

BD Rhapsody™ System

mRNA Targeted and BD® AbSeq 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
23-24123(01)	2021-12	Initial release

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Introduction

This protocol provides instructions on creating mRNA and BD[®] AbSeq single cell libraries with 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).

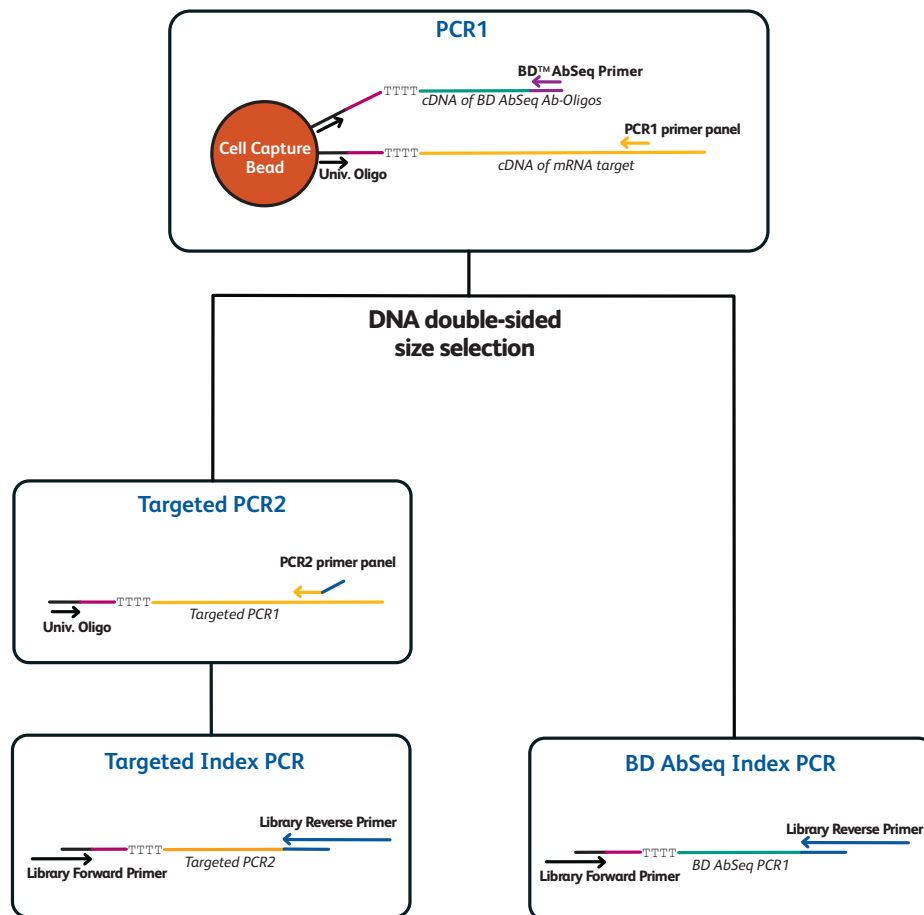
To create the libraries, the BD[®] AbSeq and BD Rhapsody[™] mRNA targets are encoded on the Enhanced Cell Capture Beads and then amplified in PCR1. After PCR1, the BD[®] AbSeq PCR1 products are separated from the mRNA targeted PCR1 products by double-sided size selection with Agencourt[®] AMPure[®] XP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

- The BD[®] AbSeq PCR1 products undergo a separate index PCR from mRNA products with library index primers.
- BD Rhapsody[™] mRNA targeted PCR1 products undergo PCR2 amplification followed by index PCR with library index primers.

After index PCR, the BD Rhapsody[™] mRNA and BD[®] AbSeq 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 BD[®] AbSeq Ab-Oligos
- 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 System 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)	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)
BD® AbSeq Primer (Cat. no. 91-1086)	12.0	14.4
Nuclease-free water (Cat. no. 650000076)	Up to 16.0	Up to 19.2
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, number of antibodies in BD® AbSeq panel, and cell number.

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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 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** (mRNA targeted PCR1 products and BD® AbSeq PCR1 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 μ L cold Bead Resuspension Buffer (Cat. no. 650000066) into the 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 AbSeq PCR1 products (~154 bp) from the longer mRNA targeted PCR1 products (350–800 bp).

In the protocol, keep both the supernatant (BD® AbSeq products) and the AMPure beads (mRNA targeted products) for purification.

Perform the purification in the post-amplification workspace.

Separating BD[®] AbSeq 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 BD[®] AbSeq PCR1 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 (BD[®] AbSeq 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 mRNA targeted PCR1 products in **Purifying mRNA targeted PCR1 products**. Purify the BD[®] AbSeq PCR1 products in Purifying the BD[®] AbSeq 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 BD[®] AbSeq PCR1 products

- 1 Pipet 100 µL AMPure XP beads into the tube with 340 µL BD[®] AbSeq PCR1 products from **step 6** of Separating BD[®] AbSeq 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, and discard.
- 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 BD[®] AbSeq PCR1 products).

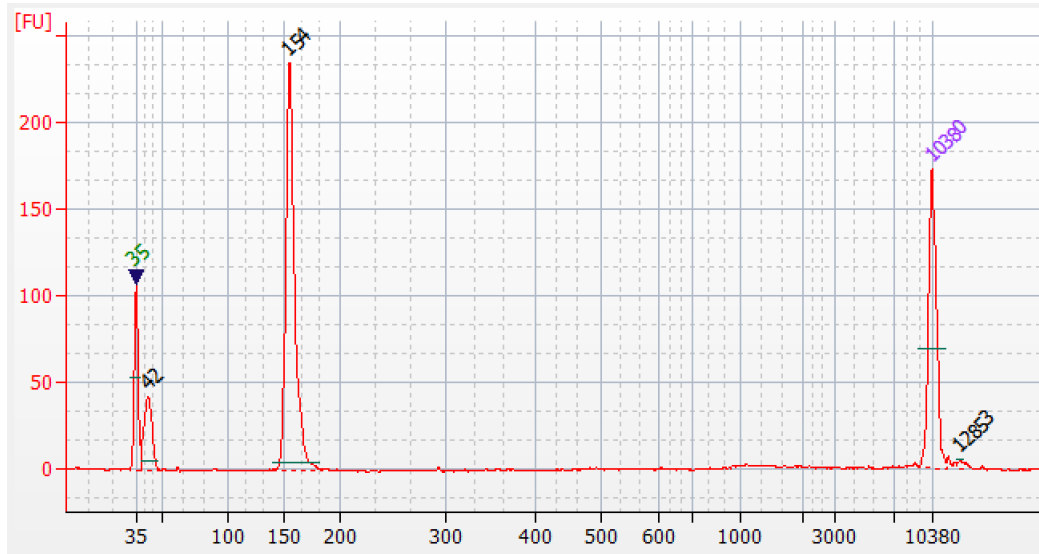
STOPPING POINT: Store at 2 °C to 8 °C before proceeding in ≤24 hours or at -25 °C to -15 °C for ≤6 months.

Quantifying BD[®] AbSeq PCR1 products

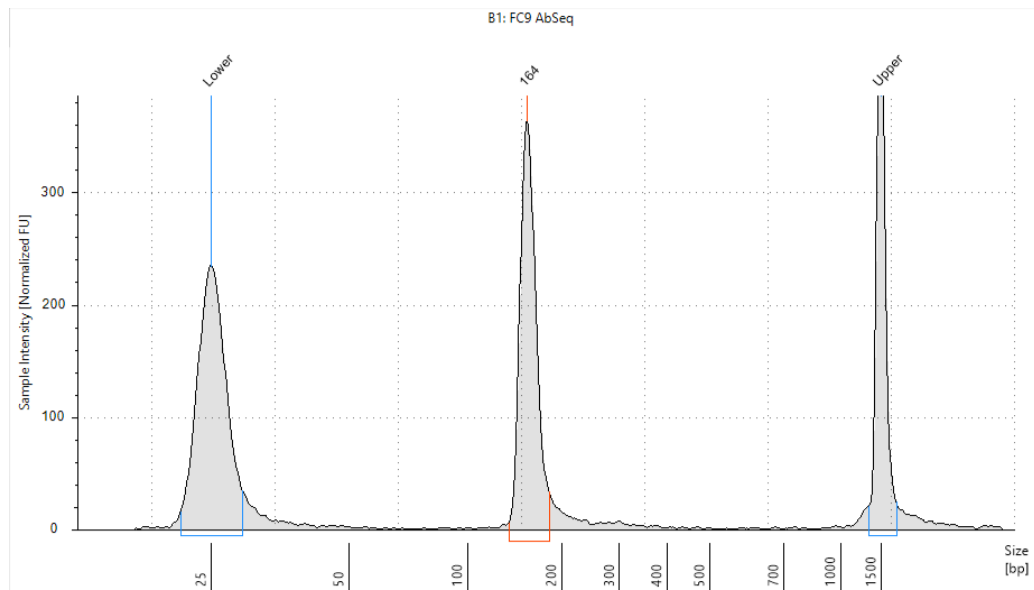
- 1 Measure the yield of the largest peak of the BD[®] AbSeq PCR1 products (~154 bp) by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. no. 5067-4626). Follow the manufacturer's instructions:

Figure 1 BD[®] AbSeq PCR1 products

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



- 2 Dilute an aliquot of BD[®] AbSeq products to 0.1–1.1 ng/μL with Elution Buffer (Cat. no. 91-1084) before index PCR of BD[®] AbSeq PCR1 products.

Performing PCR2 on the mRNA targeted PCR1 products

NOTE Only the mRNA targeted PCR1 products require PCR2 amplification. The BD[®] AbSeq PCR1 products require only index PCR.

- 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.

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.		

- 2 Gently vortex mix, briefly centrifuge, and place back on ice.
- 3 Bring PCR2 reaction mixes to post-amplification workspace.
- 4 In a new 0.2 mL PCR tube, pipet 5.0 µL purified mRNA targeted 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:**

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

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 Pipet 40 µL AMPure XP beads into tube with 50 µL the mRNA targeted PCR2 products. Pipet-mix 10 times:
- 4 Incubate at room temperature for 5 minutes.
- 5 Place tube on strip tube magnet for 3 minutes. Remove supernatant.
- 6 Keeping tube on magnet, 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 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.

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- 13 Pipet entire eluate (~30 μL) into a new 1.5 mL LoBind[®] tube (purified mRNA targeted 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 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 (Cat. no. 91-1084).

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 BD[®] AbSeq PCR1 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.

- Bring AMPure XP beads to room temperature, and vortex at high speed 1 minute until beads are fully resuspended.
- Briefly centrifuge index PCR products.
- To 50.0 μL of the index PCR products pipet:
 - mRNA targeted library: 35 μL AMPure beads.
 - BD[®] AbSeq library: 40 μL AMPure beads.
- Pipet-mix 10 times, and incubate at room temperature for 5 minutes.
- Place tubes on strip tube magnet for 3 minutes. Remove supernatant.
- 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 μ L 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 \leq 30 seconds.
- 13 For each tube, pipet entire eluate (\sim 30 μ L) 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 \leq 6 months until sequencing.

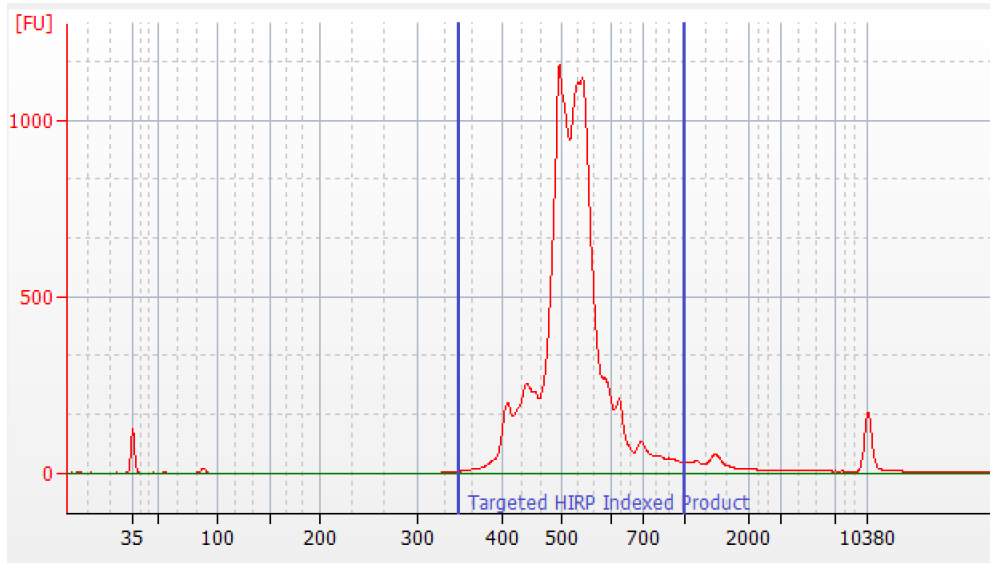
Performing quality control on the final sequencing libraries

- 1 Estimate the concentration by quantifying 2 μ L 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/ μ L.
- 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/ μ L. 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.

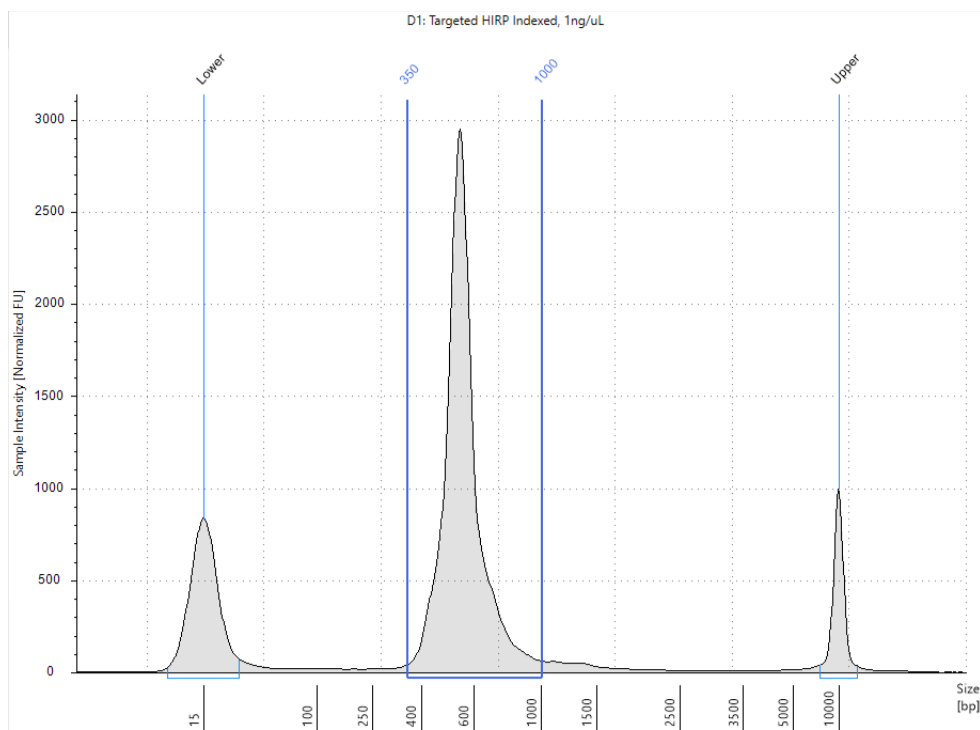
The final mRNA targeted library should show a fragment distribution on the panel used. For example, with peripheral blood mononuclear cells (PBMCs):

Figure 2 Targeted human immune response panel (HIRP) indexed product

A. Sample Bioanalyzer high-sensitivity DNA trace



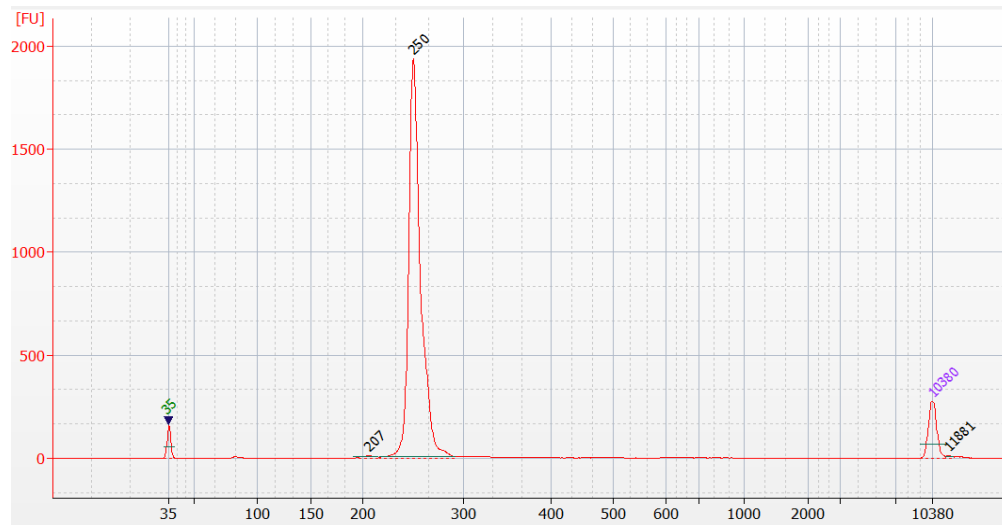
B. Sample TapeStation high-sensitivity D5000 trace



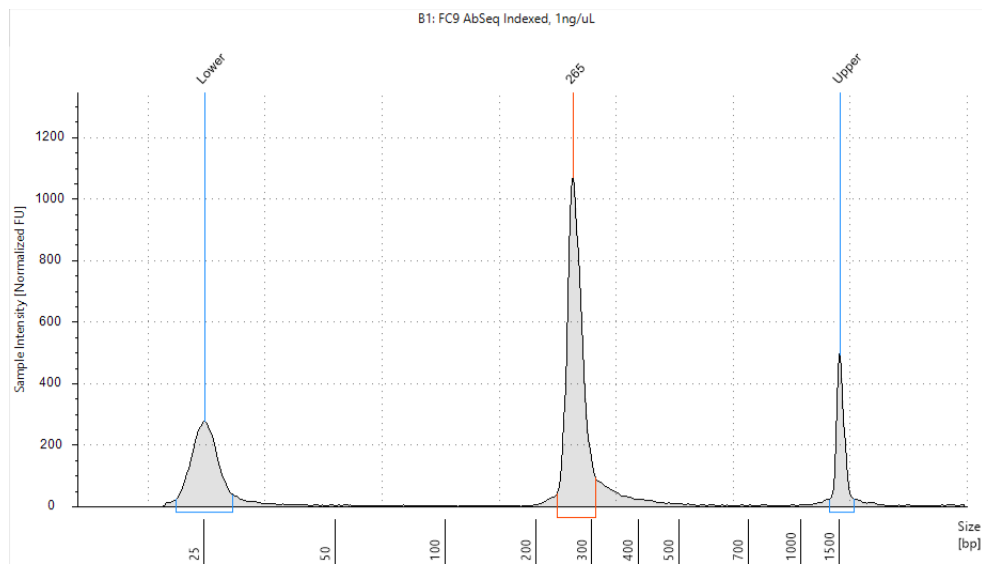
The final BD[®] AbSeq library should show a ~250 bp. For example:

Figure 3 Final BD[®] AbSeq library

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D1000 trace



NOTE 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 <i>BD® Single-Cell Multiomics Bioinformatics Handbook</i> (Doc ID: 54169)
a. To review Illumina Index 1 (i7) sequences, see Appendix A: Illumina index 1 (i7) 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 BD® AbSeq libraries:
 - The amount of sequencing needed for BD® AbSeq libraries will vary depending on application, BD® AbSeq panel plexy, and cell type. We have observed that using 40,000 sequencing reads per cell for 40-plex BD® AbSeq libraries prepared from resting PBMCs achieves an RSEC sequencing depth of ~2.

NOTE To determine the ratio of BD Rhapsody™ targeted mRNA library to AbSeq library to pool for sequencing, use the sequencing calculator available by contacting BD Biosciences technical support at scomix@bdscomix.bd.com.

NOTE Pooling > 60% AbSeq in the final sequencing pool is not recommended.

NOTE Avoid pooling >70% total AbSeq on a sequencing run as it may impact the sequencing quality of the mRNA.

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.	<ul style="list-style-type: none"> • 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. 3. If the products pass quality control, proceed to Performing index PCR to prepare final libraries on page 17 . 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.	<ul style="list-style-type: none"> • 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.
BD® AbSeq PCR1 product size too low.	<ul style="list-style-type: none"> • BD® AbSeq Primer not added to PCR1. • Too few PCR1 cycles. • Incorrect volumes of AMPure XP beads used during double-sided selection and/or volumes of AMPure XP beads swapped for mRNA and Sample Tag/BD® AbSeq products. 	Contact BD Biosciences technical support at researchapplications@bd.com .
Yield of BD® AbSeq library too low after index PCR, but yield of BD® AbSeq PCR1 products is sufficient.	Too few index PCR cycles.	Increase the number of cycles for index PCR.
	Only one primer (Library Forward or Library Reverse primer) added to index PCR mix.	Ensure that both the Library Forward Primer and Library Reverse Primer are added to the index PCR mix, and repeat index PCR.

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.

Appendix A: Illumina index 1 (i7) sequences

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