BD Rhapsody™ System

mRNA Targeted 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
23-24121(01)	2021-12	Initial release

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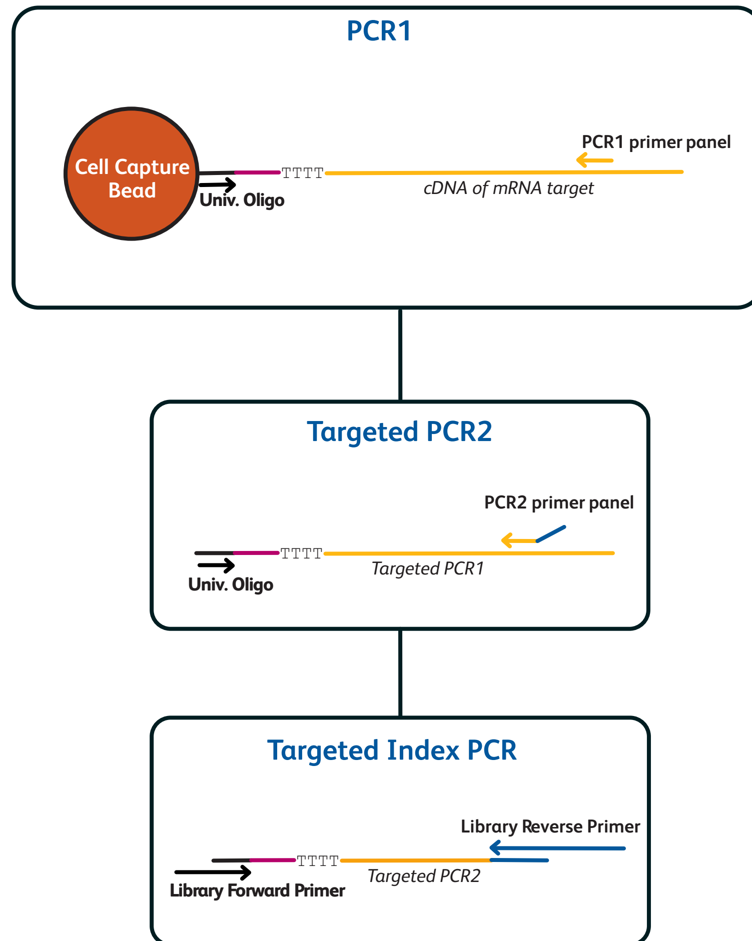
Introduction

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* (Doc ID 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063).

The cDNA of mRNA targets is first encoded on the BD Rhapsody™ Enhanced Cell Capture Beads (PN 700027881) 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 materials

- Exonuclease I-treated beads containing sample
- 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)	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)
Nuclease-free water (Cat. no. 650000076)	Up to 28.0	Up to 33.6
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
500	15
1,000	14

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
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) into the new 1.5 mL LoB ind Tube without disturbing the beads.

NOTE (Optional) Remove the tube with the Enhanced Cell Capture Beads from the 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 mRNA targeted PCR1 products

Perform the purification in the post-amplification workspace.

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 the tube with 200 µL mRNA targeted 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. Remove supernatant.

6 Keeping tube on magnet, gently add 500 µL fresh 80% ethyl alcohol, and incubate for 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 for 5 minutes.

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

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

PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. no. 91-1118)	25.0	30.0
Universal Oligo (Cat. no. 650000074)	2.0	2.4
PCR2 primer panel[a]	10.0	12.0

PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
(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 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 the tube with 50 µL PCR2 products (**step 6** of Performing PCR2 on the mRNA targeted PCR1 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 for 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 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 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, pipet reagents into a new 1.5 mL LoBind® tube on ice:

    If libraries are to be prepared from multiple samples and indexed differently, make separate index PCR mixes containing different library reverse primers for each sample.

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 mix to 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:**

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

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. 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 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 for 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 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 or 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 (final sequencing library).
14. Perform quality control before freezing sample. See **Performing quality control on the final sequencing library on page 17**.

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

Performing quality control on the final sequencing library

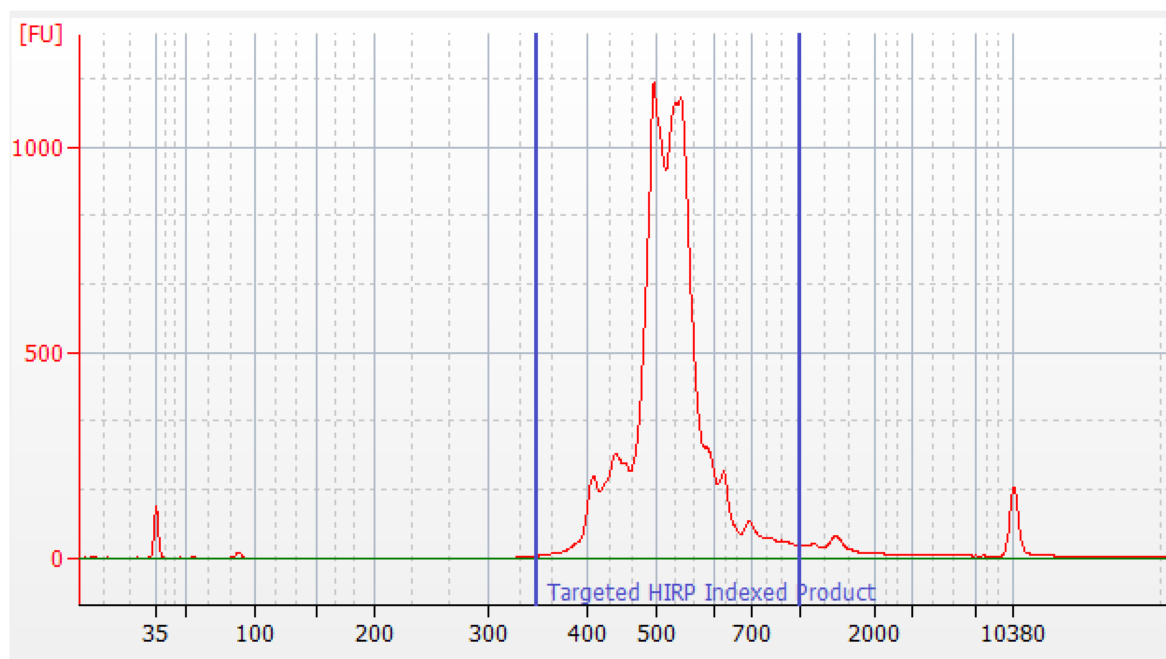
1. Estimate the concentration by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer. Follow the manufacturer's instructions. The expected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



3. If the concentration or size of the library is outside of the expected range, see **Library preparation on page 19** 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: Illumina index 1 (i7) sequences on page 21**.

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

NOTE To determine the ratio of BD Rhapsody™ targeted mRNA libraries 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.

<table>
<tr><th>Observation</th><th>Possible Causes</th><th>Recommended solutions</th></tr>
<tr><td rowspan="2">Concentration of final mRNA sequencing library too low.</td><td>Issue with PCR2 product yield or quality.</td><td>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 BD Biosciences technical support at researchapplications@bd.com.</td></tr>
<tr><td>Thermal cycler mis-programming.</td><td>Ensure that the correct thermal cycling program is used.</td></tr>
<tr><td rowspan="2">Final sequencing product size too large.</td><td>• Over-amplification during index PCR.
• Input amount of PCR2 products too high.</td><td>Repeat the index PCR with a lower input of mRNA targeted PCR2 products.</td></tr>
<tr><td>Upper and lower markers on the Agilent Bioanalyzer is incorrectly called.</td><td>Ensure that markers are correct.
Follow manufacturer’s instructions.</td></tr>
</table>

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

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