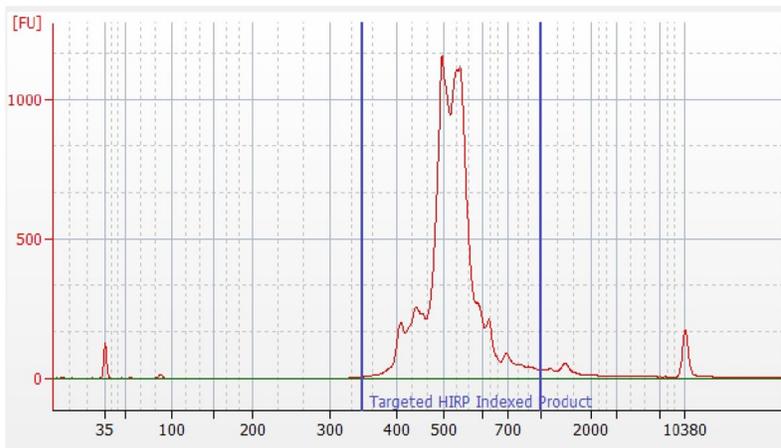


STOPPING POINT: Store at $-25\text{ }^{\circ}\text{C}$ to $-15\text{ }^{\circ}\text{C}$ for up to 6 months until sequencing.

15. Quantify the concentration of the final sequencing library with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Kit. Follow the manufacturer's instructions. The expected concentration of the libraries is $>1.5\text{ ng}/\mu\text{L}$.
16. Measure the average fragment size of the Targeted mRNA and BD® AbSeq, libraries within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit for 50–7,000 bp, 5–1,000 $\text{pg}/\mu\text{L}$ or the Agilent TapeStation. Follow the manufacturer's instructions.

Figure 3 Targeted mRNA index PCR product

A. Sample bioanalyzer high-sensitivity DNA trace - BD Rhapsody™ Immune Response Panel HS



B. Sample TapeStation high-sensitivity D5000 trace - BD Rhapsody™ Immune Response Panel HS

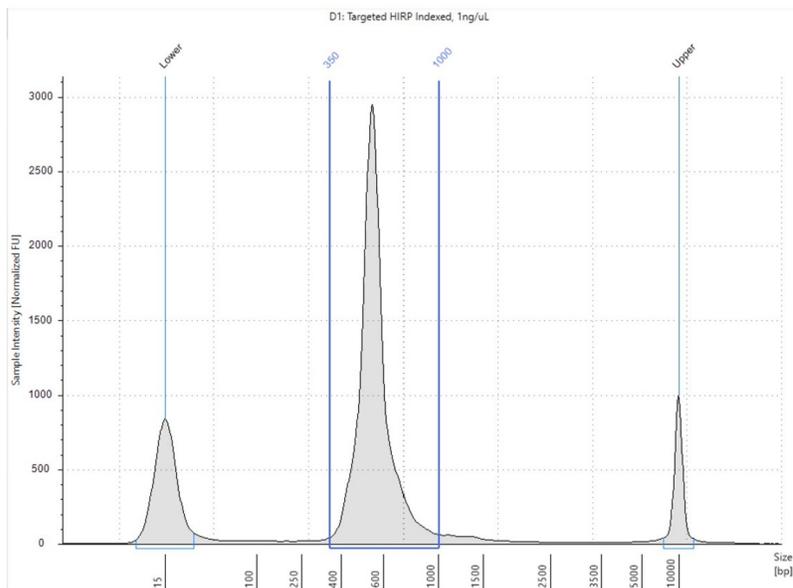
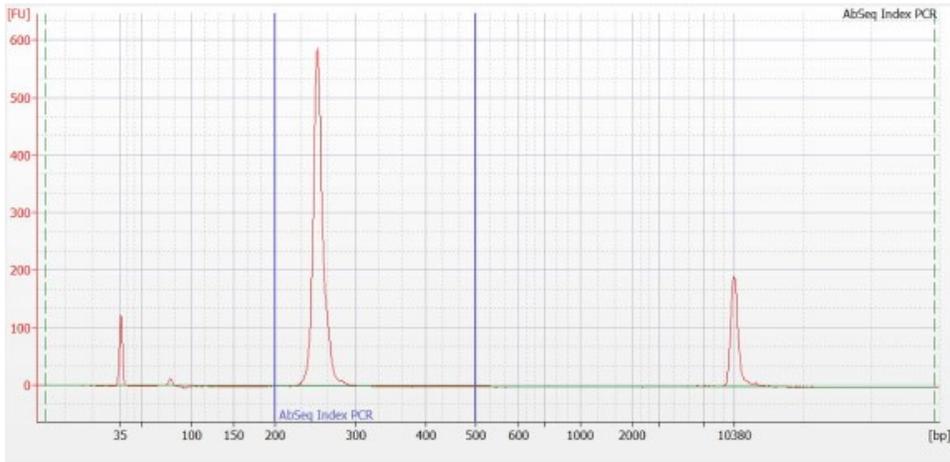
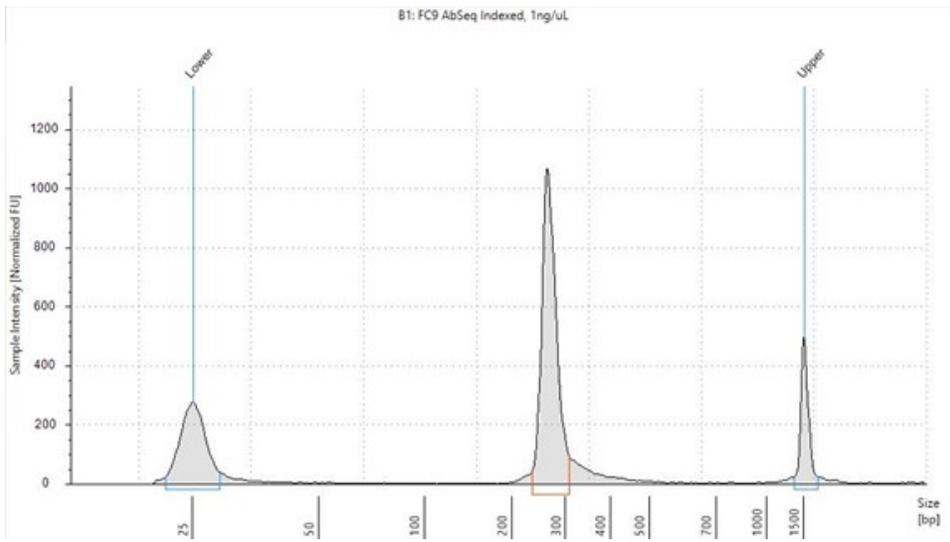


Figure 4 Final BD® AbSeq library

A. Sample bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



17. If the concentration or size of the library is outside of the expected range, see [Troubleshooting \(page 27\)](#) or contact your local Field Application Specialist or go to scomix@bdscomix.bd.com.

Sequencing

The sequencing depth for each library is dependent on application. For cell-type clustering, shallow sequencing is sufficient. However, for in-depth analysis such as comparison across multiple samples, deep sequencing is advised. We recommend meeting the requirement for recursive substitution error correction (RSEC) sequencing depth of ≥ 6 to reach the threshold of sequencing saturation where most molecules of the library have been recovered, approximately 80%. The RSEC sequencing depth and sequencing saturation are both reported by the analysis pipeline. The actual sequencing reads/cell required to achieve this depth can vary as it is dependent on the gene expression levels, number of cells, and sequencing run quality. The following reads/cell are recommended for Targeted mRNA and BD[®] AbSeq libraries.

Sequencing depth of the Targeted mRNA libraries can vary depending on whether the sample contains high or low-content RNA cells. For resting PBMCs, we recommend:

Read requirements for libraries

Gene panel	Read requirement for data analysis
BD Rhapsody™ Targeted	~2,000–20,000 reads/cell*
BD [®] AbSeq	1,000 reads/cell/AbSeq [†]

* 2,000 reads/cell can be sufficient for cell-type clustering and classification. For deeply saturated sequencing (RSEC depth >6), use 20,000 reads/cell.

[†] The amount of sequencing needed for BD[®] AbSeq libraries will vary depending on the 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 .

Required parameters

Parameter	Requirement
Platform	Illumina*
Paired-end reads	Recommend Read 1: 51 cycles; Read 2: 71 cycles; Index 1 (i5): 8 cycles; Index 2 (i7): 8 cycles
PhiX	1% recommended
Analysis	See the <i>BD[®] Single-Cell Multiomics Bioinformatics Handbook</i>

* To review Illumina Index 1 (i7) sequences, see the [Appendix \(page 30\)](#).

Sequencing recommendations

- For a NextSeq High or Mid Output run and MiniSeq High or Mid Output run, load the flow cell at a concentration between 1.5 and 1.8 pM with 1% PhiX for a sequencing run.
- For Novaseq:

Sequencing platform	Cycles	Recommended loading concentration
NovaSeq 6000 S Prime (Single Lane)	2×50, 2×100, 2×150, 2×250*	180–250 pM (XP workflow)
NovaSeq 6000 S Prime (Single Flow Cell)	2×50, 2×100, 2×150, 2×250*	350–650 pM (standard workflow)
NovaSeq 6000 S1 (Single Lane)	2×50, 2×100, 2×150*	180–250 pM (XP workflow)
NovaSeq 6000 S1 (Single Flow Cell)	2×50, 2×100, 2×150*	350–650 pM (standard workflow)
NovaSeq 6000 S2 (Single Flow Cell)	2×50, 2×100, 2×150*	350–650 pM (standard workflow)
NovaSeq 6000 S4 (Single Lane)	2×100, 2×150	180–250 pM (XP workflow)
NovaSeq 6000 S4 (Single Flow Cell)	2×100, 2×150	350–650 pM (standard workflow)
NovaSeq X 10B	2×100, 2×150	Contact local Field Application Specialist (FAS)

* NovaSeq 100 cycle kit (v1.0 or v1.5) can be used. The 100-cycle kit contains enough reagents for up to 130 cycles.

- For other sequencing platforms (e.g. Element AVITI System), follow the manufacturer's sequencing recommendations.



Avoid pooling greater than 60% total BD® AbSeq in final sequencing pool as it may impact sequencing quality of Targeted mRNA libraries.



To determine the ratio of BD Rhapsody™ Targeted mRNA library to BD® AbSeq library to pool for sequencing, use the sequencing calculator available by contacting your local Field Application Specialist (FAS) or scmix@bdscomix.bd.com.

Sequencing analysis pipeline

Contact customer support at scmix@bdscomix.bd.com for access to the latest whole transcriptome sequencing analysis pipeline.

Troubleshooting

Library preparation

Observation	Possible causes	Recommended solutions
PCR2 product yield too low.	PCR1 and PCR2 primers might have been swapped by mistake.	Ensure the correct primers are used for each step.
	cDNA synthesis might have failed due to incomplete washing of lysis buffer.	Avoid leaving behind lysis buffer or bubbles after removing lysis buffer from the tube during bead wash after retrieval from the cartridge. Use new tubes for each wash step, as described in the protocol.
	cDNA synthesis might have failed due to thermomixer not shaking during reverse transcription.	Samples need to be on the thermomixer in shake mode. Where applicable, ensure that a SmartBlock™ Thermoblock is installed on the thermomixer for 1.5-mL tubes so that the reaction can proceed at the designated temperature.
	BD Rhapsody™ Enhanced Cell Capture Beads not fully resuspended immediately before PCR1.	Gently pipet-mix BD Rhapsody™ Enhanced Cell Capture Beads in PCR1 reaction mix immediately before starting PCR1 thermal cycling to ensure uniform bead suspension.
	Thermal cycler mis-programming.	Ensure that the correct thermal cycling program is used.
	Too few PCR1 cycles.	Optimize the number of PCR cycles for the specific sample type.
	Incorrect volume of AMPure® XP magnetic beads used during PCR2 cleanup.	Use the specified volume of AMPure® XP beads.
	Incorrect solution or incorrect concentration of 80% ethyl alcohol used for washing AMPure® XP magnetic beads, resulting in premature elution of PCR products from beads.	Use 80% ethyl alcohol for washing AMPure® XP beads.

Observation	Possible causes	Recommended solutions
Concentration of final mRNA sequencing library too low.	Issue with PCR2 product yield or quality.	<ol style="list-style-type: none"> 1. Determine the product size range: <ol style="list-style-type: none"> a. Load 1 µL of purified PCR2 product at 1 ng/µL in a High Sensitivity DNA Chip on the Agilent Bioanalyzer. b. Follow the manufacturer's instructions. 2. Confirm that the mRNA tTargeted PCR2 products should show an average size range of 350–600 bp. <ul style="list-style-type: none"> • If the products pass quality control, see Step 6: Targeted mRNA and BD® AbSeq index PCR (page 20) to prepare final libraries. Repeat the index PCR. • If the products do not pass quality control, contact your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com.
	Thermal cycler mis-programming.	Ensure that the correct thermal cycling program is used.
Final sequencing product size too large	Over-amplification during index PCR or input amount of PCR2 products too high.	Repeat the index PCR with a lower input of PCR2 products.
	Upper and lower markers on the Agilent Bioanalyzer or TapeStation are incorrectly called.	Ensure that markers are correct. Follow manufacturer's instructions.
	Incorrect volume of Agencourt® AMPure® XP magnetic beads used.	Use volume specified in protocol.
BD® AbSeq PCR1 product size too low.	BD® AbSeq primer not added to PCR1 or too few PCR1 cycles. Incorrect volumes of AMPure® XP beads used during double-sided selection.	Contact your local Field Application Specialist (FAS) or 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.
	Suboptimal cluster density, or library denaturation, or both.	See troubleshooting in Illumina documentation.

Appendix

Oligonucleotides in BD Rhapsody™ Targeted mRNA Amplification Kit

The following table lists the sequences of all oligonucleotides included in the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (Cat No. 633774).

Oligonucleotide	Use	Part/ catalog no.	Sequence (5'–3')
BD Rhapsody™ Universal Oligo	Forward primer for WTA RPE PCR, Sample Tag PCR1 and PCR2, and BD® AbSeq PCR1	650000074	ACACGACGCTCTCCGATCT
BD® AbSeq Primer	Reverse primer for BD® AbSeq PCR1	91-1086	CAGACGTGTGCTCTCCGATCT
BD Rhapsody™ Library Forward Primer	Forward primer for WTA, Sample Tag, and BD® AbSeq Index PCR	91-1085	AATGATACGGCGACCACCGAGATCTACACTATAGCCT ACACTCTTCCCTACACGACGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 1	WTA, Sample Tag, and BD® AbSeq Index PCR	650000080	CAAGCAGAAGACGGCATAACGAGATAGCGTAGCGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 2		650000091	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 3		650000092	CAAGCAGAAGACGGCATAACGAGATTGCCTCTTGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 4		650000093	CAAGCAGAAGACGGCATAACGAGATTCTCTACGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T

Forward index name	i5 bases for sample sheet	
	NovaSeq, MiSeq, HiSeq 2000/2500	iSeq, MiniSeq, NexSeq, HiSeq 3000/4000
BD Rhapsody™ Library Forward Primer	TATAGCCT	AGGCTATA

Reverse index name	i7 bases for sample sheet
BD Rhapsody™ Library Reverse Primer 1 (N709)	GCTACGCT
BD Rhapsody™ Library Reverse Primer 2 (N710)	CGAGGCTG
BD Rhapsody™ Library Reverse Primer 3 (N711)	AAGAGGCA
BD Rhapsody™ Library Reverse Primer 4 (N712)	GTAGAGGA

BD® OMICS-One Dual Index Kit sequences

Forward index name	i5 bases for sample sheet	
	NovaSeq, MiSeq, HiSeq 2000/2500	iSeq, MiniSeq, NexSeq, HiSeq 3000/4000
Dual index forward primer 1	TATAGCCT	AGGCTATA
Dual index forward primer 2	ATAGAGGC	GCCTCTAT
Dual index forward primer 3	CCTATCCT	AGGATAGG
Dual index forward primer 4	GGCTCTGA	TCAGAGCC
Dual index forward primer 5	AGGCGAAG	CTTCGCCT
Dual index forward primer 6	TAATCTTA	TAAGATTA
Dual Index Forward Primer 7	CAGGACGT	ACGTCCTG
Dual Index Forward Primer 8	GTACTGAC	GTCAGTAC

Reverse index name	i7 bases for sample sheet
Dual index reverse primer 1	ATTACTCG
Dual index reverse primer 2	TCCGGAGA
Dual index reverse primer 3	CGCTCATT
Dual index reverse primer 4	GAGATTCC
Dual index reverse primer 5	ATTCAGAA
Dual index reverse primer 6	GAATTCGT
Dual index reverse primer 7	CTGAAGCT
Dual index reverse primer 8	TAATGCGC

BD® AbSeq Ab-Oligo common motifs

The antibody in each BD® AbSeq Ab-Oligo is conjugated to an oligonucleotide that contains an antibody clone-specific barcode (ABC) flanked by a poly-A tail on the 3' end and a common PCR handle (PCR primer binding site) followed by a unique molecular identifier on the 5' end, with the AbSeq Ab-Oligo sequence (5'–3') between them:

- CAGACGTGTGCTCTTCCGATCTVNNVNNNNVNNV(**Barcode sequence [ABC]**)AAAAAAAAAAAAAAAAAAAAAAAAA

The poly-A tail of the oligonucleotide allows the ABC to be captured by the BD Rhapsody™ System or other oligo-dT-based capture systems. The 5' PCR handle allows for efficient sequencing library generation for various sequencing platforms.

Viewing antibody barcode sequences

To view the ABC (barcode sequence) for an antibody:

1. Go to the [BD Biosciences](#) website for BD® AbSeq assays.
2. Click **View Products**. The page displays products pertaining to BD® AbSeq assays.
3. Click a specific product to view product details, and scroll down to the **Barcode Sequence** field.

Contact Information

Becton, Dickinson and Company

BD Biosciences

155 North McCarthy Boulevard

Milpitas, California 95035 USA

bdbiosciences.com

scomix@bdscomix.bd.com