BD Rhapsody™ System

TCR/BCR Full Length, Targeted mRNA, BD® AbSeq, and Sample Tag Library Preparation Protocol

For Research Use Only

23-24016(01)
2022-01

Becton, Dickinson and Company
BD Biosciences
2350 Qume Drive
San Jose, California 95131 USA

bdbiosciences.com
scomix@bdscomix.bd.com

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Regulatory Information

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

History

Revision	Date	Change made
23-24016(01)	2022-01	Initial release.

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Introduction

This protocol enables high-throughput single-cell transcriptome and protein analysis 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. The addition of a sample-tag library enables demultiplexing of samples that were pooled before capture on the BD Rhapsody™ system.

After staining of cell with AbSeq and Sample Tag antibodies and partitioning and lysis of cells, 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. AbSeq and Sample Tag libraries are amplified from the small products recovered from the supernatant after cleanup of larger mRNA, TCR, and BCR PCR1 products.

Required and recommended materials for cDNA synthesis and library preparation

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**
BD Rhapsody™ TCR/BCR Amplification Kit	BD Biosciences	**665345**
BD Rhapsody™ Human Immune Response Panel	BD Biosciences	**633750**
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	**A63880**
100% ethyl alcohol	Major supplier	–
Nuclease-free water	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	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
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™ Enhanced Cell Capture Beads, use low-retention filtered tips and LoBind® tubes.

  Never vortex the beads. Pipet-mix only.

- Bring AMPure XP magnetic beads to room temperature (15 °C to 25 °C) before use. See the *AMPure XP User's Guide* for information.
- Remove supernatants without disturbing AMPure XP magnetic beads.

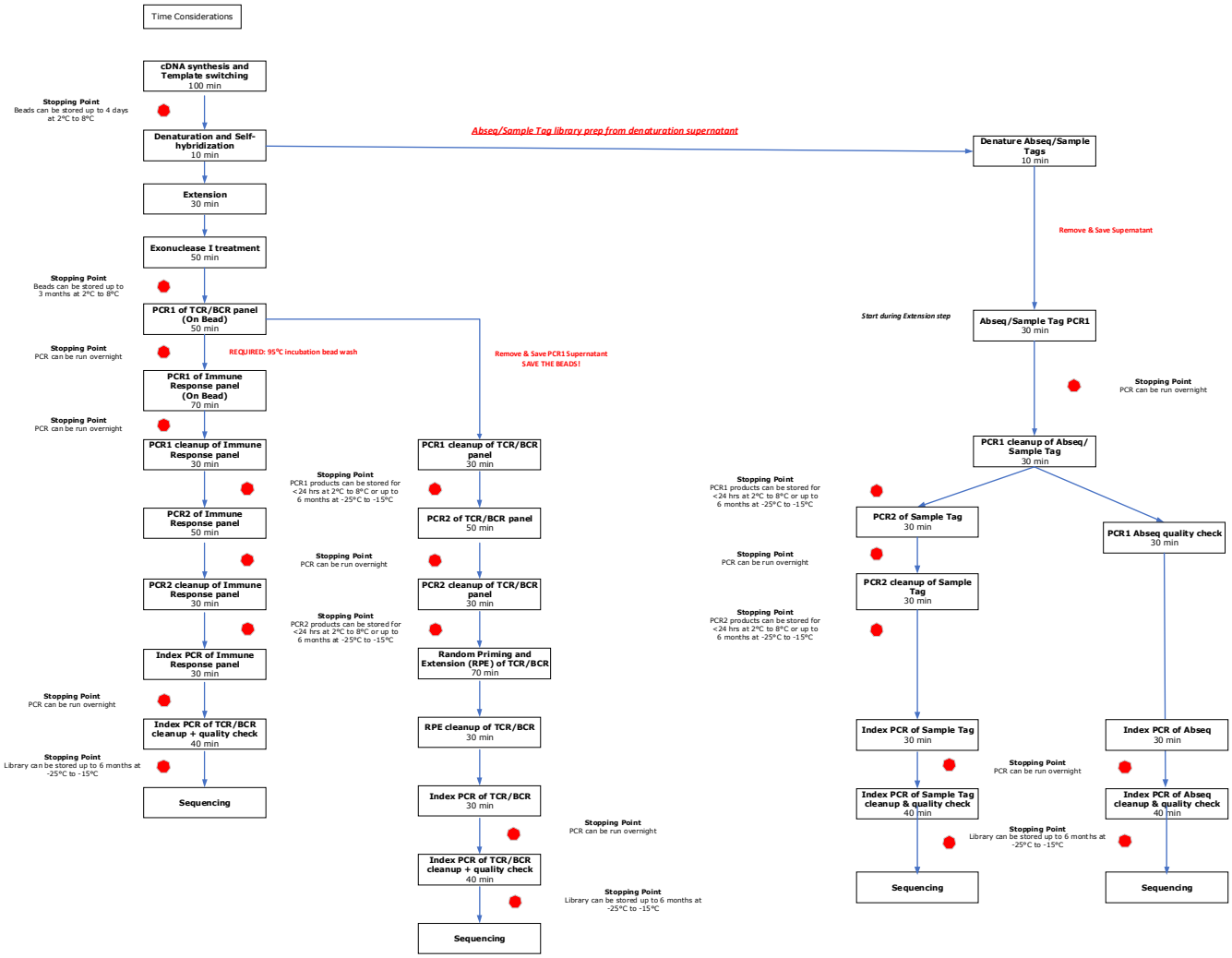
Additional documentation

- *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).

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* (Doc ID 214062)

  STOP after the section "Washing the Cell Capture Beads" and follow this protocol from **Preparing BD Rhapsody™ Enhanced Cell Capture Beads for TCR/BCR full length 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™ Enhanced Cell Capture Beads for TCR/BCR full length library amplification** and subsequent steps.

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.

Preparing BD Rhapsody™ Enhanced Cell Capture Beads for TCR/BCR full length 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. 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	98	117.6
Total	**200**	**240**

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

4 Place the tube of washed Enhanced 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 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.

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 While the bead suspension is still incubating at 1,200 rpm and 42 °C, in a new 1.5-mL LoBind® tube, pipet the following reagents.

NOTE Prepare the TSO mix approximately within 2 min before the 30 minutes incubation at 42 °C is finished.

USE IMMEDIATELY!

TSO mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
TSO	6	7.2
1M MgCl2	2	2.4
Total	**8**	**9.6**

9 Gently vortex mix, briefly centrifuge, and keep on ice.

10 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.

STOPPING POINT: BD Rhapsody™ Enhanced 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 75 µL Elution Buffer to gently resuspend the beads. Do not vortex.
- Store the beads at 2 °C to 8 °C for up to 4 days.

11 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

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

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

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

2 Place the tube of Enhanced 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 75 µL of Elution Buffer into the tube. Pipet-mix.

4 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 sample at 95 °C in a thermomixer (no shaking) for 5 minutes. Immediately after the completion of the 95 °C incubation, slightly open the lid of the tube to release air pressure within the tube.

5 Briefly centrifuge the tube, then immediately place the tube on a 1.5-mL magnet for ≤30 seconds until clear. **Keep the supernatant**. Remove the supernatant and transfer to a new 1.5-mL LoBind® tube. This contains the *AbSeq/Sample Tag supernatant products*. To minimize AbSeq/Sample Tag contamination in the TCR/BCR and Targeted mRNA libraries, ensure that all liquid is removed from the tube. Keep the supernatant tube at
4 °C until ready to proceed to **Performing AbSeq/Sample Tag PCR1 on page 14**.

6 Resuspend the beads in 1.5 mL of Hybridization Buffer.

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

8 Briefly centrifuge after 25 °C incubation. Be careful when opening the tube lid. If there are droplets on the lid, use a P10 to transfer the volume into the supernatant.

TCR/BCR extension

1 Set a thermomixer to 37 °C.

2 Ensure all reagents other than the Extension Enzyme are at room temperature.

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

TCR/BCR extension mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
TCR/BCR Extension Buffer	20	24
dNTP	20	24
TCR/BCR Extension Enzyme	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 Enhanced Cell Capture Beads on a 1.5-mL tube magnet for ≤1 minutes. Remove 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.

NOTE During Extension incubation, begin Abseq/Sample Tag PCR1. See **Performing AbSeq/Sample Tag PCR1 on page 14**. You can leave the AbSeq/Sample Tag PCR1 reaction in the thermocycler when complete. TCR/BCR PCR1 will be performed after Exonuclease I treatment. All PCR1 product purification (TCR/BCR, Targeted mRNA and AbSeq/Sample Tag) can be done at the same time.

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. Keep Exonuclease I enzyme at –25 °C to –15 °C.

1 Set one thermomixer to 37 °C and a second thermomixer 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 Enhanced Cell Capture Beads with TCR/BCR 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.

NOTE If only one thermomixer is available, allow it to equilibrate to 80 °C before starting the inactivation incubation. Place the samples on ice until that temperature is reached.

7 Incubate the bead suspension on thermomixer (no shaking) 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.

TCR/BCR, Targeted mRNA, BD® AbSeq, and Sample Tag library preparation

Before you begin

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

Performing AbSeq/Sample Tag PCR1

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

AbSeq/Sample Tag PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
BD® AbSeq Primer	10	12
Sample Tag PCR1 Primer	1	1.2
Nuclease-free water	12	14.4
Total	**133**	**159.6**

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

3 In a new 1.5-mL tube, pipet 133 µL of the AbSeq/Sample Tag PCR1 reaction mix. Add 67 µL of the AbSeq/Sample Tag product from **step 5** in **Denaturation and self-hybridization on page 11**. Pipet-mix 10 times. Do not vortex.

4 Pipet 50 µL AbSeq/Sample Tag PCR1 reaction mix into each of four 0.2-mL PCR tubes. Transfer any residual mix to one of the tubes.

5 Bring the reaction mix to the post-amplification workspace.

6 Program the thermal cycler.

AbSeq/Sample Tag PCR1 conditions

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10-11*	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞
*Suggested 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 for resting PBMCs
7,500 - 10,000	11
20,000	10

STOPPING POINT: The PCR can run overnight.

7 After PCR, briefly centrifuge the tubes.

8 Pipet-mix and combine the four reactions into a new 1.5-mL LoBind® tube, labeled *AbSeq/Sample Tag PCR1*. Keep the tube on ice.

Performing TCR/BCR and Targeted mRNA PCR1

NOTE PCR1 reactions for the TCR/BCR panel and Targeted mRNA panel are performed separately on the bead. TCR/BCR panel amplification is performed first, followed by Targeted mRNA panel amplification. There is a required 95°C bead wash step after PCR1 of TCR/BCR and before PCR1 of Immune Response panel.

NOTE Use the entire sample of beads. Sub-sampling beads is not recommended for TCR/BCR FL, Targeted mRNA, AbSeq, and Sample Tag combination assays.

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

TCR/BCR Panel PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
TCR/BCR Universal Oligo N1	10	12
Bead RT/PCR Enhancer	12	14.4
*TCR N1 Primer	2.4	2.88
*BCR N1 Primer	2.4	2.88
Nuclease-free water	73.2	87.84
Total	**200**	**240**
*__NOTE__ 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.		

Targeted mRNA Panel PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
Bead RT/PCR Enhancer	12	14.4
PCR 1 targeted mRNA primer panel	40	48
Nuclease-free water	38	45.6
Total	**200**	**240**
__NOTE__ Store on ice or at 4 °C while waiting for TCR/BCR PCR1 panel amplification to be completed.		

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

3 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.

4 Remove the tube from the magnet and resuspend the beads in 200 µL of TCR/BCR Panel PCR1 reaction mix. Do not vortex.

5 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.

6 Bring the reaction mix to the post-amplification workspace.

7 Program the thermal cycler as follows.

PCR1 conditions for TCR/BCR panel

Step	Cycles	Temperature	Time
Hot start	1	95 °C*	3 min
Denaturation	10-11**	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	∞

*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.

**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	Recommended PCR cycles for resting PBMCs
7,500 - 10,000	11
20,000	10

8 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.

9 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.

10 After PCR, briefly centrifuge the tubes.

11 Put the tubes on a strip tube magnet for >30 sec. Remove and combine supernatant in a new 1.5-mL tube. **Save the supernatant for purification of TCR/BCR PCR1 products. Keep on ice.**

NOTE The next steps describe a 95 °C bead wash that is critical for removing unwanted products from beads before adding Targeted mRNA panel PCR1 reaction mixture.

12 Keeping the tubes on the magnet, add 50 µL of Elution Buffer. Pipet-mix. Incubate on the thermocycler at 95 °C for 1 minute. (**Do not incubate for more than 1 minute.**)

13 (Spin tubes briefly if necessary.) Immediately put the tubes on a strip tube magnet and remove supernatant.

14 Add 50 µL of the Targeted mRNA panel PCR1 reaction mix to each tube.

15 Program the thermal cycler as follows.

PCR1 conditions for Targeted mRNA panel

Step	Cycles	Temperature	Time
Hot start	1	95 °C*	3 min
Denaturation	10-11**	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	∞

*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.

**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	Recommended PCR cycles for resting PBMCs
7,500 - 10,000	11
20,000	10

16 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.

17 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.

18 Pipet-mix and combine the four reactions into a new 1.5-mL LoBind® tube.

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

NOTE (Optional) Remove the tube with the Enhanced Cell Capture Beads from 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 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.

2 Bring AMPure XP beads to room temperature. Vortex on high speed for 1 minute until the beads are fully resuspended.

3 Briefly centrifuge PCR1 products.

4 To PCR1 products, pipet:

- TCR/BCR PCR1 products: 140 µL AMPure beads.
- Targeted mRNA PCR1 products: 140 µL AMPure beads.
- AbSeq/Sample Tag PCR1 products: add 280 µL AMPure beads.

5 Incubate at room temperature for 5 minutes.

6 Place tubes on strip tube magnet for 5 minutes. Remove supernatant.

7 Keeping the tube on the magnet, gently add 500 µL 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 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 for 3 minutes.

11 Remove tubes from the magnet and resuspend the bead pellet in 50 µL of Elution Buffer. Pipet- mix until the beads are fully resuspended.

12 Incubate at room temperature for 2 minutes and briefly centrifuge.

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

14 For each tube, pipet entire eluates (~50 µL) into separate new 1.5 mL LoBind® tubes separately (purified TCR/BCR, Targeted mRNA, and AbSeq PRC1 products).

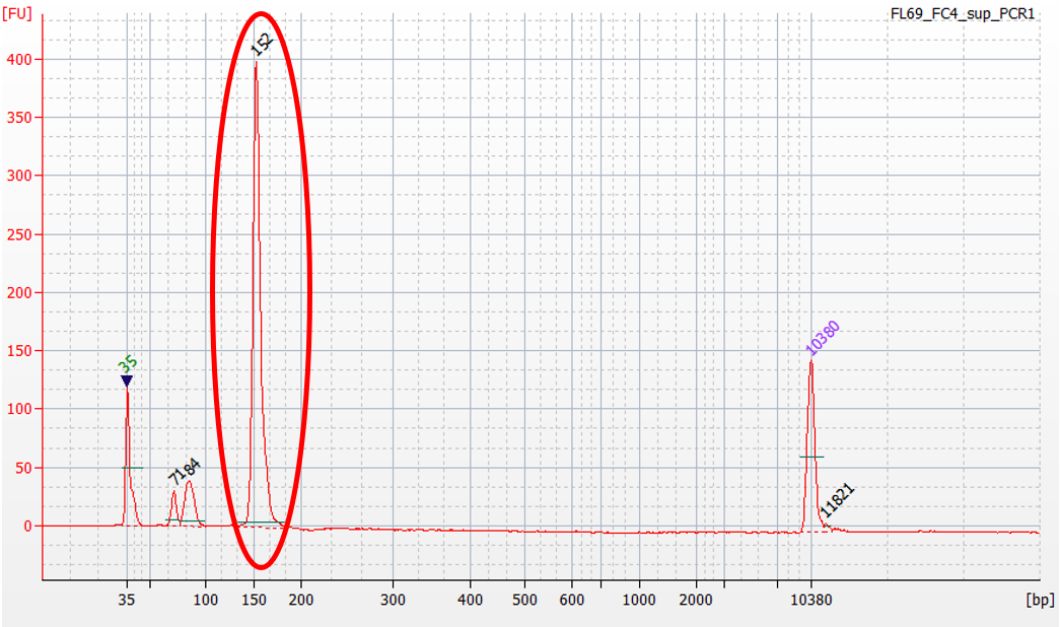
STOPPING POINT: Store at 2 °C to 8 °C before proceeding within 24 hours or at –25 °C to –15 °C for up to 6 months.

Quantifying BD® AbSeq/Sample Tag PCR1 products

1 Measure the yield of the largest peak of the BD® AbSeq/Sample Tag PCR1 product (~150 bp) by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.

2 Based on the yield of the largest peak, dilute an aliquot of BD® AbSeq/Sample Tag PCR1 product to 0.1–1.1 ng/µL with Nuclease-Free water before index PCR of BD® AbSeq PCR1 products. See **Performing index PCR to prepare final libraries on page 27**.

Figure 1 Sample Bioanalysis High Sensitivity DNA trace - AbSeq PCR1 product



		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
▶	◀	35	125.00	5,411.3	Lower Marker
2		71	51.24	1,087.6	
3		84	123.99	2,225.8	
4		152	838.65	8,334.2	
5	▶	10,380	75.00	10.9	Upper Marker
6		11,821	0.00	0.0	

Performing PCR2 on the TCR/BCR, Targeted mRNA, and Sample Tag PCR1 products

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

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

TCR/BCR PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
TCR/BCR Universal Oligo N2	2	2.4
*TCR or BCR N2 primer	6	7.2
Nuclease-free water	12	14.4
Total	**45**	**54**
*PCR2 reaction mixes for TCR and BCR are made separately.		

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**

Sample Tag PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
Sample Tag PCR2 Primer	3	3.6
Nuclease-free water	15	18
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 (from TCR/BCR, Targeted mRNA, and AbSeq/Sample Tag products) into each of the respective 45 µL of TCR or BCR, Targeted mRNA, and Sample Tag PCR2 reaction mixes.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler as follows.

TCR/BCR PCR2

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	20	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	∞

Targeted mRNA PCR2

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10	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	∞

Sample Tag PCR2

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10	95 °C	30 s
Annealing		66 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

STOPPING POINT: The PCR can run overnight.

Purifying TCR/BCR, Targeted mRNA, and Sample Tag PCR2 products

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.

2 Bring AMPure XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.

3 Briefly centrifuge the PCR2 products.

4 To 50.0 µL PCR2 products, pipet:

- TCR/BCR PCR2 products: 35 µL AMPure beads.
- Targeted mRNA PCR2 products: 40 µL AMPure beads.
- Sample Tag PCR2 products: 60 µL AMPure beads.

5 Pipet-mix 10 times and incubate at room temperature for 5 minutes.

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

7 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.

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 for 1 minute.

11 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.

12 Incubate at room temperature 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 (~50 µL) into a new 1.5-mL LoBind® tube separately (purified TCR/BCR, Targeted mRNA, and Sample Tag 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.

15 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.

Performing full length random priming on TCR/BCR libraries

Random priming

NOTE Perform TCR/BCR Random Priming the purification in the post-amplification workspace.

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

2 In pre-amplification workspace, pipet reagents into a new 1.5 mL LoBind® tube:

Random primer mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
TCR/BCR Extension Buffer	5	6
TCR/BCR Extension Primers	2.5	3
Nuclease-free water	34	40.8
Total	**41.5**	**49.8**

3 Pipet-mix the Random Primer Mix and keep at room temperature.

NOTE TCR and BCR random priming are performed separately.

4 Add 41.5 µL of Random Primer Mix + 5 µL of 1 ng/µL diluted TCR or BCR PCR2 products (5 ng total concentration). Total volume of reaction will be 46.5 µL for Random Priming.

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 reagents into a new 1.5 mL LoBind® tube:

Primer extension enzyme mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
dNTP	2	2.4
TCR/BCR Extension Enzyme	1.5	1.8
Total	**3.5**	**4.2**

8 Gently vortex mix, centrifuge, and keep at room temperature.

9 Add 3.5 µL Primer Extension Enzyme Mix to Random Priming Reaction tube to bring total volume up to 50µL. Run the following protocol on a thermocycler for Extension.

Protocol

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

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

Purifying RPE product

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.

2 Bring AMPure XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.

3 Briefly centrifuge the TCR and BCR RPE products.

4 To the TCR and BCR RPE products, add 90 µL AMPure beads.

5 Pipet-mix 10 times and incubate at room temperature for 5 minutes.

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

7 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.

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 for 1 minute.

11 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.

12 Incubate at room temperature 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 (~50 µL) into a new 1.5-mL LoBind® tube separately (purified TCR/BCR RPE products).

Performing index PCR to prepare final libraries

This section describes how to generate 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

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

Targeted mRNA and BD® AbSeq/Sample Tag index PCR mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	2	2.4

Targeted mRNA and BD® AbSeq/Sample Tag index PCR mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
*Library Reverse Primer 1 – 4	2	2.4
Nuclease-free water	18	21.6
Total	**47**	**56.4**
* For more than one library, use different Library Reverse Primers for each library.		

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

3 Bring index PCR mixes to post-amplification workspace.

4 In new 0.2 mL PCR tubes,

- For targeted mRNA libraries, dilute PCR2 products to 0.5ng/µL and pipet 3.0 µL into 47.0 µL Targeted mRNA index PCR mix.
- For AbSeq libraries, pipet 3.0 µL of 0.1–1.1 ng/µL AbSeq PCR1 product into 47 µL AbSeq Index PCR mix. (From **Quantifying BD® AbSeq/Sample Tag PCR1 products on page 21**). See **Conc. Index PCR input for AbSeq/Sample Tag libraries (ng/µL) on page 29**.
- For Sample Tag libraries, pipet 3.0 µL of 0.1–1.1 ng/µL products into 47.0 µL index PCR mix. See **Conc. Index PCR input for AbSeq/Sample Tag libraries (ng/µL) on page 29**.
- For TCR and BCR libraries, pipet 21 µL of undiluted of RPE product into 29.0 µL TCR/BCR index PCR mix.

5 Gently vortex, and briefly centrifuge.

6 Program the thermal cycler.

Targeted mRNA and TCR/BCR index PCR mix

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10	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	∞

BD® AbSeq/Sample Tag index PCR mix

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the following table. Recommended number of PCR cycles.*	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	∞
* Cycle number varies based on the concentration of the AbSeq PCR1 product and Sample Tag PCR2 product.			

Recommended number of PCR cycles

Conc. Index PCR input for AbSeq/Sample Tag libraries (ng/µL)	Recommended number of PCR cycles
0.5–1.1	6
0.25–0.5	7
0.1–0.25	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 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.

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 To 50.0 µL of the index PCR products, pipet:

- Targeted mRNA library: 35 µL AMPure beads.
- AbSeq/Sample Tag library: 40 µL AMPure beads.

Transfer 40 µL of the TCR and/or BCR index PCR product(s) to a new strip tube(s), pipet:

- TCR and BCR libraries: 26 µL AMPure beads.

5 Incubate at room temperature for 5 minutes.

6 Place 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** for a total of two washes.

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

10 Air-dry the beads at room temperature for 3 minutes.

11 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.

12 Incubate at room temperature for 2 minutes, and briefly centrifuge.

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

14 For each tube, pipet the entire eluates (~50 µL) into separate new 1.5-mL LoBind® tubes (final sequencing libraries).

15 Perform quality control before freezing samples. See **Performing quality control on the final sequencing libraries.**

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

Performing quality control on the final sequencing libraries

1 Estimate the concentration of each sample by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer. Follow the manufacturer's instructions. The expected concentration of the libraries is >1.5 ng/µL.

2 Measure the average fragment size of the targeted mRNA library within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626) for 50–7,000 bp, 5–1,000 pg/µL. Follow the manufacturer's instructions.

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

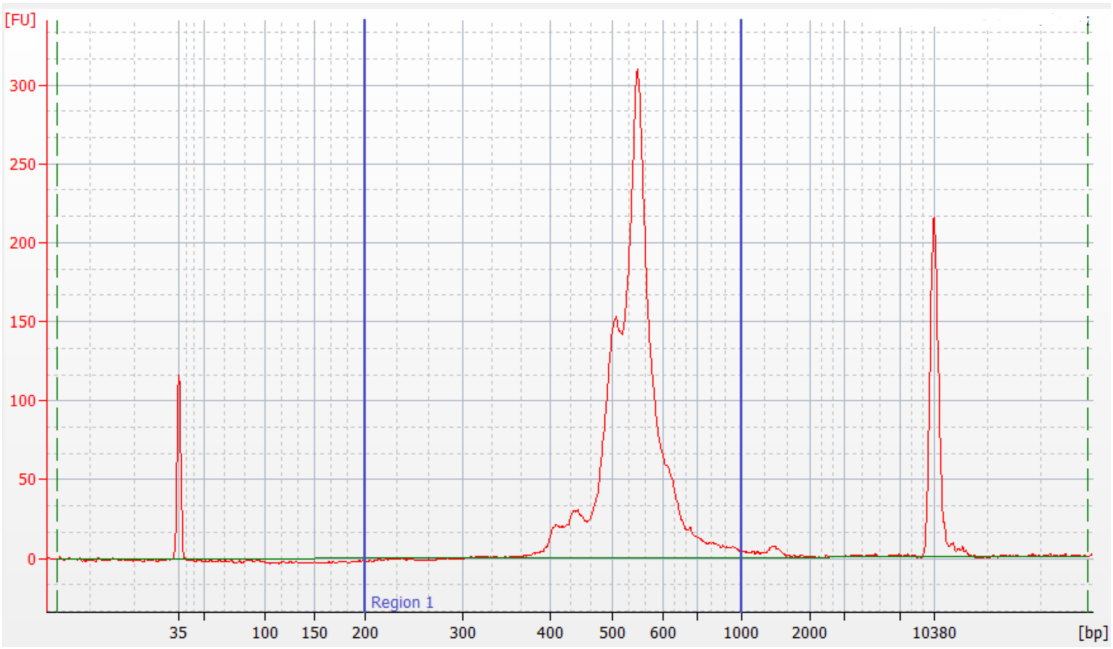


Figure 3 Sample Bioanalyzer High Sensitivity DNA trace - AbSeq index PCR product

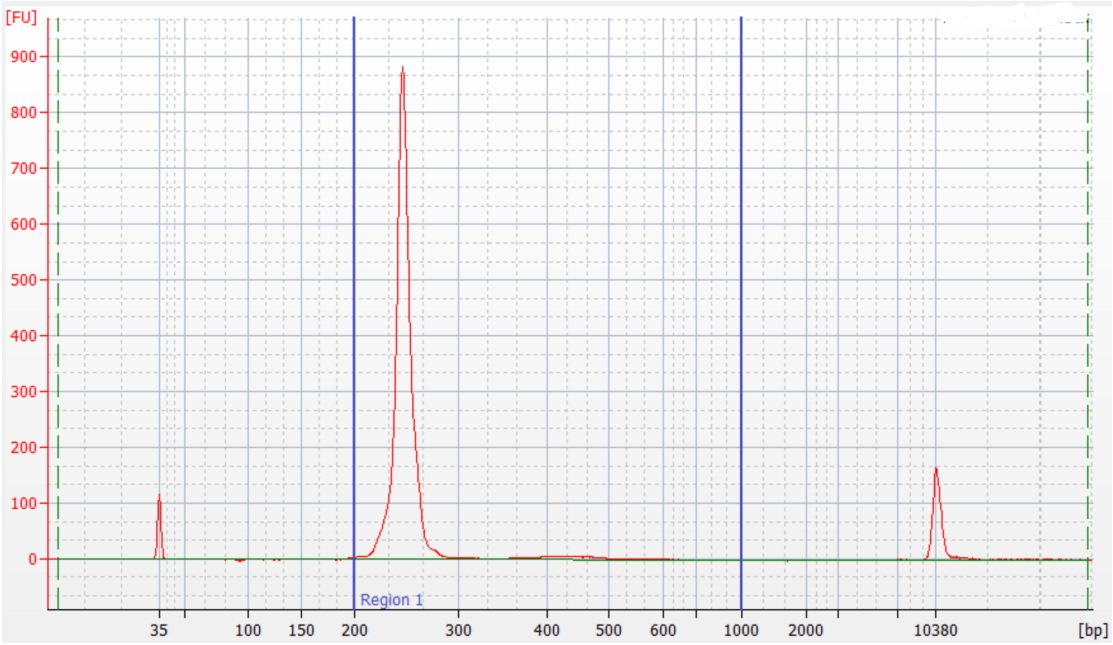


Figure 4 Sample Bioanalyzer High Sensitivity DNA trace - Sample Tag index PCR product

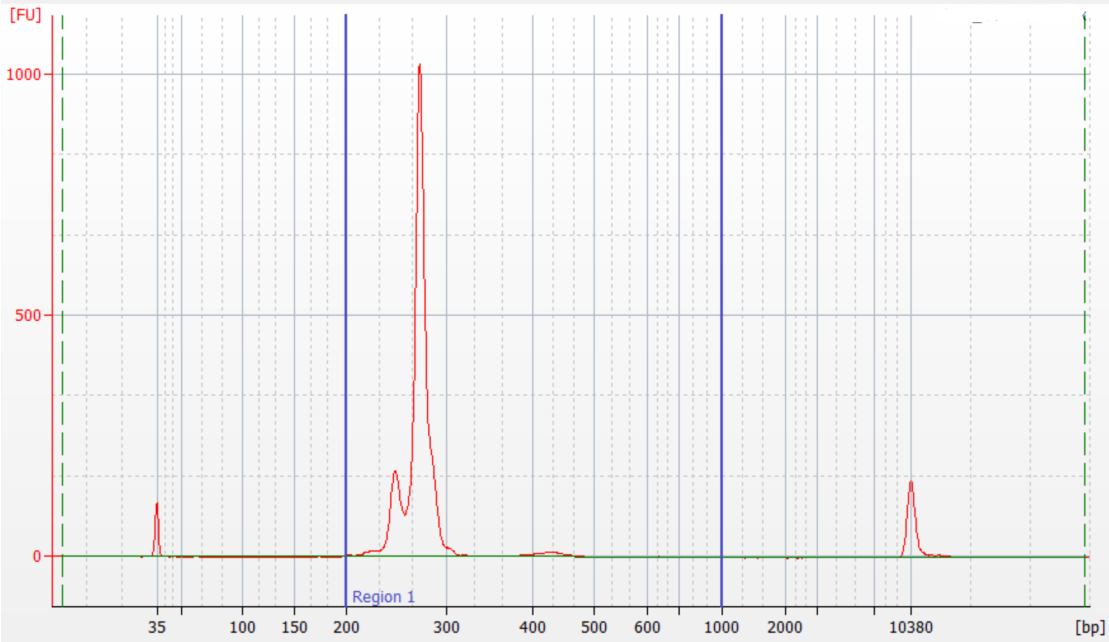


Figure 5 Sample Bioanalyzer High Sensitivity DNA trace - TCR index PCR product

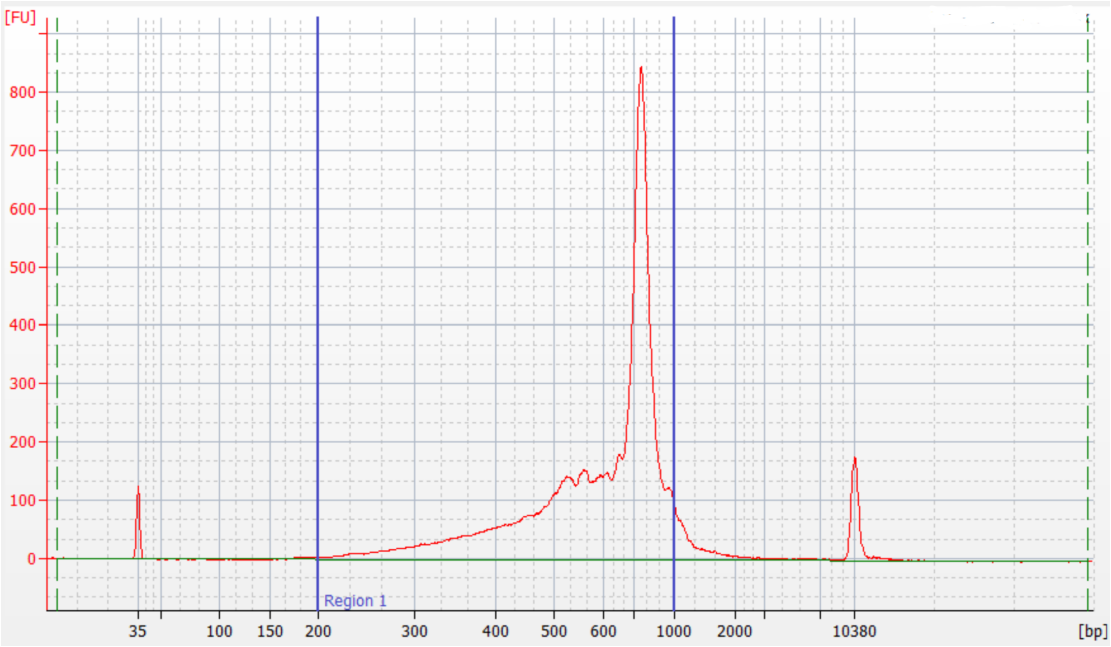
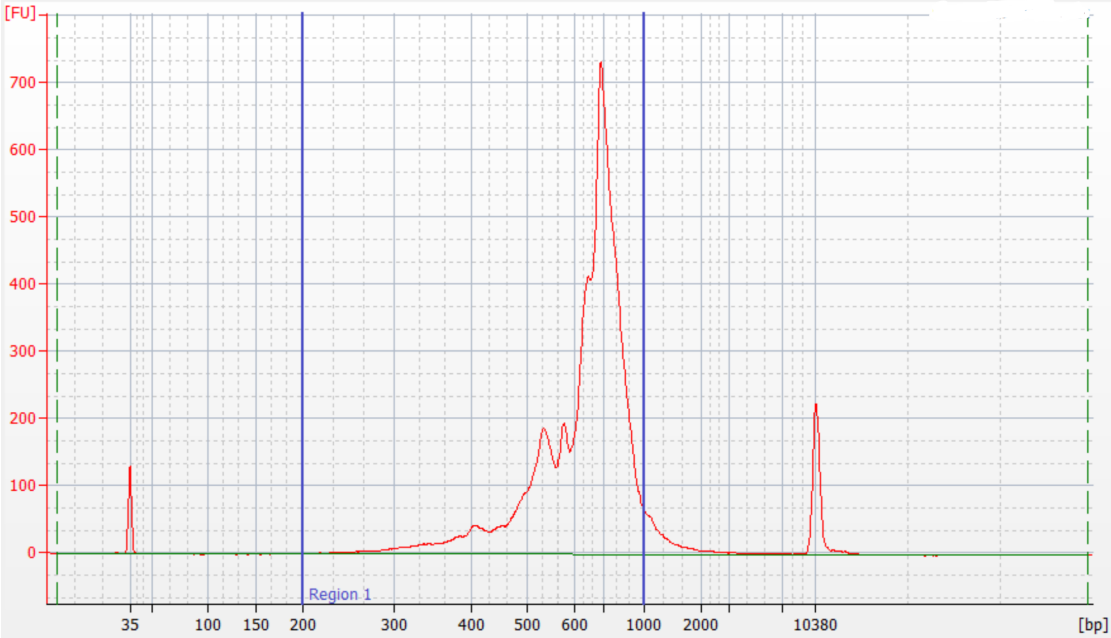


Figure 6 Sample Bioanalyzer High Sensitivity DNA trace - BCR index PCR product



Sequencing

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. We recommend 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.

Read requirements for libraries

Gene panel	Read requirement for data analysis	Adjusted reads for sequencing pooled TCR/BCR libraries on Illumina platforms***
BD Rhapsody™ Immune Response Panel Hs	~2,000-20,000 reads/cell*	N/A
Sample Tag: samples from same type of cell (combining different donor PBMCs)	120 reads/cell	N/A
Sample Tag: samples from different type of cells (combining cell lines with PBMCs)	600 reads/cell	N/A
BD Rhapsody™ AbSeq	1000 reads/cell/AbSeq**	N/A
BD Rhapsody™ TCR	~5,000 reads/T cell	~15,000 reads/T cell***
BD Rhapsody™ BCR	~5,000 reads/B cell	~15,000 reads/B cell***

*2,000 reads/cell can be sufficient for cell-type clustering and classification. For deeply saturated sequencing (RSEC depth >6), use 20,000 reads/cell.

** The amount of sequencing needed for BD® AbSeq libraries will vary depending on application, BD® AbSeq panel plexy, and cell type. We have observed that using 40,000 sequencing reads per cell for 40-plex BD® AbSeq libraries prepared from resting PBMCs achieves an RSEC sequencing depth of ~2.

***Note: Because of the long amplicon sizes, TCR and BCR libraries will not cluster as efficiently as targeted mRNA libraries. To account for this, TCR and BCR libraries should be pooled with mRNA libraries as if aiming for 3x the number of reads that are actually desired. See the following examples.

Sequencing options for AbSeq libraries

AbSeq libraries can be sequenced together or separately from targeted mRNA and TCR/BCR libraries. For optimal clustering of TCR/BCR libraries on Illumina platforms, however, we recommend sequencing AbSeq/Sample Tag libraries separately.

AbSeq/Sample Tag libraries sequenced separately (recommended)

Example of pooling AbSeq/Sample Tag using enriched T or B cells or PBMCs stained with 30-plex AbSeq

Calculating pooling ratios based on 10,000 T or B cells or 10,000 PBMCs

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
ST (combining different cell types)	600	N/A	10,000	6,000,000	2%	1 µL
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	98%	49 µL
Total				**300,000,000**	**100%**	**50 µL**

Example of pooling targeted mRNA and TCR/BCR libraries from enriched T or B cells

Calculating pooling ratios based on 10,000 T or B cells

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
mRNA Targeted	2,000	N/A	10,000	20,000,000	12%	6 µL
TCR or BCR	5,000	3	10,000	150,000,000	88%	44 µL
Total				**170,000,000**	**100%**	**50 µL**

Example of pooling targeted mRNA and TCR/BCR libraries from PBMCs

Calculating pooling ratios based on 10,000 PBMC cells, assuming 20% B cells and 40% T cells in the PBMC population

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
mRNA Targeted	2,000	N/A	10,000	20,000,000	18%	9.1 µL
TCR	5,000	3	4,000	60,000,000	55%	27.3 µL
BCR	5,000	3	2,000	30,000,000	27%	13.6 µL
Total				**110,000,000**	**100%**	**50 µL**

AbSeq/Sample Tag libraries sequenced in combination with other libraries

Example of pooling using enriched T or B cells stained with 30-plex AbSeq

Calculating pooling ratios based on 10,000 T or B cells

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 60 µL pool
mRNA Targeted	2,000	N/A	10,000	20,000,000	4%	2.5 µL
ST (combining different cell types)	600	N/A	10,000	6,000,000	1%	0.8 µL
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	63%	37.8 µL
TCR or BCR	5,000	3	10,000	150,000,000	32%	18.9 µL
Total				**476,000,000**	**100%**	**60 µL**

Example of pooling using PBMCs stained with 30-plex AbSeq

Calculating pooling ratios based on 10,000 PBMC cells, assuming 20% B cells and 40% T cells in the PBMC population

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 60 µL pool
mRNA Targeted	2,000	N/A	10,000	20,000,000	4.8%	2.9 µL
ST (combining same cell types)	600	N/A	10,000	6,000,000	1.4%	0.9 µL
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	72.1%	43.3 µL
TCR	5,000	3	4,000	60,000,000	14.4%	8.7 µL
BCR	5,000	3	2,000	30,000,000	7.2%	4.3 µL
Total				**416,000,000**	**100%**	**60 µL**

Sequencing flow cell loading and PhiX concentrations

Quantifying libraries

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

AbSeq/Sample Tag libraries

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 15% PhiX for a sequencing run.

Parameter	Requirement
Platform	Illumina: 150 cycle kit
Paired-end reads	Minimum of 51 x 75 paired read length
PhiX	Required (15%)
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook* (Doc ID: 54169)

Targeted mRNA and TCR/BCR libraries (with or without AbSeq)

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.

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 of 85 x 215* paired read length
PhiX	Required (3%)
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook* (Doc ID: 54169)
*R2 length of 215 is recommended for optimal assembly. If necessary,150 x 150 read lengths can be used.	

Appendix

Human T cell PCR1 primers

Primer name	Primer sequence (5′ - 3′)
TRAC_N1	CTGGAATAATGCTGTTGTTGAAGG
TRBC_N1	AGCCCGTAGAACTGGACTT
TRDC_N1	CTTCAAAGTCAGTGGAGTGCA
TRGC_N1	CACCGTTAACCAGCTAAATTTCATG

Human T cell PCR2 primers

Primer name	Primer sequence (5′ - 3′)
TRAC_N2	ATCAAAATCGGTGAATAGGCAGAC
TRBC_N2	GATCTCTGCTTCTGATGGCTCA
TRDC_N2	ATATCCTTGGGGTAGAATTCCTTC
TRGC_N2	GGGAAACATCTGCATCAAGTTG

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

Human B cell PCR2 primers

Primer name	Primer sequence (5′ - 3′)
IGHA_N2	CTTTCGCTCCAGGTCACACT
IGHD_N2	TGTCTGCACCCTGATATGATGG
IGHE_N2	GTCAAGGGGAAGACGGATG
IGHG_N2	AAGTAGTCCTTGACCAGGCA
IGHM_N2	ACAGGAGACGAGGGGGAAAA

Human B cell PCR2 primers

Primer name	Primer sequence (5′ - 3′)
IGKC_N2	TCAGATGGCGGGAAGATGAA
IGLC_N2	ACCAGTGTGGCCTTGTTG

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