BD Rhapsody™ System

TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA), and BD® AbSeq Library Preparation Protocol

For Research Use Only

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Regulatory Information

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

History

Revision	Date	Change made
23-24019(01)	2022-01	Initial release.

Contents

Introduction

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

After staining of cell with AbSeq and Sample Tag antibodies and partitioning and lysis of cells, cDNA is encoded on BD Rhapsody™ Enhanced Cell Capture beads using both the 3' and 5' ends of transcripts as templates. mRNA, TCR, and BCR libraries are then amplified from these on-bead cDNA libraries using a two-step nested amplification, with TCR and BCR libraries undergoing additional random priming to capture complementarity determining regions (CDR) 1, 2, and 3, as well as framework regions (FR) 1-4. AbSeq libraries are amplified from the small products recovered after cleanup of larger mRNA, TCR, and BCR PCR1 products. Note that AbSeq 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™ WTA Amplification Kit	BD Biosciences	633801
BD Rhapsody™ TCR/BCR Amplification Kit	BD Biosciences	665345
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 °C to 25 °C) and mix thoroughly before use. See the *AMPure XP User's Guide* for information.
- Remove supernatants without disturbing AMPure XP magnetic beads.

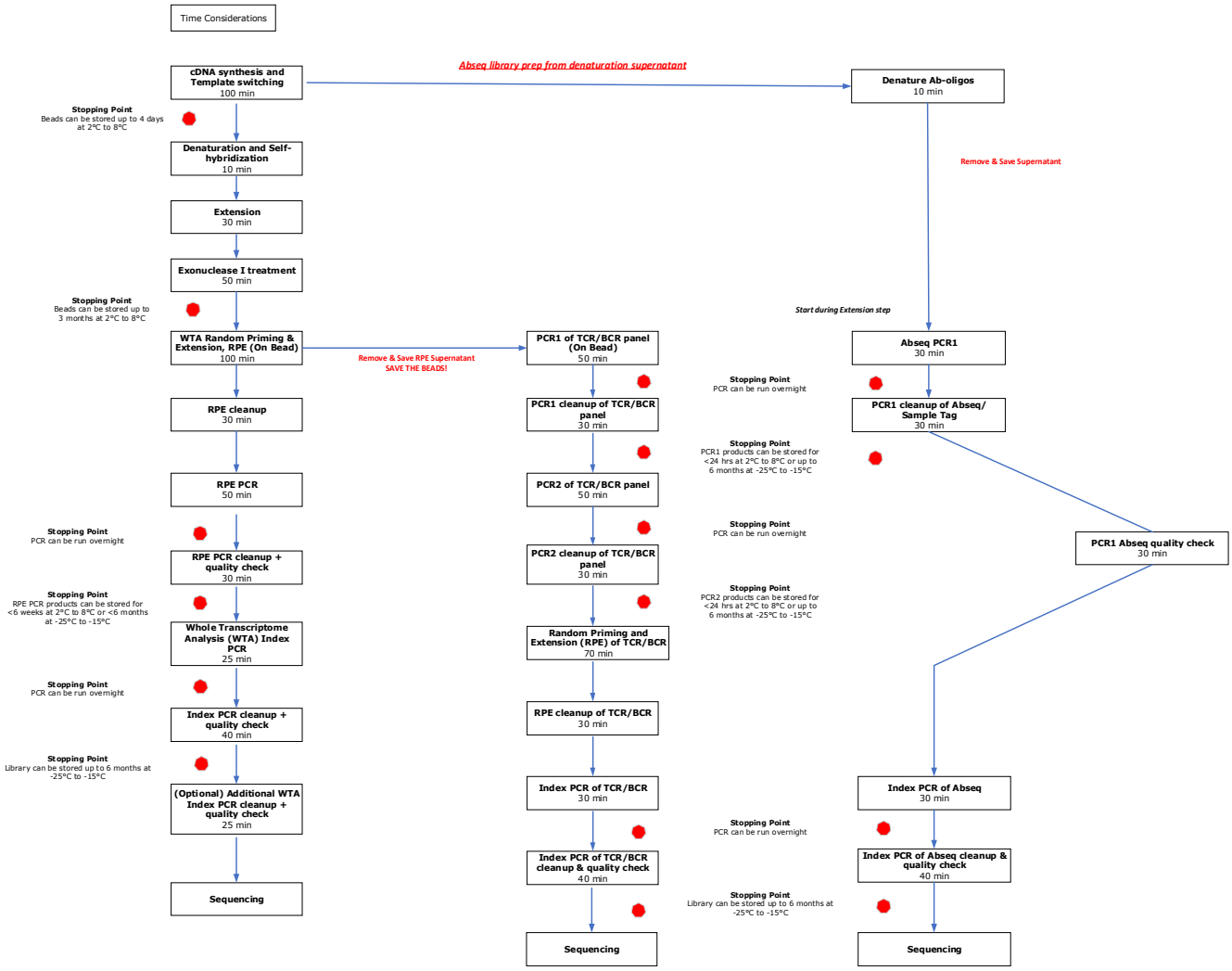
Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 214062)
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063)

Safety information

For safety information, see the *BD Rhapsody™ Single-Cell Analysis Instrument User Guide* (Doc ID 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063).

Time considerations



Procedure

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

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 214062)

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

  or

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

  STOP after the section "Washing the Cell Capture Beads" and follow this protocol from **Preparing BD Rhapsody™ Enhanced Cell Capture Beads for TCR/BCR full length, WTA, and BD® AbSeq 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 BD® AbSeq 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

cDNA/template switching mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
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 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.

NOTE Shaking is critical for this incubation!

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

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

USE IMMEDIATELY!

TSO mix

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

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

10 Add 8 µL of TSO mix to the reaction, gently pipet-mix, and incubate on the thermomixer for another 30 minutes at 1,200 rpm and 42 °C.

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

If stopping after template switching:

- Place the bead suspension on the 1.5-mL tube magnet until the solution is clear (≤1 minute).
- Carefully remove and appropriately discard the supernatant without disturbing the beads and while leaving the tube on the magnet.
- Remove the tube from the magnet, and with a low-retention tip, pipet 75 µL Elution Buffer to gently resuspend the beads. Do not vortex.
- Store the beads at 2 °C to 8 °C for up to 4 days.

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

Denaturation and self-hybridization

Thaw reagents for TCR/BCR Extension at room temperature. Keep 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 75 µL of Elution Buffer into the tube. Pipet-mix.

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

a Ensure that the beads are resuspended. Pipet-mix to resuspend, if needed.

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

5 Briefly centrifuge the tube, then immediately place the tube on a 1.5-mL magnet for ≥30 seconds until clear. **Keep the supernatant**. Remove the supernatant and transfer to a new 1.5-mL LoBind® tube. This contains the *AbSeq supernatant products*. To minimize AbSeq 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 AbSeq PCR1 on page 20**.

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 ≤2 minutes. Remove the supernatant.

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

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

NOTE During TCR/BCR Extension incubation, begin **AbSeq PCR1**. See **Performing AbSeq PCR1 on page 20**. You can leave the AbSeq PCR1 reaction in the thermocycler when complete. TCR/BCR PCR1 will be performed after **Purifying RPE PCR amplification product (single-sided cleanup) on page 18**. All PCR1 product purification (TCR/BCR and AbSeq) can be done at the same time.

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

Treating the sample with Exonuclease I

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

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

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

Exonuclease I mix

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

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

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

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

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

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

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

8 Place the tube on ice for ~1 minute.

9 Briefly spin the tube with the bead suspension.

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

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

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

12 Proceed to library preparation.

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, one thermomixer to 37 °C, and one thermomixer to 25 °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)
WTA Extension Buffer	20	24
WTA Extension Primers	20	24
Nuclease-free water	134	160.8
Total	**174**	**208.8**

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 + AbSeq 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. Remove the supernatant.

6 Briefly centrifuge the tube, then place the tube on a 1.5-mL magnet for 2 minutes. Remove and dispose of 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)
dNTP	8	12
Bead RT/PCR Enhancer	12	18
WTA Extension Enzyme	6	9
Total	**26**	**39**

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 Set the ramp rates at maximal and set "Time Mode" to "Temp Control" before the program begins.

14 Place the tube from step 12 in the thermomixer. The program takes approximately 55 minutes. Remove the tube after the program is finished.

15 Place the tube in a 1.5-mL tube magnet and remove 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. 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.

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. Remove the supernatant.

6 Keeping the tube on the magnet, gently add 1 mL of fresh 80% ethyl alcohol to the tube and incubate for 30 seconds. Remove 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 thetube.

9 Air-dry the beads at room temperature for 5 minutes or until the beads no longer look glossy.

10 Remove the tube from the magnet and resuspend the bead pellet in 40 µL of Elution Buffer. Pipet-mix the suspension at least 10 times until the beads are fully suspended.

11 Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.

12 Place the tube on the magnet until the solution is clear, usually ~30 seconds.

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

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)
PCR MasterMix	60	72
Universal Oligo	10	12
WTA Amplification Primer	10	12
Total	**80**	**96**

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.

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
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.

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.

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 without disturbing the beads.

9 Repeat **step 8** once for a total of two washes.

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

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

12 Remove the tube from the magnet and pipet 40 µL of Elution Buffer into the tube. Pipet- mix the suspension at least 10 times until the beads are fully suspended.

13 Incubate the sample at room temperature for 2 minutes. Briefly centrifuge the tube to collect the contents at the bottom.

14 Place the tube on the magnet until the solution is clear, usually ~30 seconds.

15 Pipet the eluate (~40 µL) into a new 1.5-mL LoBind® tube. The RPE PCR product is ready for WTA Index PCR.

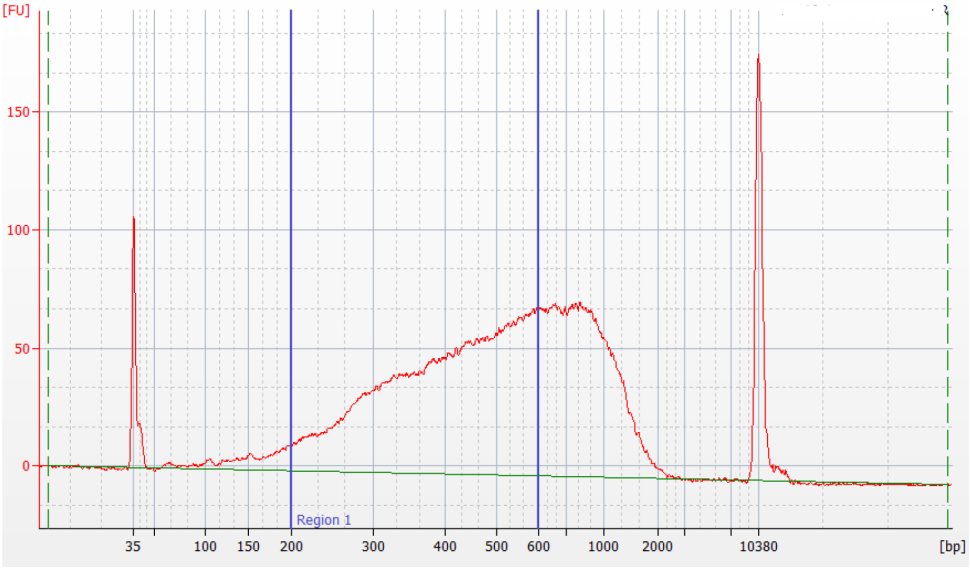
STOPPING POINT: The RPE PCR libraries can be stored at –20 °C for up to 6 months or 4 °C for up to 6 weeks.

16 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 AbSeq PCR1

This section describes how to amplify AbSeq products through PCR.

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

AbSeq PCR1 Reaction Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
BD® AbSeq Primer	10	12
Nuclease-free water	13	15.6
Total	**133**	**159.6**

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

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

4 Pipet 50 µL AbSeq 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.

Thermal cycler program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	10-11*	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

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

Recommended number of PCR cycles

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

STOPPING POINT: The PCR can run overnight.

7 After PCR, briefly centrifuge the tubes.

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

Performing TCR/BCR PCR1

1 Obtain beads from **step 19 on page 16** 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 + AbSeq combination assays.

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

PCR1 reaction mix

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

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

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. Remove the supernatant.

5 Remove the tube from the magnet and resuspend the beads in 200 µL of 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.

Thermal cycler program

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

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 °C to 8 °C in the post-amplification workspace.

Purifying TCR/BCR and AbSeq PCR1 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the TCR/BCR and AbSeq 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.

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:

- AbSeq libraries: 280 µL AMPure beads. From **step 8** in **Performing AbSeq PCR1 on page 20**.
- TCR/BCR libraries: 140 µL AMPure beads. From **step 13** in **Performing TCR/BCR PCR1 on page 21**.

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. Remove 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. Remove 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 the 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 AbSeq PCR1 products).

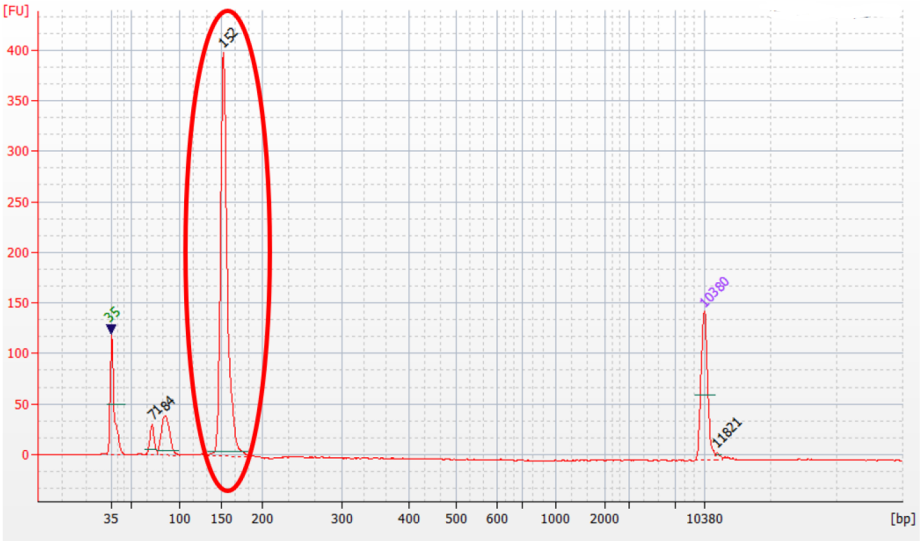
STOPPING POINT: Store at 2 °C to 8 °C before proceeding within 24 hours or at –25 °C to –15 °C for up to 6 months.

Quantifying BD® AbSeq PCR1 products

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

2 Based on the yield of the largest peak, dilute an aliquot of BD® AbSeq PCR1 product to 0.1–1.1 ng/µL with Nuclease-Free water before index PCR of BD® AbSeq PCR1 products. See **Performing TCR/BCR and AbSeq index PCR on page 29**.

Figure 2 Sample Bioanalyzer High Sensitivity DNA trace - AbSeq PCR1



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

Performing TCR/BCR PCR2 on the PCR1 products

This section describes how to amplify TCR/BCR 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.

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.		

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 PCR2 reaction mix.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler.

TCR and 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	∞

STOPPING POINT: The PCR can run overnight.

Purifying TCR/BCR PCR2 products

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

NOTE Perform PCR2 purification in the post-amplification workspace.

1 In a new 5.0-mL LoBind® tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4.0 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.

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

3 To 50 µL of PCR2 products, pipet:

- TCR/BCR libraries: 35 µL AMPure beads

4 Pipet-mix 10 times. Incubate at room temperature for 5 minutes.

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

7 Repeat **step 6** once for a total of two washes.

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

9 Air-dry the beads at room temperature for 3 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 PCR2 products).

STOPPING POINT: Store at 2 °C to 8 °C before proceeding on the same day or at –25 °C to –15 °C for up to 6 months.

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/BCR PCR2 products

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

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

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

Random primer mix

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

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

4 Add 41.5 µL of Random Primer Mix + 5 µL of 1 ng/µL diluted TCR/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 Rxn 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 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 4.0 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.

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/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. Remove 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. Remove the supernatant.

7 Repeat **step 6** once for a total of two washes.

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

9 Air-dry the beads at room temperature for 1 minute.

10 Remove tubes from the magnet and add 50 µL of Elution Buffer.

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

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

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

Performing TCR/BCR and AbSeq index PCR

This section describes how to generate TCR/BCR and AbSeq 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.

AbSeq 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 AbSeq Tag library.		

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 TCR or BCR library.		

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

3 Bring the TCR/BCR and AbSeq Index PCR mix to the 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/BCR Index PCR mix.

- For AbSeq library, pipet 3.0 µL of 0.1–1.1 ng/µL AbSeq PCR1 product into 47 µL AbSeq Index PCR mix. (From **Quantifying BD® AbSeq PCR1 products on page 24**).

5 Gently vortex, and briefly centrifuge.

6 Program the thermal cycler.

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	∞

AbSeq index PCR

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

*Cycle number varies based on the concentration of the AbSeq PCR1 product.

Recommended number of PCR cycles

Conc. Index PCR input for AbSeq 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 AbSeq index PCR products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the TCR/BCR and AbSeq 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 4.0 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.

2 Bring Agencourt 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:

- AbSeq library: 40 µL AMPure beads.

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

- TCR and BCR libraries: 26 µL AMPure beads.

5 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** 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. 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 (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 D1000. Follow the manufacturer's instructions.

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

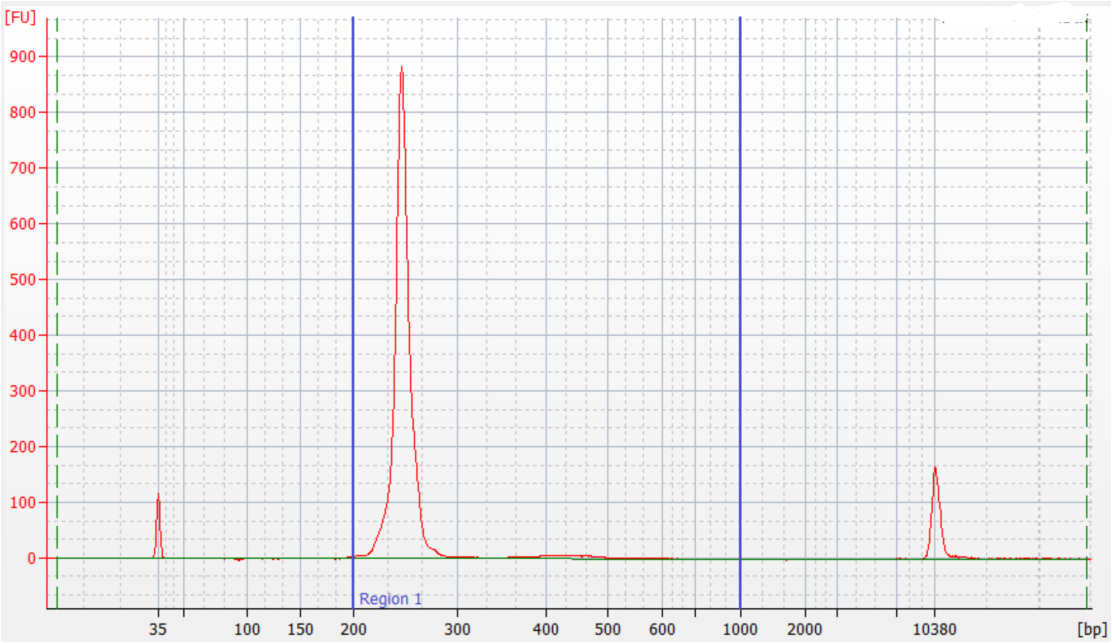


Figure 4 Sample Bioanalyzer High Sensitivity DNA trace - TCR index PCR product

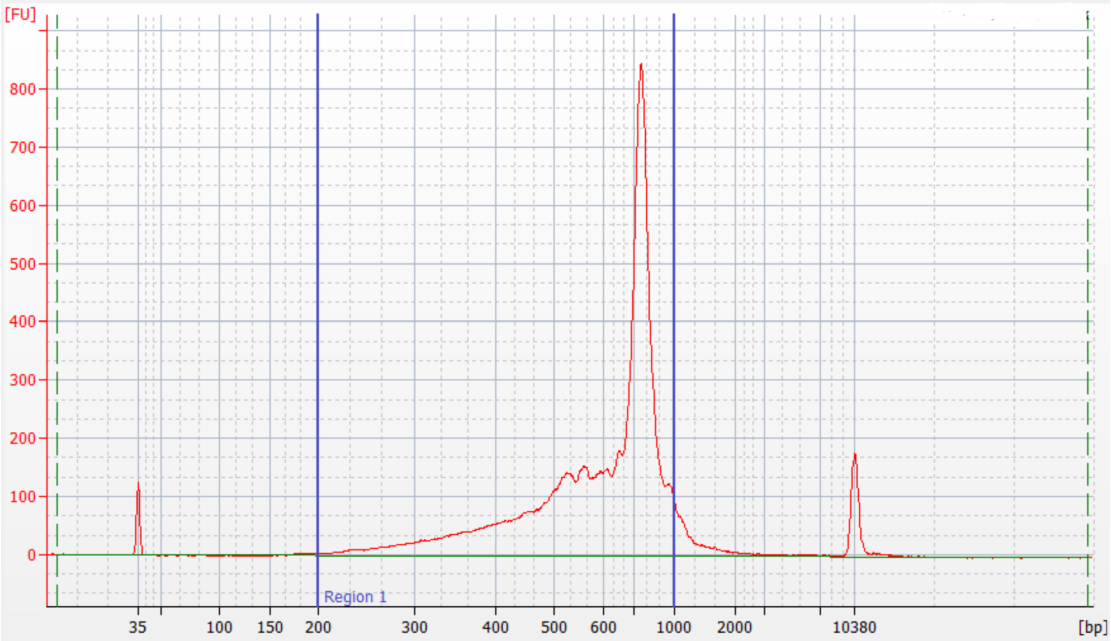
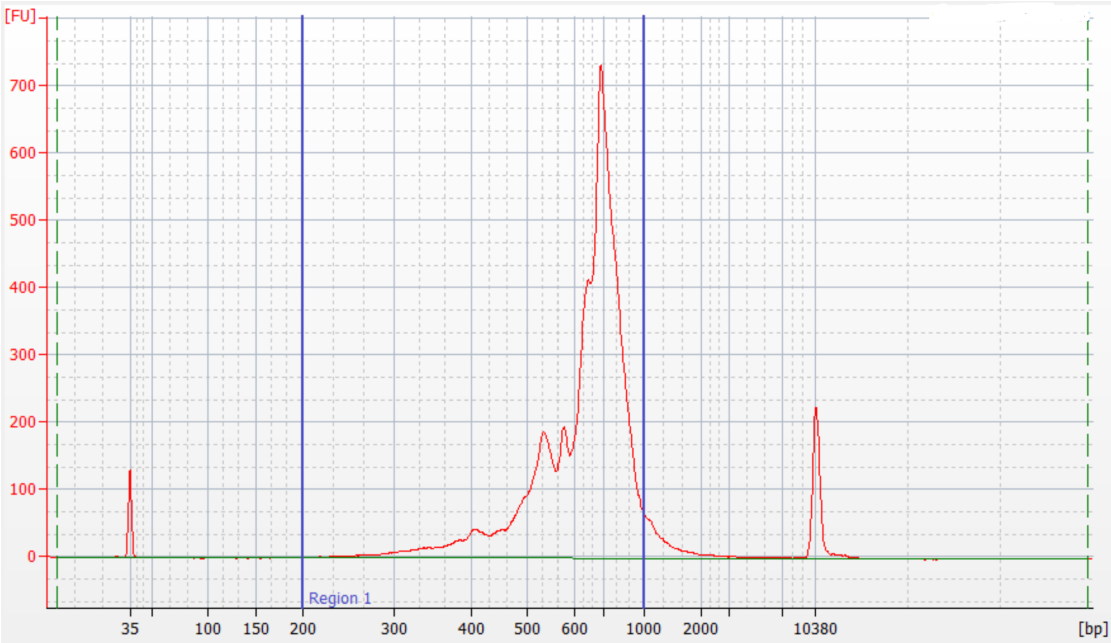


Figure 5 Sample Bioanalyzer High Sensitivity DNA trace - BCR index PCR product



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 18** 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 three-fold with Nuclease-Free water to 2 nM.

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

WTA index PCR mix

Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	5	6
*Library Reverse Primer 1 – 4	5	6
Nuclease-free water	5	6
Total	**40**	**48**
*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.

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	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.

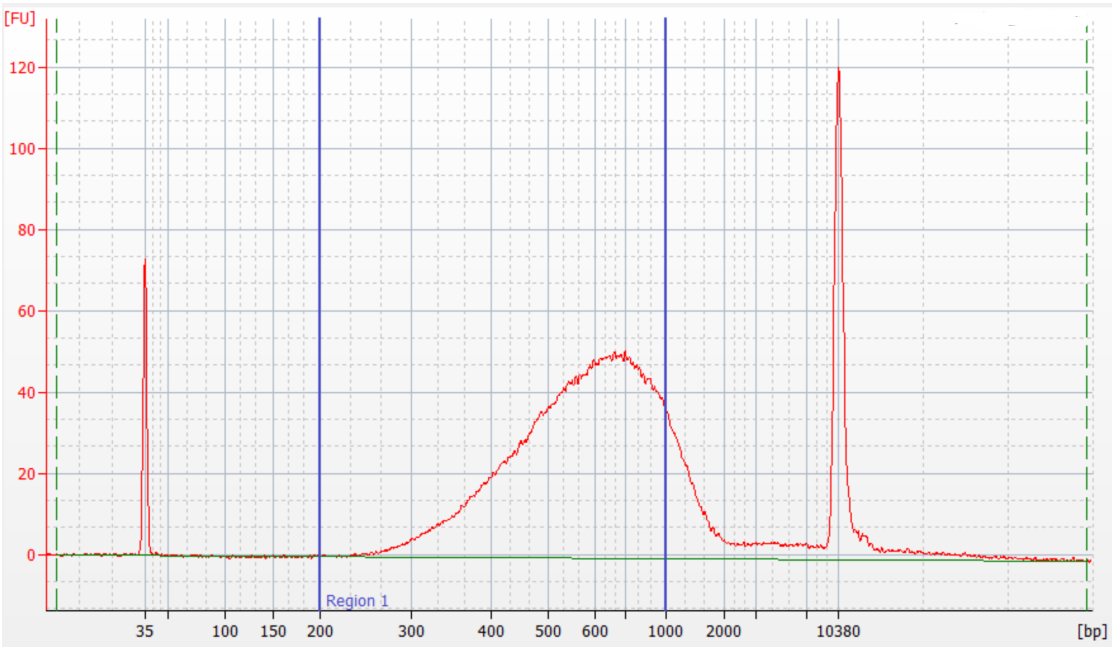
STOPPING POINT: The Index PCR libraries can be stored at –20 °C for up to 6 months until sequencing.

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 6 Sample Bioanalyzer High Sensitivity DNA trace - WTA index PCR product



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 WTA and TCR/BCR libraries 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.

   a **Library quantification**: For TCR/BCR using PBMC or DG-75/Jurkat, based on Qubit quantitation concentration and Bioanalyzer size (200 bp - 1000 bp), calculate adjusted concentration. Use adjusted concentration to pool libraries.

Sequencing recommendations

WTA library

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.

Read requirements for libraries

Gene panel	Read requirement for data analysis	Adjusted reads for sequencing pooled TCR/BCR libraries on Illumina platforms**
BD Rhapsody™ WTA	~10,000-100,000 reads/cell	N/A
BD Rhapsody™ AbSeq	1,000 reads/cell/AbSeq*	N/A
BD Rhapsody™ TCR	~5,000 reads/T cell	~25,000 reads/T cell**
BD Rhapsody™ BCR	~5,000 reads/B cell	~25,000 reads/B cell**

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

**Note: Because of the long amplicon sizes, TCR and BCR libraries will not cluster as efficiently as WTA libraries on Illumina platforms. To account for this, TCR and BCR libraries should be pooled with WTA libraries as if aiming for 5x the number of reads that are actually desired. See the following examples.

Sequencing options for AbSeq libraries

AbSeq libraries can be sequenced together or separately from WTA and TCR/BCR libraries. For optimal clustering of TCR/BCR libraries on Illumina platforms however, sequencing AbSeq separately is recommended.

AbSeq libraries sequenced separately

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

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	100%	50 µL
Total				**300,000,000**	**100%**	**50 µL**

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

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
WTA	10,000	N/A	10,000	100,000,000	29%	14 µL
TCR or BCR	5,000	5	10,000	250,000,000	71%	36 µL
Total				**350,000,000**	**100%**	**50 µL**

Example of pooling WTA and TCR/BCR libraries from PBMCs

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
WTA	10,000	N/A	10,000	100,000,000	40%	20 µL
TCR	5,000	5	4,000	100,000,000	40%	20 µL
BCR	5,000	5	2,000	50,000,000	20%	10 µL
Total				**250,000,000**	**100%**	**50 µL**

AbSeq libraries sequenced in combination with other libraries

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

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
WTA	10,000	N/A	10,000	100,000,000	15%	8 µL
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	46%	23 µL
TCR or BCR	5,000	5	10,000	250,000,000	39%	19 µL
Total				**650,000,000**	**100%**	**50 µL**

Example of pooling using PBMCs stained with 30-plex AbSeq

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
WTA	10,000	N/A	10,000	100,000,000	18.2%	9 µL
AbSeq 30-plex	30,000	N/A	10,000	300,000,000	54.5%	27 µL
TCR	5,000	5	4,000	100,000,000	18.2%	9 µL

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

Library type	Reads/cell	Correction factor for low clustering	Cell number	Reads needed	% pooling	Volume based on total 50 µL pool
BCR	5,000	5	2,000	50,000,000	9.1%	5 µL
Total				**550,000,000**	**100%**	**50 µL**

Sequencing flow cell loading and PhiX concentrations

Quantifying libraries

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

AbSeq only library

For a NextSeq High or Mid Output and MiniSeq High or Mid Output runs, load the flow cell at a concentration between 1.8-2.2 pM with 15% PhiX for a sequencing run.

Required parameters

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

WTA and TCR/BCR libraries (with or without AbSeq)

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

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

Required parameters

Parameter	Requirement
Platform	Illumina: 300 cycle kit
Paired-end reads	Minimum of 85 x 215* paired read length
PhiX	Required (3%)
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook* (Doc ID: 54169)
*R2 length of 215 is recommended for optimal assembly. If necessary 150 x150 read lengths can be used.	

Sequencing analysis pipeline

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

Appendix

Human T cell PCR1 primers

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

Human T cell PCR2 primers

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

Human B cell PCR1 primers

Primer name	Primer sequence (5′ - 3′)
IGHA_N1	CACAGTCACATCCTGGCT
IGHD_N1	GATCTCCTTCTTACTCTTGCTGG
IGHE_N1	CGCTGAAGGTTTTGTTGTCG
IGHG_N1	TGTTGCTGGGCTTGTGAT
IGHM_N1	CGTTCTTTTCTTTGTTGCCGT
IGKC_N1	TTTGTGTTTCTCGTAGTCTGCT
IGLC_N1	TGTAGCTTCTGTGGGACTTC

Human B cell PCR2 primers

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

Human B cell PCR2 primers

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