BD Rhapsody™ System

mRNA Targeted

Library Preparation Protocol

23-24121(02)
2022-11

For Research Use Only

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.

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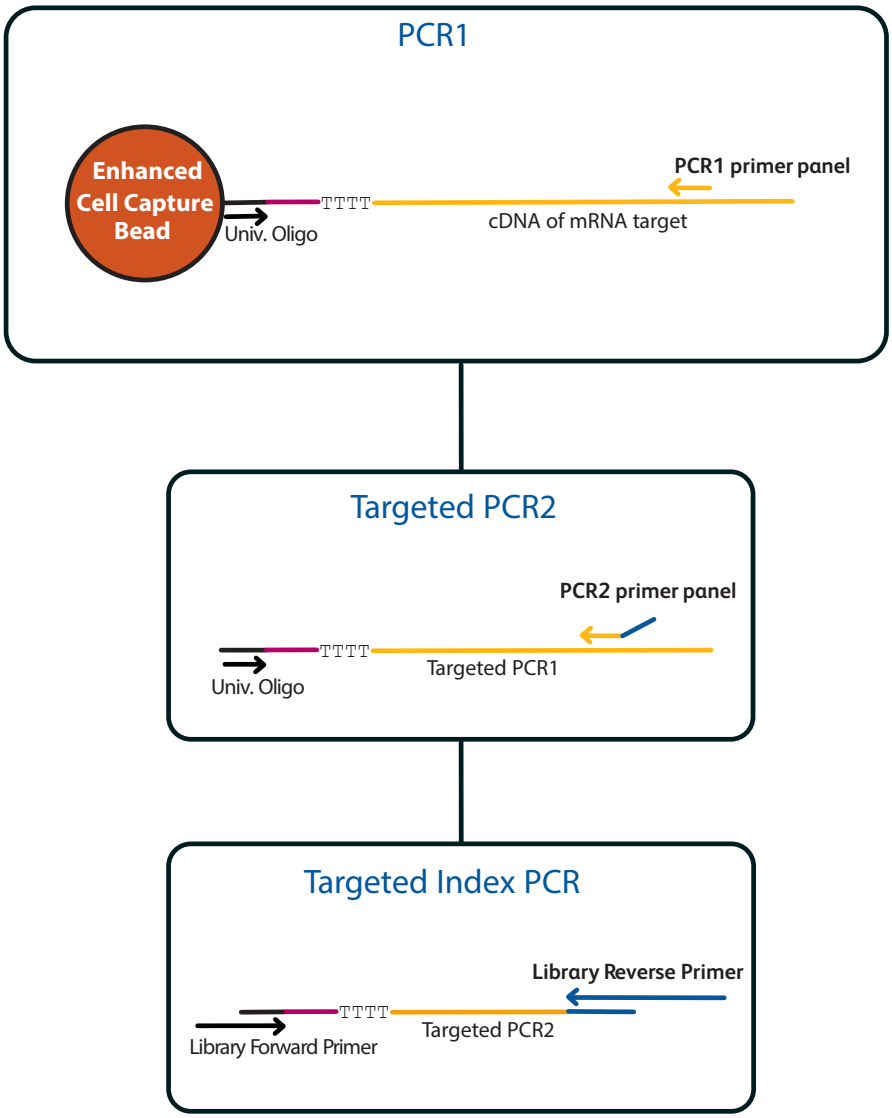
Introduction

This protocol provides instructions on creating a single cell mRNA library 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* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*.

The cDNA of mRNA targets is first encoded on the BD Rhapsody™ Enhanced Cell Capture Beads as described in the instrument user guides. This targeted library approach employs a two-step nested amplification followed by an index PCR step. The generated library can be sequenced on various Illumina sequencers.

Workflow



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

Required and recommended materials

Required reagents

Material	Supplier	Catalog no.
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit[a]	BD Biosciences	633774
Targeted mRNA PCR Panel[a,b]	BD Biosciences	various
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
Nuclease-free water	Major supplier	–
Refer to the Technical Bulletin *Ordering Additional Indexes for the BD Rhapsody™ Library Reagent Kits* to order additional indexing primers for high throughput library preparation workflows.		
a. For processing more than four libraries, two orders of this catalog number are required. b. Examples of panels - Human or Mouse Immune Response Panel. Contact your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com for custom or other available panels.		

Recommended consumables

Material	Supplier	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[a]	Corning	PCR96HSC
Or,		
MicroAmp Optical 96-Well Reaction Plate[a]	Thermo Fisher Scientific	N8010560
MicroAmp Clear Adhesive Film[a]	Thermo Fisher Scientific	4306311
DNA LoBind® tubes, 1.5-mL	Eppendorf	0030108051
DNA LoBind® tubes, 5.0-mL	Eppendorf	0030108310
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent High Sensitivity DNA	Agilent	5067-4626
Or,		
Agilent High Sensitivity D1000 ScreenTape	Agilent	5067-5584
Agilent High Sensitivity D1000 Reagents	Agilent	5067-5585
Or,		
Agilent High Sensitivity D5000 ScreenTape	Agilent	5067-5592
Agilent High Sensitivity D5000 Reagents	Agilent	5067-5593

a. Recommended for processing high throughput 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	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
6-tube magnetic separation rack for 1.5-mL tubes Or, 12-tube Magnetic Separation Rack[a] Or, Invitrogen™ DynaMag™-2 Magnet[a]	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[b]	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

a. Recommended for processing greater than 6 samples.
b. Recommended for processing high throughput library preparation workflows.

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 at room temperature (15–25 °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*
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*
- *BD® Single-Cell Multiomics Bioinformatics Handbook*

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*.

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 the following reagents into a new 1.5-mL LoBind® tube on ice.

Note: Before use of BD Rhapsody™ 10X PCR1 Custom primers and/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.

PCR1 reaction mix

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 MasterMix	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 primer panel[a,b]	40.0	48.0	192.0	384.0
(Optional) PCR1 panel supplement[a,b]	(10.0)	(12.0)	(48.0)	(96.0)
Nuclease-free water	Up to 28.0	Up to 33.6	Up to 134.4	Up to 268.8
Total	**200.0**	**240.0**	**960.0**	**1,920.0**

a. Order from BD Biosciences.
b. 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, 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 2–8 °C for up to 3 months.

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 Ensuring that the beads are fully resuspended, 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 Program the thermal cycler. **Do not use fast cycling mode**.

Program thermal cycler

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 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 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.

Suggested number of PCR cycles

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12
10,000	11
20,000	10

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.

Note: 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 tube in the thermal cycler, and unpause the thermal cycler program.

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

12 After PCR, briefly centrifuge the tubes.

13 Pipet-mix and combine the four reactions from the same sample into a new 1.5-mL LoBind® tube.

Retain the supernatant in the next step.

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

Note: (Optional) Remove the tube with the BD Rhapsody™ Enhanced Cell Capture Beads from the magnet, and pipet 200 µL cold Bead Resuspension Buffer into the tube. Pipet-mix. Do not vortex. Store beads at 2–8 °C in the post-amplification workspace.

Purifying mRNA targeted PCR1 products

Note: Perform the purification in the post-amplification workspace.

1 In a new 5.0-mL LoBind® tube, prepare 5 mL of 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 the tube for 10 seconds to mix.

Note: Make fresh 80% ethyl alcohol and use it within 24 hours. 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**

2 Bring Agencourt AMPure XP magnetic beads to room temperature. Vortex at high speed for 1 minute until the beads are fully resuspended.

3 Pipet 140 µL of AMPure beads into the tube with 200 µL mRNA targeted PCR1 products (**step 14** on page 11 of Performing PCR1). Pipet-mix 10 times.

4 Incubate at room temperature for 5 minutes.

5 Place the 1.5-mL LoBind® tube on the magnet for 5 minutes. Remove and discard the supernatant.

6 Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol into the tube, and incubate for 30 seconds. Remove and discard the supernatant.

7 Repeat **step 6** once for a total of two washes.

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

9 Air-dry the beads at room temperature for 5 minutes.

10 Remove the tube from the magnet and resuspend the bead pellet in 30 µL of Elution Buffer. Vigorously pipet-mix until the beads are uniformly dispersed. AMPure bead clumping is normal at this step and does not affect performance.

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

12 Place the tube on the magnet until the solution is clear, usually within 30 seconds.

13 Pipet the eluate (~30 µL) into a new 1.5-mL LoBind® tube (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.

Performing PCR2 on mRNA targeted PCR1 products

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

Note: 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.

PCR2 reaction mix

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 MasterMix	25.0	30.0	120.0	240.0
Universal Oligo	2.0	2.4	9.6	19.2
PCR2 primer panel[a,b]	10.0	12.0	48.0	96.0
(Optional) PCR2 panel supplement[a,b]	(2.5)	(3.0)	(12.0)	(24.0)
Nuclease-free water	Up to 8.0	Up to 9.6	Up to 38.4	Up to 76.8
Total	**45.0**	**54.0**	**216.0**	**432.0**

a. Order from BD Biosciences.
b. 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, and place back on ice.

3 Bring the PCR2 reaction mix into the post-amplification workspace.

4 In a new 0.2-mL PCR tube, pipet 5.0 µL of purified PCR1 products into 45 µL PCR2 reaction mix.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler. Do not use fast cycling mode.

Program thermal cycler

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.

STOPPING POINT: The PCR can run overnight.

Purifying mRNA targeted PCR2 products

Note: Perform 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 mRNA targeted PCR2 products.

3 Pipet 40 µL AMPure XP beads into the tube with 50 µL PCR2 products (**step 6** of Performing PCR2 on mRNA targeted PCR1 products on page 12). Pipet-mix 10 times.

4 Incubate at room temperature for 5 minutes.

5 Place the tube on the strip tube magnet for 3 minutes. Remove and discard the supernatant.

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.

7 Repeat **step 6** once for a total of two 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.

10 Remove the tube from the magnet and resuspend the bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.

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

12 Place the tube on the magnet until the solution is clear, usually within 30 seconds.

13 Pipet the entire eluate (~30 µL) into a new 1.5-mL LoBind® tube (purified mRNA targeted 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 Estimate the concentration by quantifying 2 µL of the mRNA targeted PCR2 products with a Qubit™ Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

15 Dilute an aliquot of mRNA targeted PCR2 products to 0.2–2.7 ng/µL with Elution Buffer.

Performing index PCR to prepare final libraries

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

Index PCR mix

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 MasterMix	25.0	30.0	120.0	240.0
Library Forward Primer	2.0	2.4	9.6	19.2
Library Reverse Primer 1-4[a]	2.0	2.4	–	–
Nuclease-free water	18.0	21.6	86.4	172.8
Total	**47.0**	**56.4**	**216.0**	**432.0**

a. For more than one library, use different Library Reverse Primers for each library.

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

3 Bring the index PCR mix to the post-amplification workspace.

4 In a new 0.2-mL PCR tube, pipet 3.0 µL of 0.2–2.7 ng/µL of mRNA targeted PCR2 products into 47.0 µL of index PCR mix.

5 Gently vortex, and briefly centrifuge.

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

Program thermal cycler

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)	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.

Purifying index PCR products

Note: Perform the 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 all of the index PCR products.
3. Pipet 35.0 µL AMPure XP beads into the tube with 50.0 µL index PCR products. Pipet-mix 10 times.
4. Incubate at room temperature for 5 minutes.
5. Place tube on the strip tube magnet for 3 minutes. Remove and discard the supernatant.
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.
7. Repeat **step 6** once for a total of two washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
9. Air-dry the beads at room temperature for 3 minutes.
10. Remove the tube from the magnet, and resuspend the bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
11. Incubate at room temperature for 2 minutes, and then briefly centrifuge.
12. Place the tube on the magnet until the solution is clear, usually within 30 seconds.
13. Pipet entire eluate (~30 µL) into a new 1.5-mL LoBind® tube (final sequencing library).
14. Perform quality control before freezing sample. See Performing quality control on final sequencing library on page 16.

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

Performing quality control on final sequencing library

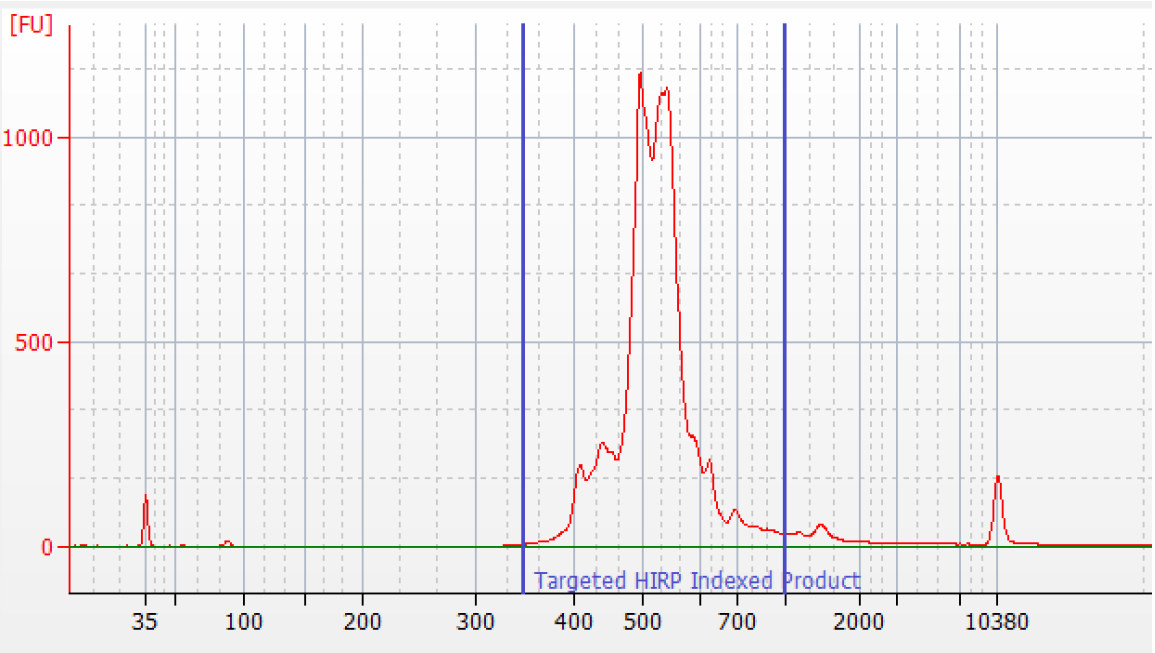
1 Estimate the concentration by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer or Agilent 4200 TapeStation. Follow the manufacturer's 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 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.

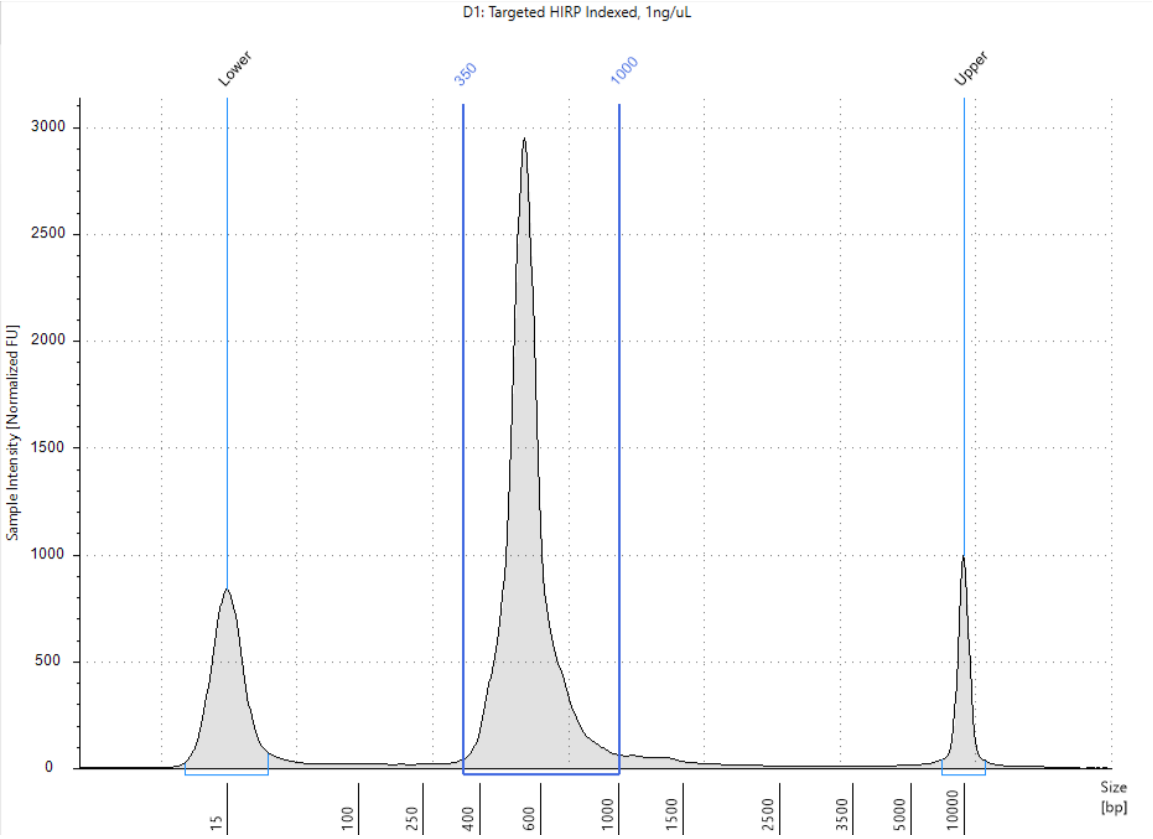
   The final mRNA targeted library should show a fragment distribution that depends on the panel used. For example, with peripheral blood mononuclear cells (PBMCs):

Figure 1 BD Rhapsody™ immune response panel Hs (human)

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D5000 trace



3 If the concentration or size of the library is outside of the expected range, see Library preparation on page 20 or contact your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com.

Sequencing

Sequencing depth is dependent on application. For cell type clustering, shallow sequencing is sufficient. For in-depth analysis, such as comparison across multiple libraries, deep sequencing is recommended. We recommend meeting the requirement for recursive substitution error correction (RSEC) sequencing depth of ≤6 in order to reach the threshold of sequencing saturation where most molecules of the library have been recovered. RSEC sequencing depth is reported by the analysis pipeline. The actual sequencing reads/cell required to achieve this depth can vary because it depends on the chosen gene panel, number of cells, and sequencing run quality.

Requirements

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.

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*
a. To review Illumina Index 1 (i7) sequences, see Appendix on page 22.	

Sequencing recommendations

- 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[a]
a. 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.	

Note: To determine the ratio of BD Rhapsody™ targeted mRNA libraries to pool for sequencing, use the sequencing calculator available by contacting your local Field Application Specialist (FAS) or 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.
	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 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. 3. If the products pass quality control, proceed to Performing index PCR to prepare final libraries on page 14. 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. 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.

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, or library denaturation, or both.	• See troubleshooting in Illumina documentation.

Appendix

Illumina index 1 (i7) sequences

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

Becton, Dickinson and Company
BD Biosciences
2350 Qume Drive
San Jose, California 95131 USA

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scomix@bdscomix.bd.com