mRNA Targeted and BD™ AbSeq Library Preparation with the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit

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).

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.

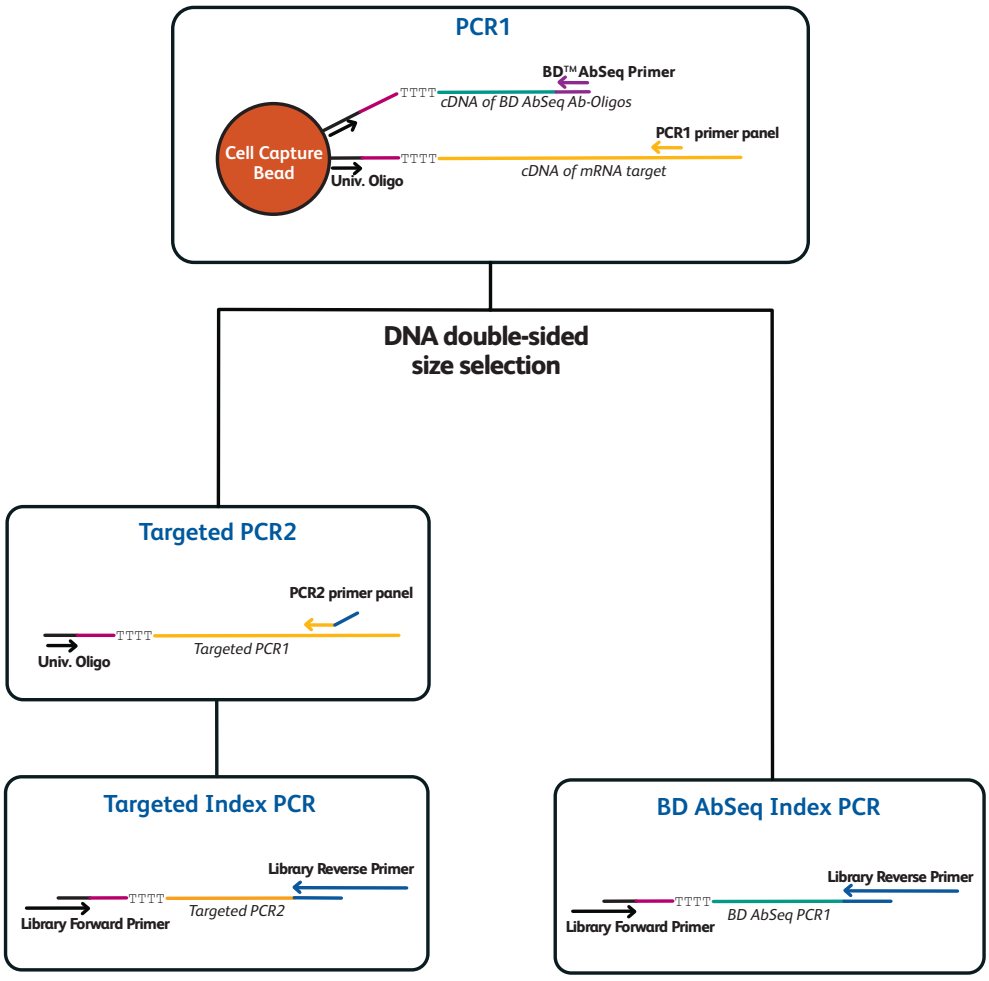
To create the libraries, the BD AbSeq and BD Rhapsody mRNA targets are encoded on Cell Capture Beads and then amplified in PCR1. After PCR1, the BD AbSeq PCR1 products are separated from the mRNA targeted PCR1 products by double-sided size selection with Agencourt® AMPure® XP magnetic beads. Size selection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

Successful preparation of mRNA and 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 an index PCR with library index primers.

After index PCR, the BD Rhapsody mRNA and BD AbSeq libraries can be combined for sequencing.

Workflow



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 labelled with BD AbSeq Ab-Oligos
- BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (Cat. No. 633774)
- PCR1 primer panel
- PCR2 primer panel
- Agencourt® AMPure® XP magnetic beads (Beckman Coulter Cat. No. A63880)
- Absolute ethyl alcohol, molecular biology grade (major supplier)
- Nuclease-free water (major supplier)
- 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.

Best practices

- Use low retention filtered pipette tips.
- When working with Cell Capture Beads, use low retention filtered tips and LoBind Tubes. **Never vortex the beads. Pipet-mix only.**
- Remove supernatants without disturbing Agencourt AMPure XP magnetic beads.

Before you begin

- Obtain the Exonuclease I-treated and inactivated 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.

Performing PCR1

1 In 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 (predesigned) primer panels are provided at 1X concentration and should not be diluted.

PCR1 reaction mix

Component	For 1 library (µL)	For 1 library + 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 subsample 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:

No. cells in PCR1	Suggested PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12
10,000	11

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 tube with the 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 post-amplification workspace.

Purifying PCR1 products by double-sided size selection

Perform double-sided AMPure bead purification to separate the shorter BD AbSeq PCR1 products (~170 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 (15°C to 25°C). Vortex high speed for 1 minute until beads are fully resuspended.

3 Pipet 140 µL AMPure beads into the 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 (15°C to 25°C) 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 (15°C to 25°C) while purifying and eluting mRNA targeted PCR1 products in Purifying mRNA targeted PCR1 products. Purify the BD AbSeq PCR1 products in Purifying 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 (15°C to 25°C) for 5 minutes.

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

6 Incubate at room temperature (15°C to 25°C) 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 (15°C to 25°C) 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 (15°C to 25°C) 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 (15°C to 25°C) for 2 minutes, and briefly centrifuge.

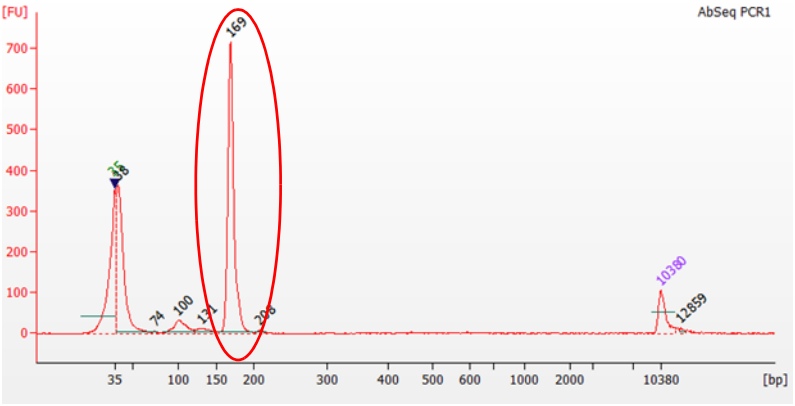
11 Place tube on magnet until solution is clear, usually ≤30 seconds.

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 (~170 bp) by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626). Follow the manufacturer's instructions:



		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
▶	◀	35	125.00	5,411.3	Lower Marker
2		38	1,436.39	57,479.7	
3		74	10.62	216.9	
4		100	191.66	2,902.8	
5		131	68.63	796.3	
6		169	1,552.22	13,924.8	
7		208	16.92	123.0	
8	▶	10.380	75.00	10.9	Upper Marker

Results | Peak Table | Region Table | Legend

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 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 (predesigned) primer panels are provided at 1X concentration and should not be diluted.

PCR2 reaction mix

Component	For 1 library (µL)	For 1 library + 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 mix into 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 (15°C to 25°C), 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 (15°C to 25°C) 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 tube on magnet, use a small-volume pipette to remove residual supernatant from tube, and discard.

9 Air-dry beads at room temperature (15°C to 25°C) for 3 minutes.

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

11 Incubate at room temperature (15°C to 25°C) for 2 minutes, and briefly centrifuge.

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

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 + 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 to post-amplification workspace.

4 In two separate, new 0.2 mL PCR tubes:
- mRNA targeted PCR2 products: Pipet 3.0 µL of 0.2–2.7 ng/µL products into 47.0 µL index PCR mix.
- 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:

Conc. index PCR input for mRNA targeted libraries (ng/µL)	Conc. index PCR input for BD AbSeq 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 (15°C to 25°C), and vortex 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.
- BD AbSeq library: 40 µL AMPure beads.

4 Pipet-mix 10 times, and incubate at room temperature (15°C to 25°C) 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 (15°C to 25°C) for 3 minutes.

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

11 Incubate at room temperature (15°C to 25°C) for 2 minutes, and briefly centrifuge.

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

13 For each tube, pipet entire eluates (~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.

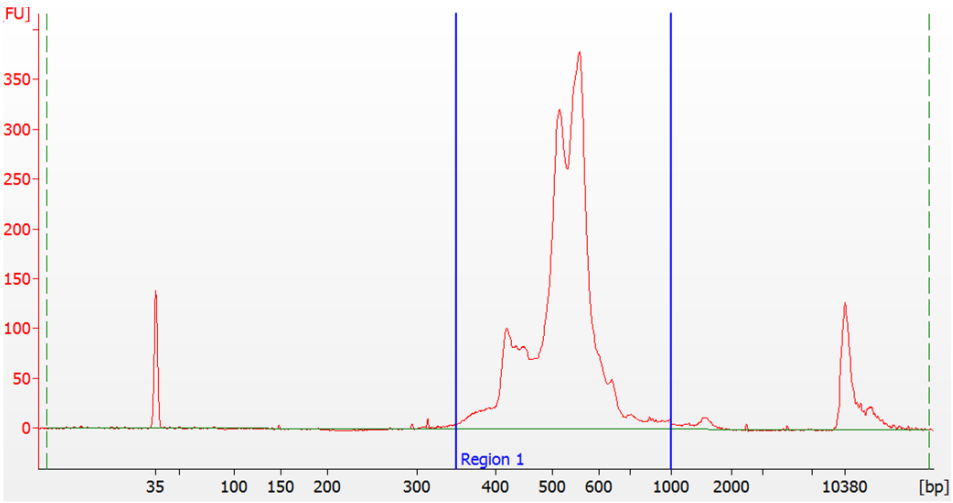
Performing quality control on the final sequencing libraries

1 Estimate the concentration of each sample 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. Follow the manufacturer's instructions.

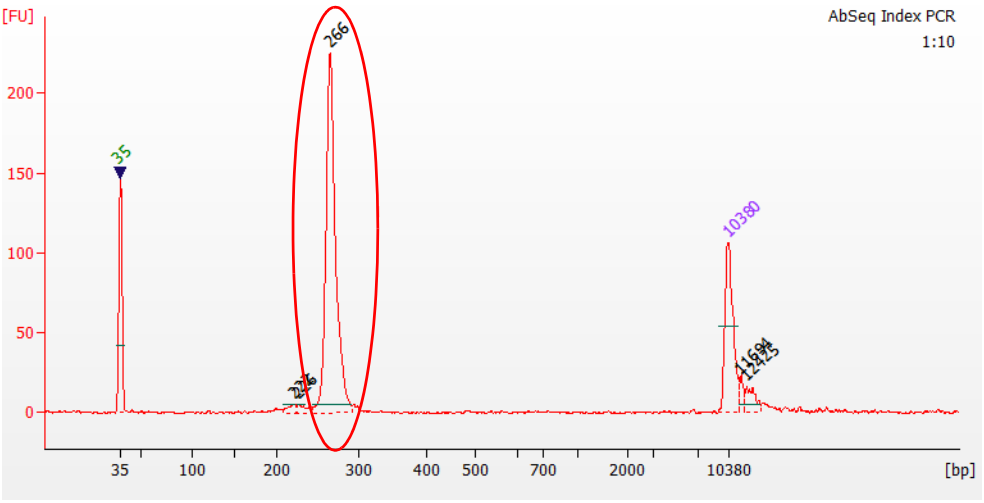
The final mRNA targeted library should show a fragment distribution that depends on the panel used. For example, with peripheral blood mononuclear cells:

BD Rhapsody™ Immune Response Panel Hs (human)



The final BD AbSeq library should show a ~270 bp. For example:

Final BD AbSeq library



Note: If the concentration or size of the library is outside of the expected range, see Troubleshooting 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	Minimum of 75 × 75 paired read length
PhiX	Required[b]
Analysis	See the *BD Single Cell Genomics Bioinformatics Handbook* (Doc ID: 54169)

a. To review Illumina Index 1 (i7) sequences, see Appendix A: Illumina Index 1 (i7) sequences.

b. BD Rhapsody targeted assays produce low-diversity libraries, so the addition of PhiX is required to provide high-quality sequencing results. For specific recommendations, see Sequencing flowcell loading and PhiX concentrations.

Recommendations

Sequencing depth

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. BD Biosciences recommends 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.

For detailed information on RSEC, contact BD Biosciences technical support at researchapplications@bd.com, and inquire about Fan J, Tsai J, Shum E. Technical Note: *Molecular Index counting adjustment methods*.

Sequencing amount for mRNA targeted libraries

This table contains recommended starting points for sequencing amount per cell with primary cells:

Gene panel	Reads/cell for clustering by cell type	Reads/cell for deep sequencing (RSEC depth ≥6)
BD Rhapsody™ Immune Response Panel Hs	~2,000	~20,000
BD Rhapsody™ T Cell Expression Panel Hs		
BD Rhapsody™ Onco-BC Panel Hs		
BD Rhapsody™ Immune Response Panel Mm		

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. BD Biosciences has 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 library to BD AbSeq library to pool for sequencing, there is a sequencing calculator available. Contact BD Biosciences technical support at researchapplications@bd.com.

Sequencing flowcell loading and PhiX concentrations

Illumina system	Sequencing flowcell loading concentration	PhiX concentration
MiSeq V2[a]	6–10 pM	10%
MiSeq V3[a]	6–10 pM	10%

Illumina system	Sequencing flowcell loading concentration	PhiX concentration
MiniSeq High or Mid Output	1–1.5 pM	20%
NextSeq High or Mid Output	1–1.5 pM	20%
HiSeq 2500[a]	7–15 pM	10%
HiSeq 3000/4000[a]	3 nM	15%

a. Sample Tag and BD AbSeq libraries have not been tested on these sequencing platforms.

- First-time users are encouraged to start at the low end of the loading concentration recommendation to avoid over-clustering.
- Dilute PhiX to the same concentration as your library before combining samples to achieve the desired final concentration of PhiX. See Illumina instructions for detailed information on preparation and storage of PhiX and optimal cluster density ranges.
- Quantify sequencing libraries as recommended or according to Illumina or service provider instructions.

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.
	Cell Capture Beads not fully resuspended immediately before PCR1	Gently pipet-mix Cell Capture Beads in PCR1 reaction mix immediately before starting PCR1 thermal cycling to ensure uniform bead suspension.
	Thermal cycler misprogramming	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 (continued)	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 products 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. Repeat the index PCR. If the products do not pass quality control, contact BD Biosciences technical support at researchapplications@bd.com.
	Thermal cycler misprogramming	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 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	• 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	Insufficient PhiX	Use the recommended concentration of PhiX with the library to be sequenced. See Sequencing flowcell loading and PhiX concentrations.
	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

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Tel 1.877.232.8995

bdbiosciences.com
ResearchApplications@bd.com

History

Revision	Date	Changes made
Doc ID 214293 Rev. 1.0	07/2018	Initial release.
Doc ID 214293 Rev. 2.0	10/2018	• Clarified instructions on preparation of index PCR master mix for each library. • For different library indexing, added statement on use of different library reverse primers than the ones supplied.
Doc ID 214293 Rev. 3.0 23-21343-00	02/2019	Revised the AMPure volumes in the following PCR purification steps: • Separating BD AbSeq PCR1 products from mRNA targeted PCR1 products • Purifying BD AbSeq PCR1 products • Purifying mRNA targeted PCR2 products • Purifying index PCR products