BD Rhapsody™ System

mRNA Whole Transcriptome Analysis (WTA) and BD® AbSeq

Library Preparation Protocol

23-24118(03)
2024-11

Regulatory information

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

History

Revision	Date	Change made
23-24118(01)	2021-12	Initial release.
23-24118(02)	2022-11	Updated for BD Rhapsody™ Enhanced Cell Capture Beads version 2.0.
23-24118(03)	2024-11	Added the BD® OMICS-One Dual Index Kit and BD Rhapsody™ Enhanced Cartridge Reagent Kit v3. Added Workflows chapter. Updated Time Considerations workflow. Updated sequencing section. Added sequencing recommendation. Added BD Rhapsody™ oligo sequences to appendix.

Contents

Introduction

This protocol provides instructions on creating single-cell whole transcriptome 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, 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*.

The cDNA of mRNA and BD® AbSeq targets is first encoded on BD Rhapsody™ Enhanced Cell Capture Beads, as described in the instrument user guides. At the same time, the barcode information from BD Rhapsody™ Enhanced Cell Capture Beads is also added to Ab-Oligos during reverse transcription, which enables amplification of the Ab-Oligos in solution. To generate the BD® AbSeq sequencing libraries, the extended Ab-Oligos are first denatured from the BD Rhapsody™ Enhanced Cell Capture Beads, which are later amplified separately through a series of PCR steps. Meanwhile, the whole transcriptome amplification library is generated directly from the BD Rhapsody™ Enhanced Cell Capture Beads using a random priming approach, followed by an index polymerase chain reaction (PCR) step. The whole transcriptome mRNA and BD® AbSeq libraries can be combined together for sequencing on various sequencers.

This protocol is intended to provide a method to screen RNA expression of single cells using a 3' whole transcriptome analysis (WTA) approach through the BD Rhapsody™ WTA Amplification Kit for samples that have been labeled using BD® AbSeq AbOligo reagents. The data set generated from this protocol can be used to generate a custom panel for subsequent 3' Targeted mRNA sequencing. Specifically, the protocol outlines how to generate whole transcriptome libraries for cell-capture inputs between 100 to 100,000 resting peripheral blood mononuclear cells (PBMCs) per sample for library generation. For cell-capture inputs between 100 to <5,000 cells per sample, there are sections in the protocol for additional cleanups. For cell types other than resting PBMCs, protocol optimization might be required by the user.

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.

BD Rhapsody™ WTA Amplification Kit (–20 ˚C)

Cap Color	Name	Quantity
	WTA extension primers	1
	WTA extension buffer	1
	WTA extension enzyme	1
	10 mM dNTP	1
	Nuclease-free water	3
	Bead RT/PCR enhancer	1
	WTA amplification primer	1
	PCR master mix	1
	Universal oligo	2
	Sample Tag PCR1 primer	2
	Sample Tag PCR2 primer	1
	BD® AbSeq PCR1 primer	1
	Library reverse primer 1–4	1 each
	Library forward primer	1
	Bead resuspension buffer	1
	Elution buffer	1

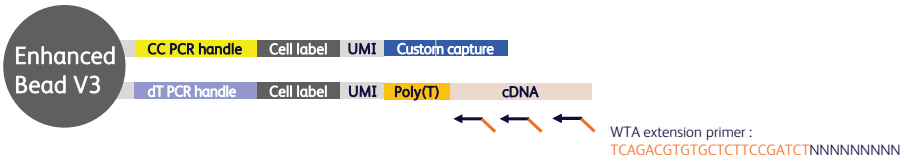
BD® OMICS-One Dual Index Kit (–20 ˚C)

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

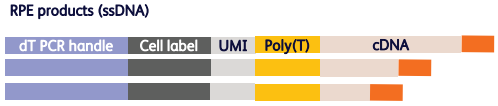
Workflows

WTA library amplification workflow

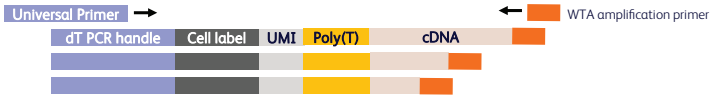
1.1 Random priming and extension (RPE) (page 13): Random priming on the bead.



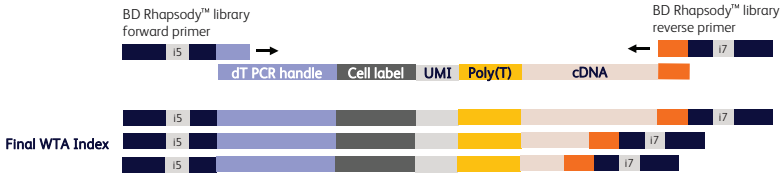
Denature off the RPE product.



1.4 RPE PCR (page 18): Amplify the RPE product.



1.6 WTA index PCR (page 22): Add adapters and indices.



BD® AbSeq library amplification workflow

2.1 BD® AbSeq PCR1 (page 28): BD® AbSeq product is amplified in PCR1.

2.4 BD® AbSeq index PCR (page 32): 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™ WTA Amplification Kit	BD Biosciences	633801
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	–

‡ 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* Or, MicroAmp Optical 96–Reaction Plate*	Corning Thermo Fisher Scientific	PCR96HSC 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 Or, Agilent High Sensitivity D1000 ScreenTape Agilent High Sensitivity D1000 Reagents Or, Agilent High Sensitivity D5000 ScreenTape Agilent High Sensitivity D5000 Reagents	Agilent Agilent Agilent Agilent Agilent	5067-4626 5067-5584 5067-5585 5067-5592 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	–
Eppendorf ThermoMixer® C*	Eppendorf	5382000023
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	–

* Two thermomixers are recommended. A heat block can be used for denaturation steps.

† 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 WTA 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.

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™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos for Intracellular CITE-seq Protocol* (doc ID 23-24664)
- *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 and storage
	WTA Library Amplification		
Pre-Amplification Workspace	Denature Ab-Oligos	10 minutes	4 °C up to 24 hours
Pre-Amplification Workspace	1.1 Random priming and extension (RPE) (page 13)	170 minutes	–
	1.2 RPE cleanup (page 16)		
Post-Amplification Workspace	1.4 RPE PCR (page 18)*	80 minutes	PCR overnight
	1.5 RPE PCR cleanup and quality check (page 19)	STOP	<6 weeks at 4 °C or <6 months at –20 °C
	1.6 WTA index PCR (page 22)	65 minutes	PCR overnight
	1.7 WTA index PCR cleanup and quality check (page 23)	STOP	<6 months at –20 °C
	(Optional) 1.8 Additional WTA index PCR cleanup (page 27)	25 minutes STOP	<6 months at –20 °C
* While the thermomixer program is running, proceed to the BD® AbSeq PCR1 task in the BD® AbSeq Library Amplification section.			
	BD® AbSeq Library Amplification		
Post-Amplification Workspace	2.1 BD® AbSeq PCR1 (page 28)	65 minutes	PCR overnight
	2.2 BD® AbSeq PCR1 cleanup (page 30), 2.3 BD® AbSeq PCR1 quality check (page 31)	STOP	<6 weeks at 4 °C or <6 months at –20 °C
	2.4 BD® AbSeq index PCR (page 32)	65 minutes	PCR overnight
	2.5 BD® AbSeq index PCR cleanup and quality check (page 33)	STOP	<6 months at –20 °C
b. After PCR program has started, continue with RPE in the WTA Library Amplification section.			

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) or the *BD Rhapsody™ System Single-Cell Labeling with BD® AbSeq Ab-Oligos for Intracellular CITE-seq Protocol* (doc ID 23-24664).

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)

This protocol is intended for the whole transcriptome amplification library generation of cell inputs between 100 to 100,000 single cells, specifically resting PBMCs. For cell inputs between 100 and 5,000 single cells, follow the extra steps outlined in the additional cleanup sections.

Ensure that the intended total cell load is between 100 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.

1. WTA library amplification

This procedure comprises the following tasks:

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents (except the enzymes) in the BD Rhapsody™ WTA Amplification Kit at room temperature (15–25 °C), then immediately place on ice.

1.1 Random priming and extension (RPE)

This section describes how to generate random priming products. First, random primers are hybridized to the cDNA on the BD Rhapsody™ Enhanced Cell Capture Beads, followed by extension with an enzyme.

Perform this procedure in the pre-amplification workspace. We recommend using a separate heat block for the 95 °C incubations.

1. Set a heat block to 95 °C and set two thermomixers to 37 °C and 25 °C, respectively.
2. In a new 1.5-mL LoBind® tube, pipet the following reagents.

Random primer 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)
●	WTA extension buffer	20.0	24.0	96.0	192.0
●	WTA extension primers	20.0	24.0	96.0	192.0
○	Nuclease-free water	134.0	160.8	643.2	1286.4
	Total	**174.0**	**208.8**	**835.2**	**1670.4**

3. Pipet-mix the random primer mix and keep at room temperature.
4. Briefly centrifuge the tube of Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads, and then complete one of the following actions.
   - If you are using a subsample of the beads, proceed to the next step.
   - If you are using the entire sample of beads, skip to step 6.
5. (Optional) To subsample the Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads:
   a. Based on the expected number of viable cells captured on the beads in the final bead resuspension volume, determine the volume of beads to subsample for sequencing.
   b. Completely resuspend the beads by pipet-mixing, then pipet the calculated volume of the 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

6. Place the tube of Exonuclease I-treated beads in bead resuspension buffer on the 1.5-mL magnet for <2 minutes or until the supernatant is clear. Remove and discard the supernatant.
7. Remove the tube from the magnet and resuspend the beads in 75 µL of elution buffer. Pipet-mix 10 times to resuspend the beads. Label a new 1.5-mL tube as AbSeq products.

   If processing more than one library, we recommended performing the BD® AbSeq denaturation one library at a time.

8. Place the tube with beads in a 95 °C heat block for 5 minutes (no shaking).

9. Briefly centrifuge the tube, then place the tube on 1.5-mL magnet for <2 minutes or until the supernatant is clear.
   a. Immediately remove the supernatant and transfer to the BD® AbSeq products tube from step 7.
   b. To minimize BD® AbSeq contamination in the WTA library, ensure that all supernatant is removed from the tube.
   c. Keep the supernatant tube on ice or at at 4 °C for up to 24 hours until ready to proceed to 2.1 BD® AbSeq PCR1 (page 28).
10. Remove the tube of BD Rhapsody™ Enhanced Cell Capture Beads from the magnet, and use a low-retention tip to pipet 200 µL of elution buffer into the tube. Pipet-mix 10 times to resuspend the beads.

    If you are processing more than one library, beads in elution buffer can be stored on ice until all tubes had been denatured.

11. Briefly centrifuge the tube, then place the tube on a 1.5-mL magnet for <2 minutes or until the supernatant is clear. Remove and dispose of the supernatant.
12. Remove the tube with the BD Rhapsody™ Enhanced Cell Capture Beads from the magnet, and use a low-retention tip to pipet 87 µL of random primer mix into the tube. Pipet-mix 10 times to resuspend the beads.

    Save the remaining volume of random primer mix for a second RPE. Keep random primer mix at room temperature.

13. Incubate the tube in the following order:
    a. 95 °C in a heat block (no shaking) for 5 minutes.
    b. Thermomixer at 1,200 rpm and at 37 °C for 5 minutes.
    c. Thermomixer at 1,200 rpm and at 25 °C for 5 minutes.

    Confirm “Time Mode” is set to “Time Control” before the program begins.

14. Briefly centrifuge the tube and keep it at room temperature.
15. Program the thermomixer.
    a. 1,200 rpm and at 25 °C for 10 minutes.
    b. 1,200 rpm and at 37 °C for 15 minutes.
    c. 1,200 rpm and at 45 °C for 10 minutes.
    d. 1,200 rpm and at 55 °C for 10 minutes.

    Confirm “Time Mode” is set to “Time Control” before the program begins.

16. In a new 1.5-mL LoBind® tube, pipet the following reagents.

Extension enzyme 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)
	dNTP	8.0	9.6	38.4	76.8
	Bead RT/PCR enhancer	12.0	14.4	57.6	115.2
	WTA extension enzyme	6.0	7.2	28.8	57.6
	Total	**26.0**	**31.2**	**124.8**	**249.6**

17. Pipet 13 µL of the extension enzyme mix into the sample tube containing the beads (for a total volume of 100 µL) and keep at room temperature until ready.

    Save the remaining volume of primer extension enzyme mix for a second RPE. Keep primer extension enzyme mix on ice.

18. Place the tube of extension enzyme mix with BD Rhapsody™ Enhanced Cell Capture Beads in the programmed thermomixer (see step 15).

    While the thermomixer program is running, begin 2.1 BD® AbSeq PCR1 (page 28).

19. Remove the tube after the program is finished. Place the tube in a 1.5-mL tube magnet for <2 minutes or until the supernatant is clear. Remove and discard the supernatant.

20. Remove the tube from the magnet and resuspend the beads in 205 µL of elution buffer using a P200 pipette.

    If processing more than one library, we recommended performing the denaturation one library at a time.

21. To denature the random priming products off the beads, pipet to resuspend the beads.

    a. Incubate the sample at 95 °C in a heat block for 5 minutes (no shaking).

    b. Immediately after completing the 95 °C incubation, slightly open the lid of the tube to release air pressure within the tube.

       Do not incubate for more than 5 minutes.

    c. Place the tube in a thermomixer at any temperature for 10 seconds at 1,200 rpm to resuspend the beads.

22. Place the tube in a 1.5-mL tube magnet for <2 minutes or until the supernatant is clear.
    a. Immediately transfer 200 µL of the supernatant containing the RPE product to a new 1.5-mL LoBind® tube.
    b. Store supernatant containing the RPE product on ice.

    If you are processing more than one library, supernatant containing RPE product can be stored on ice until all tubes had been denatured.

23. Repeat steps 12 through 21 to perform a second RPE.
24. Place the tube in a 1.5-mL tube magnet for <2 minutes or until the supernatant is clear.
    a. Immediately transfer 200 µL of the supernatant containing the RPE Product to the 1.5-mL LoBind® tube containing the supernatant from the first round of RPE (step 22) for a total of 400 µL.
    b. Store supernatant containing RPE product on ice. Discard the beads.
25. Immediately proceed to 1.2 RPE cleanup (page 16).

1.2 RPE cleanup

This section describes how to perform a single-sided AMPure® cleanup, which removes primer dimers and other small molecular weight byproducts. The final product is purified single-stranded DNA.

Perform the purification in the pre-amplification workspace. Bring Agencourt® AMPure® XP magnetic beads to room temperature.

1. In a new 15-mL conical tube, prepare 10 mL of fresh 80% (v/v) ethyl alcohol by pipetting 8 mL of absolute ethyl alcohol to 2 mL of nuclease-free water. Vortex the tube for 10 seconds.

   Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
3. If RPE product volume is <400 µL, bring volume to 400 µL with elution buffer. Pipet 720 µL of AMPure® beads into the tube containing the 400 µL of RPE product supernatant. Pipet-mix at least 10 times, then briefly centrifuge.
4. Incubate the suspension at room temperature for 10 minutes.
5. Place the tube on the 1.5-mL tube magnet for 5 minutes or until the supernatant is clear. Remove and discard the supernatant.
6. Keeping the tube on the magnet, gently pipet 1 mL of fresh 80% ethyl alcohol into the tube.
7. Incubate the sample on the magnet for 30 seconds. Remove and discard the supernatant.
8. Repeat steps 6 and 7 for a total of two ethyl alcohol washes.

9. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.
   a. For best results, briefly centrifuge the AMPure® beads while still wet and place the tube back on the magnet.
   b. Remove and discard any excess ethanol that might collect at the bottom.
10. Air dry the beads at room temperature until no longer glossy (~15–20 minutes).

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

11. Remove the tube from the magnet and resuspend the bead pellet in 40 µL of elution buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
12. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
13. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
14. Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product.
15. (Optional) For samples with low cell input (for example, starting with fewer than 5,000 PBMCs cell capture), proceed to 1.3 Additional RPE cleanup for cell input <5,000 PBMC cells (page 17) for an additional round of AMPure® XP magnetic bead purification.
16. Keep on ice until ready to proceed with 1.4 RPE PCR (page 18).

    Additional RPE cleanup is only necessary when starting with <5,000 cells captured in the 8-lane cartridge. It is not necessary when processing <5,000 cells from subsampled beads.

1.3 Additional RPE cleanup for cell input <5,000 PBMC cells

1. To the tube from 1.2 RPE cleanup (page 16), bring the purified RPE product volume up to 100 µL with nuclease-free water and transfer to a 1.5-mL LoBind® tube.

   The final volume must be exactly 100 µL to achieve the appropriate size selection of the purified RPE product.

2. Pipet-mix 10 times, then briefly centrifuge.
3. Pipet 180 µL of AMPure® beads into the tube containing 100 µL of eluted RPE product from the first round of purification.
4. Pipet-mix 10 times, then briefly centrifuge.
5. Incubate the suspension at room temperature for 5 minutes.
6. Place the suspension on the 1.5-mL tube magnet for 5 minutes or until the supernatant is clear. Remove and discard the supernatant.
7. Keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol into the tube.
8. Incubate the sample on the magnet for 30 seconds. Remove and discard the supernatant.
9. Repeat steps 7 and 8 for a total of two ethyl alcohol washes.

10. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
11. Air dry the beads at room temperature until no longer glossy.

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

12. Remove the tube from the magnet and resuspend the bead pellet in 40 µL of elution buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
13. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product after two rounds of purification.

    STOPPING POINT: Store the RPE product in a LoBind® tube on ice or at 4 °C for up to 24 hours until 1.4 RPE PCR (page 18).

1.4 RPE PCR

This section describes how to generate more RPE product through PCR amplification, resulting in multiple copies of each random-primed molecule.

1. In the pre-amplification workspace, in a new 1.5-mL LoBind® tube, pipet the following components.

RPE 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	60	72	288	576
◯	Universal oligo	10	12	48	96
◯	WTA amplification primer	10	12	48	96
	Total	**80**	**96**	**384**	**768**

2. Pipet-mix the RPE PCR mix and keep on ice.
3. Add 80 µL of the RPE PCR mix to the tube with the 40 µL of purified RPE product. Pipet-mix 10 times to create the RPE PCR reaction mix.
4. Split the mix into two 0.2 mL PCR tubes with 60 µL of RPE PCR reaction mix per tube.

5. Bring the RPE PCR reaction mix to the post-amplification workspace and run the following PCR program.

RPE PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the Recommended number of PCR cycles given in the following table*	95 °C	30 seconds
Annealing		60 °C	1 min
Extension		72 °C	1 min
Final extension	1	72 °C	2 min
Hold	1	4 °C	∞

* Recommended PCR cycles might require optimization for different cell types and cell number.

Recommended number of PCR cycles

Number of cells in RPE	Recommended PCR cycles for resting PBMCs
100	16
1,000–9,999	13
10,000	12
20,000	11
40,000	10
80,000–100,000	9

6. When the RPE PCR reaction is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

STOP **STOPPING POINT:** The PCR can run overnight.

1.5 RPE PCR cleanup and quality check

This section describes how to perform a single-sided AMPure® cleanup to remove unwanted small molecular weight products from the RPE products. The final product is purified double-stranded DNA (~200–2,000 bp).

Perform the purification in the post-amplification workspace.

1. Bring 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 pipetting 4 mL of absolute ethyl alcohol to 1 mL of nuclease-free water. Vortex the tube for 10 seconds.

   Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

3. Vortex the AMPure® XP magentic beads at high speed for 1 minute until the beads are fully resuspended.

4. Briefly centrifuge the tube with the RPE PCR product, then combine the two 60-µL RPE PCR reactions into a new 1.5-mL tube.

   The final volume must be exactly 120 µL to achieve the appropriate size selection of the purified RPE PCR product. If the volume is less than 120 µL, use elution buffer to achieve the final volume.

5. Pipet 96 µL of AMPure® XP magnetic beads into the tube containing 120 µL of RPE PCR product. Pipet-mix at least 10 times, then briefly centrifuge the samples.

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

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. Remove and discard the supernatant.
8. 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.
9. Repeat step 8 once for a total of two ethyl alcohol washes.
10. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.

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

12. Remove the tube from the magnet and pipet 40 µL of elution buffer into the tube to resuspend the bead pellet. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
13. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 µL) into a new 1.5-mL LoBind® tube. The RPE PCR product is ready for 1.6 WTA index PCR (page 22).

    STOP **STOPPING POINT:** The RPE PCR libraries can be stored at –20 °C for up to 6 months or 4 °C for up to 6 weeks.

16. Perform quality control of the RPE PCR products with the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit or the Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay.

    a. The Bioanalyzer or TapeStation trace should show a broad peak from ~200 to 2,000 bp. Use the concentration from 200 to 600 bp to calculate how much template to add into Index PCR. Refer to the blue-boxed regions in the sample trace images in the following figures.

       The Bioanalyzer or TapeStation is used to calculate molarity for the WTA library because of the distribution in fragment sizes for this library type.

       Although there are products <200 bp (BD® AbSeq contamination product) and >600 bp, these products should be removed in the double-sided cleanup after the index PCR.

Figure 1 Sample bioanalyzer high-sensitivity DNA trace - RPE PCR product trace

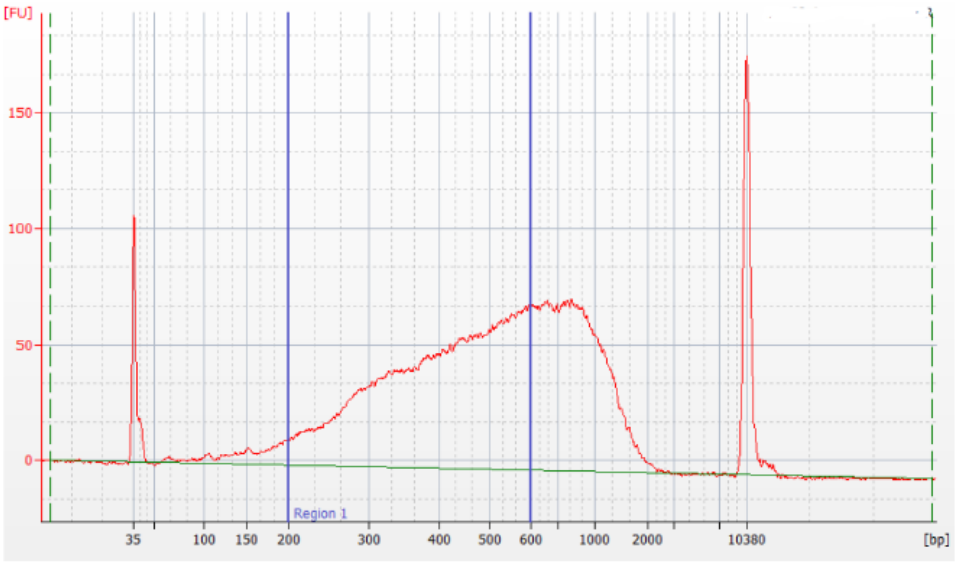
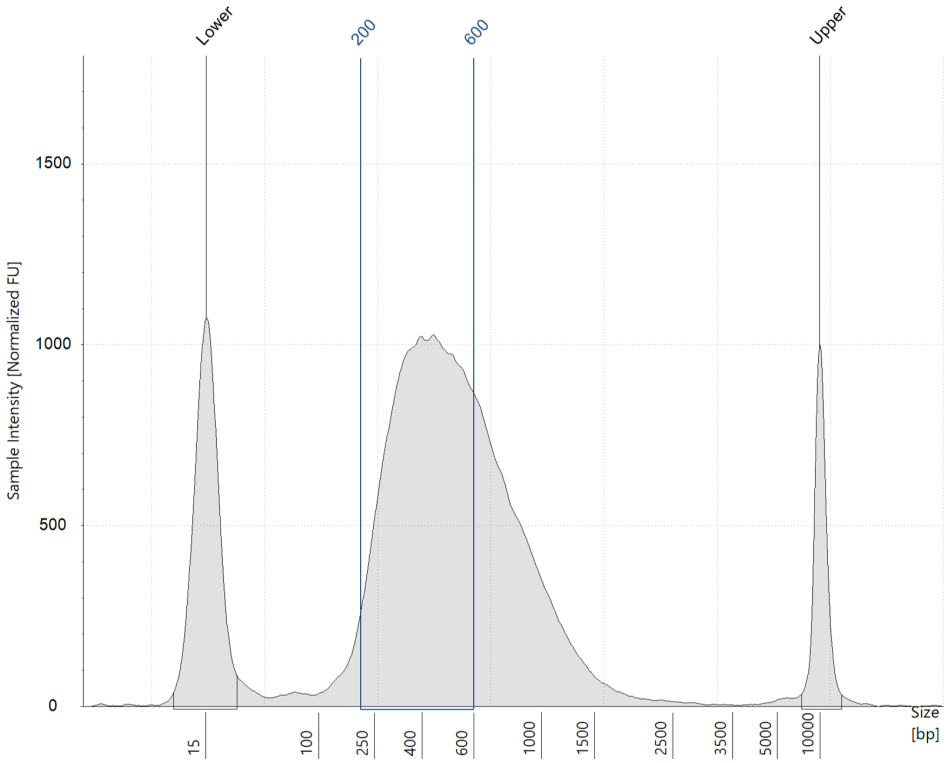


Figure 2 Sample TapeStation high-sensitivity D5000 trace



1.6 WTA index PCR

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

Perform this procedure in the post-amplification workspace.

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

1. Dilute the RPE PCR products from 1.5 RPE PCR cleanup and quality check (page 19) with nuclease-free water until the concentration of the 200–600 bp peak is 2 nM. If the product concentration is <2 nM, do not dilute. Continue to the next step.

   Example: If the Bioanalyzer measurement of the 200–600 bp peak is 6 nM, then dilute the sample threefold with nuclease-free water to 2 nM.

2. In a new 1.5-mL tube, pipet the following components to create the WTA index PCR mix.

   WTA 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	30	120	240
●	Library forward primer	5	6	24	48
●	Library reverse primer 1–4*	5	6	–	–
○	Nuclease-free water	5	6	24	48
	Total	**40**	**48**	**168**	**336**

   * For more than one library, use different library reverse primers for each library.

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

3. Pipet-mix the WTA index PCR mix and keep on ice.
4. In a new 0.2-mL PCR tube, combine WTA index PCR mix with diluted RPE PCR products as follows:
   - For one library: Combine 40 µL of WTA index PCR mix with 10 µL of 2 nM of RPE PCR product.
   - For multiple libraries: In separate tubes for each library, combine 35 µL of WTA Index PCR mix with 5 µL of the corresponding library reverse primer and 10 µL of 2 nM of RPE PCR products.
5. Pipet-mix 10 times.

6. Run the following PCR program.

Index PCR conditions for WTA

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the Recommended number of PCR cycles shown in the following table*	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	∞

* Cycle number varies based on the concentration of the RPE PCR product.

Recommended number of PCR cycles

Concentration of diluted RPE PCR products	Recommended number of PCR cycles
1 to <2 nM	9
2 nM	8

If the concentrations of diluted RPE PCR products are <1 nM, additional PCR cycles might be needed.

STOPPING POINT: The PCR can run overnight.

7. When the WTA index PCR is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

1.7 WTA index PCR cleanup and quality check

This section describes how to perform a double-sided AMPure® 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 magnetic beads to room temperature.
2. In a new 15-mL conical tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by pipetting 4 mL of absolute ethyl alcohol to 1 mL of nuclease-free water. Vortex the tube for 10 seconds.

Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

3. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
4. Add 60 µL of nuclease-free water to the WTA Index PCR product for a final volume of 110 µL.
5. Transfer 100 µL of WTA Index PCR product into a new 0.2-mL PCR tube.

The volume must be exactly 100 µL.

6. Add 60 µL of AMPure® XP magnetic beads to the 0.2-mL PCR tube from the previous step. Pipet-mix at least 10 times, then briefly centrifuge.
7. Incubate the suspension at room temperature for 5 minutes.
8. Place the suspension on the 0.2-mL strip tube magnet for 3 minutes or until the supernatant is clear.
9. While the strip tube is still on the magnet, carefully remove and transfer the 160 µL of supernatant into a new 0.2 mL strip tube without disturbing the beads.
10. Pipet 15 µL of AMPure® beads into the 0.2 mL strip tube with supernatant (from step 9). Pipet-mix at least 10 times, then briefly centrifuge.

Discard the tubes with the pelleted AMPure® beads that contain long fragments.

11. Incubate the suspension at room temperature for 5 minutes.
12. Place the suspension on a 0.2-mL tube magnet for 3 minutes or until the supernatant is clear. Remove and discard the supernatant.
13. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
14. Repeat step 13 for a total of two ethyl alcohol washes.
15. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
16. Air-dry the beads at room temperature for 30 seconds.

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

17. 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.
18. Incubate the sample at room temperature for 2 minutes.
19. Briefly centrifuge the tubes to collect the contents at the bottom.
20. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
21. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube. The WTA Index PCR eluate is the final sequencing libraries.

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

22. Quantify and perform quality control of the Index PCR libraries with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay and one of the following systems:

    - The Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit
    - The Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay

    The expected concentration from the Qubit™ Fluorometer is >1 ng/µL. The Bioanalyzer trace should show a peak from ~250 to 1,000 bp. Refer to the sample trace images in the following figures.

Figure 3 Sample bioanalyzer high-sensitivity DNA trace–WTA index PCR product

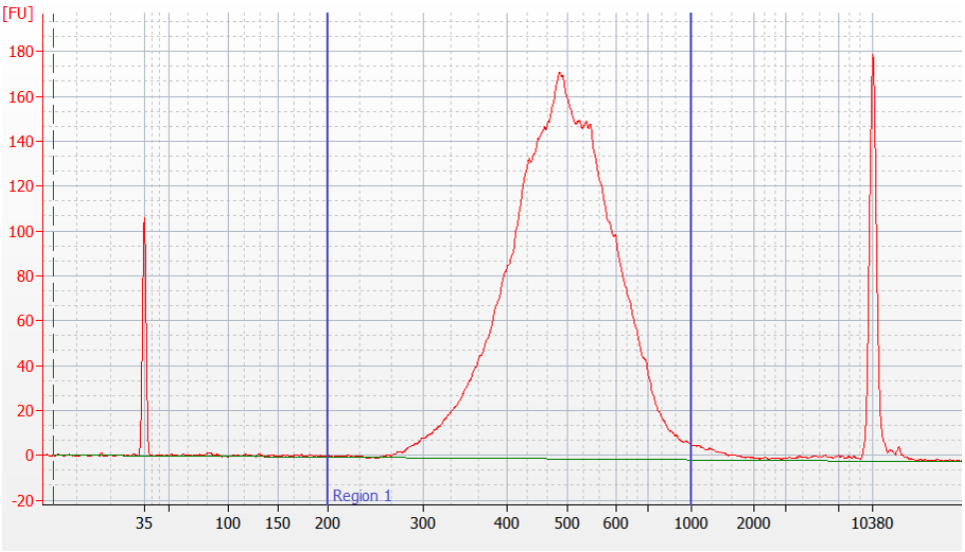


Figure 4 Sample TapeStation high-sensitivity D5000 trace–WTA index PCR product

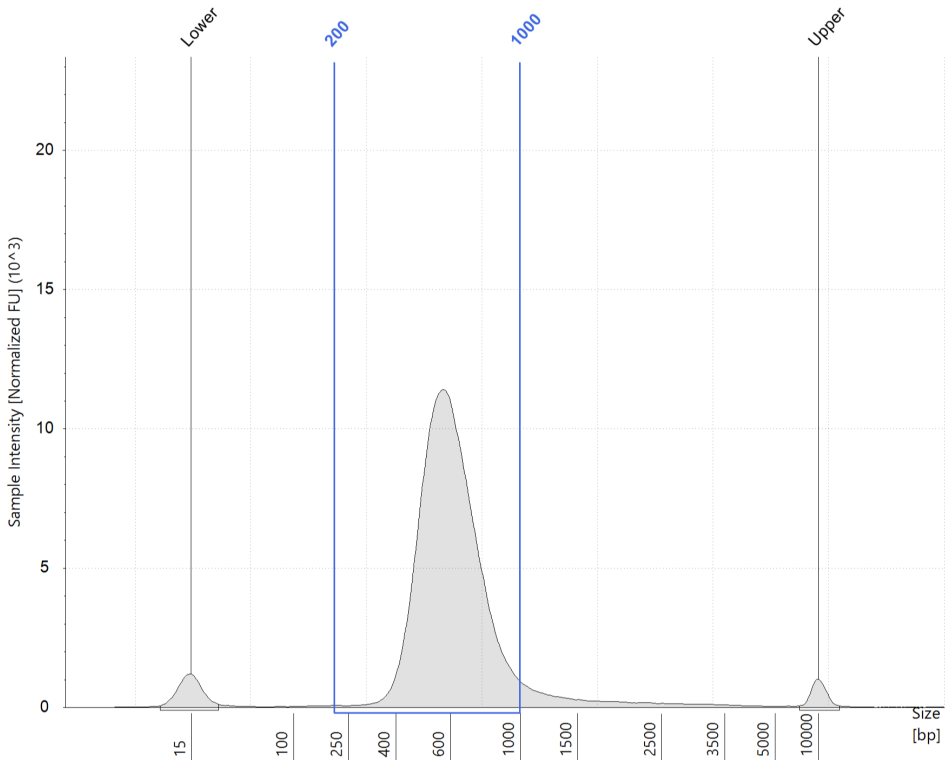
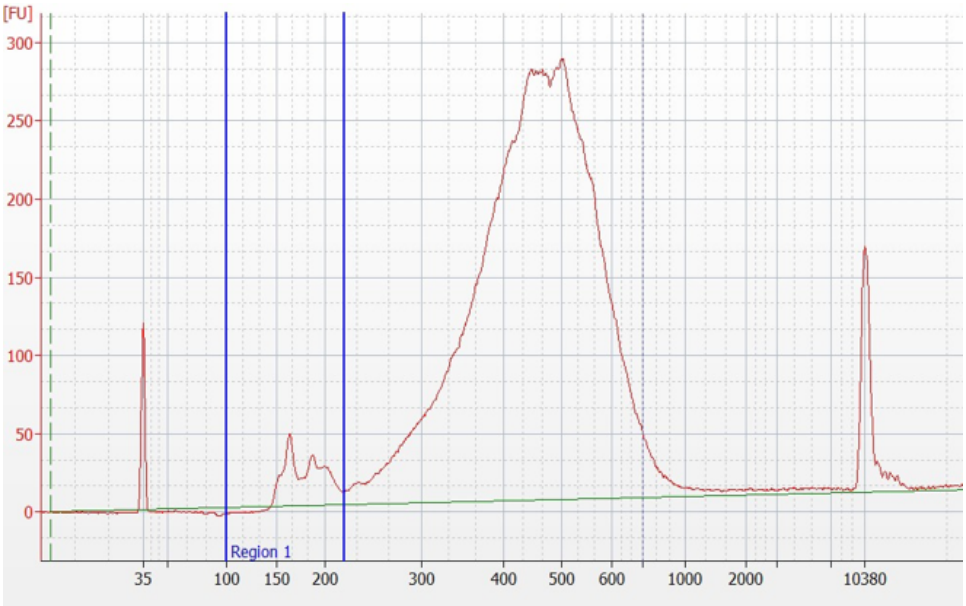


Figure 5 Sample bioanalyzer high-sensitivity DNA trace for a WTA index PCR product with an observable <270 bp peak



If smaller products (~165 bp or ~270 bp) are observed (such as the peak shown in the preceding figure), we recommend a second round of AMPure® XP magnetic bead purification. See 1.8 Additional WTA index PCR cleanup (page 27) for more information.

1.8 Additional WTA index PCR cleanup

If peaks <270 bp are observed, (as shown in the example figure traces in 1.7 WTA index PCR cleanup and quality check (page 25), perform a second round of AMPure® XP magnetic bead purification.

1. To the tube from step 21 in 1.7 WTA index PCR cleanup and quality check (page 24), bring the total purified WTA index PCR eluate volume up to 100 µL with nuclease-free water. Pipet-mix 10 times, then briefly centrifuge.

   The final volume must be exactly 100 µL to achieve the appropriate size selection of the WTA Index PCR product.

2. Pipet 75 µL of AMPure® beads into the tube containing 100 µL of eluted WTA index product from the first round of purification. Pipet-mix 10 times, then briefly centrifuge.
3. Incubate the suspension at room temperature for 5 minutes.
4. Place the suspension on the 1.5-mL tube magnet for 3 minutes or until the supernatant is clear.
5. Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol into the tube and incubate the sample on the magnet for 30 seconds. Remove and discard the supernatant.
6. Repeat step 5 for a total of two ethyl alcohol washes.
7. Keeping the tubes on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
8. Air-dry the beads at room temperature for 30 seconds.

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

9. Remove the tube from the magnet and resuspend the bead pellet in 30 µL of elution buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
10. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
11. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
12. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube. The WTA index PCR eluate is the final sequencing libraries.
13. Repeat the last step in WTA index PCR cleanup and quality check (page 25) to perform quality control.

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

2. BD® AbSeq library amplification

This section comprises the following tasks:

2.1 BD® AbSeq PCR1

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

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

BD® AbSeq PCR1 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	10.0	12.0	48.0	96.0
●	BD® AbSeq primer	10.0	12.0	48.0	96.0
○	Nuclease-free water	12.0	14.4	57.6	115.2
	Total	**132.0**	**158.4**	**633.6**	**1,267.2**

2. Pipet-mix and keep on ice.
3. In a new 1.5-mL tube, pipet 132 µL of the BD® AbSeq PCR1 mix. Add 68 µL of the BD® AbSeq product from step 9 in 1.1 Random priming and extension (RPE) (page 14). Pipet-mix 10 times to create the BD® AbSeq PCR1 reaction mix. Do not vortex.
4. Pipet 50 µL BD® AbSeq PCR1 reaction mix into each of four 0.2-mL PCR tubes. Transfer any residual mix to one of the tubes.
5. Bring the BD® AbSeq PCR1 reaction mix to the post-amplification workspace.
6. Run the following PCR program on the thermal cycler.

PCR1 conditions for BD® AbSeq panel

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	8–16*	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

* Recommended PCR cycles might need to be optimized for different cell types and cell number.

Recommended number of PCR cycles

Number of cells in PCR1	Recommended PCR cycles for resting PBMCs
100	16
1,000	14
2,500	13
5,000	12
10,000	11
20,000	10
40,000	9
80,000–100,000	8

STOPPING POINT: The PCR can be run overnight.

7. After PCR has started, continue with 1.1 Random priming and extension (RPE) (page 13).
8. After PCR, briefly centrifuge the tubes.
9. Pipet-mix and combine the four reactions into a new 1.5-mL LoBind® tube labeled AbSeq PCR1. Keep the tube on ice and proceed to 2.2 BD® AbSeq PCR1 cleanup (page 30).

2.2 BD® AbSeq PCR1 cleanup

This section describes how to perform a single-sided AMPure® cleanup to remove primer dimers from the BD® AbSeq PCR1 products. The final product is purified double-stranded DNA.

Perform the purification in the post-amplification workspace.

1. Bring 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. 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.
3. Vortex on high speed for 1 minute until the AMPure® beads are fully resuspended.
4. Briefly centrifuge the BD® AbSeq PCR1 products.

   The final volume must be exactly 200 µL to achieve the appropriate size selection of the purified BD® AbSeq PCR1 product. If the volume is less than 200 µL, use elution buffer to achieve the final volume.
5. To 200 µL of BD® AbSeq PCR1 products, pipet 280 µL AMPure® beads (from 2.1 BD® AbSeq PCR1 (page 28)).
6. Pipet-mix 10 times. Incubate at room temperature for 5 minutes.
7. Place the suspension on the 1.5-mL tube magnet for 5 minutes or until the supernatant is clear. Remove and discard the supernatant.
8. 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.
9. Repeat step 8 once for a total of two ethyl alcohol washes.
10. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 5 minutes.

    Do not overdry the AMPure® beads after the ethanol washes. Overdried beads appear cracked.
12. Remove the tube from the magnet and resuspend the bead pellet in 30 µL of elution buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended. Small clumps do not affect performance.
13. Incubate at room temperature for 2 minutes and briefly centrifuge.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified BD® AbSeq PCR1 products).

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

2.3 BD® AbSeq PCR1 quality check

1. Measure the yield of the largest peak of the BD® AbSeq PCR1 product (~160 bp) by using either the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay or the Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions. Exact size may vary due to instrument or sample purification efficiency. Select the region from 100 to 300 bp.
2. Based on the yield of the largest peak, dilute an aliquot of BD® AbSeq PCR1 product with nuclease-free water before index PCR of BD® AbSeq PCR1 products. See 2.4 BD® AbSeq index PCR (page 32).
3. **Figure 6** Sample bioanalyzer high-sensitivity DNA trace–BD® AbSeq PCR1

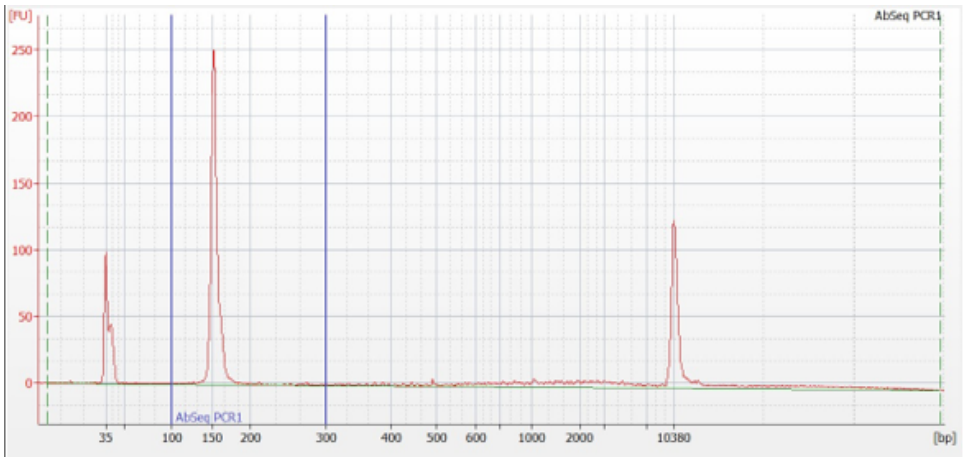
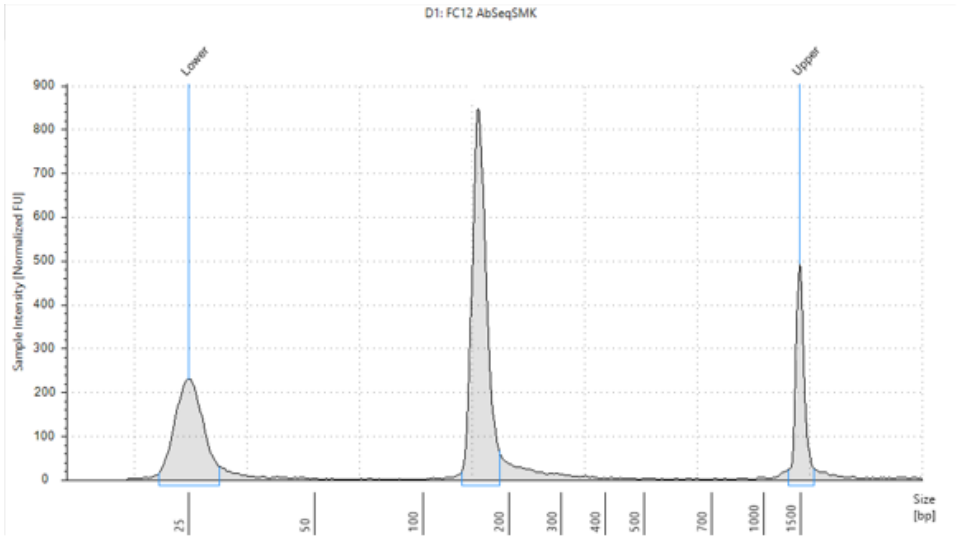


Figure 7 Sample TapeStation high-sensitivity D1000 trace



2.4 BD® AbSeq index PCR

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

For cell capture samples from multiple lanes, the same reverse primer can be used to label all the library types from one lane (for example, WTA and BD® AbSeq from Lane 1 can both be labeled with reverse primer 1, while WTA and BD® AbSeq from Lane 2 can be labeled with reverse primer 2, and so on). The kit provides 4 indexing primers, and can label all sample combinations from up to 4 lanes for the same sequencing run.

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.

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

2. Pipet-mix the BD® AbSeq index PCR mix, and keep on ice.
3. Bring the BD® AbSeq index PCR mix to post-amplification workspace.
4. In a new 0.2 mL PCR tube, combine BD® AbSeq index PCR mix with diluted BD® AbSeq PCR1 products as follows based on the number of libraries:
   - For one library, pipet 3.0 µL of 0.1–1.1 ng/µL BD® AbSeq product into 47.0 µL BD® AbSeq index PCR mix (from 2.3 BD® AbSeq PCR1 quality check (page 31)). See the Concentration Index PCR input for BD® AbSeq libraries (ng/µL) in Recommended number of PCR cycles (page 33).
   - For multiple libraries, combine 45.0 µL of BD® AbSeq index PCR mix with 2 µL of corresponding library reverse primer, and then pipet 3.0 µL of 0.1–1.1 ng/µL BD® AbSeq/ PCR1 product into the PCR tube.
5. Gently vortex, and briefly centrifuge.
6. Run the following PCR program on the thermal cycler.

Index PCR conditions for BD® AbSeq

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the Recommended number of PCR cycles in the following table	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	∞

Recommended number of PCR cycles

Concentration Index PCR input for BD® AbSeq libraries (ng/µL)	Recommended number of PCR cycles
0.5–1.1	6
0.25–0.5	7
0.1–0.25	8

STOP **STOPPING POINT:** The PCR can run overnight.

2.5 BD® AbSeq index PCR cleanup and quality check

This section describes how to perform a single-sided AMPure® cleanup to remove primer dimers from the BD® AbSeq Index PCR products. The final product is purified double-stranded DNA with full-length adapter sequences.

Perform Index PCR purification in the post-amplification workspace.

1. Bring AMPure® XP magnetic beads to room temperature.
2. In a new 15-mL LoBind® tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. 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.
3. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
4. Briefly centrifuge all the index PCR products.

   The final volume must be exactly 50 µL to achieve the appropriate size selection of the purified BD® AbSeq index PCR products. If the volume is less than 50 µL, use elution buffer to achieve the final volume.
5. Pipet 40 µL of AMPure® beads into the 0.2 mL PCR tube containing 50.0 µL of the BD® AbSeq index PCR product.

6. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
7. Place the tube on the strip tube magnet for 3 minutes or until the supernatant is clear. Remove and discard the supernatant.
8. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube, and incubate the sample on the magnet for 30 seconds. Discard the supernatant.
9. Repeat step 8 for a total of two ethyl alcohol washes.
10. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
11. Air-dry the beads at room temperature for 2 minutes.

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

12. Remove the tube from the magnet and resuspend the bead pellet in 30 µL of elution buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
13. Incubate at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the entire eluate (~30 µL) into a new 1.5-mL LoBind® tube. The index PCR product is ready for sequencing.

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

16. Perform quality control before freezing samples.
    a. Estimate the concentration by quantifying 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 >1.5 ng/µL.

       The BD® AbSeq library should show a peak of ~264 bp. Exact size may vary due to instrument or sample purification efficiency. Select the region size between 200 and 500 bp.

Figure 8 Sample bioanalyzer high-sensitivity DNA trace–BD® AbSeq index PCR product

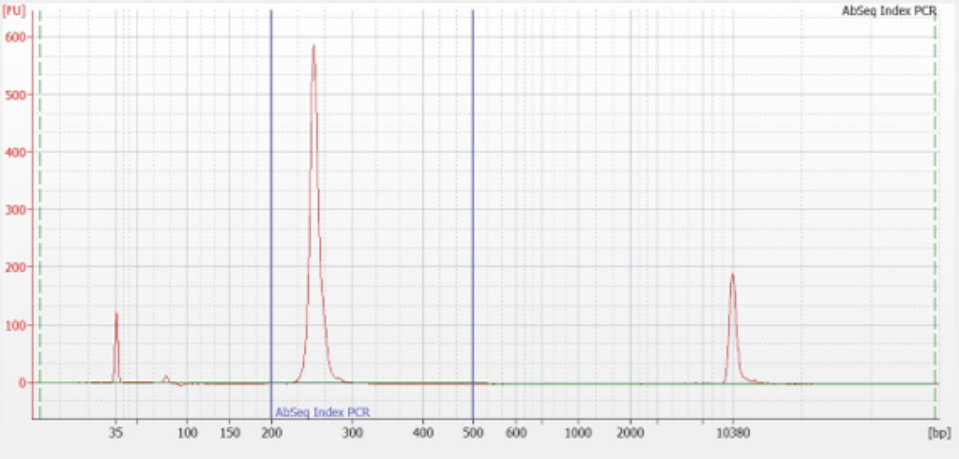
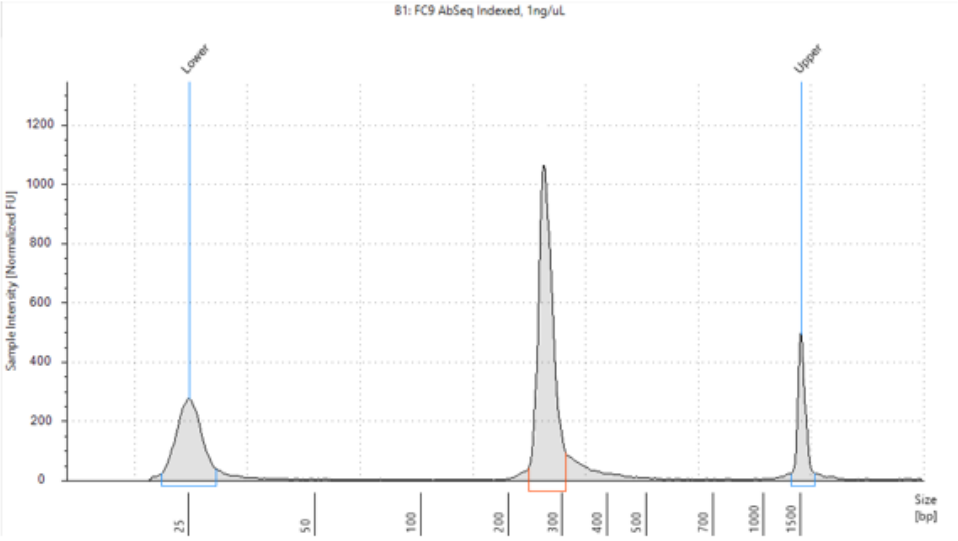


Figure 9 Sample TapeStation high-sensitivity D1000 trace–BD® AbSeq index PCR product



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 WTA mRNA andBD® AbSeq libraries.

Read requirements for libraries

Gene panel	Read requirement for data analysis
BD Rhapsody™ WTA	20,000–100,000 reads/cell
BD® AbSeq	1,000 reads/cell/AbSeq

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 *BD® Single-Cell Multiomics Bioinformatics Handbook*

* To review Illumina Index 1 (i7) sequences, see the Appendix (page 40).

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.

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

- 10,000 reads per cell for shallow sequencing. Genes per cell and UMI per cell detected is generally lower, but this can be useful for cell type identification.
- 20,000–50,000 reads per cell for moderate sequencing.
- 100,000 reads per cell for highly saturated deep sequencing to identify the majority of UMIs in the library.

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.

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

Sequencing analysis pipeline

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

Troubleshooting

Library preparation

Observation	Possible causes	Recommended solutions
Low yield of RPE-PCR.	Cell number lower than expected.	Repeat PCR using the RPE PCR product for additional cycles. Alternatively, increase index PCR cycles.
Index PCR BioAnalyzer trace of WTA library has 264 bp peak.	BD® AbSeq library contamination in mRNA library.	If peak takes up high percentage of sequencing reads (manifests as lower reads/cell than expected for WTA library, alongside higher reads/cell than expected for BD® AbSeq), perform a second round of AMPure® purification according to 1.8 Additional WTA index PCR cleanup (page 27).
Low yield of indexing PCR.	Input DNA not high enough or cycle number too low.	Repeat indexing PCR with higher cycle number. Alternatively, if RPE-PCR product was diluted before adding to indexing PCR, repeat indexing PCR with less or no dilution.
Index PCR Bioanalyzer trace of WTA library shows large amount of product larger than 600 bp.	Over-amplification during indexing PCR.	Repeat indexing PCR with lower cycle number. Alternatively, repeat indexing with diluted RPE-PCR product.
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.

Observation	Possible causes	Recommended solutions
Lower number of reads/cell than expected from mRNA.	264 bp or ~160 bp products taking reads from mRNA library.	If noise peak is seen in the 264 bp or ~160 bp range, perform a second round of AMPure® purification according to 1.8 Additional WTA index PCR cleanup (page 27).

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™ Whole Transcriptome Analysis Amplification Kit

The following table lists the sequences of all oligonucleotides included in the BD Rhapsody™ Whole Transcriptome Analysis Amplification Kit (Catalog No. 633801). Note that the BD® AbSeq primer and BD Rhapsody™ WTA amplification primer have the same sequence.

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	ACACGACGCTCTTCCGATCT
BD® AbSeq Primer	Reverse primer for BD® AbSeq PCR1	91-1086	CAGACGTGTGCTCTTCCGATCT
BD Rhapsody™ WTA Extension Primers	Random primers for WTA RPE	91-1115	TCAGACGTGTGCTCTTCCGATCTNNNNNNNNN
BD Rhapsody™ WTA Amplification Primer	Reverse primer for WTA RPE PCR	91-1116	CAGACGTGTGCTCTTCCGATCT
BD Rhapsody™ Library Forward Primer	Forward primer for WTA, Sample Tag, and BD® AbSeq Index PCR	91-1085	AATGATACGGCGACCACCGAGATCTACACTATAGCCT ACACTCTTTCCCTACACGACGCTCTTCCGAT*C*T
BD Rhapsody™ Library Reverse Primer 1	WTA, Sample Tag, and BD® AbSeq Index PCR	650000080	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 2		650000091	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 3		650000092	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T
BD Rhapsody™ Library Reverse Primer 4		650000093	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC*T

Forward index name	i5 bases for sample sheet NovaSeq, MiSeq, HiSeq 2000/2500	i5 bases for sample sheet 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	i5 bases for sample sheet 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])**AAAAAAAAAAAAAAAAAAAAAAAA

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.

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