BD Rhapsody™ System

Mouse TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA), and Sample Tag

Library Preparation Protocol

23-24365(01)
2022-12

For Research Use Only

Regulatory information

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

History

Revision	Date	Change made
23-24365(01)	2022-12	Initial release.

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. The addition of a sample-tag library enables demultiplexing of samples that were pooled before capture on the BD Rhapsody™ system.

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. Whole transcriptome mRNA libraries are amplified using random priming of the on-bead cDNA libraries. TCR and BCR libraries are then amplified from beads using a two-step nested amplification followed by additional random priming of the PCR libraries to capture complementarity determining regions (CDR) 1, 2, and 3, as well as framework regions (FR) 1-4. Note that Sample Tag libraries are amplified from the supernatant that was denatured from the beads.

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™ WTA Amplification Kit[a]	BD Biosciences	633801
BD Rhapsody™ Mouse TCR/BCR Amplification Kit[a]	BD Biosciences	666282
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
Nuclease-free water	Major supplier	–
a. For processing more than four libraries, two orders of this catalog number are required.		
Refer to the Technical Bulletin *Ordering Additional Indexes for the BD Rhapsody™ Library Reagent Kits* to order additional indexing primers for high throughput library preparation workflows.		

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	–
Axygen™ 96–Well PCR Microplates[a] Or, MicroAmp Optical 96–Reaction Plate[a]	Corning Thermo Fisher Scientific	PCR96HSC N8010560

Material	Supplier	Catalog no.
MicroAmp Clear Adhesive Film[a]	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	Agilent	5067-4626
Or,		
Agilent High Sensitivity D1000 ScreenTape	Agilent	5067-5584
Agilent High Sensitivity D1000 Reagents	Agilent	5067-5585
Or,		
Agilent High Sensitivity D5000 ScreenTape	Agilent	5067-5592
Agilent High Sensitivity D5000 Reagents	Agilent	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	New England Biolabs	S1506S
Or,		
*12-tube magnetic separation rack	New England Biolabs	S1509S
Or,		
*Invitrogen™ DynaMag™-2 magnet	Thermo Fisher Scientific	12321D
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
**Magnetic Stand–96	Thermo Fisher Scientific	AM10027
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216

Material	Supplier	Catalog no.
Agilent® 2100 Bioanalyzer Or, Agilent® 4200 TapeStation System	Agilent Technologies Agilent Technologies	G2940CAG G2991AA
Heat block	Major supplier	–
*Recommended for processing greater than six samples. **Recommended for processing high throughput library preparation workflows.		

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.

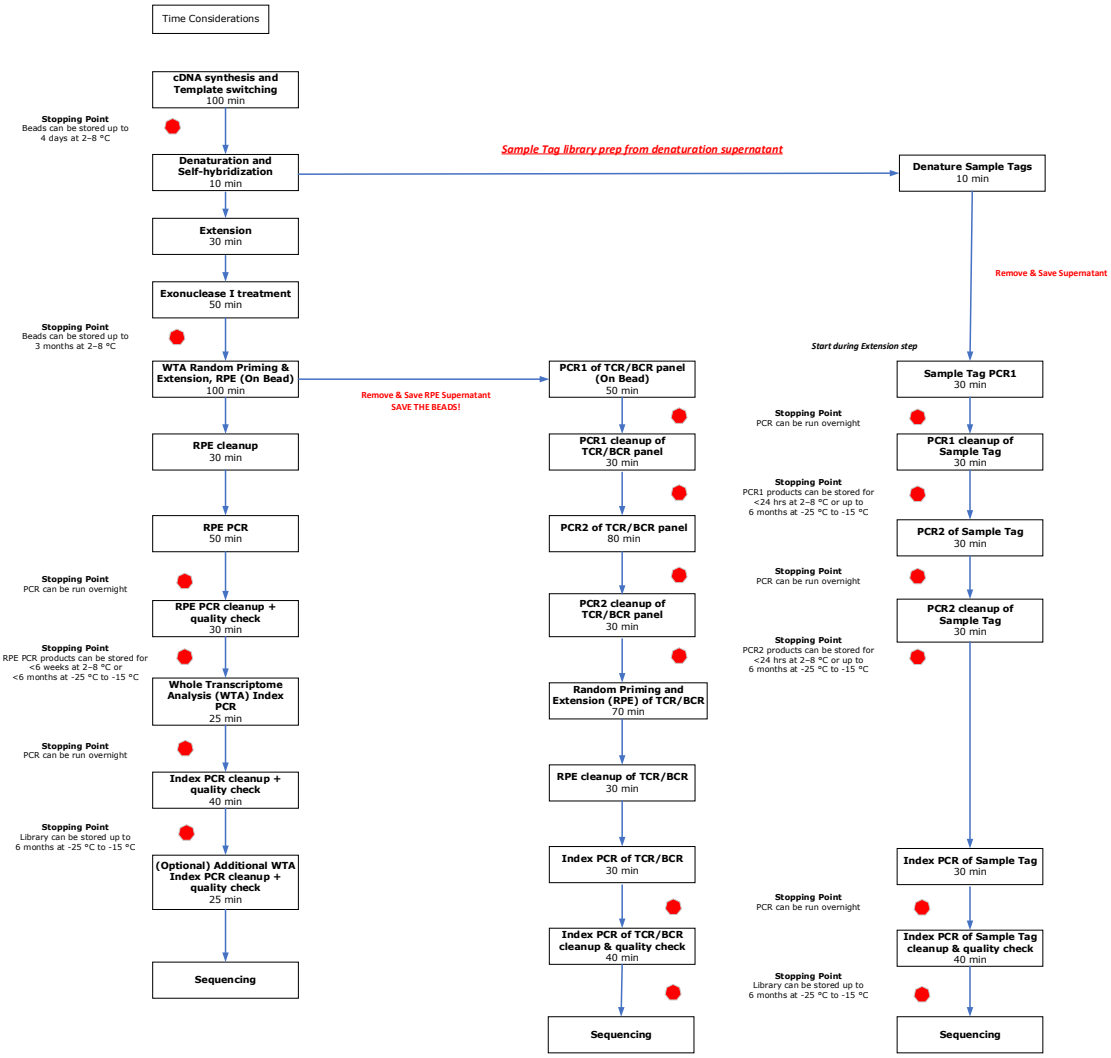
Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*
- *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 section "Washing the Cell Capture Beads" and follow this protocol from **Preparing BD Rhapsody™ Enhanced Cell Capture Beads for TCR/BCR full length, WTA, and Sample Tag library amplification** and subsequent steps.

  or

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

  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, WTA, and Sample Tag 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, WTA, and Sample Tag library amplification

cDNA synthesis and template switching

Thaw reagents (except for the enzymes) in the BD Rhapsody™ cDNA 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 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)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
RT Buffer	40	48	192	384
dNTP	20	24	96	192
RT 0.1 M DTT	10	12	48	96
Bead RT/PCR Enhancer	12	14.4	57.6	115.2
RNase Inhibitor	10	12	48	96
Reverse Transcriptase	10	12	48	96
Nuclease-free water	98	117.6	470.4	940.8
Total	**200**	**240**	**960**	**1920**

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. Discard 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)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
TSO	6	7.2	28.8	57.6
1M $MgCl_2$	2	2.4	9.6	19.2
Total	**8**	**9.6**	**38.4**	**76.8**

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 7 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 75 µL Elution Buffer to gently resuspend the beads. Do not vortex.
- Store the beads at 2–8 °C for up to 7 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 the Hybridization Buffer and reagents for TCR/BCR Extension at room temperature. Keep TCR/BCR Extension enzyme at –25 °C to –15 °C.

1 Set a thermomixer to 25 °C, and a heat block 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.

Discard 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 heat block 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 Sample Tag supernatant products. To minimize ST contamination in the TCR/BCR and WTA libraries, ensure that all liquid is removed from the tube. Keep the supernatant tube at 4 °C until ready to proceed to Performing Sample Tag PCR1 on page 18.

6 Resuspend the beads in 1.0 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)	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 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.

Note: During TCR/BCR Extension incubation, begin Sample Tag PCR1. See Performing Sample Tag PCR1 on page 18. You can leave the Sample Tag PCR1 reaction in the thermocycler when complete. TCR/BCRPCR1 will be performed after Purifying RPE PCR amplification product (single-sided cleanup) on page 16. All PCR1 product purification (TCR/BCR and 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 heat block to 80 °C.

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

Exonuclease I mix

Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)	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 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.

Performing random priming and extension (RPE) on BD Rhapsody™ Enhanced Cell Capture Beads with cDNA

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 a thermomixer to 37 °C.

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

Random Primer Mix

Kit 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	24	96	192
WTA Extension Primers	20	24	96	192
Nuclease-free water	134	160.8	643.2	1286.4
Total	**174**	**208.8**	**835.2**	**1670.4**

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

Note: Use the entire sample of beads. Sub-sampling beads is not recommended for TCR/BCR FL + WTA + Sample Tag combination assays.

4 Resuspend the Exonuclease I-treated 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. Discard 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 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

Kit 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	9.6	38.4	76.8
Bead RT/PCR Enhancer	12	14.4	57.6	115.2
WTA Extension Enzyme	6	7.2	28.8	57.6
Total	**26**	**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. Then:

Incubate the sample at 95 °C in a heat block for 5 minutes (no shaking). Immediately after the completion of 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. 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. Proceed to Purifying RPE product 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.

Note: These beads will be used for TCR/BCR target specific amplification. **DO NOT THROW AWAY!**

Purifying RPE product

This section describes how to perform a single-sided AMPure cleanup, which removes primer dimers and other small molecular weight by-products. 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 8.0 mL of absolute ethyl alcohol to 2.0 mL of nuclease-free water (from major supplier). 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 second.

13 Pipet the eluate (~40 µL) to a new PCR tube. This is the purified RPE product.

Performing 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

Kit 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.

3 Split the RPE PCR reaction mix into two PCR tubes with 60 µL of reaction mix per tube.

4 Bring the reaction 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 following table, Recommended number of PCR cycles.*	95 °C	30 s
Annealing		60 °C	1 min
Extension		72 °C	1 min
Final extension	1	72 °C	2 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 RPE PCR	Recommended PCR cycles for resting 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.

Purifying RPE PCR amplification product (single-sided cleanup)

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 4.0 mL of absolute ethyl alcohol to 1.0 mL of nuclease-free water (from major supplier). 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.

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 and discard any residual supernatant from the tube.

11 Air-dry the beads at room temperature for 3 minutes or until the beads no longer look glossy.

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.

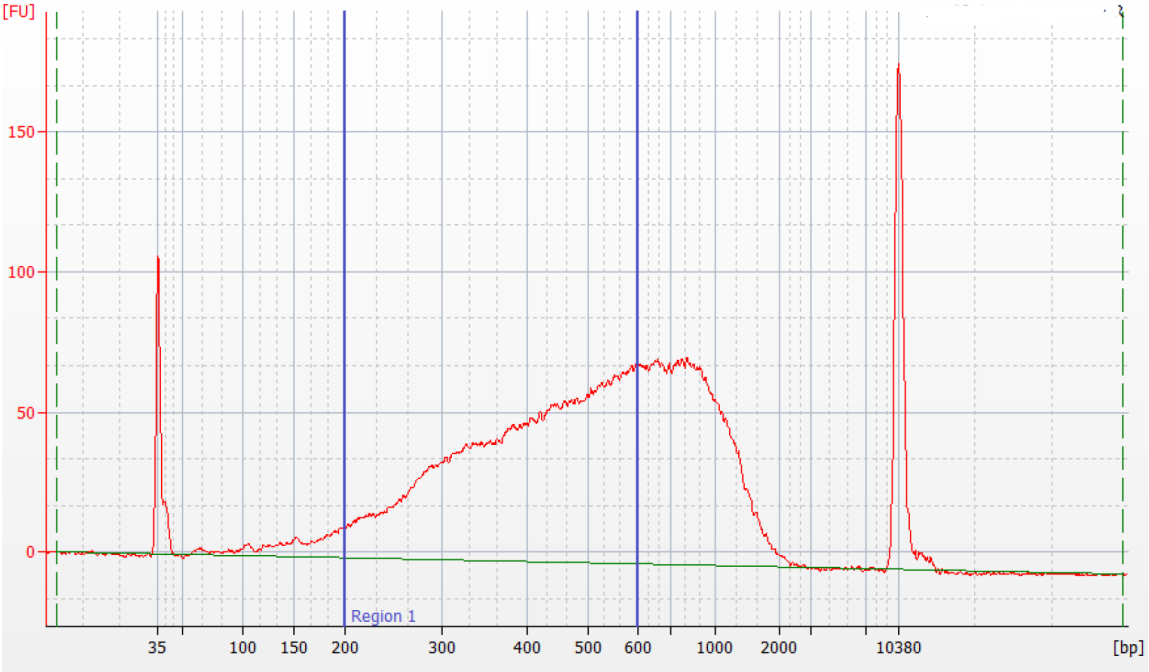
STOPPING POINT: The RPE PCR libraries can be stored at –20 °C for up to 6 months or 4 °C for up to 6 weeks.

16 Quantify and perform quality control of the RPE PCR products with a Qubit Fluorometer using the Qubit dsDNA HS Assay and 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



Performing Sample Tag PCR1

This section describes how to amplify Sample Tag products through PCR.

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

PCR1 reaction mix for Sample Tag

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	120	480	960
Universal Oligo	10	12	48	96
Sample Tag PCR1 Primer	1	1.2	4.8	9.6
Nuclease-free water	22	26.4	105.6	211.2
Total	**133**	**159.6**	**638.4**	**1276.8**

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

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

4 Pipet 50 µL 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.

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	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 *Sample Tag PCR1*. Keep the tube on ice.

Performing TCR/BCR PCR1

1 Obtain beads from Step 19 on page 14 of Performing random priming and extension (RPE) on BD Rhapsody™ Enhanced Cell Capture Beads with cDNA.

Note: Use the entire sample of beads. Sub-sampling beads is not recommended for TCR/BCR FL + WTA + Sample Tag combination assays.

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

PCR1 reaction mix TCR/BCR panel

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	120	480	960
TCR/BCR Universal Oligo N1	10	12	48	96
Bead RT/PCR Enhancer	12	14.4	57.6	115.2
*Mouse TCR N1 primer	2.4	2.88	11.52	23.04
*Mouse BCR N1 primer	2.4	2.88	11.52	23.04
Nuclease-free water	73.2	87.84	351.36	702.72
Total	**200**	**240**	**960**	**1920**

*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 reaction mix. Do not vortex.

6 Ensuring that the beads are fully resuspended, pipet 50 µL of PCR1 reaction mix with beads into each of four 0.2-mL PCR tubes. Transfer any residual mix to one of the tubes.

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

8 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

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

13 Place the 1.5-mL tube on the magnet for ≤1 minute. **Retain the supernatant that contains the TCR/BCR PCR1 products.** Carefully pipet the supernatant (TCR/BCR PCR1 products) into the new 1.5-mL LoBind® tube without disturbing the beads.

Note: (Optional) Remove the tube with the BD Rhapsody™ 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 TCR/BCR and Sample Tag PCR1 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the TCR/BCR and Sample Tag 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 4.0 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. 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 PCR1 products, pipet:

- Sample Tag libraries: 280 µL AMPure beads. From step 8 on page 19 in Performing Sample Tag PCR1.
- TCR/BCR libraries: 140 µL AMPure beads. From step 13 on page 21 in Performing 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 separately (purified TCR/BCR and Sample Tag PCR 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 TCR/BCR and Sample Tag PCR2 on the PCR1 products

This section describes how to amplify TCR/BCR and Sample Tag products through PCR. The PCR primers include partial Illumina sequencing adapters that enable the additions of full-length Illumina sequencing indices in the next PCR.

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

PCR2 reaction mixes for TCR and BCR

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
TCR/BCR Universal Oligo N2	2	2.4	9.6	19.2
*Mouse TCR N2 primer or Mouse BCR N2 primer	6	7.2	28.8	57.6
Nuclease-free water	12	14.4	57.6	115.2
Total	**45**	**54**	**216**	**432**
*PCR2 reaction mixes for TCR and BCR are made separately.				

PCR2 reaction mix for Sample Tag

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
Universal Oligo	2	2.4	9.6	19.2
Sample Tag PCR2 Primer	3	3.6	14.4	28.8
Nuclease-free water	15	18	72	144
Total	**45**	**54**	**216**	**432**

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

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

4 Pipet 5.0 µL of PCR1 products into 45 µL of TCR/BCR or Sample Tag PCR2 reaction mix.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler.

PCR2 conditions for Sample Tag

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	∞
*Suggested PCR cycles might need to be optimized for different cell types and cell number.			

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 70–55 °C = 15 °C, hence, each cycle decreases by 1 °C
	15	95 °C	30 s	
		70–55 °C	1 min	
		72 °C	1 min	
Phase II:	8	95 °C	30 s	
		55 °C	1 min	
		72 °C	1 min	
	1	72 °C	5 min	
	1	4 °C	∞	

STOPPING POINT: The PCR can run overnight.

Purifying TCR/BCR and Sample Tag PCR2 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the TCR/BCR and Sample Tag 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:
   - Sample Tag libraries: 60 µL AMPure beads
   - TCR and BCR PCR2 products: 35 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 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 entire eluate (~50 µL) into a new 1.5-mL LoBind® tube separately (purified TCR/BCR and Sample Tag 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.

14 Estimate the concentration with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

Performing random priming and extension (RPE) on TCR and BCR PCR2 products

Note: Perform TCR and BCR Random Priming the purification in the post-amplification workspace.

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 reagents into a new 1.5 mL LoBind® tube:

Random Primer Mix

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	6	24	48
TCR/BCR Extension Primers	2.5	3	12	24
Nuclease-free water	Up to 34	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.

4 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	

5 Briefly centrifuge the tube and keep at room temperature.

6 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)	For 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
dNTP	2	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**

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

8 Add 3.5 µL Primer Extension Enzyme Mix to Random Priming Rxn 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	

9 Remove tubes from Thermocycler and prepare to purify RPE product.

Purifying TCR/BCR 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. 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 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 entire eluate (~50 µL) into a new 1.5-mL LoBind® tube separately (purified TCR and BCR RPE products).

Performing TCR/BCR and Sample Tag index PCR

This section describes how to generate TCR/BCR and Sample Tag 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.

Index PCR reaction mix for Sample Tag

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	2	2.4	9.6	19.2
*Library Reverse Primer 1 - 4	2	2.4	–	–
Nuclease-free water	18	21.6	86.4	172.8
Total	**47**	**56.4**	**216**	**432**
*For more than one library, use different Library Reverse Primers for each Sample Tag library.				

Index PCR reaction mix for TCR and BCR

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	2	2.4	9.6	19.2
*Library Reverse Primer 1 - 4	2	2.4	–	–
Total	**29**	**34.8**	**129.6**	**259.2**
*For more than one library, use different Library Reverse Primers for each TCR or BCR library.				

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

3 Bring TCR/BCR and Sample Tag index PCR mix to post-amplification workspace.

4 In new 0.2 mL PCR tubes,

- For TCR/BCR libraries, pipet 21 µL of TCR/BCR RPE purified products into 29 µL of TCR and BCR Index PCR mix.

- For Sample Tag libraries, pipet 3.0 µL of 0.1–1.1 ng/µL Sample Tag PCR2 products into 47.0 µL index PCR mix. See Conc. Index PCR input for Sample Tag libraries (ng/µL) on page 28.

5 Gently vortex, and briefly centrifuge.

6 Program 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 s
Annealing		60 °C	30 s
Extension		72 °C	30 s
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

Index PCR conditions for Sample Tag

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 Sample Tag PCR2 product.			

Recommended number of PCR cycles

Conc. Index PCR input for 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 TCR/BCR and Sample Tag index PCR products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the TCR/BCR and Sample Tag 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. To 50.0 µL of the index PCR products, pipet:
   - 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 26 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. Discard 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. Discard 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. Pipet the entire eluate (~30 µL) into a new 1.5-mL LoBind® tube (final sequencing libraries).

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

15. 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 system using the Agilent High Sensitivity DNA Kit. Follow the manufacturer's instructions.

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

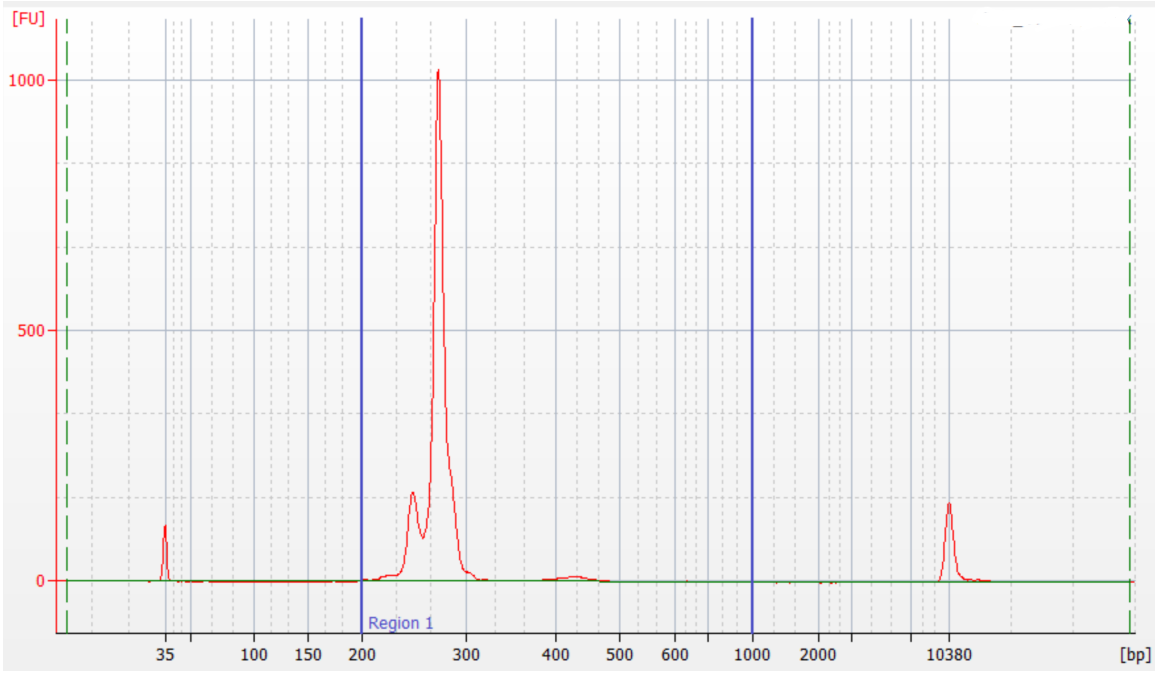


Figure 3 Sample Bioanalyzer High Sensitivity DNA trace - TCR index PCR product

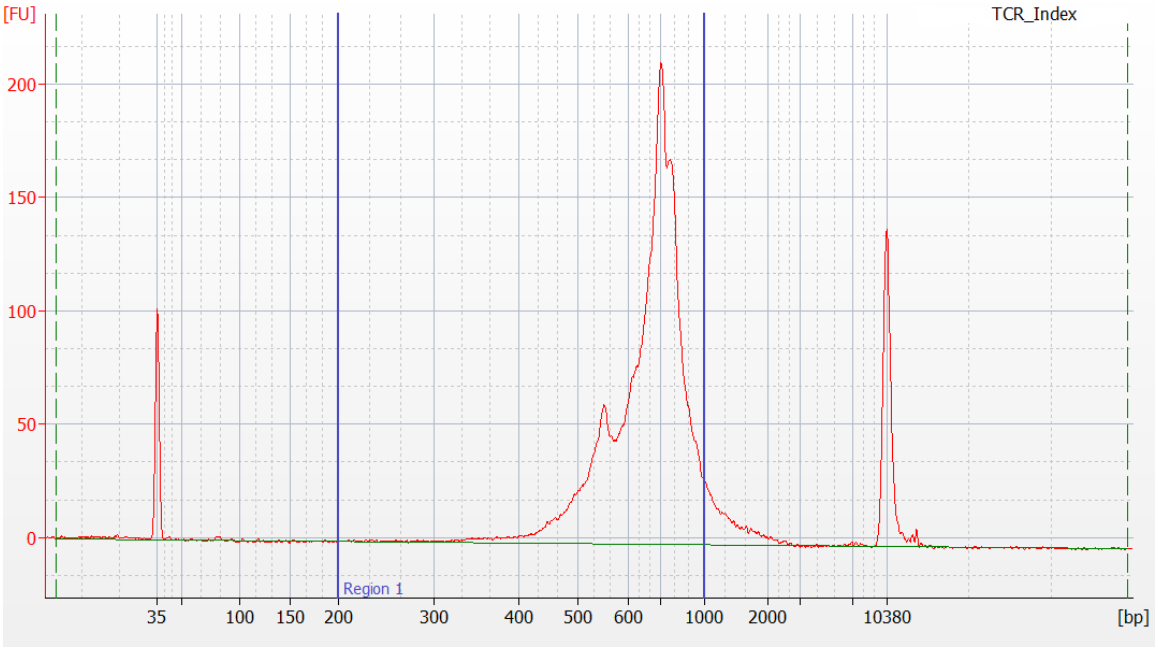
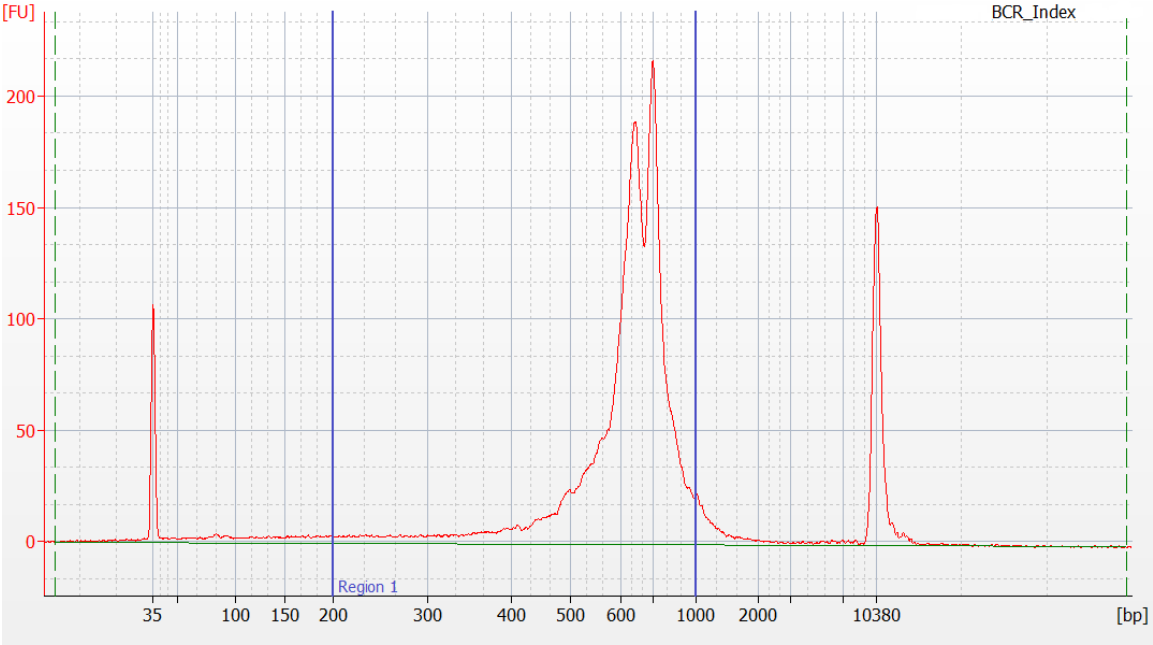


Figure 4 Sample Bioanalyzer High Sensitivity DNA trace - BCR index PCR product



Performing 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 Purifying RPE PCR amplification product (single-sided cleanup) on page 16 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 and continue.

For 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.

Index PCR reaction mix for WTA

Kit 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	5	6	–	–
Nuclease-free water	5	6	24	48
Total	**40**	**48**	**168**	**336**
*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:

   a For one sample, combine 40 µL of WTA Index PCR Mix with 10 µL of 2 nM of RPE PCR product.

   b 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 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 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.

Purifying WTA index PCR product (single-sided cleanup)

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 4.0 mL absolute ethyl alcohol, molecular biology grade, with 1.0 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 Agencourt 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. Remove 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. Remove 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.

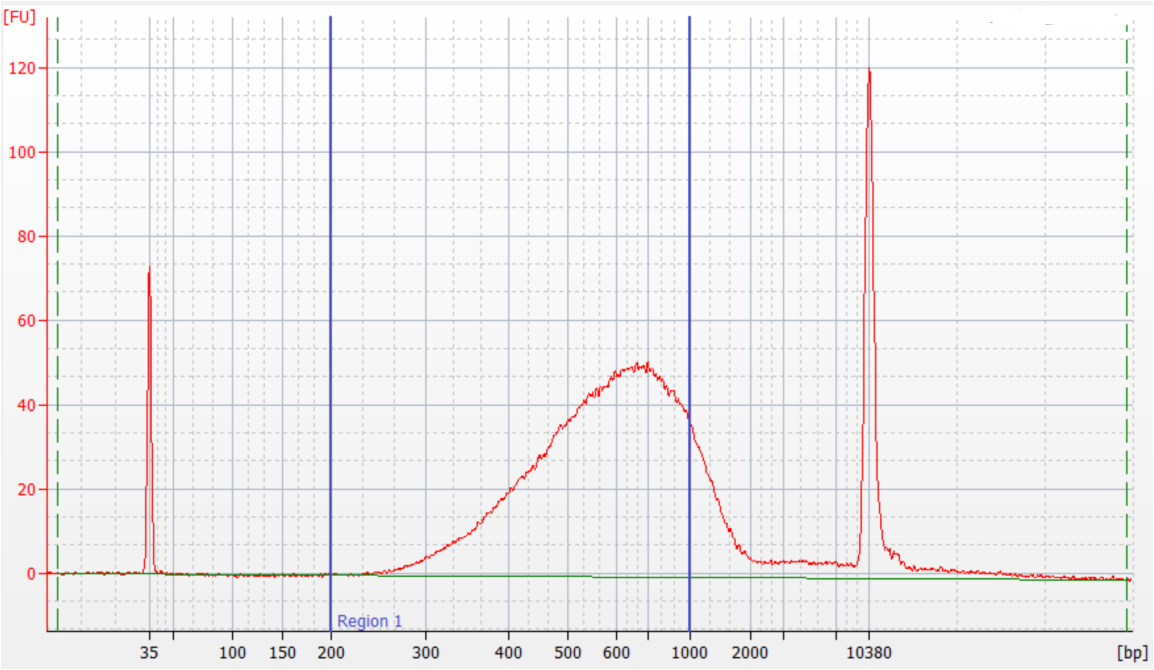
STOPPING POINT: The Index PCR libraries can be stored at –20 °C for up to 6 months until sequencing.

17 Quantify and perform quality control of the Index PCR libraries with a Qubit Fluorometer using the Qubit dsDNA HS Assay and 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 5 Sample Bioanalyzer High Sensitivity DNA trace - WTA 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 gene expression levels, number of cells, and sequencing run quality. Below are the recommended reads/cell for WTA mRNA, Sample Tag, TCR, and BCR libraries.

Read requirements for libraries

Library	Read requirement for data analysis
WTA mRNA	~10,000-100,000 reads/cell
Sample Tag: samples from the same type of cell (combining different donor PBMCs)	120 reads/cell
Sample Tag: samples from different types of cells (combining cell lines with PBMCs)	600 reads/cell
TCR	~5,000 reads/T cell
BCR	~5,000 reads/B cell

Sequencing depth can vary depending on whether the sample contains high- or low-content RNA cells. For resting PBMCs, we recommend:

- 10,000 reads per cell for shallow sequencing. Genes per cell and UMI per cell detected is generally lower but can be useful for cell type identification.
- 50,000 reads per cell for moderate sequencing.
- 100,000 reads per cell for highly saturated deep sequencing to identify the majority of UMIs in the library.

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 Sample Tag library 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 and Sample Tag libraries.

Important note: Sample Tag libraries can be sequenced together or separately from WTA mRNA and TCR/BCR libraries. However, sequencing the Sample Tag library separately will likely not be economical due to the small amount of data required for it. Examples of sequencing this library alone are not shown.

Example of pooling with no correction

In this example, a total of 5,000 enriched T cells were processed as a combination of two samples (two sample tags). These calculations assume the TCR library, and BCR library if included, sequences at 1/5 the efficiency of the WTA mRNA and Sample Tag libraries, 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)**
WTA mRNA	5,000	25,000	125,000,000	83%	144,904,077	28,981
TCR	5,000	5,000	25,000,000	16.6%	5,000,000	1,000
Sample Tag	5,000	120	600,000	0.4%	695,923	139
		Total	**150,600,000**	**100%**	**150,600,000**	

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 nor Sample Tag library. The remaining reads are allotted to the WTA mRNA and Sample Tag libraries 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 required number of reads per cell (Column C). Based on this example, 150.6 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, 150.6 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)**
WTA mRNA	5,000	25,000	125,000,000	83%	n/a	125,000,000	49.9%	125,000,000	25,000
TCR	5,000	5,000	25,000,000	26.6%	5*	125,000,000	49.9%	25,000,000	5,000
Sample Tag	5,000	120	600,000	0.4%	n/a	600,000	0.2%	600,000	120
		Total	**150,600,000****	**100%**		**250,600,000†**	**100%**	**150,600,000**	

*The 5x 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 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 (Columns D and C). The correction for library pooling did not change the amount of data generated (150.6 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 two samples of purified B and T cells, each stained with a different sample tag.

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	82%	n/a	250,000,000	49%	250,000,000	25,000
TCR	7,000	5,000	35,000,000	11%	5*	175,000,000	35%	35,000,000	5,000
BCR	3,000	5,000	15,000,000	5%	5*	75,000,000	15%	15,000,000	5,000
Sample Tag	10,000	600	6,000,000	2%	n/a	6,000,000	1%	6,000,000	600
		Total	**306,000,000****	**100%**		**506,000,000†**	**100%**	**306,000,000**	

*The 5x 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 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).
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 WTA 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, 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, 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 WTA, SMK, 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.

WTA, Sample Tag, 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.

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 x 150* paired read length Suggested: 150 x 150* paired read length
PhiX	Required (3%)
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook*
*For optimal assembly, use the sequencing configuration 85 x215 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

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

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