# BD Rhapsody™ System

## Targeted mRNA and Sample Tag

### Library Preparation Protocol

23-24122(03)
2024-11

## Copyrights

## Patents and Trademarks

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

## Regulatory information

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

## History

| Revision | Date | Change made |
|---|---|---|
| 23-24122(01) | 2021-12 | Initial release. |
| 23-24122(02) | 2022-11 | Updated for BD Rhapsody™ Enhanced Cell Capture Beads version 2.0. In the Appendix, added BD® Flex SMK sequences (page 33). |
| 23-24122(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 provides instructions for creating single-cell Targeted mRNA and Sample Tag libraries after cell capture on the BD Rhapsody™ HT Single-Cell Analysis system or the BD Rhapsody™ HT Xpress System for sequencing on various sequencers. For complete instrument procedures and safety information, see the *BD Rhapsody™ HT System Instrument User Guide* or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-free Workflow*.

To create the libraries, BD Rhapsody™ mRNA targets and Sample Tags are encoded on the BD Rhapsody™ Enhanced Cell Capture Beads and then amplified in PCR1. After PCR1, the Sample TagPCR1 products are separated from the Targeted mRNA PCR1 products by double-sided size selection with Agencourt® AMPure® XP magnetic beads. Size selection of library molecules is achieved by specific and successive use of volume ratios between DNA samples and AMPure beads.

Successful preparation of Targeted mRNA, and Sample Tag libraries requires that:

- Targeted mRNA PCR1 and Sample Tag PCR1 products undergo PCR2 amplification followed by index PCR with library index primers.
- After index PCR, the Targeted mRNAand Sample Tag libraries can be combined for sequencing.

## Symbols

The following symbols are used in this guide.

| Symbol | Description |
|---|---|
| ⚠ | Important information for maintaining measurement accuracy or data integrity. |
| 💡 | Noteworthy information. |
| STOP | Procedural stopping point. |

## Protocol kits

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

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

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

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

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

| Cap Color | Name | Quantity |
|---|---|---|
| 🔵 | PCR1 primers immune response Hs | 1 |
| 🔵 | PCR2 primers immune response Hs | 1 |

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

| Cap Color | Name | Quantity |
|---|---|---|
| 🔵 | PCR1 primers immune response Mm | 1 |
| 🔵 | PCR2 primers immune response Mm | 1 |

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

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

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

| Cap Color | Name | Quantity |
|---|---|---|
| 🔵 | PCR1 primers T cell expression Hs | 1 |
| 🔵 | PCR2 primers T cell expression Hs | 1 |

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

| Cap Color | Name | Quantity |
|---|---|---|
| 🔵 | Dual index forward primer 1–8 | 1 each |
| 🟠 | Dual index reverse primer 1–8 | 1 each |

# Workflows

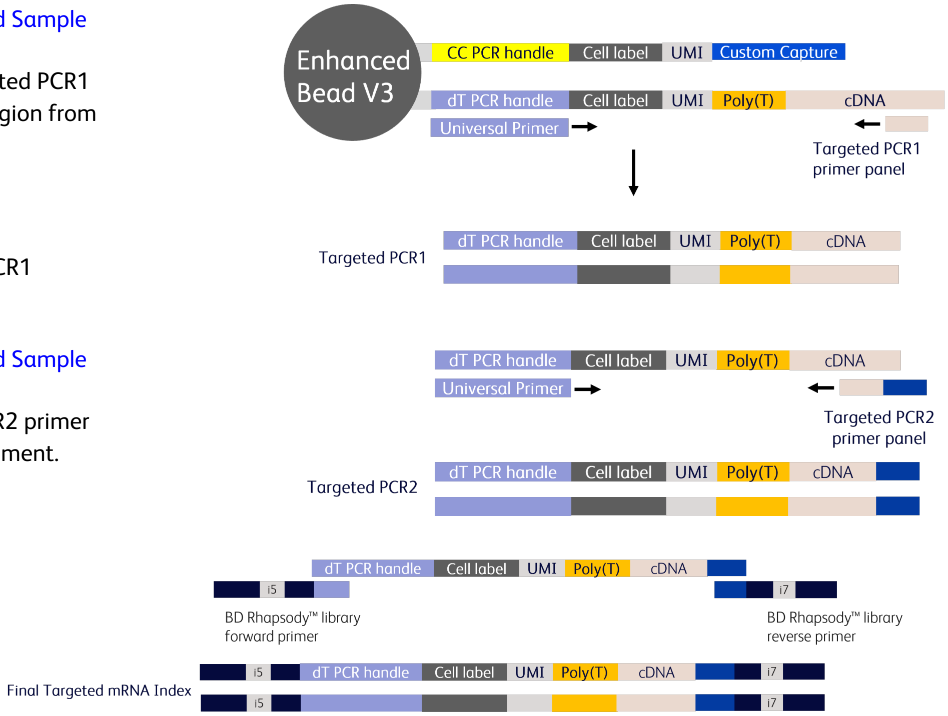
## Targeted mRNA library amplification workflow

Step 1: Targeted mRNA and Sample Tag 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 and Sample Tag PCR2 (page 18):
Amplify using Targeted PCR2 primer panel for nested PCR enrichment.

Step 5: Targeted mRNA and Sample Tag index PCR (page 20):
Add adapters and indices.

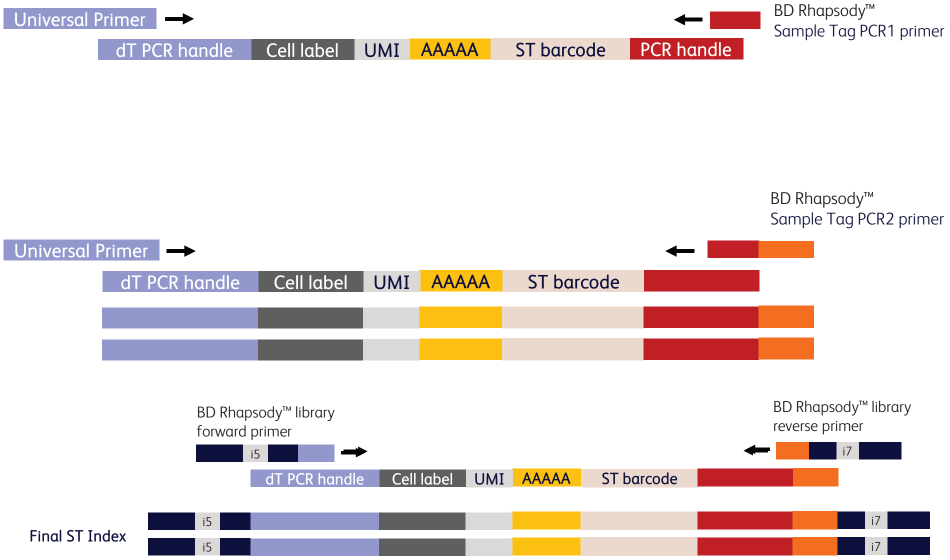


## Sample Tag library amplification workflow

Step 1: Targeted mRNA and Sample Tag PCR1 (page 13):
Sample Tag product is amplified in PCR1.

Step 3: Targeted mRNA and Sample Tag PCR2 (page 18): Sample Tag product is further amplified in PCR2.

Step 5: Targeted mRNA and Sample Tag index PCR (page 20): Add Sample Tag 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*<br>Or,<br>MicroAmp Optical 96–Reaction Plate* | Corning<br><br>Thermo Fisher Scientific | PCR96HSC<br><br>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<br>Or,<br>Agilent High Sensitivity D1000 ScreenTape<br>Agilent High Sensitivity D1000 Reagents<br>Or,<br>Agilent High Sensitivity D5000 ScreenTape<br>Agilent High Sensitivity D5000 Reagents | Agilent<br><br>Agilent<br>Agilent<br><br>Agilent<br>Agilent | 5067-4626<br><br>5067-5584<br>5067-5585<br><br>5067-5592<br>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<br>Or,<br>12-tube magnetic separation rack†<br>Or,<br>Invitrogen™ DynaMag™-2 magnet† | New England Biolabs<br>New England Biolabs<br>Thermo Fisher Scientific | S1506S<br>S1509S<br>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<br>Or,<br>Agilent® 4200 TapeStation System | Agilent Technologies<br>Agilent Technologies | G2940CAG<br>G2991AA |
| Heat block | Major supplier | – |

† Recommended for processing greater than six samples.

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

## Best practices

### Cell capture

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

### Bead handling

- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes.

  ⚠ Never vortex the beads. Pipet-mix only.

- Store BD Rhapsody™ Enhanced Cell Capture Beads at 4 °C. Do not freeze.
- Bring Agencourt® AMPure® XP magnetic beads to room temperature (15–25 °C) before use. See the *AMPure® XP User's Guide* for information.

### Libraries

- Sample Tag libraries can be sequenced separately or together with Targeted mRNA libraries.

**Master mix preparation**

- Thaw reagents (except for enzymes) at room temperature.
- Keep enzymes at –25 °C to –15 °C until ready for use.
- Return reagents to correct storage temperature as soon as possible after preparing the master mix.

**Supernatant handling**

- Read this protocol carefully before beginning each section. Note which steps require you to keep supernatant to avoid accidentally discarding required products.
- Remove supernatants without disturbing AMPure® XP magnetic beads.
- Make and use fresh 80% ethyl alcohol within 24 hours. Adjust the volume of 80% ethyl alcohol depending on the number of libraries.

**Bead amplification**

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

## Additional documentation

- *BD Rhapsody™ HT Single-Cell Analysis System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24252)
- *BD Rhapsody™ HT Xpress System Single-Cell Capture and cDNA Synthesis Protocol* (doc ID 23-24253)
- *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kits Protocol* (doc ID 23-21340)
- *BD Rhapsody™ System Single-Cell Labeling with BD® Flex Single-Cell Multiplexing Kits Protocol* (doc ID 23-24311)
- *BD Rhapsody™ Sequence Analysis Pipeline User's Guide* (doc ID 23-24580)

## Safety information

For safety information, refer to the *BD Rhapsody™ HT Single-Cell Analysis System Instrument User Guide* (doc ID 23-24607) or the *BD Rhapsody™ HT Xpress System Instrument User Guide for Scanner-Free Workflow* (doc ID 23-24256).

## Time considerations

| **Station** | **Workflow** | **Timing** | **Stopping Point & Storage** |
|---|---|---|---|
| | **Targeted mRNA and Sample Tag Library Amplification** | | |
| Post-Amplification Workspace | Step 1: Targeted mRNA and Sample Tag PCR1 (page 13) | 100 minutes | PCR overnight |
| | Step 2: Targeted mRNA and Sample Tag PCR1 cleanup (page 16) | STOP | <24 hours at 4 °C or<br><6 months at –20 °C |
| | Step 3: Targeted mRNA and Sample Tag PCR2 (page 18) | 80 minutes | PCR overnight |
| | Step 4: Targeted mRNA and Sample Tag PCR2 cleanup (page 19) | STOP | <24 hours at 4 °C or<br><6 months at –20 °C |
| | Step 5: Targeted mRNA and Sample Tag index PCR (page 20) | 70 minutes | PCR overnight |
| | Step 6: Targeted mRNA and Sample Tag index PCR cleanup and quality check (page 22) | STOP | <6 months at –20 °C |

# Procedure

Continue this procedure after staining the antibodies as described in the *BD Rhapsody™ System Single-Cell Labeling with BD® Single-Cell Multiplexing Kits Protocol* or the *BD Rhapsody™ System Single-Cell Labeling with BD® Flex Single-Cell Multiplexing Kits Protocol*.

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system using either of the following guides for cell capture, reverse transcription, and Exonuclease treatment:

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

# Targeted mRNA and Sample Tag library amplification

This procedure comprises the following tasks:

- Step 1: Targeted mRNA and Sample Tag PCR1 (page 13)
- Step 2: Targeted mRNA and Sample Tag PCR1 cleanup (page 16)
- Step 3: Targeted mRNA and Sample Tag PCR2 (page 18)
- Step 4: Targeted mRNA and Sample Tag PCR2 cleanup (page 19)
- Step 5: Targeted mRNA and Sample Tag index PCR (page 20)
- Step 6: Targeted mRNA and Sample Tag index PCR cleanup and quality check (page 22)

## Before you begin

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

## Step 1: Targeted mRNA and Sample Tag 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) |
| 🟣 | Sample Tag PCR1 primer | 1.2 | 1.44 | 5.76 | 11.52 |
| ○ | Nuclease-free water | 26.8<br>(16.8) | 32.16<br>(20.16) | 128.64<br>(80.64) | 257.28<br>(161.28) |
|  | **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 and Sample Tag PCR1 cleanup

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

⚠ Keep both the supernatant (Sample Tag products) and the AMPure beads (Targeted mRNA products) for purification.

💡 Perform PCR1 purification in the post-amplification workspace.

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

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

    **80% Ethyl Alcohol**

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

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

    ⚠ The final volume must be exactly 200 µL to achieve the appropriate size selection of the purified PCR1 product. If the volume is less than 200 µL, use elution buffer to achieve the final volume.

5. Pipet 140 µL of AMPure® beads into the 200 µL of Targeted mRNA and SampleTag PCR1 products. Pipet-mix 10 times, then briefly centrifuge the samples.
6. Incubate at room temperature for 5 minutes.
7. Place the 1.5-mL LoBind® tube on the magnet for 5 minutes or until the supernatant is clear.

8. Keeping the tube on the magnet, transfer the 340 µL of supernatant (Sample Tag PCR1 products) to a new 1.5-mL tube without disturbing the beads (Targeted mRNA PCR1 products).
9. Store the supernatant (step 8) on ice while purifying and eluting the Targeted mRNA products. Purify the Sample Tag PCR1 products after purifying the Targeted mRNA PCR1 products.
10. Keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant without disturbing the beads.
11. Repeat step 10 once for a total of two ethyl alcohol washes.
12. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
13. Air-dry the beads at room temperature for 5 minutes.

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

14. Remove the tube from the magnet and pipet 30 µL of elution buffer to resuspend the bead pellet. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.
15. Incubate at room temperature for 2 minutes and briefly centrifuge.
16. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
17. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified Targeted mRNA PCR1 products).
18. To purify Sample Tag PCR1 products, pipet 100 µL of AMPure® beads into the tube with 340 µL supernatant from step 8. Pipet-mix 10 times, then briefly centrifuge the samples.
19. Incubate at room temperature for 5 minutes.
20. Place the 1.5-mL LoBind® tube on the magnet for 5 minutes or until the supernatant is clear. Remove and discard the supernatant without disturbing the beads.
21. Keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove and discard the supernatant without disturbing the beads.
22. Repeat step 21 for a total of two ethyl alcohol washes.
23. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
24. Air-dry the beads at room temperature for 5 minutes.

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

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

28. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified Sample Tag PCR1 products).

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

## Step 3: Targeted mRNA and Sample Tag 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<br>(5.5) | 9.6<br>(6.6) | 38.4<br>(26.4) | 76.8<br>(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.

**Sample Tag 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 |
| ● | Sample Tag PCR2 primer | 3.0 | 3.6 | 14.4 | 28.8 |
| ○ | Nuclease-free water | 15.0 | 18.0 | 72.0 | 144.0 |
|  | **Total** | **45.0** | **54.0** | **216.0** | **432.0** |

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. In two separate and new 0.2-mL PCR tubes:
   - Targeted mRNA PCR1 products: Pipet 5.0 µL products into 45.0 µL Targeted mRNA PCR2 reaction mix.
   - Sample Tag PCR1 products: Pipet 5.0 µL products into 45.0 µL Sample Tag 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 | ∞ |

**PCR2 conditions for Sample Tag**

| Step | Cycles | Temperature | Time |
|---|---|---|---|
| Hot start | 1 | 95 °C | 3 min |
| Denaturation | 10 | 95 °C | 30 seconds |
| Annealing | | 66 °C | 30 seconds |
| 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 and Sample Tag 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
   - Sample Tag library: 60 µL 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) of each sample into two separate and new 1.5-mL LoBind® tubes (purified Targeted mRNA and Sample Tag PCR2 products).

   STOP **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 and Sample Tag PCR2 products with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.

## Step 5: Targeted mRNA and Sample Tag index PCR

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

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

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

   **Targeted mRNA and Sample Tag index PCR mix**

| Cap | Component | For 1 library (µL) | For 1 library with 20% overage (µL) | For 4 libraries with 20% overage (µL) | For 8 libraries with 20% overage (µL) |
|---|---|---|---|---|---|
| ○ | PCR master mix | 25.0 | 30.0 | 120.0 | 240.0 |
| 🔴 | Library forward primer | 2.0 | 2.4 | 9.6 | 19.2 |
| 🔴 | Library reverse primer 1–4* † | 2.0 | 2.4 | – | – |
| ○ | Nuclease-free water | 18.0 | 21.6 | 86.4 | 172.8 |
|  | **Total** | **47.0** | **56.4** | **216.0** | **432.0** |

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

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

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

2. Gently vortex mix and briefly centrifuge. Pipet 47 µL of Targeted mRNA or Sample Tag index PCR mix into one 0.2-mL PCR tube for each library, and place back on ice.
3. Bring the Index PCR mixes to post-amplification workspace.
4. In separate and new 0.2-mL PCR tubes:
   - Targeted mRNA PCR2 products: Pipet 3.0 µL of 0.2–2.7 ng/µL products into 47.0 µL index PCR mix.
   - Sample Tag PCR2 products: Pipet 3.0 µL of 0.1–1.1 ng/µL products into 47.0 µL index PCR mix.
5. Gently vortex, and briefly centrifuge.
6. Run the following PCR program on the thermal cycler.

   **Index PCR conditions for Targeted mRNA**

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

* See suggested PCR cycles.

**Suggested PCR cycles**

| Concentration index PCR input for mRNA Targeted libraries (ng/µL) | Concentration index PCR input for Sample Tag libraries (ng/µL) | Suggested PCR cycles |
|---|---|---|
| 1.2–2.7 | 0.5–1.1 | 6 |
| 0.6–1.2 | 0.25–0.5 | 7 |
| 0.2–0.6 | 0.1–0.25 | 8 |

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

## Step 6: Targeted mRNA and Sample Tag index PCR cleanup and quality check

Perform PCR purification in the post-amplification workspace.

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

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

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

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

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

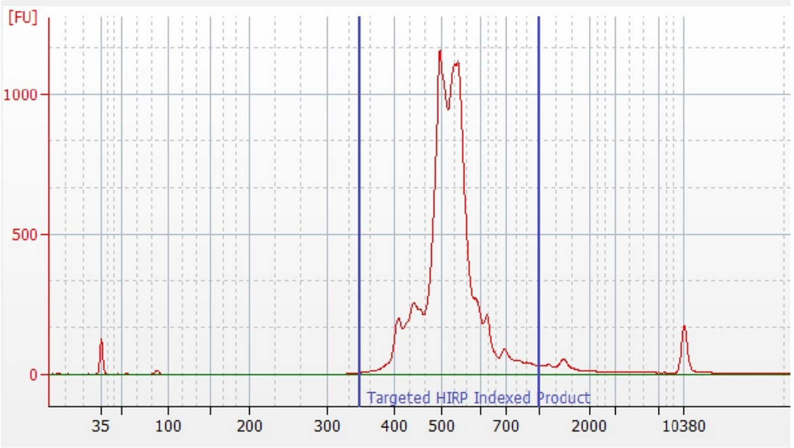
13. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
14. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (final sequencing library).

STOP **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 and Sample Tag, libraries within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit for 50–7,000 bp, 5–1,000 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

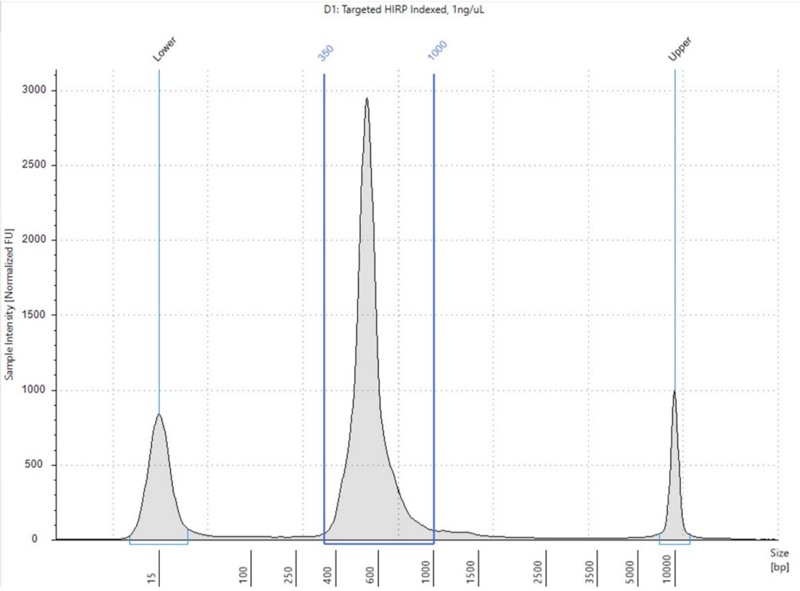
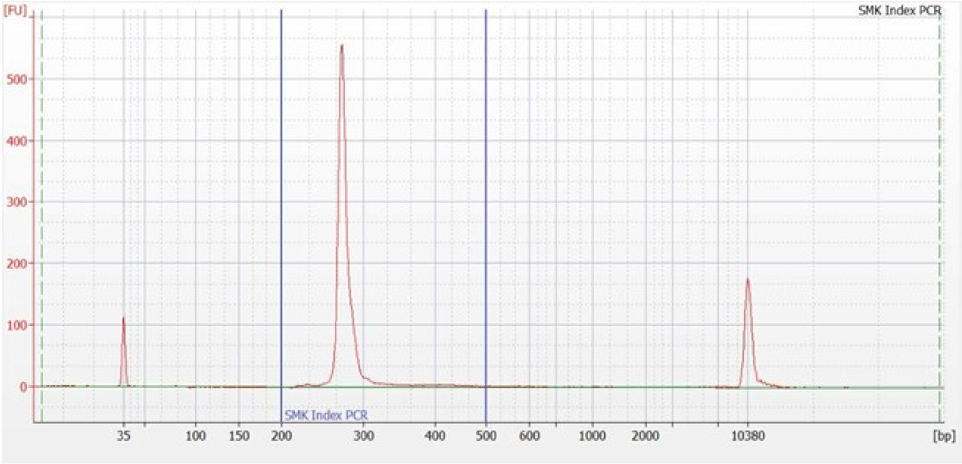
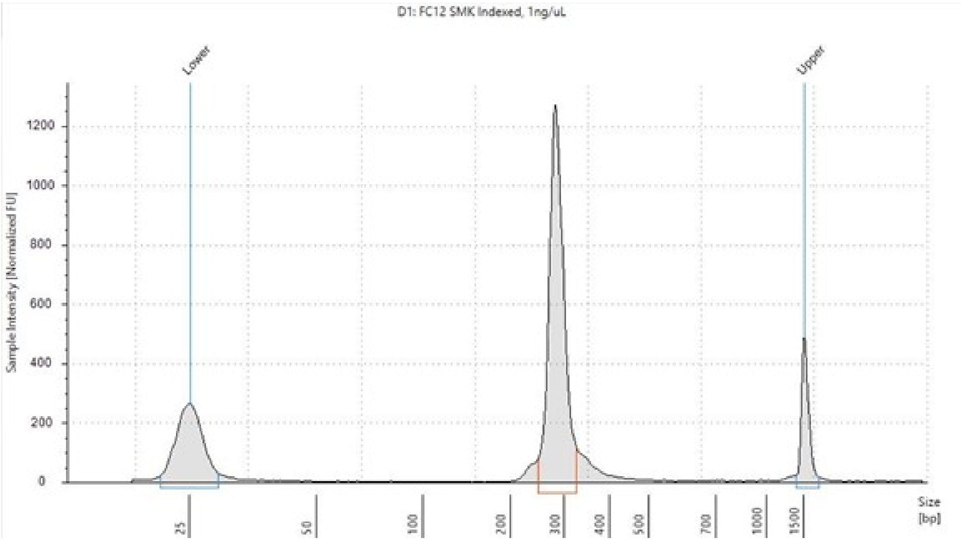


**Figure 2** Sample Tag index PCR product

A. Sample bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



17. If the concentration or size of the library is outside of the expected range, see Troubleshooting (page 26) or contact your local Field Application Specialist or go to scomix@bdscomix.bd.com.

# Sequencing

The sequencing depth for each library is dependent on application. For cell-type clustering, shallow sequencing is sufficient. However, for in-depth analysis such as comparison across multiple samples, deep sequencing is advised. We recommend meeting the requirement for recursive substitution error correction (RSEC) sequencing depth of ≥6 to reach the threshold of sequencing saturation where most molecules of the library have been recovered, approximately 80%. The RSEC sequencing depth and sequencing saturation are both reported by the analysis pipeline. The actual sequencing reads/cell required to achieve this depth can vary as it is dependent on the gene expression levels, number of cells, and sequencing run quality. The following reads/cell are recommended for Targeted mRNA and Sample Tag 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* |
| Sample Tag | 600 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 29).

## 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 Sample Tag 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.<br>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.<br>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:<br>a. Load 1 µL of purified PCR2 product at 1 ng/µL in a High Sensitivity DNA Chip on the Agilent Bioanalyzer.<br>b. Follow the manufacturer's instructions.<br>2. Confirm that the mRNA tTargeted PCR2 products should show an average size range of 350–600 bp.<br>• If the products pass quality control, see Step 5: Targeted mRNA and Sample Tag index PCR (page 20) to prepare final libraries. Repeat the index PCR.<br>• 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. |
| Yield of Sample Tag library too low after index PCR (<1 ng/µL). | Sample Tag labeling incubation time too short. | Ensure that the cells were labeled with Sample Tags correctly and that the correct incubation time was used. |
| | PCR1 and PCR2 primers swapped. | Ensure that correct primer is used for each step. |
| | 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. |
| | Too few index PCR cycles. | Increase the number of index PCR cycles. |

| Observation | Possible causes | Recommended solutions |
|---|---|---|
| Expected size of Sample Tag products is too short (<276 bp). | Upper and lower markers on the Agilent Bioanalyzer or Agilent TapeStation are incorrectly called. | Ensure that the markers are correct. Follow the manufacturer's instructions. |
| | Inefficient Sample Tag labeling. | Ensure that the cells were labeled with Sample Tags correctly and that the correct incubation time was used. |
| | Sample Tags were not amplified in PCR steps due to incorrect primers used. | Perform PCR2 again. See .<br>Analyze products using the Agilent Bioanalyzer or the Agilent TapeStation and look for a ~182 bp peak that corresponds to Sample Tag PCR2 products. |

## 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. |
| High proportion of undetermined Sample Tag calls in sequencing results. | Insufficient sequencing of the Sample Tag Library. | 1. Set pooled samples of the same cell type to 120 reads/cell.<br>2. Set pooled samples of different cell types to 600 reads/cell.<br>3. Repeat sequencing of Sample Tag library. If issue persists, contact your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com. |
| | Insufficient washes after labeling cells with Sample Tags. | Follow the washing steps in this protocol. |
| | BD Rhapsody™ Cartridge overloaded with cells. | Follow the cell loading steps in the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*. |

# Appendix

## Oligonucleotides in BD Rhapsody™ Targeted mRNA and BD® AbSeq 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™ Sample Tag PCR1 Primer | Reverse primer for Sample Tag PCR1 | 91-1088 | GTTGTCAAGATGCTACCGTT |
| BD Rhapsody™ Sample Tag PCR2 Primer | Reverse primer for Sample Tag PCR2 | 91-1089 | CAGACGTGTGCTCTTCCGATCTGTTGTCAAGATGCTACCGTT |
| BD Rhapsody™ Library Forward Primer | Forward primer for WTA, Sample Tag, and BD® AbSeq Index PCR | 91-1085 | AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGACGCTCTTCCGAT*C*T |
| BD Rhapsody™ Library Reverse Primer 1 | WTA, Sample Tag, and BD® AbSeq Index PCR | 650000080 | CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T |
| BD Rhapsody™ Library Reverse Primer 2 | | 650000091 | CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T |
| BD Rhapsody™ Library Reverse Primer 3 | | 650000092 | CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T |
| BD Rhapsody™ Library Reverse Primer 4 | | 650000093 | CAAGCAGAAGACGGCATACGAGATTCCTCTACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*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 |

## Sample Tag sequences

### Human Sample Tag sequences

Each Human Sample Tag is a human universal antibody conjugated with a unique oligonucleotide sequence to allow for sample identification. Each Sample Tag has common 5' and 3' ends, with the Sample Tag sequence between them:

- GTTGTCAAGATGCTACCGTTCAGAG**(Sample Tag sequence)**AAAAAAAAAAAAAAAAAAAAAAAAA

| Sample Tag | Sample Tag sequence |
|---|---|
| Sample Tag 1–Human | ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG |
| Sample Tag 2–Human | TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG |
| Sample Tag 3–Human | CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT |
| Sample Tag 4–Human | ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT |
| Sample Tag 5–Human | CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG |
| Sample Tag 6–Human | TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC |
| Sample Tag 7–Human | TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT |
| Sample Tag 8–Human | CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT |
| Sample Tag 9–Human | GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG |
| Sample Tag 10–Human | GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC |
| Sample Tag 11–Human | CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC |
| Sample Tag 12–Human | GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG |

## Mouse Immune Sample Tag sequences

Each Mouse Immune Sample Tag is an Anti-Mouse CD45, Clone 30-F11 antibody conjugated with a unique oligonucleotide sequence to allow for sample identification. Each Sample Tag has common 5' and 3' ends, with the Sample Tag sequence between them:

- GTTGTCAAGATGCTACCGTTCAGAG**(Sample Tag sequence)**AAAAAAAAAAAAAAAAAAAAAAAAA

| Sample Tag | Sample Tag sequence |
|---|---|
| Sample Tag 1–Mouse Immune | AAGAGTCGACTGCCATGTCCCCTCCGCGGGTCCGTGCCCCCCAAG |
| Sample Tag 2–Mouse Immune | ACCGATTAGGTGCGAGGCGCTATAGTCGTACGTCGTTGCCGTGCC |
| Sample Tag 3–Mouse Immune | AGGAGGCCCCGCGTGAGAGTGATCAATCCAGGATACATTCCCGTC |
| Sample Tag 4–Mouse Immune | TTAACCGAGGCGTGAGTTTGGAGCGTACCGGCTTTGCGCAGGGCT |
| Sample Tag 5–Mouse Immune | GGCAAGGTGTCACATTGGGCTACCGCGGGAGGTCGACCAGATCCT |
| Sample Tag 6–Mouse Immune | GCGGGCACAGCGGCTAGGGTGTTCCGGGTGGACCATGGTTCAGGC |
| Sample Tag 7–Mouse Immune | ACCGGAGGCGTGTGTACGTGCGTTTCGAATTCCTGTAAGCCCACC |
| Sample Tag 8–Mouse Immune | TCGCTGCCGTGCTTCATTGTCGCCGTTCTAACCTCCGATGTCTCG |
| Sample Tag 9–Mouse Immune | GCCTACCCGCTATGCTCGTCGGCTGGTTAGAGTTTACTGCACGCC |
| Sample Tag 10–Mouse Immune | TCCCATTCGAATCACGAGGCCGGGTGCGTTCTCCTATGCAATCCC |
| Sample Tag 11–Mouse Immune | GGTTGGCTCAGAGGCCCCAGGCTGCGGACGTCGTCGGACTCGCGT |
| Sample Tag 12–Mouse Immune | CTGGGTGCCTGGTCGGGTTACGTCGGCCCTCGGGTCGCGAAGGTC |

## BD® Flex SMK sequences

Each Flex Sample Tag is an anti-PE antibody conjugated with a unique oligonucleotide sequence to allow for sample identification. Each Sample Tag has common 5' and 3' ends, with the Sample Tag sequence between them:

- GTTGTCAAGATGCTACCGTTCAGAG**(Sample Tag sequence)**AAAAAAAAAAAAAAAAAAAAAAAAA

### BD® Flex Single-Cell Multiplexing Kit A (Catalog No. 633849)

⚠ Not for use together with Hu SMK Tags 1–6 (Catalog No. 633781) in the same experiment.

| **Sample Tag** | **Sample Tag sequence** | **Notes** |
|---|---|---|
| Sample Tag 1–Flex | ATTCAAGGGCAGCCGCGTCACGATTGGATACGACTGTTGGACCGG | Barcode sequence is the same as human SMK Sample Tag 1 |
| Sample Tag 2–Flex | TGGATGGGATAAGTGCGTGATGGACCGAAGGGACCTCGTGGCCGG | Barcode sequence is the same as human SMK Sample Tag 2 |
| Sample Tag 3–Flex | CGGCTCGTGCTGCGTCGTCTCAAGTCCAGAAACTCCGTGTATCCT | Barcode sequence is the same as human SMK Sample Tag 3 |
| Sample Tag 4–Flex | ATTGGGAGGCTTTCGTACCGCTGCCGCCACCAGGTGATACCCGCT | Barcode sequence is the same as human SMK Sample Tag 4 |
| Sample Tag 5–Flex | CTCCCTGGTGTTCAATACCCGATGTGGTGGGCAGAATGTGGCTGG | Barcode sequence is the same as human SMK Sample Tag 5 |
| Sample Tag 6–Flex | TTACCCGCAGGAAGACGTATACCCCTCGTGCCAGGCGACCAATGC | Barcode sequence is the same as human SMK Sample Tag 6 |

## BD® Flex Single-Cell Multiplexing Kit B (Catalog No. 633850)

Not for use together with Hu SMK Tags 7–12 (Catalog No. 633781) in the same experiment.

| Sample Tag | Sample Tag sequence | Notes |
|---|---|---|
| Sample Tag 7–Flex | TGTCTACGTCGGACCGCAAGAAGTGAGTCAGAGGCTGCACGCTGT | Barcode sequence is the same as human SMK Sample Tag 7 |
| Sample Tag 8–Flex | CCCCACCAGGTTGCTTTGTCGGACGAGCCCGCACAGCGCTAGGAT | Barcode sequence is the same as human SMK Sample Tag 8 |
| Sample Tag 9–Flex | GTGATCCGCGCAGGCACACATACCGACTCAGATGGGTTGTCCAGG | Barcode sequence is the same as human SMK Sample Tag 9 |
| Sample Tag 10–Flex | GCAGCCGGCGTCGTACGAGGCACAGCGGAGACTAGATGAGGCCCC | Barcode sequence is the same as human SMK Sample Tag 10 |
| Sample Tag 11–Flex | CGCGTCCAATTTCCGAAGCCCCGCCCTAGGAGTTCCCCTGCGTGC | Barcode sequence is the same as human SMK Sample Tag 11 |
| Sample Tag 12–Flex | GCCCATTCATTGCACCCGCCAGTGATCGACCCTAGTGGAGCTAAG | Barcode sequence is the same as human SMK Sample Tag 12 |

## BD® Flex Single-Cell Multiplexing Kit C (Catalog No. 633851)

Not for use together with Ms SMK Tags 1–6 (Catalog No. 633793) in the same experiment.

| **Sample Tag** | **Sample Tag sequence** | **Notes** |
|---|---|---|
| Sample Tag 13–Flex | AAGAGTCGACTGCCATGTCCCCTCCGCGGGTCCGTGCCCCCCAAG | Barcode sequence is the same as mouse SMK Sample Tag 1 |
| Sample Tag 14–Flex | ACCGATTAGGTGCGAGGCGCTATAGTCGTACGTCGTTGCCGTGCC | Barcode sequence is the same as mouse SMK Sample Tag 2 |
| Sample Tag 15–Flex | AGGAGGCCCCGCGTGAGAGTGATCAATCCAGGATACATTCCCGTC | Barcode sequence is the same as mouse SMK Sample Tag 3 |
| Sample Tag 16–Flex | TTAACCGAGGCGTGAGTTTGGAGCGTACCGGCTTTGCGCAGGGCT | Barcode sequence is the same as mouse SMK Sample Tag 4 |
| Sample Tag 17–Flex | GGCAAGGTGTCACATTGGGCTACCGCGGGAGGTCGACCAGATCCT | Barcode sequence is the same as mouse SMK Sample Tag 5 |
| Sample Tag 18–Flex | GCGGGCACAGCGGCTAGGGTGTTCCGGGTGGACCATGGTTCAGGC | Barcode sequence is the same as mouse SMK Sample Tag 6 |

## BD® Flex Single-Cell Multiplexing Kit D (Catalog No. 633852)

Not for use together with Ms SMK Tags 7–12 (Catalog No. 633793) in the same experiment.

| Sample Tag | Sample Tag sequence | Notes |
|---|---|---|
| Sample Tag 19–Flex | ACCGGAGGCGTGTGTACGTGCGTTTCGAATTCCTGTAAGCCCACC | Barcode sequence is the same as mouse SMK Sample Tag 7 |
| Sample Tag 20–Flex | TCGCTGCCGTGCTTCATTGTCGCCGTTCTAACCTCCGATGTCTCG | Barcode sequence is the same as mouse SMK Sample Tag 8 |
| Sample Tag 21–Flex | GCCTACCCGCTATGCTCGTCGGCTGGTTAGAGTTTACTGCACGCC | Barcode sequence is the same as mouse SMK Sample Tag 9 |
| Sample Tag 22–Flex | TCCCATTCGAATCACGAGGCCGGGTGCGTTCTCCTATGCAATCCC | Barcode sequence is the same as mouse SMK Sample Tag 10 |
| Sample Tag 23–Flex | GGTTGGCTCAGAGGCCCCAGGCTGCGGACGTCGTCGGACTCGCGT | Barcode sequence is the same as mouse SMK Sample Tag 11 |
| Sample Tag 24–Flex | CTGGGTGCCTGGTCGGGTTACGTCGGCCCTCGGGTCGCGAAGGTC | Barcode sequence is the same as mouse SMK Sample Tag 12 |

# Contact Information

**Becton, Dickinson and Company**
**BD Biosciences**
155 North McCarthy Boulevard
Milpitas, California 95035 USA

bdbiosciences.com
scomix@bdscomix.bd.com