BD Rhapsody™ System

Mouse TCR/BCR Next and mRNA Whole Transcriptome Analysis (WTA)

Library Preparation Protocol

23-24516(01)
2024-01

For Research Use Only

Regulatory information

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

History

Revision	Date	Change made
23-24516(01)	2024-01	Initial release.

Contents

Introduction

This protocol enables high throughput single-cell transcriptome alongside TCR and BCR profiling of individual cells captured on the BD Rhapsody™ system, providing instructions for amplifying Illumina-compatible single-cell barcoded mRNA, TCR, and BCR libraries.

cDNA is encoded on BD Rhapsody™ Enhanced Cell Capture Beads using both the 3' and 5' ends of transcripts as templates. mRNA, TCR, and BCR libraries are then amplified from these on-bead cDNA libraries using a two-step nested amplification, with TCR and BCR libraries undergoing additional random priming to capture complementarity determining regions (CDR) 1, 2, and 3, as well as framework regions (FR) 1-4.

Protocol kits

Before you begin, ensure that you have the correct kits for this protocol. Matching cap colors indicate you have the correct kit, along with the catalog numbers found in the Required and recommended materials section.

BD Rhapsody™ cDNA Kit

Cap Color	Name	Quantity
	RT Buffer	1
	RT 0.1M DTT	1
	Reverse Transcriptase	1
	dNTP	1
	RNAse Inhibitor	1
	Bead RT/PCR Enhancer	1
	10X Exonuclease I Buffer	1
	Exonuclease I	1
	Nuclease-free water	2
	Bead Resuspension Buffer	1

BD Rhapsody™ Enhanced Cartridge Reagent Kit V3

Cap Color	Name	Quantity
	BD Rhapsody™ HT Enhanced Cell Capture Beads V3	4
	Sample Buffer	1
	Cartridge Wash Buffer 1	1
	Cartridge Wash Buffer 2	1
	Lysis Buffer	4
	Bead Wash Buffer	1
	Waste Collection Container	4
	1M DTT	1

BD Rhapsody™ WTA Amplification Kit

Cap Color	Name	Quantity
	WTA Extension Primers	1
	WTA Extension Buffer	1
	WTA Extension Enzyme	1
	10 mM dNTP	1
	Nuclease-free water	3
	Bead RT/PCR Enhancer	3
	WTA Amplification Primer	1
	PCR Master Mix	1
	Universal Oligo	2
	Sample Tag PCR1 Primer	1
	Sample Tag PCR2 Primer	1
	BD® AbSeq PCR1 Primer	1
	Library Reverse Primer 1-4	1 each
	Library Forward Primer	2
	Bead Resuspension Buffer	3
	Elution Buffer	2

BD Rhapsody™ TCR/BCR Next Amplification Kit

Cap Color	Name	Quantity
	TCR/BCR Extension Primers	1
	TCR/BCR Extension Buffer	1
	TCR/BCR Extension Enzyme	1
	10 mM dNTP	2
	Nuclease-free water	2
	Bead RT/PCR Enhancer	1
	TSO Next	1
	TCR N1 Primer - mouse	1
	TCR N2 Primer - mouse	1
	BCR N1 Primer - mouse	1
	BCR N2 Primer - mouse	1
	PCR Master Mix	1
	TCR/BCR Universal Oligo N1	1
	TCR/BCR Universal Oligo N2	1
	Elution Buffer	2
	1M $MgCl_2$	1
	Hybridization Buffer	4

Symbols

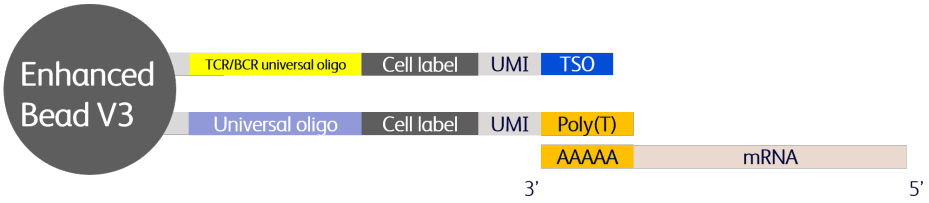
The following symbols are used in this guide.

Symbol	Description
⚠	Indicates a crucial step or stopping point.

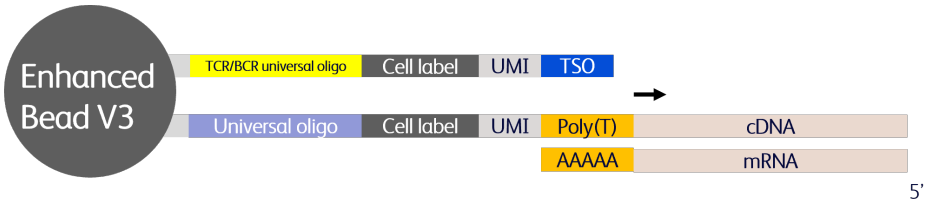
Workflows

Reverse transcription, template switching, and Exonuclease I treatment workflow

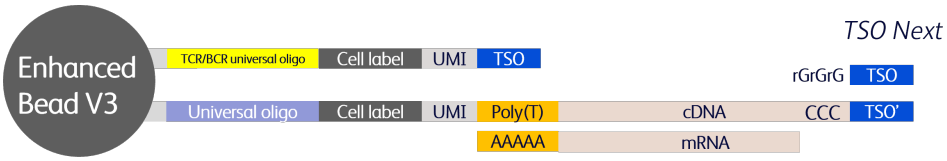
Cell lysis in microwell: The mRNA oligos are captured by poly(T).



Reverse transcription: Complementary DNA (cDNA) is synthesized from captured mRNA.

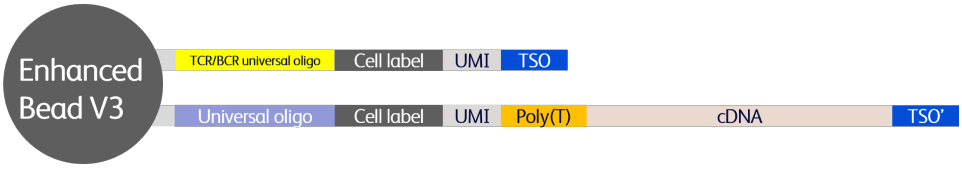


Template-switching oligo priming: TSO is added to 3' end of cDNA on the bead.

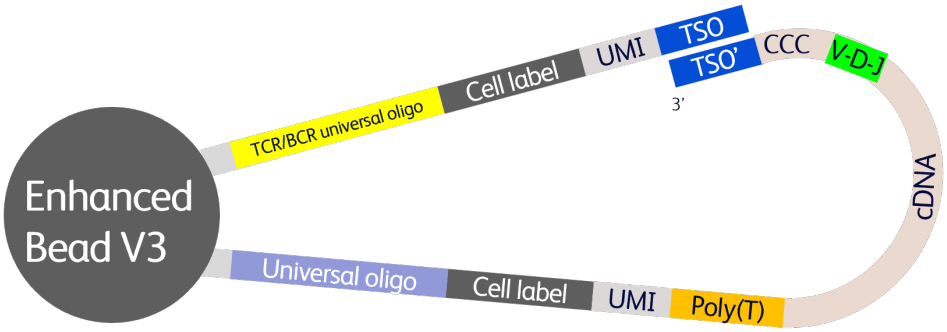


Denaturation

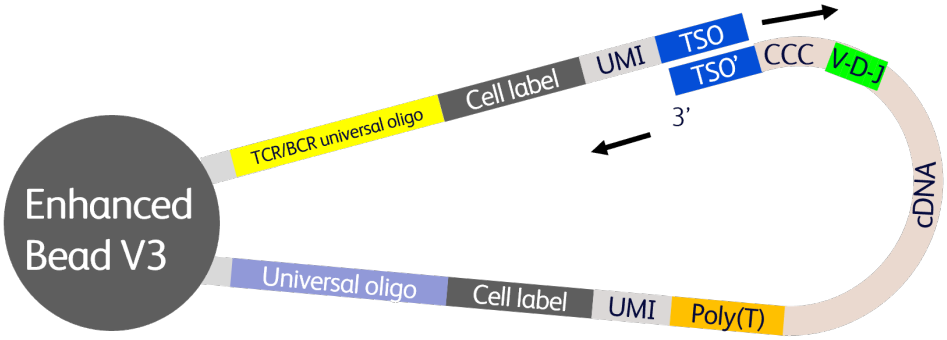
- **Supernatant**: The template is denatured off the bead. Discard the supernatant.
- **Bead**: Single-stranded DNA is generated on beads to prepare for self-hybridization.



Self-hybridization: Resuspend the beads in pre-warmed hybridization buffer, then gradually cool down to allow the TSO' to hybridize to the TSO capture strand.

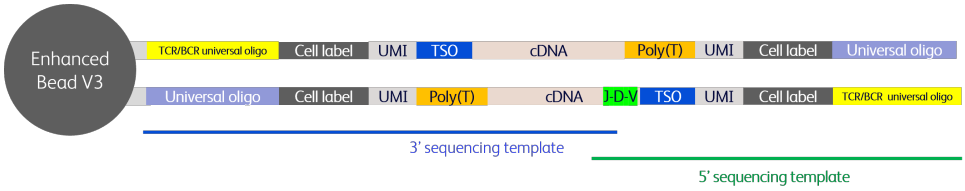


Extension: Copy the TCR/BCR universal oligo onto the 3' end of cDNA strand.



Exonuclease I: Cleave unused oligo capture sequences off the bead.

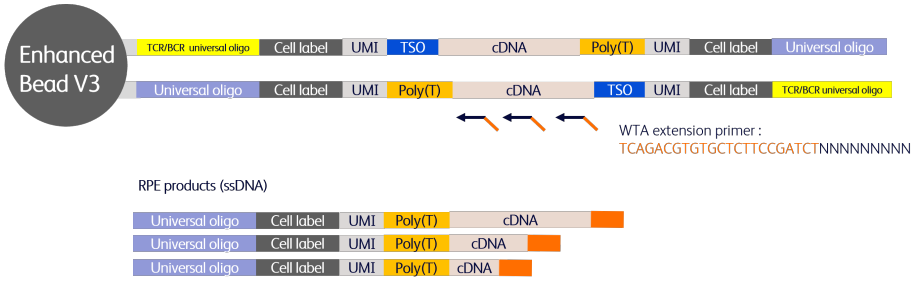
Final bead layout:
3' sequencing template: mRNA library
5' sequencing template: TCR/BCR library



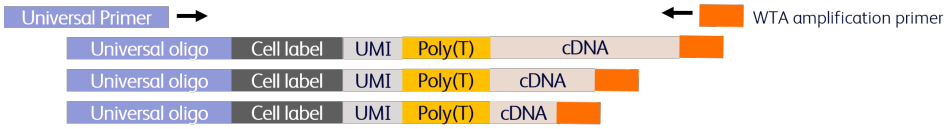
WTA library amplification workflow

WTA RPE: Random priming on the bead.

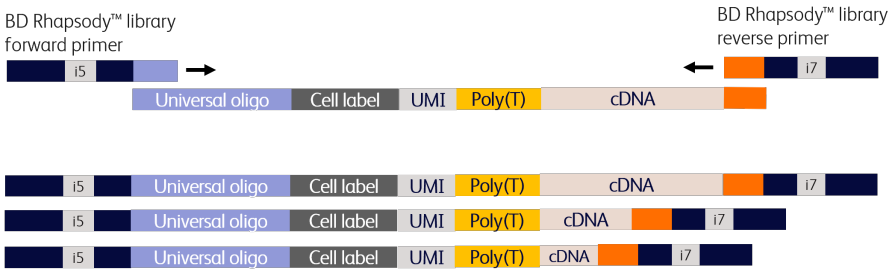
Denature off the RPE product.



WTA RPE
PCR: Amplify the RPE product.

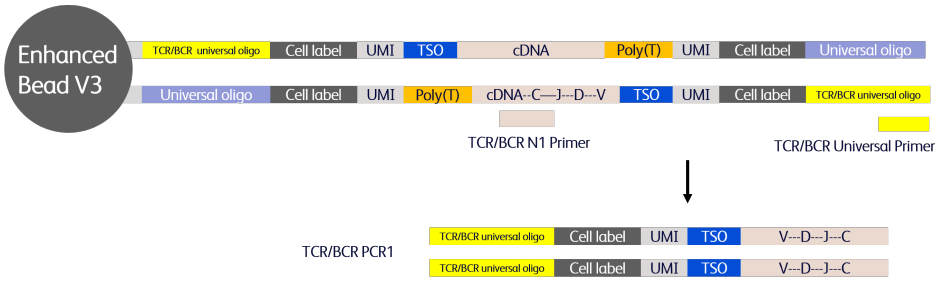


WTA Index PCR: Add Illumina adapters and indices.



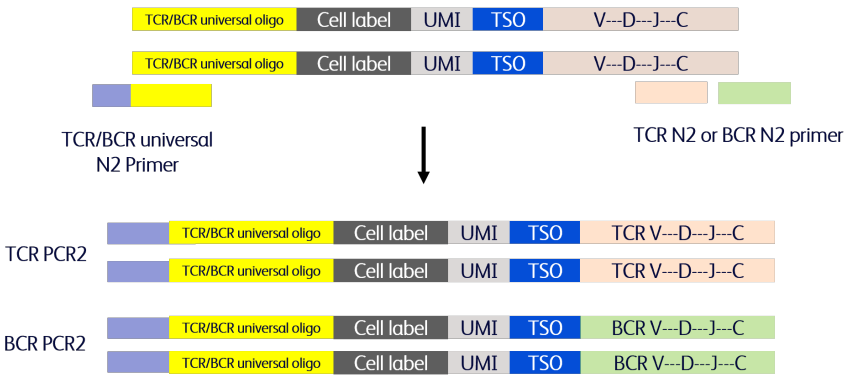
TCR/BCR library amplification workflow

TCR/BCR
PCR1: TCR/BCR universal primer and TCR/BCR N1 primer copy target region from the bead.

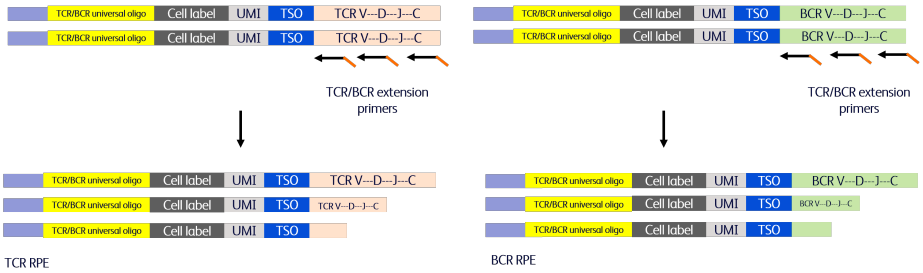


Amplify in solution and collect supernatant as TCR/BCR PCR1 product.

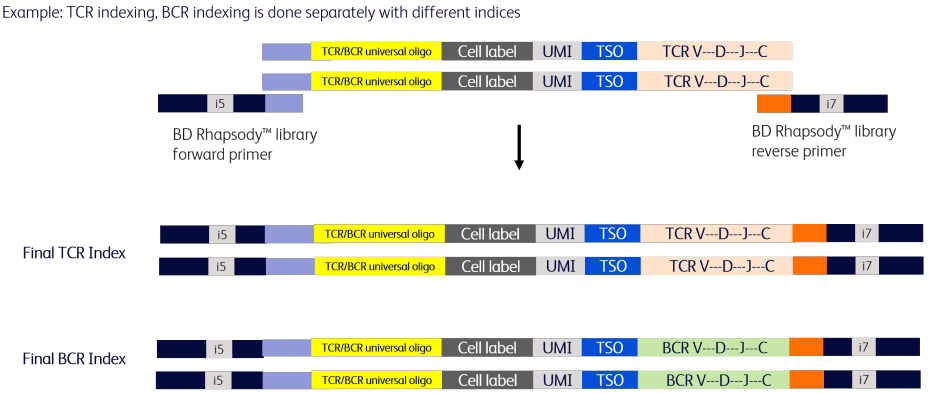
TCR/BCR
PCR2: TCR/BCR universal N2 primer adds sequencing handle. TCR N2 or BCR N2 primer for nested PCR enrichment.



TCR/BCR RPE for fragmentation: Random priming is used for fragmentation for sequencing, full-length VDJ is assembled bioinformatically.



TCR/BCR Index PCR: Add Illumina adapters and indices.



Required and recommended materials

Required reagents

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

Material	Supplier	Catalog no.
BD Rhapsody™ cDNA Kit[a]	BD Biosciences	633773
BD Rhapsody™ Enhanced Cartridge Reagent Kit V3[b]	BD Biosciences	667052
BD Rhapsody™ WTA Amplification Kit[a]	BD Biosciences	633801
BD Rhapsody™ Mouse TCR/BCR Next Amplification Kit[a]	BD Biosciences	667059
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol, molecular biology grade	Major supplier	–
Nuclease-free water	Major supplier	–

a. For processing more than four libraries, two orders of this catalog number are required.

b. The Enhanced Cartridge Reagent Kit V3 must be used before beginning this protocol.

Note: For additional indexing primers for high throughput library preparation workflows, the BD Rhapsody™ Dual Index Kit (Catalog no. 667237) is required.

Recommended consumables

Material	Supplier	Part number/Catalog no.
Pipettes (P10, P20, P200, P1000)	Major supplier	–
Low-retention, filtered pipette tips	Major supplier	–
0.2-mL PCR 8-strip tubes	Major supplier	–
Axygen® 96–Well PCR Microplates[a] Or, MicroAmp Optical 96–Reaction Plate[a]	Corning Thermo Fisher Scientific	PCR96HSC N8010560
MicroAmp Clear Adhesive Filma	Thermo Fisher Scientific	4306311
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
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent High Sensitivity DNA Kit Or, Agilent High Sensitivity D1000 ScreenTape Agilent High Sensitivity D1000 Reagents Or, Agilent High Sensitivity D5000 ScreenTape Agilent High Sensitivity D5000 Reagents	Agilent Agilent Agilent Agilent Agilent	5067-4626 5067-5584 5067-5585 5067-5592 5067-5593

a. Recommended for processing high throughput library preparation workflows.

Equipment

Material	Supplier	Catalog no.
Microcentrifuge for 1.5–2.0-mL tubes	Major supplier	–
Microcentrifuge for 0.2-mL tubes	Major supplier	–
Vortexer	Major supplier	–
Digital timer	Major supplier	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
6-tube magnetic separation rack for 1.5-mL tubes Or, 12-tube magnetic separation rack[a] Or, Invitrogen™ DynaMag™-2 magnet[a]	New England Biolabs New England Biolabs Thermo Fisher Scientific	S1506S S1509S 12321D
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
Magnetic Stand–96[b]	Thermo Fisher Scientific	AM10027
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Agilent® 2100 Bioanalyzer Or, Agilent® 4200 TapeStation System	Agilent Technologies Agilent Technologies	G2940CAG G2991AA
Heat block	Major supplier	–

a. Recommended for processing greater than six samples.

b. Recommended for processing high throughput library preparation workflows.

Best practices

⚠ **Important**: The BD Rhapsody™ Enhanced Cartridge Reagent Kit V3 (Catalog no. 667052) must be used for this protocol. The BD Rhapsody™ TCR/BCR Next Amplification Kit (Catalog no. 667058) is not compatible with the BD Rhapsody™ Enhanced Cartridge Reagent Kit (Catalog no. 664887).

Cell capture

- Ensure that the intended total cell load is 7,500–20,000. Cell loads outside this recommended range may require protocol optimization and might yield suboptimal results.
- For best results, ensure that cells have high viability before proceeding with cell capture.

Bead handling

- When working with BD Rhapsody™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® Tubes.

  Never vortex the beads. Pipet-mix only.

- Store BD Rhapsody™ Enhanced Cell Capture Beads at 4 °C. Do not freeze.
- Bring Agencourt AMPure® XP magnetic beads to room temperature (15–25 °C) before use. See the *AMPure® XP User's Guide* for information.

Master mix preparation

- Thaw reagents (except for enzymes) at room temperature.
- Keep enzymes at –25 °C to –15 °C until ready for use.
- Return reagents to correct storage temperature as soon as possible after preparing the master mix.

Denaturation and self-hybridization

- Remove supernatant promptly after 95 °C denaturation step (≤30 seconds after placing on magnet).
- Ensure that Hybridization Buffer is pre-heated at 80 °C for at least 20 minutes before resuspending beads in step 7 of 1.2 Denaturation and self-hybridization. Using cold or room temperature Hybridization Buffer may negatively impact self-hybridization efficiency.

Supernatant handling

- Read the protocol carefully before beginning each section. Note which steps require you to keep supernatant to avoid accidentally discarding required products.
- Remove supernatants without disturbing AMPure® XP magnetic beads.
- Make and use fresh 80% ethyl alcohol within 24 hours. Adjust the volume of 80% ethyl alcohol depending on the number of libraries.

Bead amplification

- Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension. Start the thermocycler program immediately after mixing.
- Save beads after the first amplification step (2.1 Random priming and extension (RPE)). They must be used again for the second bead amplification step (3.1 TCR/BCR PCR1).

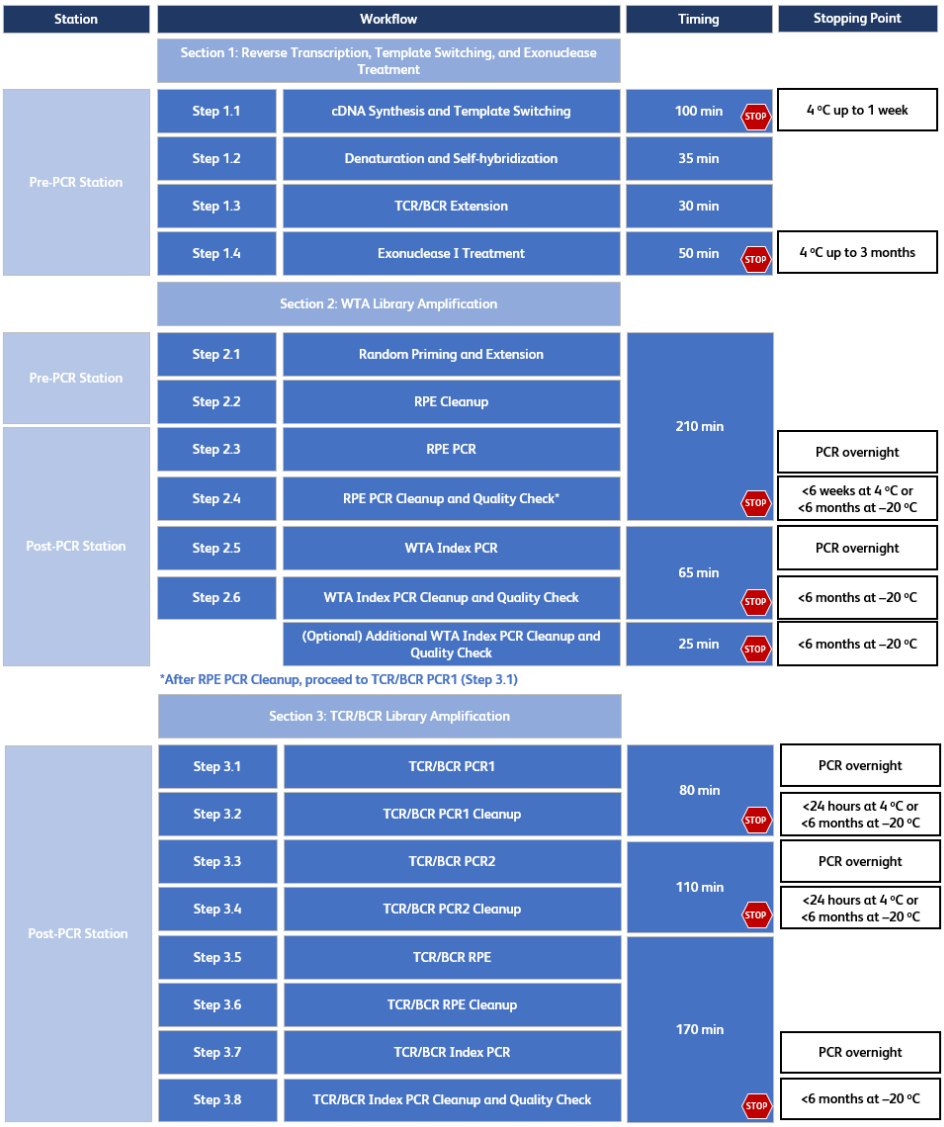
Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*

Safety information

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

Time considerations



Procedure

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system following either the:

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*

  STOP after the "Washing BD Rhapsody™ Enhanced Cell Capture Beads" section and follow this protocol from Reverse transcription, template switching, and Exonuclease I treatment and subsequent steps.

  or

- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*

STOP after the "Washing BD Rhapsody™ Enhanced Cell Capture Beads" section and follow this protocol from Reverse transcription, template switching, and Exonuclease I treatment and subsequent steps.

⚠ **Important: The BD Rhapsody™ Enhanced Cartridge Reagent Kit V3 (Catalog no. 667052) must be used for this protocol.**

Ensure that the intended total cell load is between 7,500–20,000 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.

1. Reverse transcription, template switching, and Exonuclease I treatment

1.1 cDNA synthesis and template switching

Thaw reagents (except for the enzymes) in the BD Rhapsody™ cDNA Kit and necessary reagents from the BD Rhapsody™ TCR/BCR Next Amplification Kit at room temperature. 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. If performing self-hybridization on the same day, set a second thermomixer to:
   - 1,200 rpm and at 80 °C for 3 minutes.
   - 1,200 rpm and at 25 °C for 1 minute.

   Note: The thermomixer set to 80 °C will be used as a heat block to warm the Hybridization Buffer, and then used with programmed cooling during 1.2 Denaturation and self-hybridization.
3. In a new 1.5-mL LoBind® tube, pipet the following reagents.

cDNA/template switching mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
	RT Buffer	40.0	48.0	192.0	384.0
	dNTP	20.0	24.0	96.0	192.0
	RT 0.1 M DTT	10.0	12.0	48.0	96.0
	Bead RT/PCR Enhancer	12.0	14.4	57.6	115.2
	RNase Inhibitor	10.0	12.0	48.0	96.0
	Reverse Transcriptase	10.0	12.0	48.0	96.0

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	Nuclease-free water	98.0	117.6	470.4	940.8
	Total	**200.0**	**240.0**	**960.0**	**1920.0**

4. Gently vortex mix, briefly centrifuge, and place back on ice.
5. Place the tube of washed BD Rhapsody™ Enhanced Cell Capture Beads on a 1.5-mL tube magnet for ≥2 minutes. Discard the supernatant.
6. Remove the tube from the magnet and pipet 200 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.
7. Transfer the bead suspension to a new 1.5-mL LoBind® tube.
8. Incubate the bead suspension on the thermomixer at 1,200 rpm and 42 °C for 30 minutes.

   ⚠ **Important: Shaking is critical for this incubation.**
9. While the bead suspension is still incubating at 1,200 rpm and 42 °C, pipet the following reagents in a new 1.5-mL LoBind® tube.

   Note: Prepare the TSO Mix approximately within 2 minutes before the 30 minutes incubation at 42 °C is finished.

   ⚠ **Important: Use immediately.**

   TSO Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
●	TSO Next	6.0	7.2	28.8	57.6
●	1M $MgCl_2$	2.0	2.4	9.6	19.2
	Total	**8.0**	**9.6**	**38.4**	**76.8**

10. Gently vortex mix, briefly centrifuge, and keep on ice.
11. Add 8 µL of TSO Mix to the reaction, gently pipet-mix, and incubate on the thermomixer for another 30 minutes at 1,200 rpm and 42 °C.

    ⚠ **Important**: If you are performing self-hybridization on the same day, complete steps 3 and 4 from 1.2 Denaturation and self-hybridization now.
12. Place the bead suspension on the 1.5-mL tube magnet until the solution is clear (≤1 minute). Discard the supernatant.
13. Remove the tube from the magnet and pipet 200 µL of Elution Buffer into the tube. Pipet-mix. Place on ice.

OPTIONAL STOPPING POINT: BD Rhapsody™ Enhanced Cell Capture Beads can be stored up to 7 days at 2–8 °C after template switching.

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

1.2 Denaturation and self-hybridization

Thaw the Hybridization Buffer at room temperature.

1. Set a heat block to 95 °C.
2. Program the thermomixer.
   a. 1,200 rpm and at 80 °C for 3 minutes.
   b. 1,200 rpm and at 25 °C for 1 minute.

   Note: If you performed cDNA synthesis on the same day, this is the same thermomixer from step 2 of 1.1 cDNA synthesis and template switching, and the thermomixer is already programmed.

   ⚠ **Important**: Confirm "Time Mode" on the thermomixer is set to "Temperature Control" to ensure that the 25 °C temperature is reached before the 1 minute at 25 °C (step b) begins.
3. Prepare Hybridization Buffer for self-hybridization. Aliquot 1.2 mL Hybridization Buffer into a new 1.5-mL LoBind® tube and place the tube in the pre-heated 80 °C thermomixer (from step 2) without shaking.
4. Keep the tube of Hybridization Buffer in the 80 °C thermomixer until ready to use, at least 20 minutes before resuspending beads in step 7 of this section.
5. To denature, incubate the tube in the following order:
   a. Ensure that the beads are resuspended. Pipet-mix to resuspend, if needed.
   b. Incubate the tube at 95 °C in a heat block for 5 minutes.
   c. Immediately after the completion of the 95 °C incubation, slightly open the lid of the tube to release air pressure within the tube.
6. Immediately place the tube on the magnet for ≤30 seconds until clear. Discard the supernatant.
7. Resuspend the beads in 1.0 mL of pre-heated 80 °C Hybridization Buffer, and immediately place in the pre-programed thermomixer from step 2. Start the program.

   Note: Incubation will take approximately 25 minutes.
8. After the hybridization step, place tube on ice for at least 1 minute while TCR/BCR Extension Mix is being prepared.

1.3 TCR/BCR extension

Thaw the reagents for TCR/BCR Extension at room temperature. Keep TCR/BCR Extension enzyme at –25 °C to –15 °C.

1. Set a thermomixer to 37 °C.
2. Ensure all reagents other than the TCR/BCR Extension enzyme are at room temperature.
3. In a new 1.5-mL LoBind® tube, pipet the following reagents.

TCR/BCR Extension Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	TCR/BCR Extension Buffer	20	24	96	192
●	dNTP	20	24	96	192
○	TCR/BCR Extension Enzyme	10	12	48	96
○	Nuclease-free water	150	180	720	1440
	Total	**200**	**240**	**960**	**1920**

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 BD Rhapsody™ Enhanced Cell Capture Beads on a 1.5-mL tube magnet for ≤2 minutes. Discard the supernatant.
7. Remove the tubes from magnet and resuspend using 200 µL of TCR/BCR 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.

1.4 Exonuclease I treatment

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

1. Set one thermomixer to 37 °C and a heat block to 80 °C.
2. In a new 1.5-mL LoBind® tube, pipet the following reagents.

Exonuclease I Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
●	10X Exonuclease I Buffer	20	24	96	192
●	Exonuclease I	10	12	48	96
○	Nuclease-free water	170	204	816	1632
	Total	**200**	**240**	**960**	**1920**

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

4. Place the tube of BD Rhapsody™ Enhanced Cell Capture Beads with TCR/BCR Extension Mix on a 1.5-mL tube magnet for ≤1 minute. Discard 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 in the 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. Discard 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 2–8 °C for up to 3 months.
12. Proceed to library preparation.

2. WTA library amplification

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents in the BD Rhapsody™ WTA Amplification Kit at room temperature (15–25 °C), then immediately place on ice.

2.1 Random priming and extension (RPE)

This section describes how to generate random priming products. First, random primers are hybridized to the cDNA on the BD Rhapsody™ Enhanced Cell Capture Beads, followed by extension with an enzyme.

Note: Perform this procedure in the pre-amplification workspace.

1. Set a heat block to 95 °C and set two thermomixers to 37 °C and 25 °C, respectively.
2. In a new 1.5-mL LoBind® tube, pipet the following reagents.

Random Primer Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
	WTA Extension Buffer	20.0	24.0	96.0	192.0
	WTA Extension Primers	20.0	24.0	96.0	192.0
	Nuclease-free water	134.0	160.8	643.2	1286.4
	Total	**174.0**	**208.8**	**835.2**	**1670.4**

3. Pipet-mix the Random Primer Mix and keep at room temperature.
4. Resuspend the Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads with a pipette.
5. Place the tube of Exonuclease I-treated beads in Bead Resuspension Buffer on the 1.5-mL magnet for 2 minutes. Remove the supernatant.
6. Briefly centrifuge the tube, then place the tube on a 1.5-mL magnet for 2 minutes. Discard the supernatant.
7. Remove the tube with the BD Rhapsody™ Enhanced Cell Capture Beads from the magnet, and use a low-retention tip to pipet 174 µL of Random Primer Mix into the tube. Pipet-mix 10 times to resuspend the beads.
8. Incubate the tube in the following order:
   a. 95 °C in a heat block (no shaking) for 5 minutes.
   b. Thermomixer at 1,200 rpm and at 37 °C for 5 minutes.
   c. Thermomixer at 1,200 rpm and at 25 °C for 15 minutes.
9. Briefly centrifuge the tube and keep it at room temperature.
10. In a new 1.5-mL LoBind® tube, pipet the following reagents.

Extension Enzyme Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
	dNTP	8.0	9.6	38.4	76.8
	Bead RT/PCR Enhancer	12.0	14.4	57.6	115.2
	WTA Extension Enzyme	6.0	7.2	28.8	57.6
	Total	**26.0**	**31.2**	**124.8**	**249.6**

11. Pipet-mix the Extension Enzyme Mix.
12. Pipet 26 µL of the Extension Enzyme Mix into the sample tube containing the beads (for a total volume of 200 µL) and keep at room temperature until ready.
13. Program the thermomixer.
    a. 1,200 rpm and at 25 °C for 10 minutes.
    b. 1,200 rpm and at 37 °C for 15 minutes.
    c. 1,200 rpm and at 45 °C for 10 minutes.
    d. 1,200 rpm and at 55 °C for 10 minutes.

    ⚠ **Important**: Confirm “Time Mode” is set to “Time Control” before the program begins.
14. Place the tube from step 12 in the thermomixer. Remove the tube after the program is finished.
15. Place the tube in a 1.5-mL tube magnet and discard the supernatant.

16. Remove the tube from the magnet and resuspend the beads in 205 µL of Elution Buffer using a P200 pipette.
17. To denature the random priming products off the beads, pipet to resuspend the beads.
    a. Incubate the sample at 95 °C in a heat block for 5 minutes (no shaking).
    b. Immediately after completing the 95 °C incubation, slightly open the lid of the tube to release air pressure within the tube.

    Note: Do not incubate for more than 5 minutes.
18. Place the tube in a 1.5-mL tube magnet.
    a. Immediately transfer 200 µL of the supernatant containing the Random Primer Extension Product (RPE Product) to a new 1.5-mL LoBind® tube and keep at room temperature.
    b. Proceed to 2.2 RPE cleanup in the following section.
19. Pipet 200 µL of cold Bead Resuspension Buffer to the tube with leftover beads. Gently resuspend the beads by pipet-mixing only. Do not vortex. Store the beads on ice or at 4 °C in the pre-amplification workspace until needed.

    ⚠ **Important**: These beads will be used for target specific amplification during 3. TCR/BCR library amplification. **DO NOT THROW AWAY!**

2.2 RPE cleanup

This section describes how to perform a single-sided AMPure® cleanup, which removes primer dimers and other small molecular weight byproducts. The final product is purified single-stranded DNA.

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

1. In a new 15-mL conical tube, prepare 10 mL of fresh 80% (v/v) ethyl alcohol by pipetting 8 mL of absolute ethyl alcohol to 2 mL of nuclease-free water. Vortex the tube for 10 seconds.

   Note: Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.
2. Bring Agencourt® AMPure® XP magnetic beads to room temperature. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
3. Pipet 320 µL of AMPure® XP magnetic beads into the tube containing the 200 µL of RPE product supernatant. Pipet-mix at least 10 times, then briefly centrifuge.
4. Incubate at room temperature for 10 minutes.
5. Place the tube on the 1.5-mL tube magnet for 5 minutes. Discard the supernatant.
6. Keeping the tube on the magnet, gently add 1 mL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
7. Repeat step 6 for a total of two washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
9. Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.

10. Remove the tube from the magnet and resuspend the bead pellet in 40 µL of Elution Buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
11. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
12. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
13. Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product.
14. Keep on ice until ready to proceed with 2.3 RPE PCR.

2.3 RPE PCR

This section describes how to generate more RPE product through PCR amplification, so that there are multiple copies of each random-primed molecule.

1. In the pre-amplification workspace, in a new 1.5-mL LoBind® tube, pipet the following components.

RPE PCR Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
◯	PCR MasterMix	60	72	288	576
◯	Universal Oligo	10	12	48	96
◯	WTA Amplification Primer	10	12	48	96
	Total	**80**	**96**	**384**	**768**

2. Add 80 µL of the RPE PCR Mix to the tube with the 40 µL of purified RPE product. Pipet-mix 10 times to create the RPE PCR Reaction Mix.
3. Split the mix into two PCR tubes with 60 µL of RPE PCR Reaction Mix per tube.

4. Bring the RPE PCR Reaction Mix to the post-amplification workspace and run the following PCR program.

RPE PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the Recommended number of PCR cycles table[a]	95 °C	30 seconds
Annealing		60 °C	1 min
Extension		72 °C	1 min
Final extension	1	72 °C	2 min
Hold	1	4 °C	∞

a. Recommended PCR cycles might need to be optimized for different cell types and cell number.

Recommended number of PCR cycles

Number of cells in RPE	Recommended PCR cycles for PBMCs
7,500	13
10,000	12
20,000	11

5. When the RPE PCR reaction is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

2.4 RPE PCR cleanup and quality check

This section describes how to perform a single-sided AMPure® cleanup to remove unwanted small molecular weight products from the RPE products. The final product is purified double-stranded DNA (~200–2,000 bp).

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

1. Combine the two 60-µL RPE PCR reactions into a new 1.5-mL tube.
2. Briefly centrifuge the tube with the RPE PCR product.

   ⚠ **Important**: It is critical for the final volume to be exactly 120 µL to achieve the appropriate size selection of the purified RPE PCR product.

3. In a new 15-mL conical tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by pipetting 4 mL of absolute ethyl alcohol to 1 mL of nuclease-free water. Vortex the tube for 10 seconds.

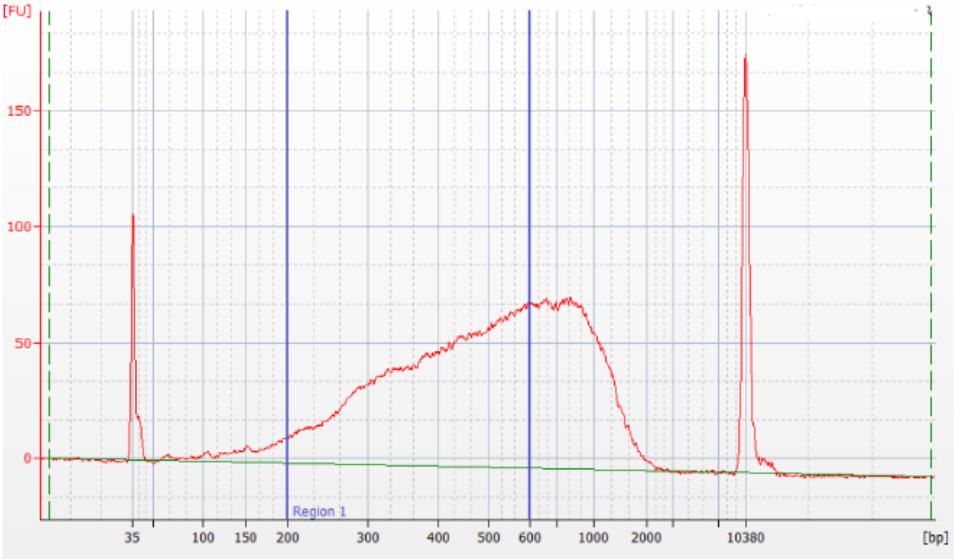
   Note: Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

4. Bring AMPure® XP magnetic beads to room temperature. Vortex the AMPure® XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.
5. Pipet 96 µL of AMPure® XP magnetic beads into the tube containing 120 µL of RPE PCR product. Pipet-mix at least 10 times, then briefly centrifuge the samples.
6. Incubate at room temperature for 5 minutes.
7. Place the 1.5-mL LoBind® tube on the magnet for 5 minutes. Discard the supernatant.
8. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant without disturbing the beads.
9. Repeat step 8 once for a total of two washes.
10. Keeping the tube on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
11. Air-dry the beads at room temperature for 3 minutes.
12. Remove the tube from the magnet and pipet 40 µL of Elution Buffer into the tube. Pipet-mix the suspension at least 10 times until the beads are fully suspended.
13. Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the eluate (~40 µL) into a new 1.5-mL LoBind® tube. The RPE PCR product is ready for WTA Index PCR.

    ⚠ **STOPPING POINT**: The RPE PCR libraries can be stored at –20 °C for up to 6 months or 4 °C for up to 6 weeks.

16. Perform quality control of the RPE PCR products by quantifying 2 µL of the final sequencing library with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay and with the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit.
    a. The expected concentration from the Qubit™ Fluorometer is ~0.5 to 10 ng/µL.
    b. The Bioanalyzer trace should show a broad peak from ~150 to 2,000 bp. Use the concentration from 200 to 600 bp to calculate how much template to add into Index PCR. Refer to the blue-boxed regions in the sample trace images in Figure 1.

Figure 1 Sample Bioanalyzer High Sensitivity DNA trace - RPE PCR product trace



2.5 WTA index PCR

This section describes how to generate mRNA libraries compatible with the Illumina sequencing platform, by adding full-length Illumina sequencing adapters and indices through PCR.

Note: Perform this procedure in the post-amplification workspace.

1. Dilute the RPE PCR products from 2.4 RPE PCR cleanup and quality check with nuclease-free water such that the concentration of the 200–600 bp peak is 2 nm. If the product concentration is <2 nm, do not dilute. Continue to the next step.

   Example: If the Bioanalyzer measurement of the 200–600 bp peak is 6 nm, then dilute the sample threefold with nuclease-free water to 2 nm.

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

WTA Index PCR Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	PCR MasterMix	25	30	120	240
●	Library Forward Primer	5	6	24	48
●	Library Reverse Primer 1–4[a]	5	6	–	–
○	Nuclease-free water	5	6	24	48
	Total	**40**	**48**	**168**	**336**

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

3. Gently vortex mix, briefly centrifuge, and place back on ice.
4. In a new 0.2-mL PCR tube, combine WTA Index PCR Mix with diluted RPE PCR products as follows:
   - For one sample, combine 40 µL of WTA Index PCR Mix with 10 µL of 2 nm of RPE PCR product.
   - For multiple samples, combine 35 µL of WTA Index PCR Mix with 5 µL of Library Reverse Primer and 10 µL of 2 nm of RPE PCR products.
5. Pipet-mix 10 times.
6. Run the following PCR program.

Index PCR conditions for WTA

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the Recommended number of PCR cycles table[a]	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	30 seconds
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

a. Cycle number varies based on the concentration of the RPE PCR product.

Recommended number of PCR cycles

Concentration of diluted RPE PCR products	Recommended number of PCR cycles
1 to <2 nm	9
2 nm	8

If the concentrations of diluted RPE PCR products are <1 nm, additional PCR cycles might be needed.

⚠ **STOPPING POINT**: The PCR can run overnight.

7. When the WTA Index PCR is complete, briefly centrifuge to collect the contents at the bottom of the tubes.

2.6 WTA index PCR cleanup and quality check

This section describes how to perform a single-sided AMPure® cleanup for Illumina sequencing. The final product is purified double-stranded DNA with full-length Illumina adapter sequences.

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

1. Add 60 µL of nuclease-free water to the WTA Index PCR product for a final volume of 110 µL.
2. Transfer 100 µL of WTA Index PCR product into a new 0.2-mL PCR tube.
3. 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.

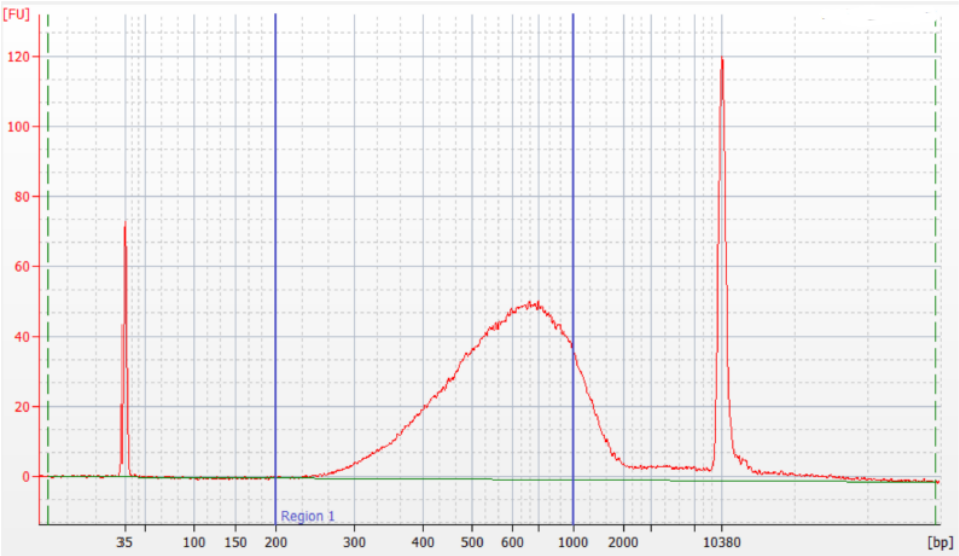
Note: Make fresh 80% ethyl alcohol, and use it within 24 hours.

4. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until the beads are fully resuspended.
5. Add 65 µL of AMPure® XP magnetic beads to the 0.2-mL PCR tube from step 2.
6. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
7. Place the tube on the strip tube magnet for 3 minutes. Discard the supernatant.
8. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
9. Repeat step 8 for a total of two washes.
10. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
11. Leave the tubes open on the magnet to dry the AMPure® XP magnetic beads at room temperature for ~1 minute. Do not over-dry the AMPure® XP magnetic beads.
12. Pipet 30 µL of Elution Buffer into the tubes and pipet-mix to completely resuspend the AMPure® XP magnetic beads.
13. Incubate the samples at room temperature for 2 minutes.
14. Briefly centrifuge the tubes to collect the contents at the bottom.
15. Place the tubes on the magnet until the solution is clear, usually ~30 seconds.
16. Pipet the eluate (~30 µL) into new 1.5-mL LoBind® tubes. The WTA Index PCR eluate is the final sequencing libraries.

    ⚠ **STOPPING POINT**: The Index PCR libraries can be stored at –20 °C for up to 6 months until sequencing.

17. Perform quality control of the Index PCR libraries by quantifying 2 µL of the final sequencing library with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay and with the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit.
    a. The expected concentration from the Qubit™ Fluorometer is >1 ng/µL.
    b. The Bioanalyzer trace should show a peak from ~300 to 2,000 bp.

Figure 2 Sample Bioanalyzer High Sensitivity DNA trace - WTA index PCR product



3. TCR/BCR library amplification

Before you begin

- Obtain Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents in the BD Rhapsody™ Mouse TCR/BCR Next Amplification Kit at room temperature, and then place on ice.

3.1 TCR/BCR PCR1

1. Obtain beads from Step 19 of 2.1 Random priming and extension (RPE).
2. In the pre-amplification workspace, pipet the following reagents into a new 1.5-mL LoBind® tube.

TCR/BCR PCR1 Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	PCR MasterMix	100.00	120.00	480.00	960.00
●	TCR/BCR Universal Oligo N1	10.00	12.00	48.00	96.00
●	Bead RT/PCR Enhancer	12.00	14.40	57.6	115.20
●	Mouse TCR N1 Primer[a]	2.40	2.88	11.52	23.04
●	Mouse BCR N1 Primer[a]	2.40	2.88	11.52	23.04
○	Nuclease-free water	73.20	87.84	351.36	702.72
	Total	**200.00**	**240.00**	**960.00**	**1920.00**

a. If only doing TCR or BCR amplification, replace N1 primer volume with nuclease-free water. For example, if only doing TCR amplification, replace BCR N1 primer with nuclease-free water.

3. Gently vortex mix, briefly centrifuge, and place back on ice.
4. Briefly spin the tube with the bead suspension. Place the tube of beads in Bead Resuspension Buffer on a 1.5-mL magnet for ≤1 minute. Discard the supernatant.
5. Remove the tube from the magnet and resuspend the beads in 200 µL of TCR/BCR PCR1 Mix to create the TCR/BCR PCR1 Reaction Mix. Do not vortex.
6. Ensuring that the beads are fully resuspended, pipet 50 µL of TCR/BCR PCR1 Reaction Mix with beads into each of four 0.2-mL PCR tubes. Transfer any residual mix to one of the tubes.
7. Bring the TCR/BCR PCR1 Reaction Mix to the post-amplification workspace.
8. Run the following PCR program on the thermal cycler.

PCR1 conditions for TCR/BCR panel

Step	Cycles	Temperature	Time
Hot start	1	95 °C[a]	3 min
Denaturation	10-11[b]	95 °C	30 seconds
Annealing		60 °C	1 min
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

a. To avoid beads settling due to prolonged incubation time on the thermal cycler before the denaturation step, it is critical to pause the instrument at 95 °C before loading the samples. Different thermal cyclers might have different pause time settings. In certain brands of thermal cyclers, however, we have observed a step-skipping error with the pause/unpause functions. To ensure that the full 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. Recommended PCR cycles might need to be optimized for different cell types and cell number.

Recommended number of PCR cycles

Number of cells in PCR1	Recommended PCR cycles
7,500 - 10,000	11
20,000	10

9. Ramp the heated lid and heat block of the post-amplification thermal cycler to ≥95 °C by starting the thermal cycler program and then pausing it.

   Note: Do not proceed to thermal cycling until each tube is gently mixed by pipette to ensure uniform bead suspension.

10. 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.

11. After PCR, briefly centrifuge the tubes.

12. Put the tubes on a strip tube magnet for >30 seconds. Remove and carefully combine the supernatant in a new 1.5-mL LoBind® tube without disturbing the beads.

    ⚠ **Important**: Save the supernatant for purification of TCR/BCR PCR1 products. Keep on ice until proceeding to TCR/BCR PCR2.

13. Place the 0.2-mL PCR tubes on a magnet for ≤1 minute. Retain the supernatant.

14. For each sample, collect and combine the supernatant from the four 0.2-mL PCR tubes into one new 1.5-mL LoBind® tube.

15. For each sample, add 50 µL of cold Bead Resuspension Buffer to each 0.2-mL PCR tube, and then combine the beads into a new 1.5-mL LoBind® tube.

16. Store the beads at 2–8 °C in the post-amplification workspace.

3.2 TCR/BCR PCR1 cleanup

This section describes how to perform a single-sided AMPure® cleanup to remove primer dimers from the TCR/BCR PCR1 products. The final product is purified double-stranded DNA.

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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2. Bring the AMPure® XP magnetic beads to room temperature. Vortex on high speed for 1 minute until the beads are fully resuspended.
3. To 200 µL of TCR/BCR PCR1 products, pipet 140 µL AMPure® beads (from step 13 in 3.1 TCR/BCR PCR1).
4. Pipet-mix 10 times. Incubate at room temperature for 5 minutes.
5. Place the 1.5-mL LoBind® tube on the magnet for 5 minutes. Discard the supernatant.
6. Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
7. Repeat step 6 once for a total of two washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
9. Air-dry the beads at room temperature for 5 minutes.
10. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of Elution Buffer. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.
11. Incubate at room temperature for 2 minutes and briefly centrifuge.
12. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
13. Pipet the eluate (~50 µL) into a new 1.5-mL LoBind® tube (purified TCR/BCR PCR1 products).

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

3.3 TCR/BCR PCR2

This section describes how to amplify TCR/BCR products through PCR.

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

TCR and BCR PCR2 Mixes

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	PCR MasterMix	25.0	30.0	120.0	240.0
🔵	TCR/BCR Universal Oligo N2	2.0	2.4	9.6	19.2
🔴 🟢	Mouse TCR N2 Primer or Mouse BCR N2 Primer [a]	6.0	7.2	28.8	57.6
○	Nuclease-free water	12.0	14.4	57.6	115.2
	Total	**45.0**	**54.0**	**216.0**	**432.0**

a. PCR2 mixes for TCR and BCR are made separately.

2. Gently vortex mix, briefly centrifuge, and place back on ice. Pipet 45 µL of PCR2 Mix into one 0.2-mL PCR tube for each library.
3. Bring the TCR PCR2 Mix and the BCR PCR2 Mix to the post-amplification workspace.
4. Pipet 5.0 µL of PCR1 products into 45 µL of PCR2 Mix for each library to create the TCR PCR2 Reaction Mix and BCR PCR2 Reaction Mix, respectively.
5. Gently vortex and briefly centrifuge.
6. Run the following PCR program on the thermal cycler.

PCR2 conditions for TCR and BCR

Step	Cycles	Temperature	Time	
Phase I:	1	95 °C	3 min	Press Option > **Auto Delta Starting cycle** > "2" Delta > "1 degree" > Done The temperature decreases by 1 °C each cycle, from 75 °C to 56 °C.
	15	95 °C	30 seconds	
		70–56 °C	1 min	
		72 °C	1 min	
Phase II:	8	95 °C	30 seconds	
		60 °C	1 min	
		72 °C	1 min	
	1	72 °C	5 min	
	1	4 °C	∞	

⚠ **STOPPING POINT**: The PCR can run overnight.

3.4 TCR/BCR PCR2 cleanup

This section describes how to perform a single-sided AMPure® cleanup to remove primer dimers from the TCR and BCR PCR2 products. The final product is purified double-stranded DNA.

Note: Perform PCR2 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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.
3. To 50.0 µL PCR2 products, pipet 35 µL of AMPure® beads.
4. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
5. Place the tube on the strip tube magnet for 3 minutes. Discard the supernatant.
6. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
7. Repeat step 6 once for a total of two washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.

9. Air-dry the beads at room temperature for 3 minutes.
10. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
11. Incubate at room temperature for 2 minutes and briefly centrifuge.
12. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
13. Pipet the eluate (~50 µL) into a new 1.5-mL LoBind® tube.

    ⚠ **STOPPING POINT**: Store at 2–8 °C before proceeding on the same day, or at –25 °C to –15 °C for up to 6 months.
14. Estimate the concentration by quantifying 2 µL of the final sequencing library with a Qubit™ Fluorometer using the Qubit™ dsDNA HS Assay Kit. Follow the manufacturer's instructions.

3.5 TCR/BCR RPE

1. Dilute an aliquot of the TCR and BCR PCR2 products with nuclease-free water to 1.0 ng/µL.

   Note: If PCR2 concentration is <1 ng/µL, increase the volume of PCR2 product needed to ensure 5 ng total concentration and decrease the volume of water in the Random Primer Mix accordingly.
2. In pre-amplification workspace, pipet the following reagents into a new 1.5 mL LoBind® tube:

Random Primer Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
◯	TCR/BCR Extension Buffer	5.0	6.0	24.0	48.0
◯	TCR/BCR Extension Primers	2.5	3.0	12.0	24.0
◯	Nuclease-free water	Up to 34.0	Up to 40.8	Up to 163.2	Up to 326.4
	Total	**41.5**	**49.8**	**199.2**	**398.4**

3. Pipet-mix the Random Primer Mix and keep at room temperature. Pipet 41.5 µL of Random Primer Mix into one 0.2-mL PCR tube for each library.
4. Add 5 µL of 1.0 ng/µL purified TCR or BCR PCR2 products into each 0.2-mL PCR tube containing Random Primer Mix.
5. Perform denaturation and random priming on thermocycler using the following program:

Program

Temperature	Time	Cycles
95 °C	5 min	1
37 °C	5 min	
25 °C	15 min	

6. Briefly centrifuge the tube and keep at room temperature.
7. In pre-amplification workspace, pipet the following reagents into a new 1.5 mL LoBind® tube:

Primer Extension Enzyme Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
	dNTP	2.0	2.4	9.6	19.2
	TCR/BCR Extension Enzyme	1.5	1.8	7.2	14.4
	Total	**3.5**	**4.2**	**16.8**	**33.6**

8. Gently vortex mix, centrifuge, and place on ice.
9. Add 3.5 µL Primer Extension Enzyme Mix to the Random Priming Reaction tube to bring total volume up to 50 µL. Run the following protocol on a thermocycler for Extension:

Program

Temperature	Time	Cycles
25 °C	10 min	1
37 °C	15 min	
45 °C	10 min	
55 °C	10 min	

10. Remove the tubes from the thermocycler and prepare to purify RPE product.

3.6 TCR/BCR RPE cleanup

Note: Perform 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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.
3. To the TCR and BCR RPE products, add 90 µL AMPure® beads.
4. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
5. Place the tube on the strip tube magnet for 3 minutes. Discard the supernatant.
6. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
7. Repeat step 6 once for a total of two washes.
8. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
9. Air-dry the beads at room temperature for 1 minute.
10. Remove tubes from the magnet and add 50 µL of Elution Buffer.
11. Incubate at room temperature for 2 minutes and briefly centrifuge.
12. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
13. Pipet the entire eluate (~50 µL) into a new 1.5-mL LoBind® tube separately (purified TCR and BCR RPE products).

3.7 TCR/BCR index PCR

This section describes how to generate TCR/BCR libraries compatible with the Illumina sequencing platform, by adding full-length Illumina sequencing adapters and indices through PCR.

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

TCR/BCR Index PCR Mix

Cap	Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
○	PCR MasterMix	25.0	30.0	120.0	240.0
●	Library Forward Primer[a]	2.0	2.4	9.6	19.2
●	Library Reverse Primer[a]	2.0	2.4	–	–
	Total	**29.0**	**34.8**	**129.6**	**259.2**

a. For more than one library, use different Library Reverse Primers for each TCR or BCR library. If needed, use the BD Rhapsody™ Dual Index Kit to process additional samples.

2. Gently vortex mix, briefly centrifuge, and place back on ice.
3. Bring the TCR/BCR Index PCR Mix to post-amplification workspace.

4. In new 0.2 mL PCR tubes, pipet 21 µL of TCR/BCR RPE purified products into 29 µL of TCR/BCR Index PCR Mix.
5. Gently vortex, and briefly centrifuge.
6. Run the following PCR program on the thermal cycler.

Index PCR conditions for TCR/BCR

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10	95 °C	30 seconds
Annealing		60 °C	30 seconds
Extension		72 °C	30 seconds
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

⚠ **STOPPING POINT**: The PCR can run overnight.

3.8 TCR/BCR index PCR cleanup and quality check

This section describes how to perform a single-sided AMPure® cleanup to remove primer dimers from the TCR/BCR index PCR products. The final product is purified double-stranded DNA with full-length Illumina adapter sequences.

Note: Perform Index PCR 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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol, and use it within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.
2. Bring AMPure® XP beads to room temperature and vortex at high speed for 1 minute until the beads are fully resuspended.
3. Briefly centrifuge all the index PCR products.
4. Transfer 40 µL of the TCR and/or BCR index PCR products to a new strip tube or tubes.
5. Pipet 26 µL of AMPure® beads.
6. Pipet-mix 10 times and incubate at room temperature for 5 minutes.
7. Place the tube on the strip tube magnet for 3 minutes. Discard the supernatant.
8. Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
9. Repeat step 7 for a total of two washes.

10. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
11. Air-dry the beads at room temperature for 3 minutes.
12. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
13. Incubate at room temperature for 2 minutes, and briefly centrifuge.
14. Place the tube on the magnet until the solution is clear, usually ~30 seconds.
15. Pipet the entire eluate (~50 µL) into a new 1.5-mL LoBind® tube (final sequencing libraries).
16. Perform quality control before freezing samples.
    a. 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.
    b. Measure the average fragment size of the TCR/BCR library 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.

Figure 3 Sample Bioanalyzer High Sensitivity DNA trace - TCR index PCR product

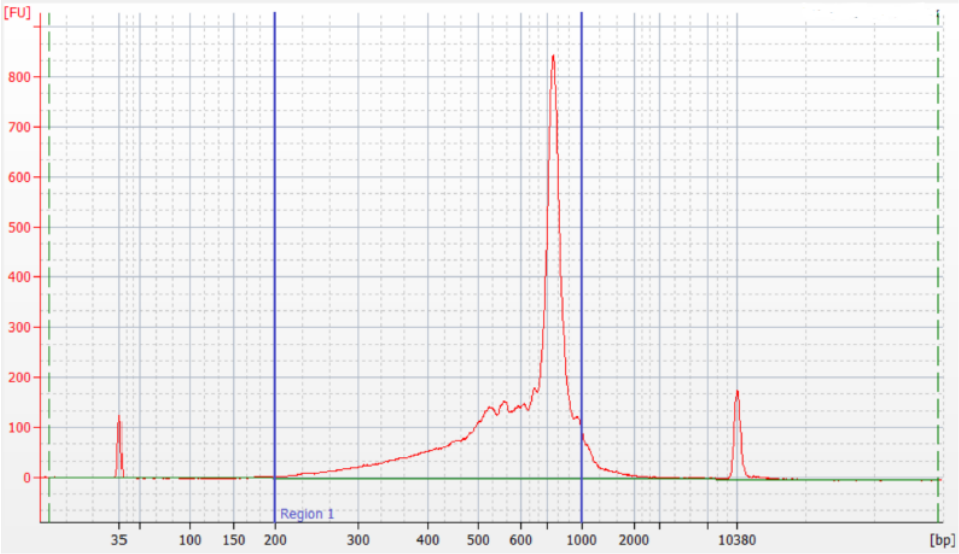
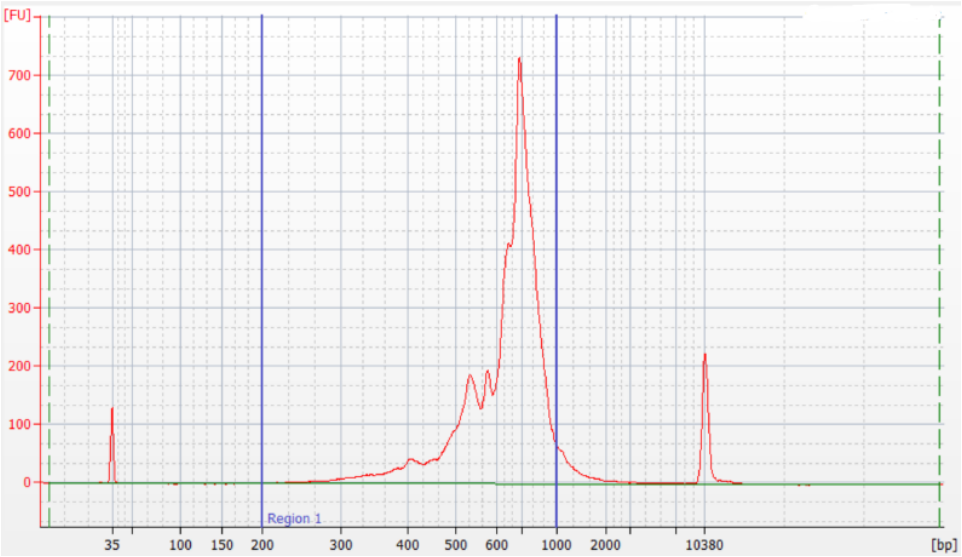


Figure 4 Sample Bioanalyzer High Sensitivity DNA trace - BCR index PCR product



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

Sequencing

The sequencing depth for each library is dependent on application. For cell-type clustering, shallow sequencing is sufficient. However, for in-depth analysis, such as comparison across multiple samples, deep sequencing is advised. We recommend meeting the requirement for recursive substitution error correction (RSEC) sequencing depth of ≥6 to reach the threshold of sequencing saturation where most molecules of the library have been recovered, approximately 80%. The RSEC sequencing depth and sequencing saturation are both reported by the analysis pipeline. The actual sequencing reads/cell required to achieve this depth can vary as it is dependent on the gene expression levels, number of cells, and sequencing run quality. The following reads/cell are recommended for WTA mRNA, TCR, and BCR libraries.

Read requirements for libraries

Library	Read requirement for data analysis
WTA mRNA	10,000–100,000 reads/cell
TCR	~5,000 reads/T cell
BCR	~5,000 reads/B cell

Pooling libraries for sequencing

The efficiency of sequencing on Illumina instruments is influenced by many conditions, library size being one of them. The TCR and BCR libraries are ~200–300 bp larger than the WTA mRNA library and ~600 bp larger than the which will cause them to produce less sequencing data if pooled in a 1:1 ratio with the other libraries. To overcome the difference in sequencing efficiency, more DNA of the TCR and BCR libraries needs to be included in the pool than would be expected when calculating ratios based on read depth. The following tables show

examples of different pooling strategies and the expected sequencing outcome, with and without correction for the size of the TCR and BCR libraries. Validation data indicates a 5X volume correction factor is needed for sequencing TCR and BCR libraries with WTA mRNA libraries.

Example of pooling with no correction

In this example, a total of 5,000 enriched T cells were processed. These calculations assume the TCR library, and BCR library if included, sequences at 1/5 the efficiency of the WTA mRNA library, supported by internal testing.

Pooling for WTA mRNA and TCR libraries

A	B	C	D	E	F	G
Library type	**Number of cells**	**Expected reads/cell**	**Reads needed**	**Pooling ratio**	**Sequencing results**	**Sequencing results (reads/cell)**
WTA mRNA	5,000	25,000	125,000,000	83%	145,000,000	29,000
TCR	5,000	5,000	25,000,000	17%	5,000,000	1,000
Total			**150,000,000**	**100%**	**150,000,000**	–

After sequencing, the reads/cell for the TCR library (Column G) does not match with and are much lower than the expectation (Column C), because it does not sequence as efficiently as the WTA mRNA library. The remaining reads are allotted to the WTA mRNA library resulting in more reads than required. To obtain the desired number of reads/cell for each library, a correction factor is required for pooling calculations.

Example of pooling with correction

In this example, the same sample as in the previous one was pooled using a correction factor of 5 for the TCR library to overcome the differences in sequencing efficiency. The amount of data needing to be generated (Column D) is based on the cell number (Column B) and expected number of reads per cell (Column C). Based on this example, 150 million reads are needed to achieve the appropriate read depths. Changing the pooling ratios by correcting for the lower TCR sequencing efficiency will help ensure the correct amount of data is generated for each library. This modified pooling scheme, however, does not change the total amount of data needing to be generated, which is 150 million reads.

Pooling for WTA mRNA and TCR libraries

A	B	C	D	E	F	G	H	I	J
Library type	**Number of cells**	**Expected reads/cell**	**Reads needed**	**Pooling ratio before correction**	**Correction**	**Reads needed for pooling**	**Pooling ratio with correction**	**Sequencing results**	**Sequencing results (reads/cell)**
WTA mRNA	5,000	25,000	125,000,000	83%	N/A	125,000,000	50%	125,000,000	25,000
TCR	5,000	5,000	25,000,000	17%	5[a]	125,000,000	50%	25,000,000	5,000
Total			**150,000,000[b]**	**100%**	–	**250,000,000[c]**	**100%**	**150,000,000**	–

a. The 5X correction factor is a recommended starting point and some fine tuning may be required to achieve the optimal library balance.

b. Total amount of data to be requested from the sequencing facility plus 3% PhiX.

c. Read total only for pooling purposes.

After sequencing, the total amount of data generated (Column I) as well as the reads/cell for each library (Column J) are as expected (Columns D and C respectively). The correction for library pooling did not change the amount of data generated (150 million reads) but helped ensure the data was spread out appropriately to each library.

Example of pooling with a mixed population

The following table shows the pooling logic for a mixed population of cells.

Pooling for WTA mRNA, TCR, and BCR libraries

A	B	C	D	E	F	G	H	I	J
Library type	**Number of cells**	**Expected reads/cell**	**Reads needed**	**Pooling ratio before correction**	**Correction**	**Reads needed for pooling**	**Pooling ratio with correction**	**Sequencing results**	**Sequencing results (reads/cell)**
WTA mRNA	10,000	25,000	250,000,000	88%	N/A	250,000,000	59%	250,000,000	25,000
TCR	4,000	5,000	20,000,000	7%	5[a]	100,000,000	23%	20,000,000	5,000
BCR	3,000	5,000	15,000,000	5%	5[a]	75,000,000	18%	15,000,000	5,000
Total			**285,000,000[b]**	**100%**	–	**425,000,000[c]**	**100%**	**406,000,000**	–

a. The 5X correction factor is a recommended starting point and some fine tuning may be required to achieve the optimal library balance.

b. Total amount of data to be requested from the sequencing facility plus 3% PhiX.

c. Read total only for pooling purposes.

Additional considerations

- The 5X volume correction factor is a recommended starting place for pooling these libraries. This may need to be adjusted to accommodate different types of flow cells (for example, patterned vs non-patterned).
- It can be easier to achieve the desired sequencing depth when sequencing multiple TCR or BCR libraries alone since all the libraries are the same size. Pooling will not require a correction and will only be dependent on the number of cells and the reads/cell. This scheme, however, would require 10–15% PhiX, rather than the 3% PhiX when sequencing with the WTA mRNA library.
- All libraries derived from the same cartridge can be indexed with the same Illumina indices or reverse index primer from the BD Rhapsody™ reagents. The primary analysis pipeline can differentiate the library types (for example, WTA mRNA vs TCR) based on their structure and sequences. Demultiplexing statistics are reported from the pipeline, but should these statistics be desired prior to running the pipeline, then unique indices will be required for each library.

For additional support with pooling and sequencing, reach out to your local Field Application Specialist (FAS) or scomix@bdscomix.bd.com.

Sequencing flow cell loading and PhiX concentrations

Quantifying libraries

Calculate the molar concentration of WTA and TCR/BCR libraries using Qubit™ quantitation concentration (ng/µL) and average Bioanalyzer size (200–1000 bp). For TCR/BCR libraries, the expected Qubit™ concentration should be >1.5 ng/µL. Use the calculated molar concentrations to pool libraries.

WTA and TCR/BCR libraries (with or without BD® AbSeq/Sample Tag)

For a NextSeq High or Mid Output and MiniSeq High or Mid Output runs, load the flow cell at a concentration between 1.4–1.8 pm with 3% PhiX. For other sequencers follow Illumina recommendations for loading concentration and use 3% PhiX.

Note: If using less than 10,000 reads/cell for the WTA library, increase PhiX percentage to 5–10% to account for lower library diversity.

Set up sequencing run on Illumina® BaseSpace. Enter the pooled libraries as one sample if libraries were made with the same Library Forward primer but with different i7 indices.

Required parameters

Parameter	Requirement
Platform	Illumina: 300 cycle kit
Paired-end reads	Minimum: 65 × 150[a] paired read length Recommended: 150 × 150[a] paired read length
PhiX	Required (3%)
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook*

a. For optimal assembly, use the sequencing configuration 85 × 215 paired read length.

Sequencing analysis pipeline

Contact customer support at scomix@bdscomix.bd.com for access to the latest whole transcriptome sequencing analysis pipeline.

Appendix

Illumina Index 1 (i7) sequences

Library Reverse Primer	Sequence
1	GCTACGCT
2	CGAGGCTG
3	AAGAGGCA
4	GTAGAGGA

Illumina Index 2 (i5) sequence

Primer	Sequence
Library Forward Primer	TATAGCCT

Mouse T cell PCR1 primers

Primer name	Primer sequence (5'–3')
Ms_TRAC_N1	TTTTCGGCACATTGATTTGGGAG
Ms_TRBC_N1	CTCAGGCAGTAGCTATAATTGCT
Ms_TRDC_N1	CAATCTTCTTGGATGATCTGAGACT
Ms_TRGC1-TRGC2_N1	GGAAAGAACTTTTCAAGGAGACAAAGG

Mouse T cell PCR2 primers

Primer name	Primer sequence (5'–3')
Ms_TRAC_N2	AGGTTCTGGGTTCTGGATGT
Ms_TRBC_N2	CAATCTCTGCTTTTGATGGCTC
Ms_TRDC_N2	GTAGAAATCTTTCACCAGACAAGC
Ms_TRGC1-TRGC2_N2	TTGGGGGAAATGTCTGCA
Ms_TRGC4_N2	ATAGTAGGCTTGGGAGAAAAGTCTGA

Mouse B cell PCR1 primers

Primer name	Primer sequence (5'–3')
Ms_IGHA_N1	AACTGGCTGCTCATGGTGTA
Ms_IGHD_N1	AAGTGTGGTTGAGGTTCAGTTCTG
Ms_IGHE_N1	GAAGTTCACAGTGCTCATGTTC
Ms_IGHG1_N1	CAGAGTGTAGAGGTCAGACT
Ms_IGHG2A-IGHG2C_N1	TCGAGGTTACAGTCACTGAG
Ms_IGHG2B_N1	GATCCAGAGTTCCAAGTCACAG
Ms_IGHG3_N1	TACGTTGCAGATGACAGTCT
Ms_IGHM_N1	TGGATGACTTCAGTGTTGTTCTG
Ms_IGKC_N1	TGTAGGTGCTGTCTTTGCTG
Ms_IGLC1_N1	CTGTAACTGCTATGCCTTTCCC
Ms_IGLC2-IGLC3_N1	TTGGTGGGATTTGAAGTGTCC

Mouse B cell PCR2 primers

Primer name	Primer sequence (5'–3')
Ms_IGHA_N2	TGTCAGTGGGTAGATGGTGG
Ms_IGHD_N2	CTGACTTCCAATTACTAAACAGCC
Ms_IGHE_N2	TAGAGCTGAGGGTTCCTGATAG
Ms_IGHG1_N2	CAGTGGATAGACAGATGGGGGT
Ms_IGHG2A-IGHG2C_N2	ATGGGGCTGTTGTTTTGG
Ms_IGHG2B_N2	GTGGATAGACTGATGGGGGTGTT
Ms_IGHG3_N2	AGGGAAGTAGCCTTTGACAAG
Ms_IGHM_N2	GACATTTGGGAAGGACTGACTC
Ms_IGKC_N2	AGATGTTAACTGCTCACTGGATG
Ms_IGLC1_N2	GTTAGTCTCGAGCTCTTCAGA
Ms_IGLC2-IGLC3_N2	CAGTGTGGCTTTGTTTTCCT

Contact Information

Becton, Dickinson and Company
BD Biosciences
155 North McCarthy Boulevard
Milpitas, California 95035 USA

bdbiosciences.com
scomix@bdscomix.bd.com