BD Rhapsody™ System

Library Preparation Protocol

TCR/BCR Full Length and Targeted mRNA

23-24013(02)
2022-07

For Research Use Only

Regulatory information

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

History

Revision	Date	Change made
23-24013(01)	2022-01	Initial release.
23-24013(02)	2022-07	Updated Time considerations workflow. Updated Sequencing section. Added additional library pooling and sequencing recommendation. In Appendix, added Illumina Index 1 (i7) sequences.

Contents

Introduction

This protocol enables high-throughput single-cell transcriptome 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.

After 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.

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

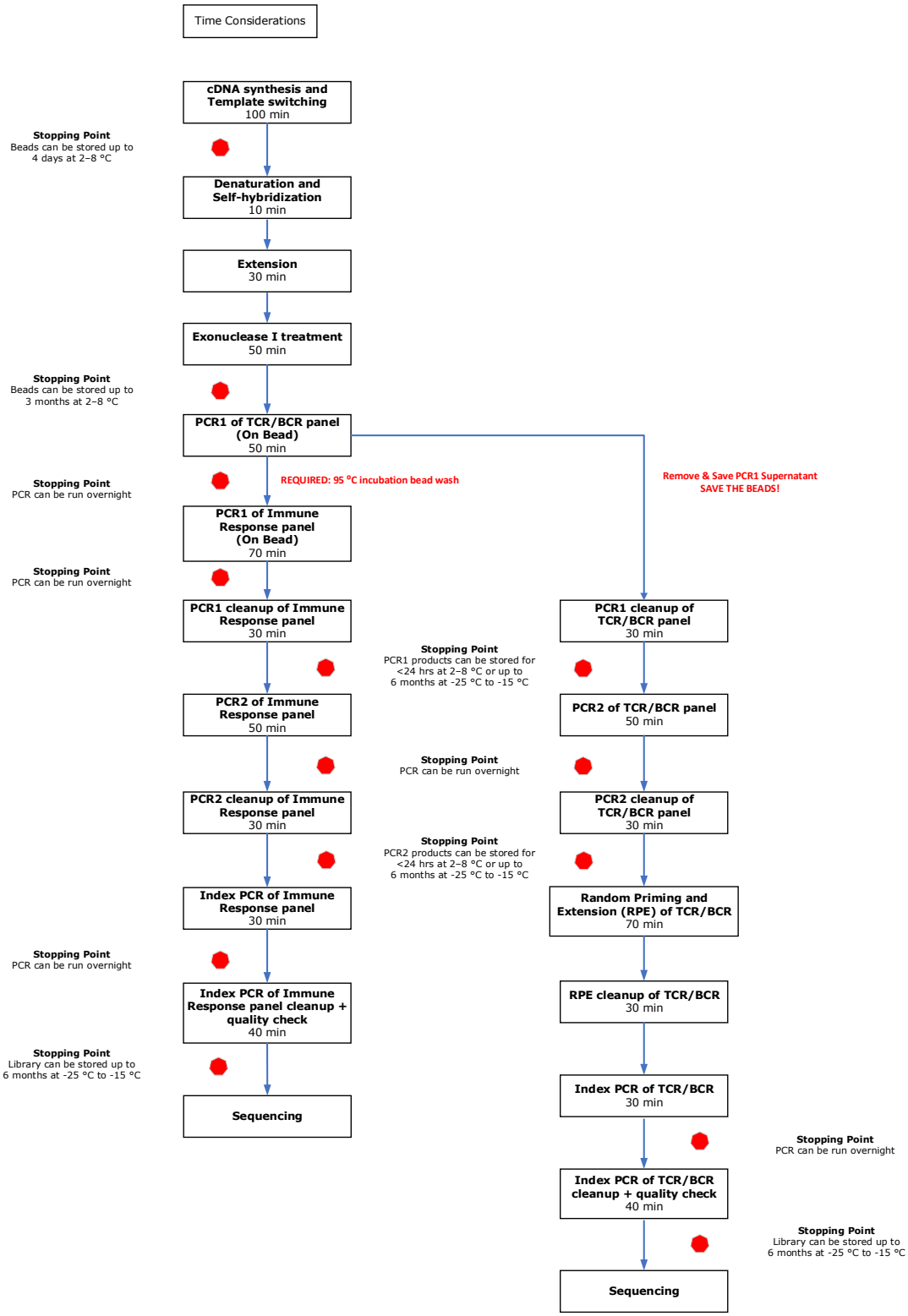
Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (23-21336)
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (23-21332)

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* (23-21336) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (23-21332).

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* (23-21336)

  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* (23-21332)

  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 minutes 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 2–8 °C after template switching.

If stopping after template switching:

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

11 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.

Denaturation and self-hybridization

Thaw reagents for TCR/BCR Extension at room temperature. Keep TCR/BCR 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 200 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.

c Briefly centrifuge the tube after 95 °C incubation.

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

6 Resuspend the beads in 1.5 mL of 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 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

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.

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 2–8 °C for up to 3 months.

12 Proceed to library preparation.

TCR/BCR and Targeted mRNA 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 PCR1

Note: PCR1 reactions for the Targeted mRNA panel and TCR/BCR 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.

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

PCR1 reaction mix for TCR/BCR panel

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**
*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.		

PCR1 reaction mix for Targeted mRNA panel

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
PCR1 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 Proceed as follows:

- Entire sample: Skip to **step 5**.
- Sub-sample: Proceed to **step 4**.

4 Sub-sample the Exonuclease I-treated beads:

a Based on the number of wells with viable cells and a bead detected by the BD Rhapsody™ scanner or the number of cells targeted for capture in the cartridge, determine the volume of beads to sub-sample for targeted sequencing.

b Briefly spin the tube with the bead suspension. Pipet-mix to completely resuspend the beads, and pipet the calculated volume of bead suspension into a new 1.5-mL LoBind® tube.

The remaining beads can be stored at 2–8 °C for up to 3 months.

5 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. Remove the supernatant.

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

7 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.

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

9 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	Suggested PCR cycles for resting PBMCs
7,500 - 10,000	11
20,000	10

Suggested number of PCR cycles for sub-sampled Exonuclease I-treated beads

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12

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

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

11 For each 0.2-mL PCR tube, gently pipet-mix, immediately place the tube in thermal cycler, and unpause the thermal cycler program.

STOPPING POINT: The PCR can run overnight, but proceed with purification within 24 hours after PCR.

12 After PCR, briefly centrifuge the tubes.

13 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 PCR products from the beads before addition of Targeted mRNA panel PCR1 reaction mixture.

14 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.)

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

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

17 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	Suggested PCR cycles for resting PBMCs
7,500 - 10,000	11
20,000	10

Suggested number of PCR cycles for sub-sampled Exonuclease I-treated beads

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12

18 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.

19 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.

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

21 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 2–8 °C in the post-amplification workspace.

Purifying PCR1 products

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

1 In a new 5.0-mL LoBind® tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by combining 4.0 mL absolute ethyl alcohol, molecular biology grade, with 1.0 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

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

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

3 Pipet 140 µL of AMPure beads into the tube with 200 µL PCR1 products of each TCR/BCR and Targeted mRNA panels. Pipet-mix 10 times.

4 Incubate at room temperature for 5 minutes.

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

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

7 Keeping the tube on the magnet, gently add 500 µL of fresh 80% ethyl alcohol 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 3 minutes.

11 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.

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 eluate (~50 µL) into a new 1.5-mL LoBind® tube separately (purified targeted mRNA and 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.

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

Note: Targeted mRNA and TCR/BCR 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**

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

3 Bring the PCR2 reaction mix to the post-amplification workspace.

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

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler.

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	∞

STOPPING POINT: The PCR can run overnight.

Purifying TCR/BCR and Targeted mRNA 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.

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 targeted mRNA and TCR/BCR PCR2 products).

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.

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 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 50 µL. 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 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
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 library, dilute PCR2 products to 0.5 ng/µL and pipet 3.0 µL into 47.0 µL index PCR mix.
- For TCR and BCR libraries, pipet 21 µL of undiluted of RPE product into 29.0 µL index PCR mix.

5 Gently vortex, and briefly centrifuge.

6 Program the thermal cycler.

Targeted mRNA and TCR/BCR index PCR

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	∞

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.

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, 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 1 Sample Bioanalyzer High Sensitivity DNA trace - mRNA Targeted index PCR product

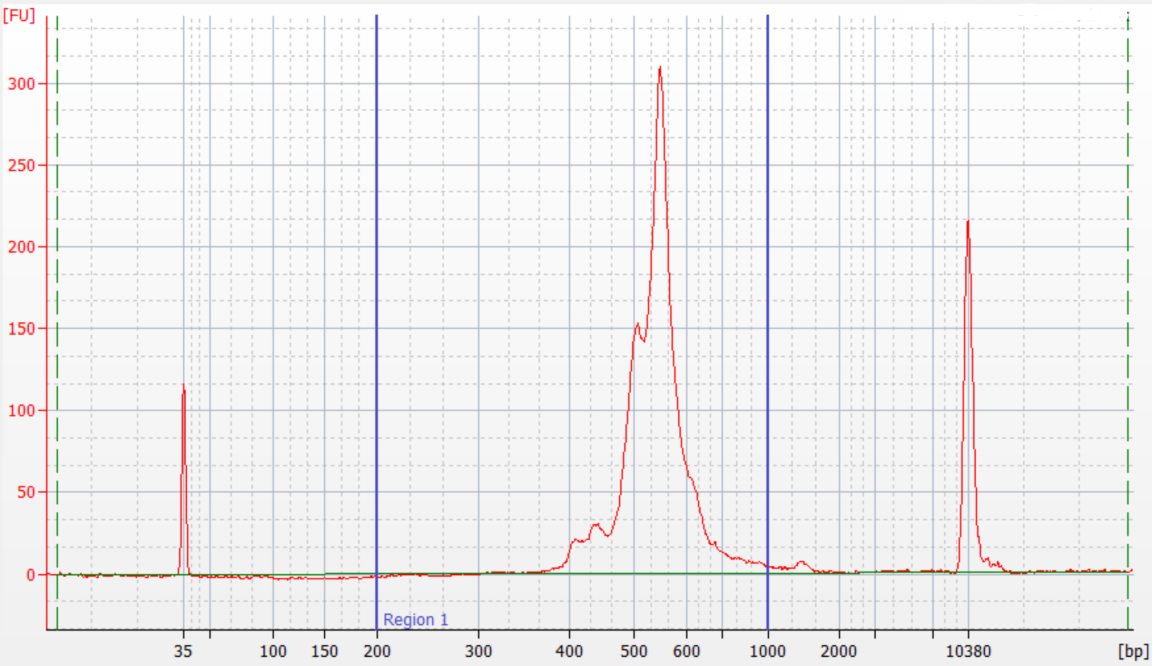


Figure 2 Sample Bioanalyzer High Sensitivity DNA trace - TCR index PCR product

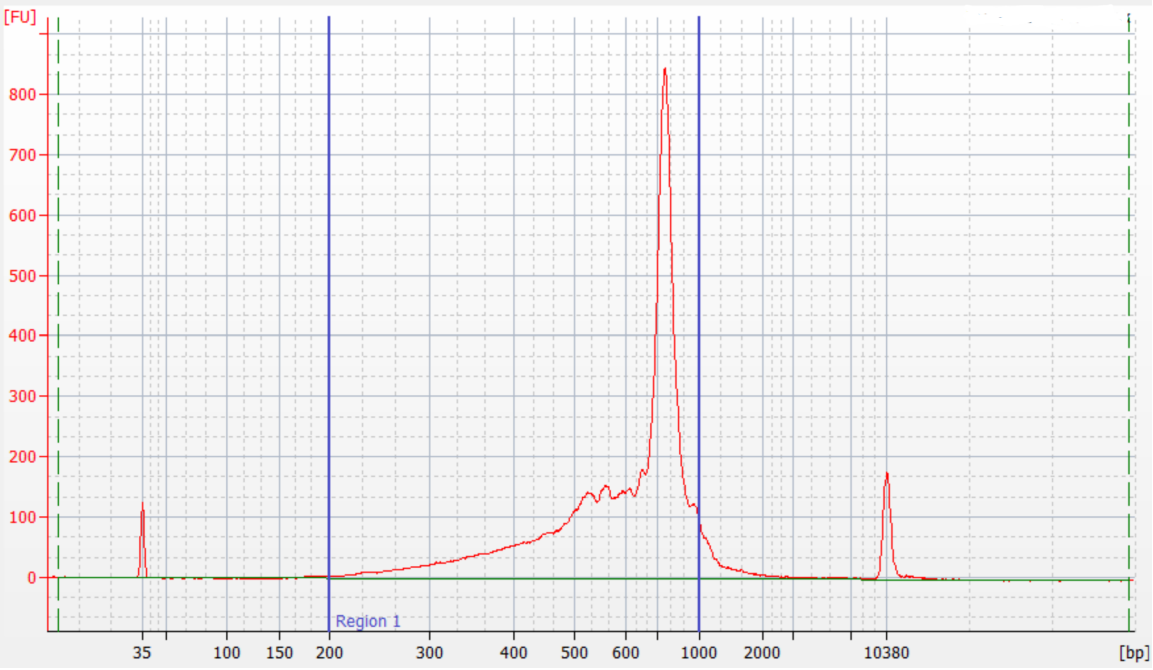
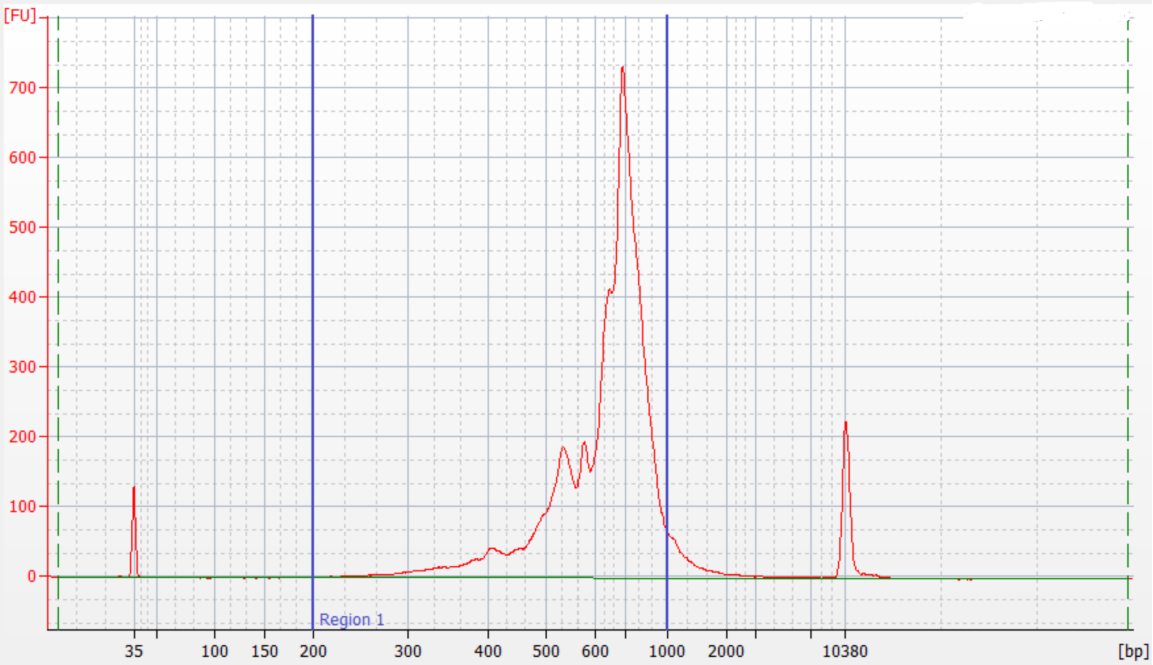


Figure 3 Sample Bioanalyzer High Sensitivity DNA trace - BCR index PCR product



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 chosen gene panel, number of cells, and sequencing run quality. Below are the recommended reads/cell for targeted mRNA, TCR, and BCR libraries.

Read requirements for libraries

Library	Read requirement for data analysis
Targeted mRNA	~2,000-20,000 reads/cell*
TCR	~5,000 reads/T cell
BCR	~5,000 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.	

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 targeted mRNA library and this will cause them to produce less sequencing data if pooled in a 1:1 ratio with the targeted mRNA library. 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 3x volume correction factor is needed for sequencing TCR and BCR libraries with a targeted mRNA one.

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 1/3 the efficiency of the targeted mRNA library, supported by internal testing.

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)**
Targeted mRNA	5,000	2,000	10,000,000	28%	26,666,667	5,333
TCR	5,000	5,000	25,000,000	72%	8,333,333	1,667
		Total	**35,000,000**	**100%**	**35,000,000**	

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 targeted mRNA library. The remaining reads are allotted to the targeted mRNA library resulting in almost three times 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 3 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 required number of reads per cell (Column C). Based on this example, 35 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, 35 million reads.

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)**
Targeted mRNA	5,000	2,000	10,000,000	29%	n/a	10,000,000	12%	10,000,000	2,000
TCR	5,000	5,000	25,000,000	71%	3*	75,000,000	88%	25,000,000	5,000
		Total	**35,000,000****	**100%**		**85,000,000†**	**100%**	**35,000,000**	

*The 3x correction factor is a recommended starting point and some fine tuning may be required to achieve the optimal library balance.

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

†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 (Column D and C, respectively). The correction for library pooling did not change the amount of data generated (35 million reads), but helped ensure the data was spread out appropriately to each library.

Example of pooling with a mixed population

The table below shows the pooling logic for a mixed population of cells such as PBMCs assuming 40% T cells and 30% B cells.

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)**
Targeted mRNA	10,000	5,000	50,000,000	59%	n/a	50,000,000	32%	50,000,000	5,000
TCR	4,000	5,000	20,000,000	24%	3*	60,000,000	39%	20,000,000	5,000
BCR	3,000	5,000	15,000,000	18%	3*	45,000,000	29%	15,000,000	5,000
		Total	**85,000,000****	**100%**		**155,000,000†**	**100%**	**85,000,000**	

*The 3x correction factor is a recommended starting point and some fine tuning may be required to achieve the optimal library balance.

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

†Read total only for pooling purposes.

Additional considerations

1. The 3x 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).
2. 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% when sequencing with the targeted mRNA library.
3. 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, targeted 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, please 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 targeted mRNA 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.

Targeted mRNA and TCR/BCR libraries

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* (23-21713)
*R2 length of 215 is recommended for optimal assembly. If necessary 150 x 150 read lengths can be used.	

Appendix

Illumina Index 1 (i7) sequences

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

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
IGKC_N2	TCAGATGGCGGGAAGATGAA
IGLC_N2	ACCAGTGTGGCCTTGTTG

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