BD Rhapsody™ System

mRNA Targeted and Sample Tag Library Preparation Protocol

For Research Use Only

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Regulatory Information

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

History

Revision	Date	Change made
23-24122(01)	2021-12	Initial release

Contents

Introduction

This protocol provides instructions on creating single cell mRNA and Sample Tag libraries after cell capture on the BD Rhapsody™ Single-Cell Analysis system or the BD Rhapsody™ Express Single-Cell Analysis system for sequencing on Illumina sequencers. For complete instrument procedures and safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063).

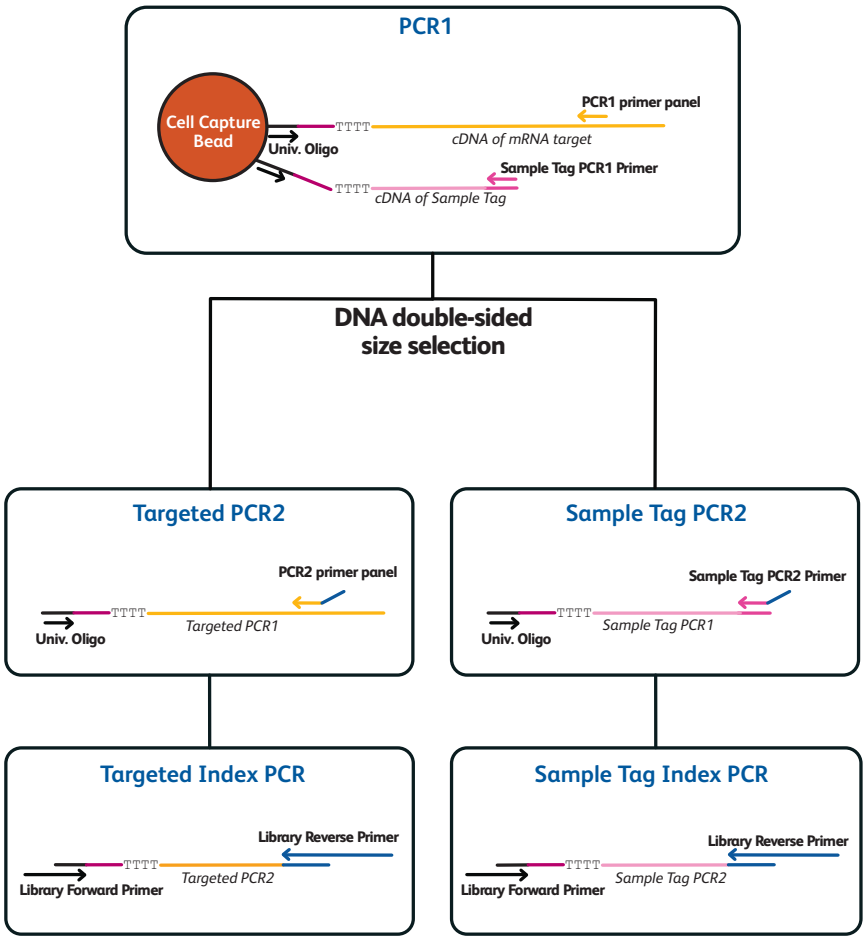
To create the libraries, Sample Tags and BD Rhapsody™ mRNA targets are encoded on the Enhanced Cell Capture Beads and then amplified in PCR1. After PCR1, the Sample Tag PCR1 products are separated from the mRNA targeted PCR1 products by double-sided size selection with Agencourt® AMPure® XP magnetic 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 mRNA and Sample Tag libraries requires that:

- The Sample Tag PCR1 products undergo a separate index PCR from mRNA products with library index primers.
- mRNA targeted PCR1 products and Sample Tag PCR1 products undergo PCR2 amplification followed by index PCR with library index primers.

After index PCR, the mRNA and Sample Tag libraries can be combined for sequencing.

Workflow



NOTE Univ. Oligo: Universal Oligo; region (dark purple) between universal oligo and poly(dT): cell label and Unique Molecular Identifier.

Required materials

- Exonuclease I-treated beads containing samples labeled with Sample Tags
- BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (Cat. no. 633774)

Kit component	Part number	Cap color
Nuclease-free water	650000076	Neutral
Bead RT/PCR Enhancer	91-1082	Black
PCR MasterMix	91-1083	White
Elution Buffer	91-1084	Pink
Universal Oligo	650000074	White
Library Forward Primer	91-1085	Red
Library Reverse Primer 1	650000080	Red
Library Reverse Primer 2	650000091	Red
Library Reverse Primer 3	650000092	Red
Library Reverse Primer 4	650000093	Red
Bead Resuspension Buffer	650000066	Black
Sample Tag PCR1 Primer	91-1088	Purple
Sample Tag PCR2 Primer	91-1089	Purple
BD® AbSeq Primer	91-1086	Green

- PCR1 primer panel
- PCR2 primer panel
- Agencourt® AMPure® XP magnetic beads (Beckman Coulter Life Sciences, Cat. no. A63880)
- Absolute ethyl alcohol, molecular biology grade (major supplier)
- Nuclease-free water (major supplier)

  NOTE The kit provides enough to prepare the PCR MasterMixes. You will need to purchase additional nuclease-free water for the AMPure purification steps.

- 6-Tube Magnetic Separation Rack for 1.5- mL tubes (New England Biolabs, Cat. no. S1506S)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. no. Q32851)

For a complete list of materials, see appropriate instrument user guide.

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents in the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (Cat. no. 633774) at room temperature (15 °C to 25 °C), and then place on ice.

Best practices

- Use low-retention filtered pipette tips.
- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes. **Never vortex the beads. Pipet-mix only.**
- Bring AMPure XP magnetic beads to room temperature before use.
- Remove supernatants without disturbing AMPure XP magnetic beads.

Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 214062)
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063)
- *BD® Single-Cell Multiomics Bioinformatics Handbook* (Doc ID 54169)

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* (Doc ID: 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID: 214063).

Procedure

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system following either the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* for cell capture, reverse transcription, and Exonuclease treatment.

Performing PCR1

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

Before use of BD Rhapsody™ 10X PCR1 Custom primers (Cat. no. 633743) and/or BD Rhapsody™ 10X PCR1 Supplement primers (Cat. no. 633742), 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.

PCR1 reaction mix

Component	For 1 library (µL) with 10% overage (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. no. 91-1083)	100.0	120.0
Universal Oligo (Cat. no. 650000074)	20.0	24.0
Bead RT/PCR Enhancer (Cat. no. 91-1082)	12.0	14.4
PCR1 primer panel[a]	40.0	48.0
(Optional) PCR1 panel supplement[a]	(10.0)	(12.0)
Sample Tag PCR1 Primer (Cat. no. 91-1088)	1.2	1.4
Nuclease-free water (Cat. no. 650000076)	Up to 26.8	Up to 32.2
Total	**200.0**	**240.0**

a. Order from BD Biosciences.

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Proceed as follows:

- Entire sample: Skip to **step 5**.
- Sub-sample: Proceed to **step 4**.

4 Sub-sample the Exonuclease I-treated beads:

   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 beads can be stored at 2 °C to 8 °C for ≤3 months.

5 Place tube of Exonuclease I-treated beads in Bead Resuspension Buffer (Cat. no. 650000066) on 1.5 mL magnet for <2 minutes.

   Remove supernatant.

6 Remove tube from magnet, and resuspend beads in 200 µL PCR1 reaction mix. Do not vortex.

7 Ensuring that the beads are fully resuspended, pipet 50 µL 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 reaction mix to the post-amplification workspace.

9 Program the thermal cycler. Do not use fast cycling mode:

Step	Cycles	Temperature	Time
Hot start	1	95 °C[a]	3 min
Denaturation	11-15[b]	95 °C	30 s
Annealing		60 °C	3 min
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

a. To avoid beads settling due to prolonged incubation time on 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, BD Biosciences has observed a step-skipping error with the pause/unpause functions. To ensure that the full three-minute denaturation is not skipped, verify that the pause/unpause functions are working correctly on your thermal cycler. To avoid the step-skipping problem, a one-minute 95 °C pause step can be added immediately before the three-minute 95 °C denaturation step.

b. Suggested PCR cycles might need to be optimized for different cell types and cell number.

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
1,000	14
2,500	13
5,000	12
10,000	11
20,000	10

10 Ramp heated lid and heat block of 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 tube in thermal cycler, and unpause the thermal cycler program.

STOPPING POINT: The PCR can run overnight but proceed with purification ≤24 hours after PCR.

12 After PCR, briefly centrifuge tubes.

13 Pipet-mix and combine the four reactions 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** (targeted mRNA PCR1 products and Sample Tag products) into the new 1.5 mL LoBind® tube without disturbing the beads.

NOTE (Optional) Remove the tube with the Enhanced Cell Capture Beads from magnet, and pipet 200 µL cold Bead Resuspension Buffer (Cat. no. 650000066) into tube. Pipet-mix. Do not vortex. Store beads at 2 °C to 8 °C in the post-amplification workspace.

Purifying PCR1 products by double-sided size selection

Perform double-sided AMPure bead purification to separate the shorter Sample Tag PCR1 products (~170 bp) from the longer mRNA targeted PCR1 products (350–800 bp).

In the protocol, keep both the supernatant (Sample Tag products) and the AMPure beads (mRNA targeted products) during purification workflow.

Perform the purification in the post-amplification workspace.

Separating sample tag PCR1 products from mRNA targeted PCR1 products

1 In a new 5.0 mL LoBind® tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4.0 mL absolute ethyl alcohol, molecular biology grade (major supplier) with 1.0 mL nuclease-free water (major supplier). Vortex tube for 10 seconds to mix.

   Make fresh 80% ethyl alcohol, and use it in ≤24 hours.

2 Bring Agencourt AMPure XP magnetic beads (Beckman Coulter Cat. no. A63880) to room temperature. Vortex at high speed for 1 minute until beads are fully resuspended.

3 Pipet 140 µL AMPure XP beads into a tube with 200 µL mRNA targeted PCR1 products and Sample Tag products (**step 14** of Performing PCR1). Pipet-mix 10 times.

4 Incubate at room temperature for 5 minutes.

5 Place 1.5 mL LoBind® tube on magnet for 5 minutes.

6 Keeping tube on magnet, transfer the 340 µL supernatant (Sample Tag PCR1 products) to a new 1.5 mL tube without disturbing beads (mRNA targeted PCR1 products).

7 Store the supernatant (step 6) at room temperature while purifying and eluting the mRNA targeted products in **Purifying mRNA targeted PCR1 products**. Purify the Sample Tag PCR1 products after purifying the mRNA targeted PCR1 products.

Purifying mRNA targeted PCR1 products

1 Keeping tube on magnet, gently add 500 µL fresh 80% ethyl alcohol to the tube of AMPure beads bound with mRNA targeted PCR1 products, and incubate 30 seconds. Remove supernatant.

2 Repeat step 1 once for two washes.

3 Keeping tube on magnet, use a small-volume pipette to remove residual supernatant from tube, and discard.

4 Air-dry beads at room temperature for 5 minutes.

5 Remove tube from magnet, and resuspend bead pellet in 30 µL Elution Buffer (Cat. no. 91-1084) into tube. Vigorously pipet-mix until beads are uniformly dispersed. AMPure bead clumping is normal at this step and does not affect performance.

6 Incubate at room temperature for 2 minutes, and briefly centrifuge.

7 Place tube on magnet until solution is clear, usually ≤30 seconds.

8 Pipet the eluate (~30 µL) into a new 1.5 mL LoBind® tube (purified mRNA targeted PCR1 products).

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

Purifying sample tag PCR1 products

1 Pipet 100 µL AMPure XP beads into the tube with 340 µL Sample Tag PCR1 products from **step 6** of Separating Sample Tag PCR1 products from mRNA targeted PCR1 products. Pipet-mix 10 times.

2 Incubate at room temperature for 5 minutes.

3 Place on magnet for 5 minutes.

4 Keeping tube on magnet, remove supernatant.

5 Keeping tube on magnet, gently add 500 µL fresh 80% ethyl alcohol, and incubate 30 seconds. Remove supernatant.

6 Repeat **step 5** once for two washes.

7 Keeping tube on magnet, use a small-volume pipette to remove residual supernatant from tube, and discard.

8 Air-dry beads at room temperature for 5 minutes.

9 Remove tube from magnet, and resuspend bead pellet in 30 µL Elution Buffer (Cat. no. 91-1084). Vigorously pipet-mix until beads are uniformly dispersed. Small clumps do not affect performance.

10 Incubate at room temperature for 2 minutes, and briefly centrifuge.

11 Place tube on magnet until solution is clear, usually ≤30 seconds.

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

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

Performing PCR2 on the PCR1 products

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

Before use of BD Rhapsody™ 10X PCR2 Custom primers and/or BD Rhapsody™ 10X PCR2 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.

mRNA targeted PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. no. 91-1083)	25.0	30.0
Universal Oligo (Cat. no. 650000074)	2.0	2.4
PCR2 primer panel[a]	10.0	12.0
(Optional) PCR2 panel supplement[a]	(2.5)	(3.0)
Nuclease-Free Water (Cat. no. 650000076)	Up to 8.0	Up to 9.6
Total	**45.0**	**54.0**

a. Order from BD Biosciences.

Sample Tag PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. no. 91-1083)	25.0	30.0
Universal Oligo (Cat. no. 650000074)	2.0	2.4
Sample Tag PCR2 Primer (Cat. no. 91-1089)	3.0	3.6
Nuclease-Free Water (Cat. no. 650000076)	15.0	18.0
Total	**45.0**	**54.0**

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Bring PCR2 reaction mixes to post-amplification workspace.

4 In two separate, new 0.2 mL PCR tubes:

a mRNA targeted PCR1 products: Pipet 5.0 µL products into 45 µL mRNA targeted PCR2 reaction mix.

b Sample Tag PCR1 products: Pipet 5.0 µL products into 45 µL Sample Tag PCR2 reaction mix.

5 Gently vortex, and briefly centrifuge.

6 For mRNA targeted PCR 1 products, program the thermal cycler. **Do not use fast cycling mode:**

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

a. Cycle number might require optimization according to cell number and type.

For Sample Tag PCR2, program the thermal cycler. **Do not use fast cycling mode:**

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10[a]	95 °C	30 s
Annealing		66 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

a. Cycle number might require optimization according to cell number and type.

STOPPING POINT: The PCR can run overnight.

Purifying mRNA targeted and sample tag PCR2 products

Perform purification in the post-amplification workspace.

1 Bring AMPure XP beads to room temperature, and vortex at high speed 1 minute until beads are fully resuspended.

2 Briefly centrifuge mRNA targeted PCR2 products.

3 To 50.0 µL PCR2 products, pipet:

- mRNA targeted PCR2 products: 40 µL AMPure beads.
- Sample Tag PCR2 products: 60 µL AMPure beads.

4 Pipet-mix 10 times, and incubate at room temperature for 5 minutes.

5 Place each tube on strip tube magnet for 3 minutes. Remove supernatant.

6 Keeping tube on magnet, for each tube, gently add 200 µL fresh 80% ethyl alcohol into tube, and incubate 30 seconds. Remove supernatant.

7 Repeat **step 6** once for two washes.

8 Keeping each tube on magnet, use a small-volume pipette to remove residual supernatant from tube, and discard.

9 Air-dry beads at room temperature for 3 minutes.

10 Remove each tube from magnet, and resuspend each bead pellet in 30 µL Elution Buffer (Cat. no. 91-1084). Pipet-mix until beads are fully resuspended.

11 Incubate at room temperature for 2 minutes, and briefly centrifuge.

12 Place each tube on magnet until solution is clear, usually ≤30 seconds.

13 Pipet entire eluate (~30 µL) of each sample into two separate new 1.5 mL LoBind® tubes (purified mRNA targeted PCR2 and Sample Tag PCR2 products).

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

14 Estimate the concentration of each sample by quantifying 2 µL of the targeted mRNA PCR2 and Sample Tag PCR2 products with a Qubit™ Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

15 Dilute an aliquot of the products with Elution Buffer (Cat. no. 91-1084):

- mRNA targeted PCR2 products: 0.2–2.7 ng/µL.
- Sample Tag PCR2 products: 0.1–1.1 ng/µL.

Performing index PCR to prepare final libraries

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

For a single cartridge or sample, consider using the same index for all libraries for that cartridge or sample. If libraries are to be indexed differently, make separate index PCR mixes containing different library reverse primers for each library type.

Index PCR mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. no. 91-1083)	25.0	30.0
Library Forward Primer (Cat. no. 91-1085)	2.0	2.4
Library Reverse Primer 1-4 (Cat. no. 650000080, 650000091-93)	2.0	2.4
Nuclease-free water (Cat. no. 650000076)	18.0	21.6
Total	**47.0**	**56.4**

2 Gently vortex mix, briefly centrifuge, and place back on ice.

3 Bring index PCR mixes into post-amplification workspace.

4 In two separate and new 0.2 mL PCR tubes:

a mRNA targeted PCR2 products: Pipet 3.0 µL of 0.2–2.7 ng/µL products into 47.0 µL index PCR mix.

b 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 Program the thermal cycler. **Do not use fast cycling mode:**

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	6-8[a]	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	30 s
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

a. Suggested PCR cycles.

Suggested PCR cycles

Concentration index PCR input for 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.

Purifying index PCR products

Perform the purification in the post-amplification workspace.

1 Bring AMPure XP beads to room temperature, and vortex at high speed 1 minute until beads are fully resuspended.

2 Briefly centrifuge index PCR products.

3 To 50.0 µL of the index PCR products pipet:

- mRNA targeted library: 35 µL AMPure beads.
- Sample Tag library: 40 µL AMPure beads.

4 Pipet-mix 10 times, and incubate at room temperature for 5 minutes.

5 Place tubes on strip tube magnet for 3 minutes. Remove supernatant.

6 Keeping tube on magnet, for each tube, gently add 200 µL fresh 80% ethyl alcohol into tube, and incubate 30 seconds. Remove supernatant.

7 Repeat **step 6** once for two washes.

8 Keeping tubes on magnet, use a small-volume pipette to remove residual supernatant from tube, and discard.

9 Air-dry beads at room temperature for 3 minutes.

10 Remove tubes from magnet, and resuspend each pellet in 30 µL Elution Buffer (Cat. no. 91-1084). Pipet-mix until beads are fully resuspended.

11 Incubate at room temperature for 2 minutes, and briefly centrifuge.

12 Place tubes on magnet until solution is clear, usually ≤30 seconds.

13 For each tube, pipet entire eluate (~30 µL) into two separate new 1.5 mL LoBind® tubes (final sequencing libraries).

14 Perform quality control before freezing samples. See **Performing quality control on the final sequencing libraries**.

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

Performing quality control on the final sequencing libraries

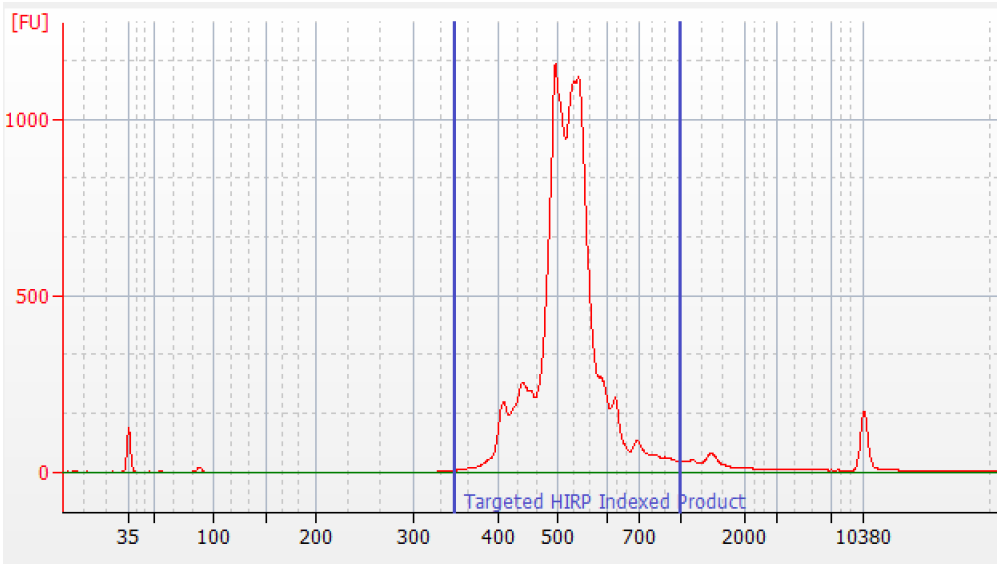
1 Estimate the concentration by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer. Follow the manufacturer's instructions. The expected concentration of the libraries is >1.5 ng/µL.

2 Measure the average fragment size of the mRNA targeted library within the size range of 350–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. no. 5067-4626) for 50–7,000 bp, 5–1,000 pg/µL. The Bioanalyzer is used to calculate molarity for the targeted library because of the distribution of fragment sizes for this library type. Follow the manufacturer's instructions.

The Sample Tag library should show a peak of -270 bp. The BD Rhapsody™ mRNA targeted library shows a fragment distribution that depends on the panel used. For example, with peripheral blood mononuclear cells (PBMCs):

Figure 1 Targeted human immune response panel (HIRP) indexed product

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D5000 trace

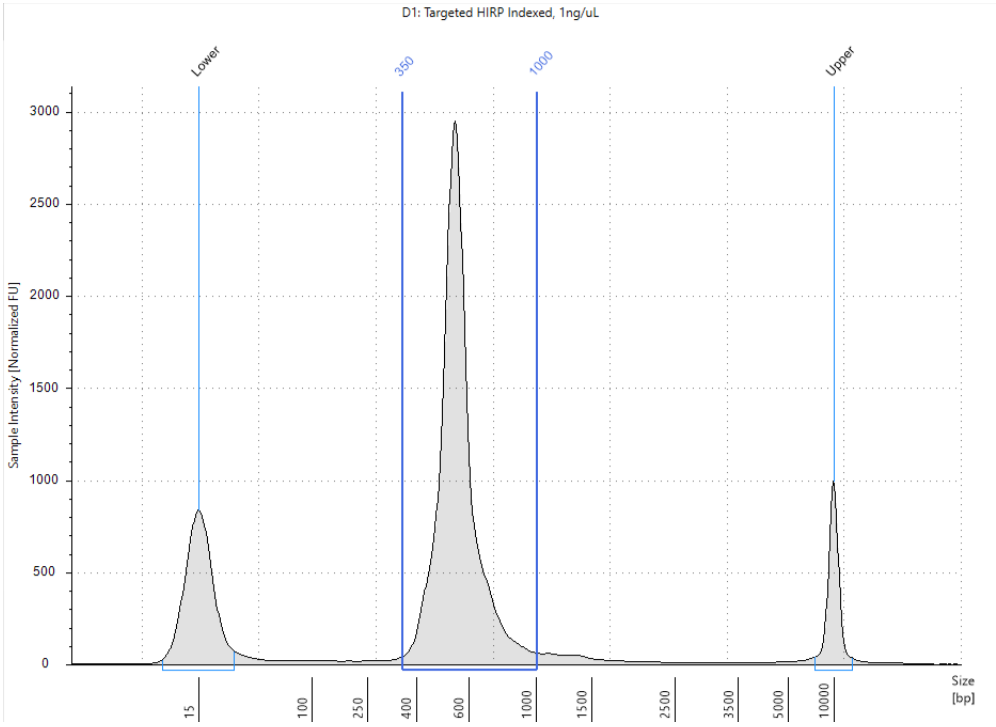
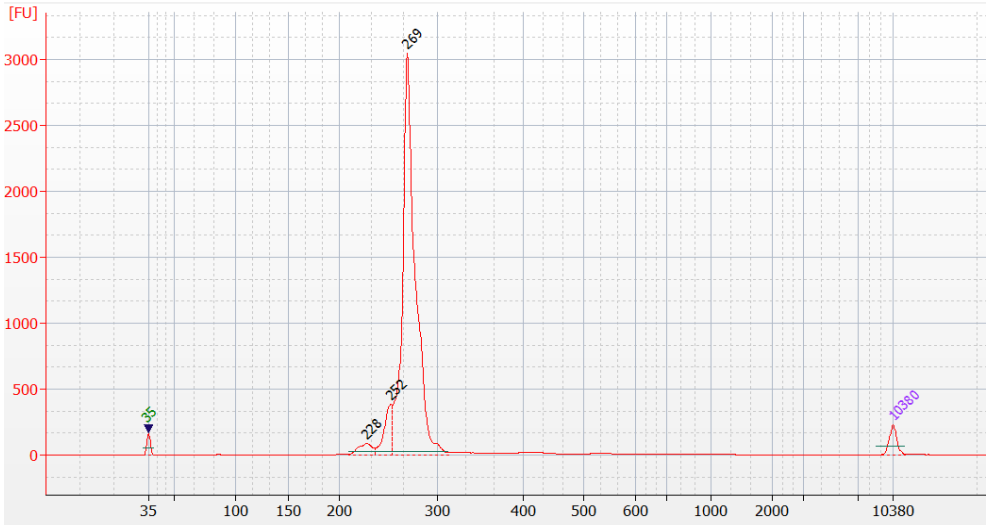
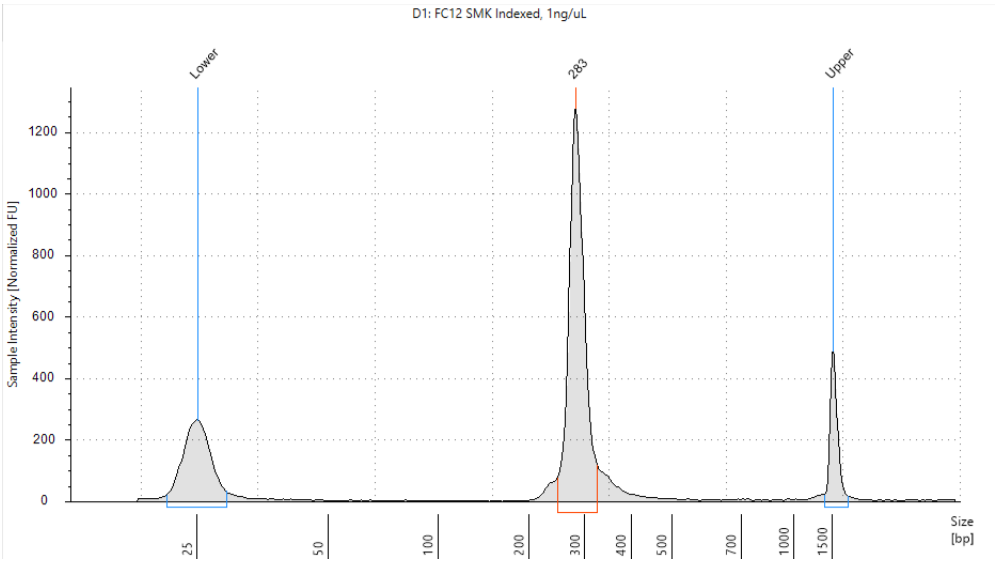


Figure 2 SMK indexed product

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



3 If the concentration or size of the library is outside of the expected range, see **Library preparation on page 23** or contact BD Biosciences technical support at researchapplications@bd.com.

Sequencing

Requirements

- Run setup for Illumina® BaseSpace and sample sheet sequencing. Enter the pooled libraries as one sample if both libraries were made with the same Library Reverse primer or if both libraries share the same i7 index.
- Required parameters:

Parameter	Requirement
Platform	Illumina[a]
Paired-end reads	Recommend Read 1: 51 cycles; Read 2: 71 cycles
PhiX	1% recommended
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook* (Doc ID: 54169)

a. To review Illumina Index 1 (i7) sequences, see **Appendix A: Sample sequences on 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 1.5-1.8 pM with 1% PhiX for a sequencing run.
- 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:
  - 2,000 reads per cell for clustering by cell type identification
  - 20,000 reads per cell for deep sequencing
- Sequencing amount for Sample Tag libraries:
  - Pooling samples of the same type: 120 reads/cell; for example, combining different donor PBMCs.
  - Pooling different sample types: 600 reads/cell; for example, combining Jurkat cells with PBMCs.

NOTE 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 BD Biosciences technical support at scomix@bdscomix.bd.com.

Troubleshooting

Library preparation

Observation	Possible causes	Recommended solutions
PCR2 product yield too low.	PCR1 and PCR2 primers might have been swapped by mistake.	Ensure that 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.
	Enhanced Cell Capture Beads not fully resuspended immediately before PCR1.	Gently pipet-mix 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 Agencourt 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 Agencourt 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: – Load 1 µL of purified PCR2 product at 1 ng/µL in a High Sensitivity DNA Chip on the Agilent Bioanalyzer. – Follow the manufacturer's instructions. 2. Confirm that the mRNA targeted PCR2 products should show an average size range of 350–600 bp and the Sample Tag PCR2 products should show an average size of ~190 bp. 3. If the products pass quality control, proceed to **Performing index PCR to prepare final libraries on page 16**. Repeat the index PCR. If the products do not pass quality control, contact BD Biosciences technical support at researchapplications@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. • Input amount of PCR2 products too high.	Repeat the index PCR with a lower input of mRNA targeted PCR2 products.
	Upper and lower markers on the Agilent Bioanalyzer is 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.

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.	Suboptimal cluster density and/or library denaturation.	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: 120 reads/cell. – Pooled samples of different cell types: 600 reads/cell. 2. Repeat sequencing. If issue persists, contact BD Biosciences technical support at researchapplications@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 instrument user guides.

Appendix A: Sample sequences

Sample tag sequences

Human sample tag

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 and the Sample Tag sequence:

GTTGTCAAGATGCTACCGTTCAGAG[**Sample Tag sequence**]AAAAAAAAAAAAAAAAAAAAAAAA

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

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 and the Sample Tag sequence:

GTTGTCAAGATGCTACCGTTCAGAG[**Sample Tag sequence**]AAAAAAAAAAAAAAAAAAAAAAAA

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

Illumina index 1 (i7) sequences

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

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