BD Rhapsody™ System

VDJ CDR3 Library Preparation Protocol

For Research Use Only

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Regulatory Information

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

History

Revision	Date	Change made
23-22202-00	1/2020	Initial release

Contents

Introduction

This protocol provides instructions on creating a single-cell mRNA library with the BD Rhapsody™ Single-Cell Analysis System or the BD Rhapsody™ Express Single-Cell Analysis System for sequencing on Illumina® sequencers. It is also intended to provide a method to enable a high-throughput detection of VDJ recombination events by generating sequencer-ready libraries specific to the CDR3-specific antigen binding regions of B and T cells. To create the library, cDNA of gene targets are encoded on BD Rhapsody™ Cell Capture Beads. 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.

IMPORTANT The protocol described herein has not been thoroughly tested or optimized and is provided as is and without warranty. Any use of this protocol is at the risk of the user. BD Biosciences reserves the right to change specifications at any time. Information in the protocol is subject to change without notice. BD Biosciences assumes no responsibility for any errors or omissions. In no event shall BD Biosciences be liable for any damages in connection with or arising from the use of this protocol.

Required and recommended materials

Required reagents

Store the reagents at the storage temperature specified on the label.

Material	Supplier	Catalog No.
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit	BD Biosciences	633774
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
10 mM Tris-HCl with 0.05% Tween-20, pH 8.0 (Tris-Tween20)	Teknova	T1485
Nuclease-free water	Major Supplier	–
Template switch oligo, TSO (5' T TTT TTT TTT TTT TTT TTT TTT TTT rG rG rG 3'). Stock concentration of TSO is 100 µM in 10 mM Tris, 0.1 mM EDTA, pH 8.0	Major Supplier	
Klenow Fragment (3' -> 5' exo-) (includes NEBuffer 2)	New England Biolabs	M0212L
10 mM dNTP	New England Biolabs	N0447L
1 M MgCl2	Major Supplier	–
1 M Tris-HCl, pH 8.0 (diluted to 50 mM Tris-HCl for the assay)	Major Supplier	–

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	–
15-mL conical tube	Major Supplier	–
DNA LoBind Tubes, 1.5 mL	Eppendorf	0030108051
DNA LoBind Tubes, 5.0 mL	Eppendorf	0030108310
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856

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	–
Heat block	Major Supplier	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
SmartBlock Thermoblock 1.5 mL to fit on the ThermoMixer® C	Eppendorf	5360000038
6-tube magnetic separation rack for 1.5-mL tubes	New England Biolabs	S1506S
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Agilent® 2100 Bioanalyzer Or, Agilent® 4200 TapeStation System	Agilent Technologies Agilent Technologies	G2940CAG G2991AA

Best practices

- Use low-retention filtered pipette tips.
- When working with BD Rhapsody 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)

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* (Doc ID: 214062)

  STOP after the section "Washing the Cell Capture Beads" and follow this protocol from Preparing BD Rhapsody™ Cell Capture Beads for VDJ library amplification and subsequent steps.

  Or,

- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID: 214063)

  STOP after the section "Washing the Cell Capture Beads" and follow this protocol from Preparing BD Rhapsody™ Cell Capture Beads for VDJ library amplification and subsequent steps.

Ensure that the intended total cell load is between 1,000–10,000 single cells for this protocol. Cell load below or above this recommended range may not be suitable for current protocol configuration. Then proceed as described in the following procedure.

Preparing BD Rhapsody™ Cell Capture Beads for VDJ library amplification

cDNA synthesis and template switching

Thaw reagents (except for the enzymes) in the BD Rhapsody cDNA Kit (Cat. No. 633773) at room temperature (15°C to 25°C). Keep enzymes at –25°C to –15°C.

NOTE This section should be performed in the pre-amplification workspace.

1 Set a thermomixer to 42°C.

2 In a new 1.5-mL LoBind tube, pipet the following reagents.

cDNA/Template switching mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
RT Buffer	40	48
dNTP	20	24
RT 0.1 M DTT	10	12
Bead RT/PCR Enhancer	12	14.4
RNase Inhibitor	10	12
Reverse Transcriptase	10	12
Nuclease-free water	92	110.4
Total	**194**	**232.8**

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

4 Place the tube of washed Cell Capture Beads on a 1.5-mL tube magnet for ≥2 minutes. Remove the supernatant.

5 Remove the tube from the magnet and pipet 194 µL of cDNA mix into the beads. Pipet-mix.

NOTE Keep the prepared cDNA mix with beads on ice until the suspension is transferred in the next step.

6 Transfer the bead suspension to a new 1.5-mL LoBind tube.

7 Incubate the bead suspension on the thermomixer at 1,200 rpm and 42°C for 30 minutes.

Shaking is critical for this incubation.

8 Add 6 µL of template switch oligo (100 uM 5' T TTT TTT TTT TTT TTT TTT TTT TTT rG rG rG 3') and 2 µL of 1 M $MgCl_2$ to the reaction mix, and incubate on the thermomixer for another 30 minutes at 1,200 rpm and 42°C.

STOPPING POINT: BD Rhapsody Cell Capture Beads can be stored up to 4 days at 2°C to 8°C after template switching.

If stopping after template switching:

- Place the bead suspension on the 1.5-mL tube magnet until the solution is clear (≤1 minute).
- Carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
- Remove the tube from the magnet, and with a low-retention tip, pipet 200 µL of Tris-Tween20 to gently resuspend the beads. Do not vortex.
- Store the beads at 2°C to 8°C for up to 4 days.

9 During incubation, prepare 5 mL of 50 mM Tris-HCl. Keep at room temperature.

NOTE 50 mM Tris-HCl is from 1 M Tris-HCl diluted with nuclease-free water (i.e., 1:20 dilution).

10 If using the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*, view the Rhapsody scanner image analysis to see if the analysis metrics passed.

Denaturation and self-hybridization

Prepare 5 mL of 50 mM Tris-HCl and keep at room temperature. Thaw reagents for Klenow extension at room temperature (15°C to 25°C). Keep Klenow fragment exo- enzyme at –25°C to –15°C.

If the BD Rhapsody Cell Capture Beads were stored after template switching, briefly centrifuge and proceed to step 4.

1 Set one thermomixer to 37°C, a second thermomixer to 25°C, and a third thermomixer or heat block to 95°C.

2 Place the tube of Cell Capture Beads with cDNA mix on a 1.5-mL tube magnet for ≤1 minute. Remove the supernatant.

3 Remove the tube from the magnet and pipet 200 µL of Tris-Tween20 buffer into the tube. Pipet-mix.

4 To denature, incubate the tube in the following order:

- Ensure that the beads are resuspended. Pipet-mix to resuspend, if needed.
- Incubate the sample at 95°C in a heat block (no shaking) for 5 minutes.
- Place the tube in a thermomixer at any temperature for 10 seconds at 1,200 rpm to resuspend the beads.

5 Immediately place the tube on the magnet for ≤30 seconds until clear. Remove the supernatant.

6 Resuspend the beads in 1.5 mL of 50 mM Tris-HCl.

7 Incubate the bead suspension on the thermomixer at 1,200 rpm and 25°C for 2 minutes.

Klenow extension

1 Set a thermomixer to 37°C.

2 Ensure all reagents other than the Klenow fragment exo- are at room temperature.

3 In a new 1.5-mL LoBind tube, pipet the following reagents.

Klenow extension mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
10X Klenow Buffer	20	24
dNTP	20	24
Klenow fragment exo-	10	12
Nuclease-free water	150	180
Total	**200**	**240**

4 Gently vortex mix, briefly centrifuge, and keep at room temperature.

5 Briefly spin the tube with the bead suspension.

6 Place the tube of Cell Capture Beads on a 1.5-mL tube magnet for ≤2 minutes. Remove the supernatant.

7 Remove the tubes from magnet and resuspend using 200 µL of Klenow extension mix. Pipet-mix.

8 Incubate the bead suspension on a thermomixer at 1,200 rpm and 37°C for 30 minutes.

9 Briefly spin the tube with the beads suspension and place the tube on ice.

Treating the sample with Exonuclease I

Thaw reagents for Exonuclease I treatment at room temperature (15°C to 25°C). Keep Exonuclease I enzyme at –25°C to –15°C.

1 Set one thermomixer to 37°C and a second thermomixer or heat block to 80°C.

2 In a new 1.5-mL LoBind tube, pipet the following reagents.

Exonuclease I mix

Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)
10X Exonuclease I Buffer	20	24
Exonuclease I	10	12
Nuclease-free water	170	204
Total	**200**	**240**

3 Gently vortex mix, briefly centrifuge, and keep at room temperature.

4 Place the tube of Cell Capture Beads with Klenow Extension mix on a 1.5-mL tube magnet for ≤1 minute. Remove the supernatant.

5 Remove the tube from the magnet and pipet 200 µL Exonuclease I mix into the tube. Pipet-mix.

6 Incubate the bead suspension on thermomixer at 1,200 rpm and 37°C for 30 minutes.

7 Incubate the bead suspension on thermomixer (no shaking) or heat block at 80°C for 20 minutes.

8 Place the tube on ice for ~1 minute.

9 Briefly spin the tube with the bead suspension.

10 Place the tube on the magnet for ≤1 minute until clear. Remove the supernatant.

11 Remove the tube from the magnet, and pipet 200 µL of cold Bead Resuspension Buffer into the tube. Pipet-mix.

STOPPING POINT: Exonuclease I-treated beads can be stored at 2°C to 8°C for up to 3 months.

12 Proceed to library preparation.

Targeted mRNA, TCR, and BCR library preparation

This protocol provides instructions on creating BD Rhapsody targeted mRNA, TCR, and BCR libraries with the BD Rhapsody Single-Cell Analysis system or the BD Rhapsody Express Single-Cell Analysis system for sequencing on Illumina sequencers.

Required materials

Material	Supplier	Catalog No.
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit	BD Biosciences	633774
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
10 mM Tris-HCl with 0.05% Tween-20, pH 8.0 (Tris-Tween20)	Teknova	T1485
DNA Suspension Buffer, pH 8.0, DNase/RNase Tested, PCR Grade	Teknova	T0221
Nuclease-free water	Major supplier	–
BD Rhapsody™ panel	BD Biosciences	Various
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent DNA High Sensitivity Kit Or, Agilent High Sensitivity D5000 ScreenTape Agilent High Sensitivity D5000 Reagents	Agilent Technologies Agilent Technologies Agilent Technologies	5067-4626 5067-5592 5067-5593

Human T cell PCR1 primers

Primer Name	Primer Sequence (5' – 3')
TRAC_N1	CTGGAATAATGCTGTTGTTGAAGG
TRBC_N1	AGCCCGTAGAACTGGACTT
TRDC_N1	CTTCAAAGTCAGTGGAGTGCA
TRGC_N1	CACCGTTAACCAGCTAAATTTCATG

Pooling Human T cell PCR1 primer panel

Name	Primer Stock Conc. (µM)	Volume per primer (µL)	DNA Suspension Buffer (µL)	Final volume (µL)
TRAC_N1 TRBC_N1 TRDC_N1 TRGC_N1	100	25.00	400.00	500

Human T cell PCR2 primers

Primer Name	Primer Sequence (5' – 3')
TRAC_N2	CAGACGTGTGCTCTTCCGATCTATCAAAATCGGTGAATAGGCAGAC
TRBC_N2	CAGACGTGTGCTCTTCCGATCTGATCTCTGCTTCTGATGGCTCA
TRDC_N2	CAGACGTGTGCTCTTCCGATCTATATCCTTGGGGTAGAATTCCTTC
TRGC_N2	CAGACGTGTGCTCTTCCGATCTGGGAAACATCTGCATCAAGTTG

Pooling Human T cell PCR2 primer panel

Name	Primer Stock Conc. (µM)	Volume per primer (µL)	DNA Suspension Buffer (µL)	Final volume (µL)
TRAC_N2 TRBC_N2 TRDC_N2 TRGC_N2	100	25.00	400.00	500

Human B cell PCR1 primers

Primer Name	Primer Sequence (5' – 3')
IGHA_N1	CACAGTCACATCCTGGCT
IGHD_N1	GATCTCCTTCTTACTCTTGCTGG
IGHE_N1	CGCTGAAGGTTTTGTTGTCG
IGHG_N1	TGTTGCTGGGCTTGTGAT
IGHM_N1	CGTTCTTTTCTTTGTTGCCGT
IGKC_N1	TTTGTGTTTCTCGTAGTCTGCT
IGLC_N1	TGTAGCTTCTGTGGGACTTC

Pooling Human B cell PCR1 primer panel

Name	Primer Stock Conc. (µM)	Volume per primer (µL)	DNA Suspension Buffer (µL)	Final volume (µL)
IGHA_N1 IGHD_N1 IGHE_N1 IGHG_N1 IGHM_N1 IGKC_N1 IGLC_N1	100	15.00	420.00	525

Human B cell PCR2 primers

Primer Name	Primer Sequence (5' – 3')
IGHA_N2	CAGACGTGTGCTCTTCCGATCTCTTTCGCTCCAGGTCACACT
IGHD_N2	CAGACGTGTGCTCTTCCGATCTTGTCTGCACCCTGATATGATGG
IGHE_N2	CAGACGTGTGCTCTTCCGATCTGTCAAGGGGAAGACGGATG
IGHG_N2	CAGACGTGTGCTCTTCCGATCTAAGTAGTCCTTGACCAGGCA
IGHM_N2	CAGACGTGTGCTCTTCCGATCTACAGGAGACGAGGGGGAAAA
IGKC_N2	CAGACGTGTGCTCTTCCGATCTTCAGATGGCGGGAAGATGAA
IGLC_N2	CAGACGTGTGCTCTTCCGATCTACCAGTGTGGCCTTGTTG

Pooling Human B cell PCR2 primer panel

Name	Primer Stock Conc. (µM)	Volume per primer (µL)	DNA Suspension Buffer (µL)	Final volume (µL)
IGHA_N2 IGHD_N2 IGHE_N2 IGHG_N2 IGHM_N2 IGKC_N2 IGLC_N2	100	75.00	N/A	525

Before you begin

- Obtain Exonuclease I-treated BD Rhapsody Cell Capture Beads.
- Thaw the reagents in the BD Rhapsody Targeted mRNA and AbSeq Amplification Kit at room temperature (15°C to 25°C), and then place on ice.

Performing PCR1

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

PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	20	24
Bead RT/PCR Enhancer	12	14.4
PCR1 targeted mRNA primer panel	40	48
TCR PCR1 primer panel	2.4	2.88
BCR PCR1 primer panel	4.2	5.04
Nuclease-free water	21.4	25.68
Total	**200**	**240**

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 Briefly spin the tube with the bead suspension. 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 up to 3 months.

5 Briefly spin the tube with the bead suspension. Place the tube of Exonuclease I-treated beads in Bead Resuspension Buffer on a 1.5-mL magnet for ≤1 minute. Remove 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 as follows. Do not use fast cycling mode.

Step	Cycles	Temperature	Time
Hot start	1	95°C[a]	3 min
Denaturation	11–14[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, BD Biosciences has observed a step-skipping error with the pause/unpause functions. To ensure that the full 3-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 1-minute 95°C pause step can be added immediately before the 3-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
1,000	14
2,500	13
5,000	12
10,000	11

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.

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 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 into a new 1.5-mL LoBind tube.

14 Place the 1.5-mL tube on the magnet for ≤1 minute. **Retain the supernatant.** Carefully pipet the supernatant (PCR1 products) into the new 1.5-mL LoBind tube without disturbing the beads.

NOTE (Optional) Remove the tube with the Cell Capture Beads from the magnet and pipet 200 µL of cold Bead Resuspension Buffer into the tube. Pipet-mix. Do not vortex. Store the beads at 2°C to 8°C in the post-amplification workspace.

Purifying 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, with 1.0 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

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

2 Bring Agencourt AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex on high speed for 1 minute until the beads are fully resuspended.

3 Pipet 140 µL of AMPure beads into the tube with 200 µL PCR1 products. Pipet-mix 10 times.

4 Incubate at room temperature (15°C to 25°C) for 5 minutes.

5 Place the 1.5-mL LoBind tube on the magnet for 5 minutes.

6 Keeping the tube on the magnet, remove the supernatant without disturbing the beads.

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

8 Repeat step 7 once for a total of two washes.

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

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

11 Remove the tube from the magnet and resuspend the bead pellet in 30 µL of Tris-Tween20. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.

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

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

14 Pipet the eluate (~30 µL) into a new 1.5-mL LoBind tube (purified targeted mRNA, TCR, and BCR PCR1 products).

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

Performing PCR2 on the targeted mRNA, TCR, and BCR PCR1 products

NOTE Targeted mRNA, TCR, and BCR products are amplified separately in PCR2.

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

Targeted mRNA PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
PCR2 targeted mRNA primer panel	10	12
Nuclease-free water	8	9.6
Total	**45**	**54**

TCR PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
TCR PCR2 primer panel	6	7.2
Nuclease-free water	12	14.4
Total	**45**	**54**

BCR PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
BCR PCR2 primer panel	2	2.4
Nuclease-free water	16	19.2
Total	**45**	**54**

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

3 Bring the PCR2 mixes into the post-amplification workspace.

4 In a new 0.2-mL PCR tube, pipet 5.0 µL of purified PCR1 products (which contains targeted mRNA, TCR, and BCR products) into each of the respective 45 µL of targeted mRNA, TCR, or BCR PCR2 reaction mixes.

5 Gently vortex and briefly centrifuge.

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

Targeted mRNA PCR2

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.

TCR and BCR PCR2

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

STOPPING POINT: The PCR can run overnight.

Purifying targeted mRNA, TCR, and BCR PCR2 products

Purify targeted mRNA PCR2 products first, then continue with purifying TCR and BCR PCR2 products.

NOTE Perform purification in the post-amplification workspace.

Purifying targeted mRNA PCR2 products

1. In a new 5.0-mL LoBind tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

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

2. Bring AMPure XP beads to room temperature (15°C to 25°C) and vortex at high speed for 1 minute until beads are fully resuspended.

3. Briefly centrifuge the PCR2 products.

4. Pipet 40 µL of AMPure XP beads into a tube with 50 µL of targeted mRNA PCR2 products. Pipet-mix 10 times.

5. Incubate at room temperature (15°C to 25°C) for 5 minutes.

6. Place the tube on the strip tube magnet for 3 minutes. Remove the supernatant.

7. Keeping the targeted mRNA tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Remove the supernatant.

8. Repeat step 7 once for a total of two washes.

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

10 Air-dry the beads at room temperature (15°C to 25°C) for 1 minute.

11 Remove the tube from the magnet and resuspend the bead pellet in 30 µL of Tris-Tween20. Pipet-mix until the beads are fully resuspended.

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

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

14 Pipet the entire eluate (~30 µL) into a new 1.5-mL LoBind tube (purified targeted mRNA 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 up to 6 months.

Purifying TCR and BCR PCR2 products by double-sided selection

1 To the TCR and BCR PCR2 products, pipet 55 µL of nuclease-free water. Transfer 100 µL of diluted PCR2 product to a new 0.2-mL tube.

2 Add 50 µL of AMPure XP beads to 100 µL of diluted PCR2 product. Pipet-mix 10 times.

3 Incubate at room temperature (15°C to 25°C) for 5 minutes.

4 Place the tube on the strip tube magnet for 3 minutes.

5 Keeping the tube on the magnet, transfer the 150 µL of supernatant (TCR and BCR PCR2 products) to a new 0.2-mL tube without disturbing the beads.

6 To the TCR and BCR PCR2 supernatant, add 10 µL of AMPure XP beads. Pipet-mix 10 times.

7 Incubate at room temperature (15°C to 25°C) for 5 minutes.

8 Place the tube on the strip tube magnet for 1 minute. Remove the supernatant.

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

10 Repeat step 9 once for a total of two washes.

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

12 Air-dry the beads at room temperature (15°C to 25°C) for 30 seconds.

13 Remove the tube from the magnet and resuspend the bead pellet in 30 µL of Tris-Tween20. Pipet-mix until the beads are fully resuspended.

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

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

16 For each sample, pipet the entire eluate (~30 µL) into a separate, new 1.5-mL LoBind tube (purified TCR and BCR 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 up to 6 months.

17 Estimate the concentration by quantifying 2 µL of the PCR2 products with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

18 Dilute an aliquot of the PCR2 products with Tris-Tween20:

- Targeted mRNA PCR2 products: 0.2-2.7 ng/µL
- TCR and BCR PCR2 products: 0.5 ng/µL

Performing Index PCR to prepare final libraries

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

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, prepare separate index PCR mixes containing different library reverse primers for each library type.

Targeted mRNA Index PCR mix

Kit Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	2	2.4
Library Reverse Primer 1–4	2	2.4
Nuclease-free water	18	21.6
Total	**47**	**56.4**

TCR/BCR Index PCR mix

Kit Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	2	2.4
Library Reverse Primer 1–4	2	2.4
Total	**29**	**34.8**

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

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

4 In new 0.2-mL PCR tubes,

a For targeted mRNA library, pipet 3.0 µL of 0.2–2.7 ng/µL PCR2 product into 47.0 µL of index PCR mix.

b For TCR and BCR libraries, pipet 21.0 µL of 0.5 ng/µL PCR2 products into 29.0 µL of index PCR mix.

5 Gently vortex and briefly centrifuge.

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

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	Targeted mRNA: 6–8. See the following table Suggested number of PCR cycles TCR/BCR: 7	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	∞

Suggested number of PCR cycles

Conc. index PCR input for targeted mRNA 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 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, with 1.0 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

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

2 Bring the AMPure XP beads to room temperature (15°C to 25°C), and vortex on high speed for 1 minute until the beads are fully resuspended.

3 Briefly centrifuge the index PCR products.

4 To 50.0 µL of the index PCR products, pipet:

- Targeted mRNA libraries: 35 µL of AMPure beads
- TCR and BCR libraries: 30 µL of AMPure beads

5 Incubate at room temperature (15°C to 25°C) for 5 minutes.

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

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

8 Repeat step 7 once for a total of two washes.

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

10 Air-dry the beads at room temperature (15°C to 25°C) for 1 minute.

11 Remove the tubes from the magnet and resuspend the bead pellet in 30 µL of Tris-Tween20. Pipet-mix until the beads are fully resuspended.

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

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

14 For each sample, pipet the entire eluate (~30 µL) into a separate new 1.5-mL LoBind tube (final sequencing libraries).

15 Perform quality control before freezing the samples. See Performing quality control on the final sequencing libraries in the following section.

STOPPING POINT: Store at –25°C to –15°C for up to 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 targeted mRNA, TCR, and BCR libraries within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit for 50–7,000 bp, 5–1,000 pg/µL. Follow the manufacturer's instructions. Refer to the sample trace images.

Figure 1 BD Rhapsody Immune Response Panel Hs (human)

Fragment distribution of ~450–700 bp.

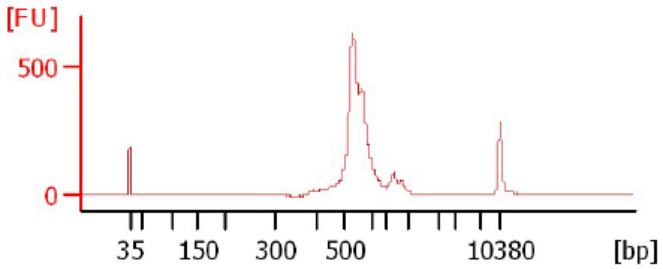


Figure 2 BD Rhapsody TCR Library

Fragment distribution of ~600–1,000 bp.

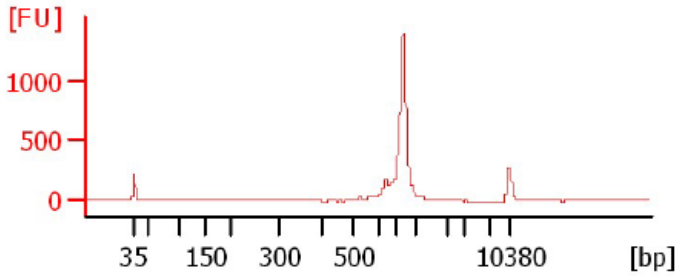
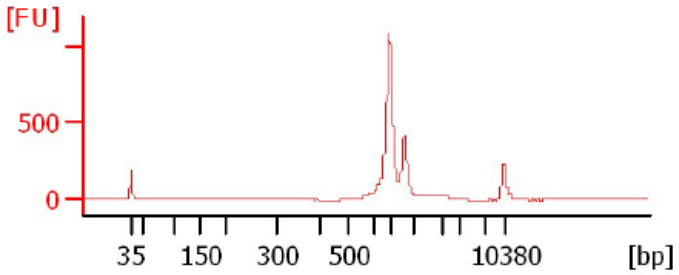


Figure 3 BD Rhapsody BCR Library

Fragment distribution of ~600–1,000 bp.



Sequencing

Requirements

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

Parameter	Requirement
Platform	Illumina: 300 cycle kit
Paired-end reads	Minimum of 75 × 225 paired read length
PhiX	Required
Analysis	Contact customer support at scomix@bdscomix.bd.com for access to the latest BD Rhapsody sequence analysis pipeline with built-in support for VDJ.

Recommendations

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.

Sequencing amount for VDJ libraries

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

Gene panel	Reads/cell	Reads/cell
BD Rhapsody targeted mRNA Panel Hs	~2,000 (for clustering by cell type)	~20,000 (for deep sequencing, RSEC depth ≥6)
BD Rhapsody TCR	~3,000	N/A
BD Rhapsody BCR	~3,000	N/A

Sequencing flowcell loading and PhiX concentrations

For a NextSeq High or Mid Output and MiniSeq High or Mid Output runs, load the flow cell at a concentration between 1.8–2.2 pM with 10% PhiX for a sequencing run.

For library pooling

Library	Pooling ratio	Pooling volume (20 µL)
BD Rhapsody targeted mRNA panel	1	2
BD Rhapsody TCR	5.5	11
BD Rhapsody BCR	3.5	7