BD Rhapsody™ System

Targeted mRNA

Library Preparation Protocol

23-24121(03)
2024-11

Regulatory information

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

History

Revision	Date	Change made
23-24121(01)	2021-12	Initial release.
23-24121(02)	2022-11	Updated for BD Rhapsody™ Enhanced Cell Capture Beads version 2.0.
23-24121(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

This protocol enables high throughput single-cell transcriptome profiling of individual cells captured on the BD Rhapsody™ system and provides instructions for amplifying single-cell barcoded mRNA libraries for sequencing on various sequencers. cDNA is encoded on BD Rhapsody™ Enhanced Cell Capture Beads using the 3' ends of transcripts as templates. mRNA libraries are then amplified from these on-bead cDNA libraries using a two-step nested amplification.

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

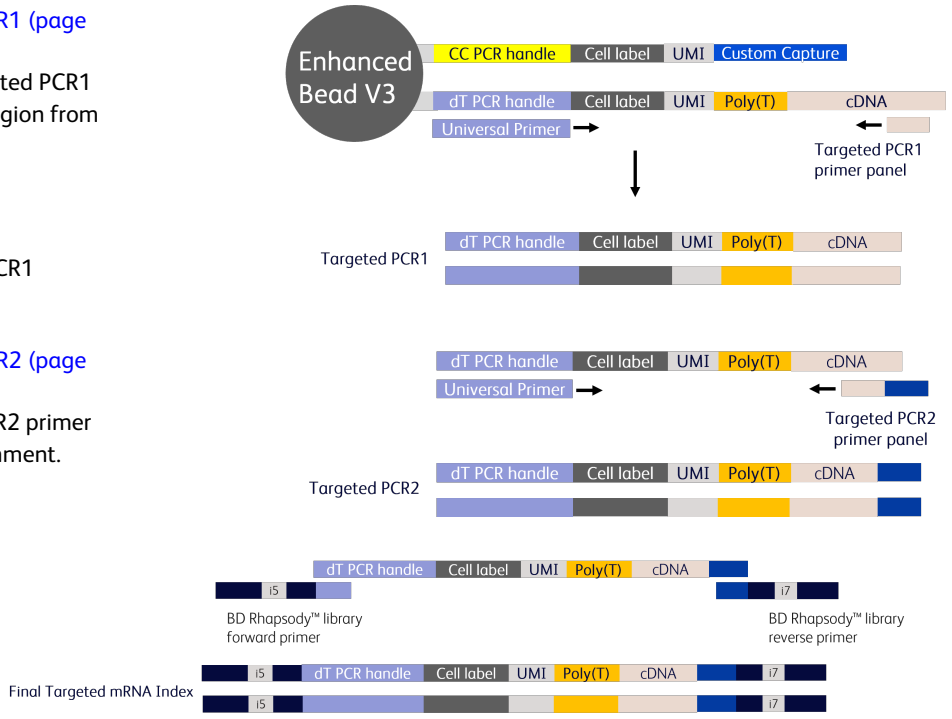
Targeted mRNA library amplification workflow

Step 1: Targeted mRNA 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 3: Targeted mRNA PCR2 (page 16):
Amplify using Targeted PCR2 primer panel for nested PCR enrichment.

Step 5: Targeted mRNA index PCR (page 18):
Add 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* 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	–
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.

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™ 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 Library Amplification		
Post-Amplification Workspace	Step 1: Targeted mRNA PCR1 (page 13)	100 minutes	PCR overnight
	Step 2: Targeted mRNA PCR1 cleanup (page 15)	STOP	<24 hours at 4 °C or <6 months at –20 °C
	Step 3: Targeted mRNA PCR2 (page 16)	80 minutes	PCR overnight
	Step 4: Targeted mRNA PCR2 cleanup (page 17)	STOP	<24 hours at 4 °C or <6 months at –20 °C
	Step 5: Targeted mRNA index PCR (page 18)	70 minutes	PCR overnight
	Step 6: Targeted mRNA index PCR cleanup and quality check (page 19)	STOP	<6 months at –20 °C

Procedure

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 library amplification

This procedure comprises the following tasks:

- Step 1: Targeted mRNA PCR1 (page 13)
- Step 2: Targeted mRNA PCR1 cleanup (page 15)
- Step 3: Targeted mRNA PCR2 (page 16)
- Step 4: Targeted mRNA PCR2 cleanup (page 17)
- Step 5: Targeted mRNA index PCR (page 18)
- Step 6: Targeted mRNA index PCR cleanup and quality check (page 19)

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 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)
○	Nuclease-free water	28.0 (18.0)	33.6 (21.6)	134.4 (86.4)	268.8 (172.8)
	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.

    STOP **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 PCR1 cleanup

Keep both the supernatant (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 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. Remove and discard the supernatant without disturbing the beads.
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.

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

12. 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.
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 Targeted mRNA 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.

Step 3: 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.

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	∞

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

Step 4: 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 5: Targeted mRNA index PCR

This section describes how to generate Targeted mRNA 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 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 index PCR mix into one 0.2-mL PCR tube for each library, and place back on ice.
3. Bring the Index PCR mix to post-amplification workspace.
4. In new 0.2 mL PCR tubes, pipet 3.0 µL of 0.2–2.7 ng/µL products into 47.0 µL of Targeted mRNA 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)	Suggested PCR cycles
1.2–2.7	6
0.6–1.2	7
0.2–0.6	8

STOPPING POINT: The PCR can run overnight.

Step 6: Targeted mRNA 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. Pipet 35 µL of AMPure® beads into 50 µL of the Targeted mRNA index PCR products.
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).

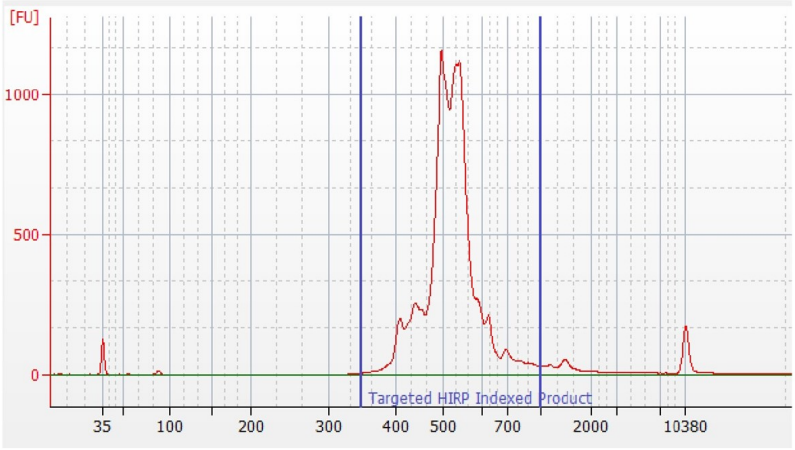
STOPPING POINT: Store at –25 °C to –15 °C for up to 6 months until sequencing.

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 ng/µL.

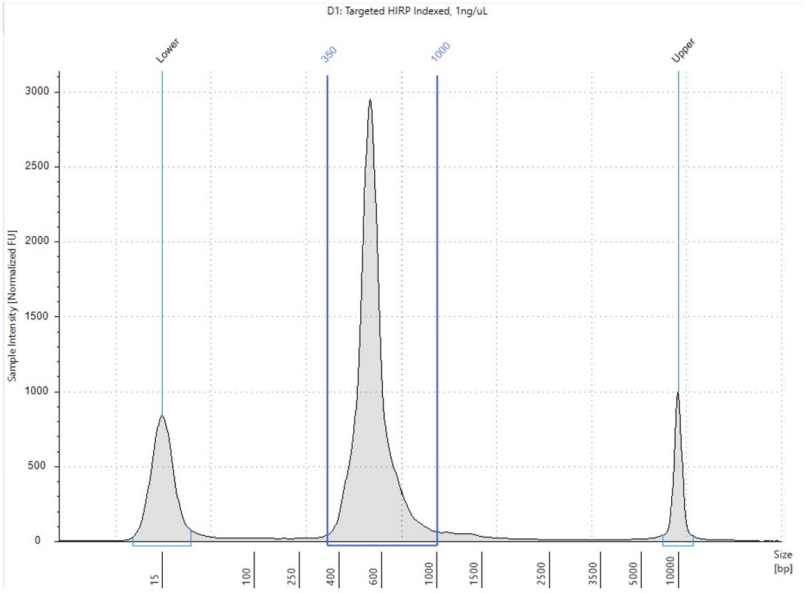
16. Measure the average fragment size of the Targeted mRNA, library within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit for 50–7,000 bp, 5–1,000 pg/µL or the Agilent TapeStation. Follow the manufacturer's instructions.

Figure 1 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



17. If the concentration or size of the library is outside of the expected range, see Troubleshooting (page 24) 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 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*

* 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.

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 26).

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.

To determine the ratio of BD Rhapsody™ Targeted mRNA 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
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.	1. Determine the product size range: 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. • If the products pass quality control, see Step 5: Targeted mRNA index PCR (page 18) 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.

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	ACACGACGCTCTTCCGATCT
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

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