BD Rhapsody™ System

mRNA Whole Transcriptome Analysis (WTA) and AbSeq Library Preparation Protocol

For Research Use Only

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Regulatory Information

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

History

Revision	Date	Change made
23-21751-00	10/2019	Initial release

Contents

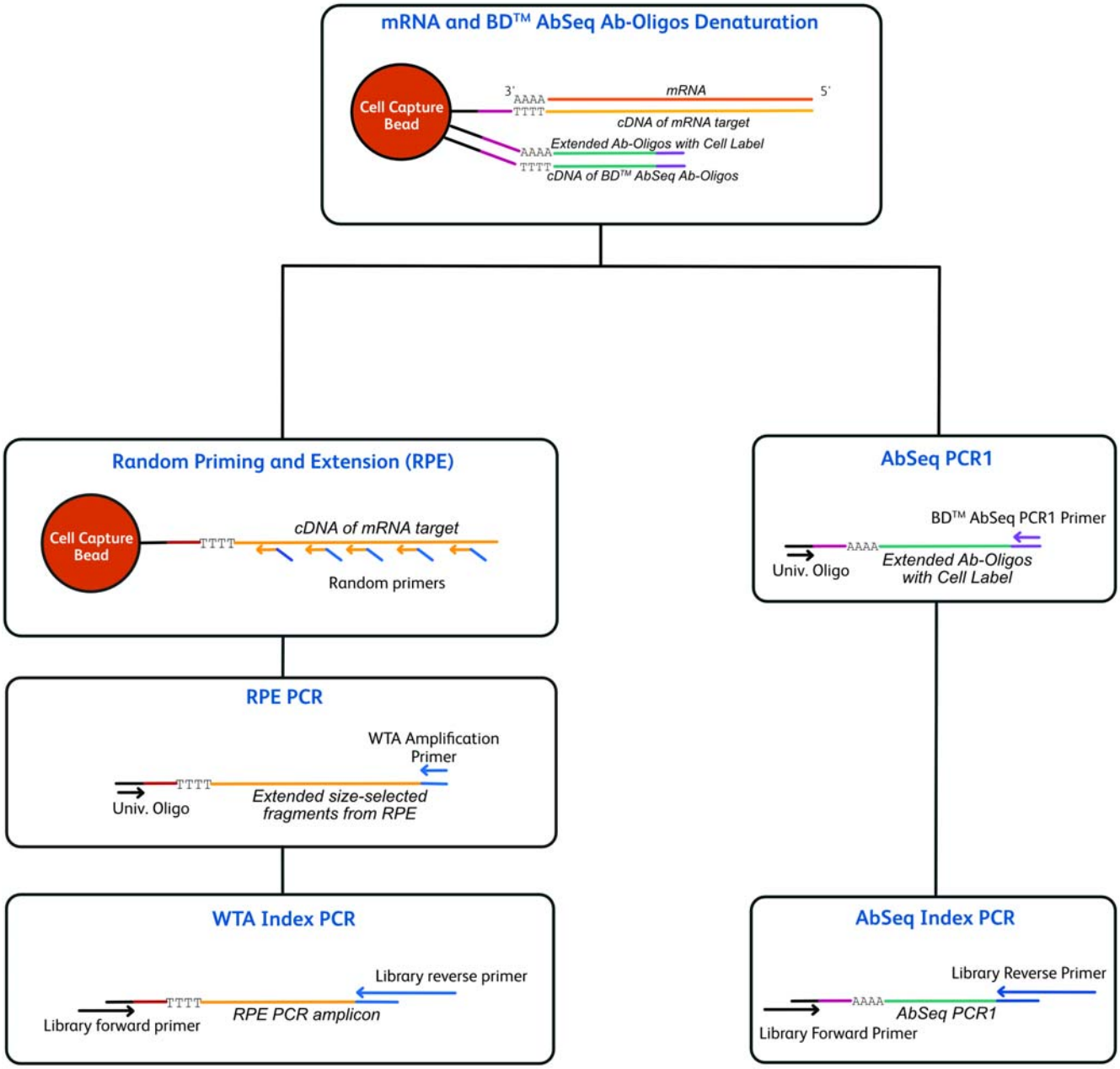
Introduction

This protocol provides instructions on creating single cell whole transcriptome mRNA and AbSeq protein-detection libraries after cell capture on the BD Rhapsody™ Single-Cell Analysis System or the BD Rhapsody™ Express Single-Cell Analysis System for sequencing on Illumina sequencers. For complete instrument procedures and safety information, see the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID 214063).

The cDNA of mRNA and AbSeq targets is first encoded on BD Rhapsody™ Cell Capture Beads, as described in the instrument user guides. At the same time, the barcode information from BD Rhapsody Cell Capture Beads is also added to AbOligos during reverse transcription, which enables amplification of the AbOligos in a separate PCR reaction. To generate the AbSeq sequencing libraries, the extended AbOligos are first denatured from the BD Rhapsody Cell Capture Beads, and are later amplified through a series of PCR steps. Meanwhile, the whole transcriptome amplification library is generated directly from the BD Rhapsody Cell Capture Beads using a random priming approach, followed by an index PCR step. Both the whole transcriptome mRNA and AbSeq libraries can be combined together for sequencing on various Illumina sequencers.

This protocol is intended to provide a method to screen RNA expression of single cells using a 3' whole transcriptome analysis (WTA) approach through the BD Rhapsody™ WTA Amplification Kit for samples that have also been labeled using BD® AbSeq AbOligos. The mRNA data set generated from this protocol can be used to generate a custom mRNA Primer panel for subsequent 3' targeted mRNA sequencing. Specifically, the WTA protocol outlines how to generate whole transcriptome libraries for BD Rhapsody Cell Capture Beads inputs between 1,000 to 10,000 resting PBMCs per sample for library generation. For BD Rhapsody Cell Capture Beads inputs between 1,000 to <5,000 cells per sample, there are additional sections in the protocol, Purifying RPE product on page 14 and Purification of the WTA Index PCR product (dual-sided cleanup) on page 23. For cell types other than resting PBMCs, protocol optimization might be required by the user.

Workflow


mRNA and BD™ AbSeq Ab-Oligos Denaturation
Cell Capture Bead
3'
AAAA
TTTT
mRNA
5'
cDNA of mRNA target
Extended Ab-Oligos with Cell Label
AAAA
TTTT
cDNA of BD™ AbSeq Ab-Oligos
Random Priming and Extension (RPE)
Cell Capture Bead
TTTT
cDNA of mRNA target
Random primers
AbSeq PCR1
BD™ AbSeq PCR1 Primer
AAAA
Univ. Oligo
Extended Ab-Oligos with Cell Label
RPE PCR
WTA Amplification Primer
TTTT
Univ. Oligo
Extended size-selected fragments from RPE
WTA Index PCR
Library reverse primer
TTTT
Library forward primer
RPE PCR amplicon
AbSeq Index PCR
Library Reverse Primer
AAAA
Library Forward Primer
AbSeq PCR1

Required materials

- Exonuclease I-treated BD Rhapsody Cell Capture Beads containing sample
- BD Rhapsody™ WTA Amplification Kit (Cat. No. 633801)

Kit component	Part number	Cap color
Nuclease-free water	650000076	Clear
WTA Extension Buffer	91-1114	Blue
WTA Extension Primers	91-1115	Blue
10 mM dNTP	650000077	Orange
Bead RT/PCR Enhancer	91-1082	Black
WTA Extension Enzyme	91-1117	Blue
PCR MasterMix	91-1118	White
Universal Oligo	650000074	White
BD™ AbSeq Primer	91-1086	Green
WTA Amplification Primer	91-1116	White
Elution Buffer	91-1084	Pink
Bead Resuspension Buffer	650000066	Black
Library Forward Primer	91-1085	Red
Library Reverse Primer 1	650000080	Red
Library Reverse Primer 2	650000091	Red
Library Reverse Primer 3	650000092	Red
Library Reverse Primer 4	650000093	Red
Sample Tag PCR1 Primer	91-1088	Purple
Sample Tag PCR2 Primer	91-1089	Purple

- Agencourt® AMPure® XP magnetic beads (Beckman Coulter Life Sciences, Cat. No. A63880)
- Absolute ethyl alcohol, molecular biology grade (major supplier)
- Nuclease-free water (major supplier)

  NOTE The kit provides enough to prepare the PCR MasterMix. You will need to purchase additional nuclease-free water for the AMPure purification steps.

- 6-Tube Magnetic Separation Rack for 1.5-mL tubes (New England Biolabs, Cat. No. S1506S)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32851)

- Agilent® DNA High Sensitivity Kit (Agilent Technologies, Cat. No. 5067-4626)

  OR

  - Agilent® High Sensitivity D5000 ScreenTape (Agilent Technologies, Cat. No. 5067-5592)
  - Agilent® High Sensitivity D5000 Reagents (Agilent Technologies, Cat. No. 5067-5593)

  OR

  - Agilent® High Sensitivity D1000 ScreenTape (Agilent Technologies, Cat. No. 5067-5584)
  - Agilent® High Sensitivity D1000 Reagents (Agilent Technologies, Cat. No. 5067-5585)

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody Cell Capture Beads. Use the Cell Capture Beads within 48 hours of performing Exonuclease I treatment.
- Thaw reagents in the BD Rhapsody WTA Amplification Kit at room temperature (15°C to 25°C), then immediately place on ice.

Best practices

- Use filtered pipette tips.
- When working with Cell Capture Beads, use low-retention filtered tips and LoBind Tubes. **Never vortex the beads. Pipet-mix only.**
- Bring AMPure XP magnetic beads to room temperature and mix thoroughly before use. See the *AMPure XP User's Guide* for information.
- Remove supernatants without disturbing the AMPure XP magnetic beads.

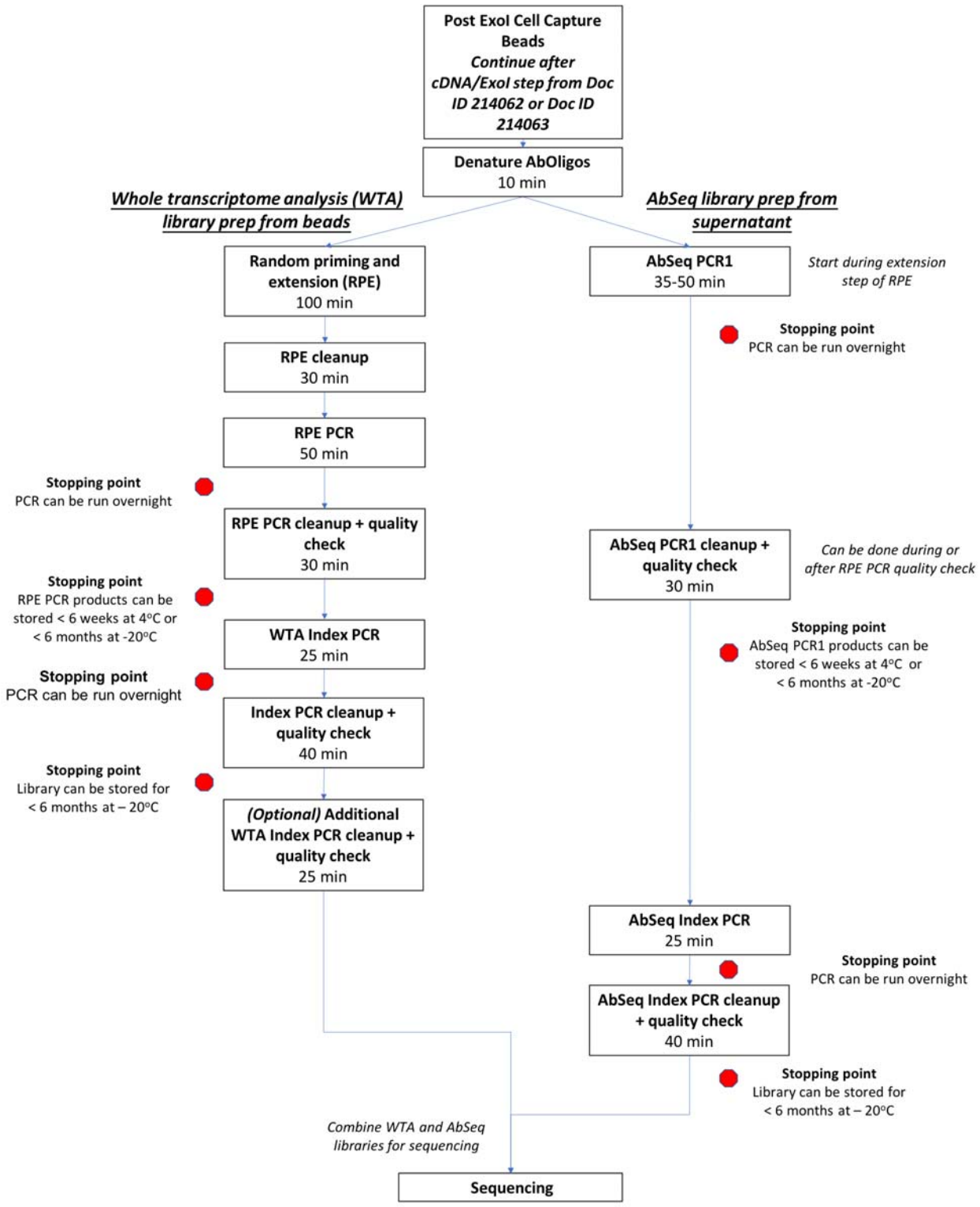
Additional documentation

- *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



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

This section describes how to generate random priming products. First, AbOligos with cell barcode and UMI information from BD Rhapsody Cell Capture Beads are denatured off of the beads and saved for AbSeq amplification. Then, random primers are hybridized to the cDNA on the BD Rhapsody Cell Capture Beads, followed by polymerase extension.

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)	For 2 libraries with 10% overage (µL)
WTA Extension Buffer (Cat. No. 91-1114)	20	24	44
WTA Extension Primers (Cat. No. 91-1115)	20	24	44
Nuclease-free water (Cat. No. 650000076)	134	160.8	294.8
Total	**174**	**208.8**	**382.8**

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

4 Choose between using the entire sample or a subsample of the Exonuclease I-treated BD Rhapsody Cell Capture Beads. If using the entire sample of beads, skip to step 6. If using a subsample, proceed to step 5.

5 (Optional) Subsample the Exonuclease I-treated BD Rhapsody Cell Capture Beads:

a Based on the expected number of viable cells captured on the beads in the final bead resuspension volume, determine the volume of beads to subsample for sequencing.

b Completely resuspend the beads by pipet-mixing, then pipet the calculated volume of the bead suspension into a new 1.5-mL LoBind tube. If needed, bring the total volume up to 200 µL with Bead Resuspension Buffer.

NOTE The remaining beads can be stored in Bead Resuspension Buffer at 4°C for up to 3 months.

6 Resuspend the Exonuclease I-treated BD Rhapsody Cell Capture Beads with a pipette.

7 Place the tube of Exonuclease I-treated beads in Bead Resuspension Buffer on the 1.5-mL magnet for 2 minutes. Remove the supernatant.

8 Remove the tube from the magnet and resuspend the beads in 75 µL of Elution Buffer. Pipet-mix 10 times to resuspend the beads.

9 Place the tube with the beads in a 95°C heat block for 5 minutes (no shaking).

10 Label a new 1.5-mL tube as *AbSeq supernatant products*.

11 Briefly centrifuge the tube, then immediately place the tube on a 1.5-mL magnet for <2 minutes. Remove the supernatant and transfer to the *AbSeq supernatant products* tube. To minimize AbSeq contamination in the WTA library, ensure that all liquid is removed from the tube. Keep the supernatant tube at 4°C for up to 24 hours until ready to proceed to Performing AbSeq PCR1 on page 12.

12 Remove the tube with the BD Rhapsody Cell Capture Beads from the magnet, and use a low-retention tip to pipet 200 µL of Elution Buffer into the tube. Pipet-mix 10 times to resuspend the beads.

13 Briefly centrifuge the tube, then place the tube on a 1.5-mL magnet for <2 minutes. Remove and dispose of the supernatant.

14 Remove the tube with the BD Rhapsody 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.

15 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

16 Briefly centrifuge the tube and keep it at room temperature.

17 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 50% overage (µL)	For 2 libraries with 30% overage (µL)
10 mM dNTP (Cat. No. 650000077)	8	12	20
Bead RT/PCR Enhancer (Cat. No. 91-1082)	12	18	31
WTA Extension Enzyme (Cat. No. 91-1117)	6	9	16
Total	**26**	**39**	**67**

18 Pipet-mix the Extension Enzyme Mix.

19 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.

20 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.

21 Place the tube from step 19 in the thermomixer. Remove the tube after the program is finished.

NOTE While the thermomixer program is running, begin AbSeq PCR1. See Performing AbSeq PCR1 in the following section.

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

23 Remove the tube from the magnet and resuspend the beads in 205 µL of Elution Buffer using a P200 pipette.

24 To denature the random priming products off the beads, pipet to resuspend the beads. Then:

a Incubate the sample at 95°C in a heat block for 5 minutes (no shaking).

b Place the tube in a thermomixer at any temperature for 10 seconds at 1,200 rpm to resuspend the beads.

25 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 on page 14.

26 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 at 4°C in the pre-amplification workspace for up to 3 months.

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.

AbSeq PCR1 Reaction Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. No. 91-1118)	100	120
Universal Oligo (Cat. No. 650000074)	10	12
Bead RT/PCR Enhancer (Cat. No. 91-1082)	12	14.4
AbSeq PCR1 Primer (Cat. No. 91-1086)	10	12
Total	**132**	**158.4**

2 Gently vortex mix and briefly centrifuge.

3 In a new 1.5-mL tube, pipet 132 µL of the AbSeq PCR1 reaction mix. Add 68 µL of the AbSeq product from step 11 in Performing random priming and extension (RPE) on BD Rhapsody Cell Capture Beads with cDNA on page 10. Pipet-mix 10 times.

4 Pipet 50 µL AbSeq 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. Do not use fast cycling mode:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	11–15*	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.

Suggested number of PCR cycles

Number of cells in PCR1	Suggested PCR cycles for resting PBMCs
500	15
1,000	14
2,500	13
5,000	12
10,000	11

STOPPING POINT: The PCR can run overnight.

7 After PCR has started, proceed to step 21 in Performing random priming and extension (RPE) on BD Rhapsody Cell Capture Beads with cDNA on page 10.

8 After PCR, briefly centrifuge the tubes.

9 Pipet-mix and combine the four reactions into a new 1.5-mL LoBind tube, labeled *AbSeq PCR1*. Proceed to Purifying AbSeq PCR1 products on page 19.

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. An additional cleanup is recommended for low cell input (<5,000 cells) to ensure maximum removal of the unwanted small molecular weight products before the next PCR.

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 (15°C to 25°C). Vortex the AMPure XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.

3. Pipet 360 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 the suspension at room temperature for 10 minutes.

5. Place the suspension 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.

7. Incubate the sample on the magnet for 30 seconds. Remove the supernatant.

8. Repeat the 80% ethyl alcohol wash for a total of two washes.

9. Keeping the tube on the magnet, use a P20 pipette to remove and discard any residual supernatant from the tube.

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

11. 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.

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

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

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

    NOTE For samples with low cell input (eg, starting with fewer than 5,000 PBMCs), proceed to step 15 for an additional round of AMPure XP magnetic purification.

Additional RPE purification steps for cell input <5,000 PBMC cells

15 To the tube from step 14, bring the purified RPE product volume up to 100 µL with nuclease-free water and transfer to a 1.5-mL LoBind tube.

IMPORTANT It is critical for the final volume to be exactly 100 µL to achieve the desired size selection of the purified RPE product.

16 Pipet-mix 10 times, then briefly centrifuge.

17 Pipet 180 µL of AMPure XP magnetic beads into the tube containing 100 µL of eluted RPE product from the first round of purification.

18 Pipet-mix 10 times, then briefly centrifuge.

19 Repeat step 4 through step 14 once more, resulting in a total of two rounds of purification.

20 Elute into a new PCR tube (~40 µL).

STOPPING POINT: Store the RPE product in a LoBind tube on ice or at 4°C for up to 24 hours until PCR.

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 2 libraries with 10% overage (µL)
PCR MasterMix (Cat. No. 91-1118)	60	72	132
Universal Oligo (Cat. No. 650000074)	10	12	22
WTA Amplification Primer (Cat. No. 91-1116)	10	12	22
Total	**80**	**96**	**176**

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.

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	Refer to the following table, Suggested 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.

Suggested number of PCR cycles

Number of cells in RPE PCR	Suggested PCR cycles for resting PBMCs
1,000–9,999	13
10,000	12

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

Purification of the 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 that the cleanup for WTA with AbSeq is different from the cleanup for WTA performed alone, in order to minimize the AbSeq signal carried over into the WTA product. If performing WTA alone, follow the cleanup outlined in *BD Rhapsody™ System mRNA Whole Transcriptome Analysis (WTA) Library Preparation Protocol* (23-21711-00).

NOTE Perform the purification in the post-amplification workspace.

1 Combine the two RPE PCR reactions into a new 1.5-mL tube.

2 Briefly centrifuge the tube with the RPE PCR product.

3 Bring AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex the AMPure XP magnetic beads at high speed for 1 minute until the beads are fully resuspended.

4 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.

5 Incubate the suspension at room temperature for 5 minutes.

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

7 Keeping the tube on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the tube.

8 Incubate the sample for 30 seconds on the magnet. Remove the supernatant.

9 Repeat the 80% ethyl alcohol wash 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 5 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 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 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 either of the following systems:

- Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit

- Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay

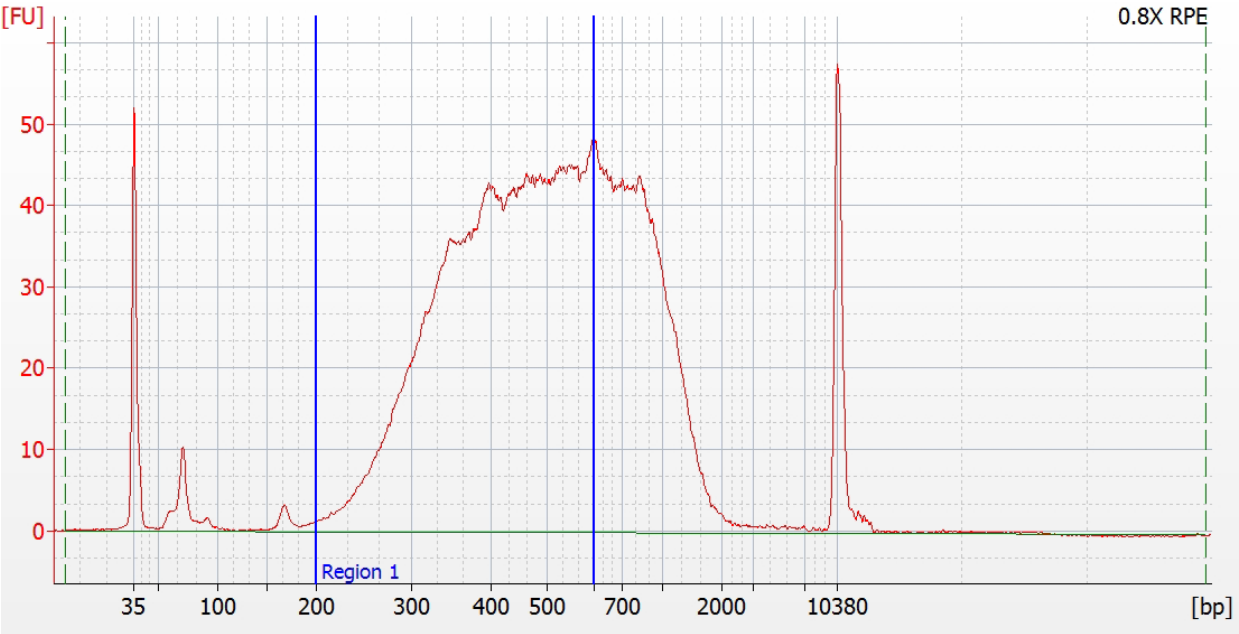
a The expected concentration from the Qubit Fluorometer is ~0.5 to 10 ng/µL.

b The Bioanalyzer/TapeStation 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 on page 18.

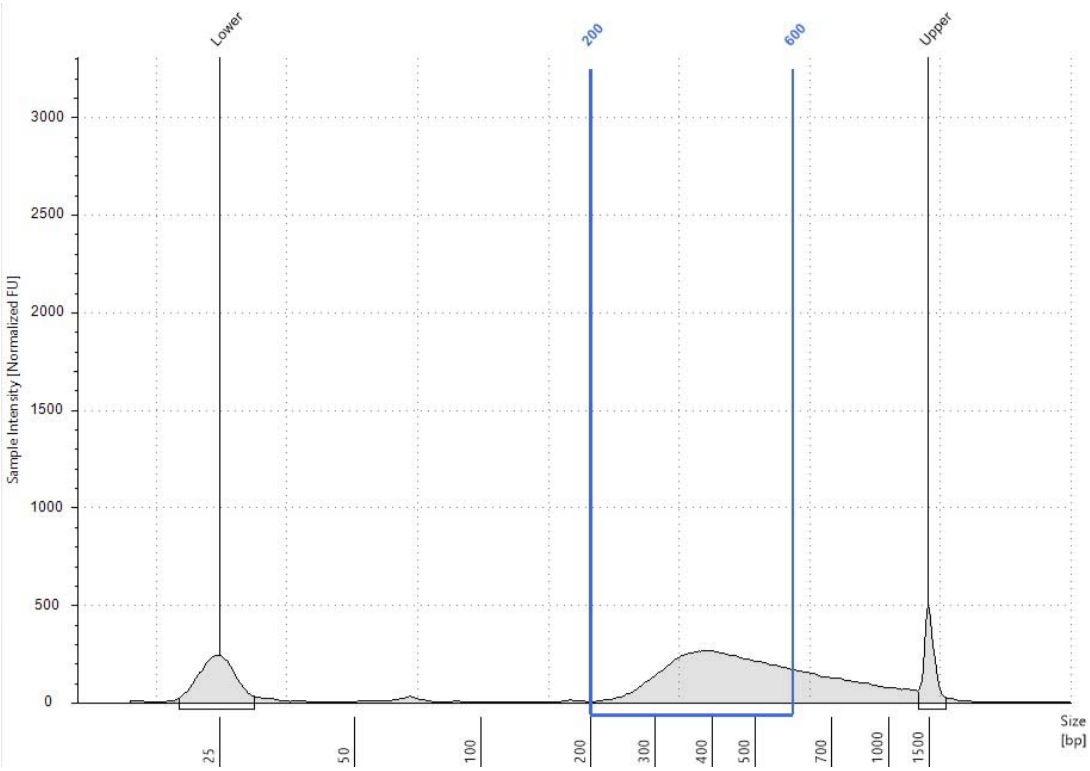
NOTE Although there are products ~170 bp (AbSeq contamination product) and >600 bp, these products should be removed in the double-sided cleanup after the next PCR.

Figure 1 RPE PCR product traces

A. Sample Bioanalyzer High Sensitivity DNA trace



B. Sample TapeStation High Sensitivity D1000 trace



Purifying AbSeq PCR1 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the 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 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.

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

2. Bring the AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex at high speed for 1 minute until the beads are fully resuspended.

3. Pipet 280 µL AMPure XP beads into a tube with 200 µL AbSeq PCR1 from Performing AbSeq PCR1 on page 12. Pipet-mix 10 times.

4. Incubate at room temperature (15°C to 25°C) 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, and incubate for 30 seconds. Remove the supernatant.

7. Repeat step 6 once for two washes.

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

9. Air-dry the beads at room temperature (15°C to 25°C) for 5 minutes.

10. Remove the tube from the magnet and resuspend the bead pellet in 30 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 (15°C to 25°C) for 2 minutes, then briefly centrifuge.

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

13. Pipet the eluate (~30 µL) into a new 1.5-mL LoBind tube (purified 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.

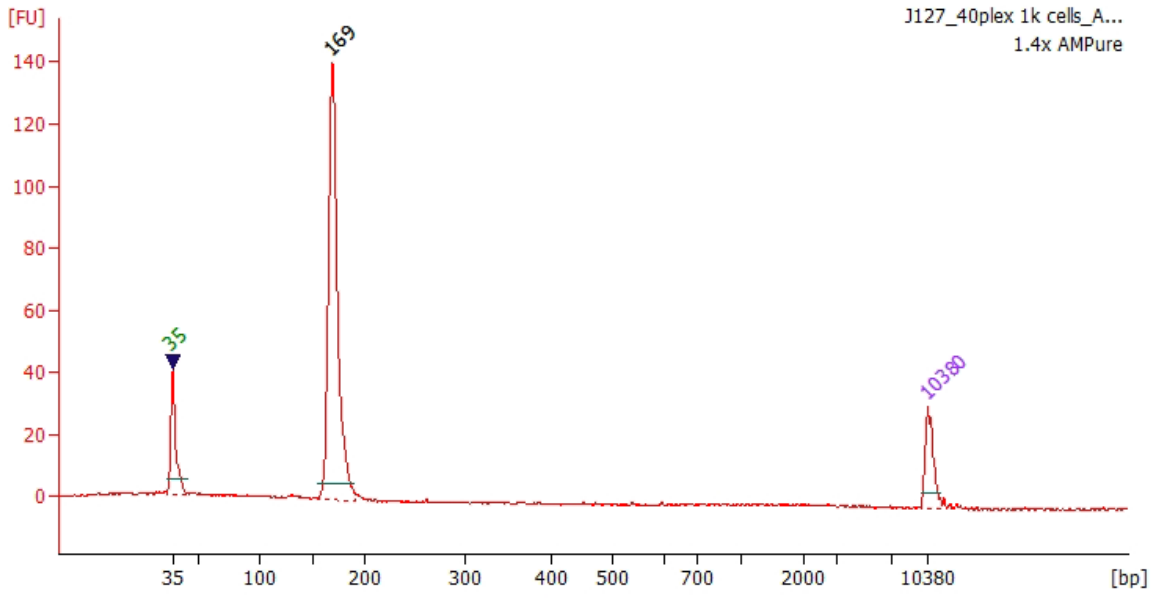
Quantifying BD AbSeq PCR1 products

1 Measure the yield of the BD AbSeq PCR1 products (~170 bp)—size varies on different instruments—by using the Agilent Bioanalyzer with the High Sensitivity Kit or the Agilent TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions.

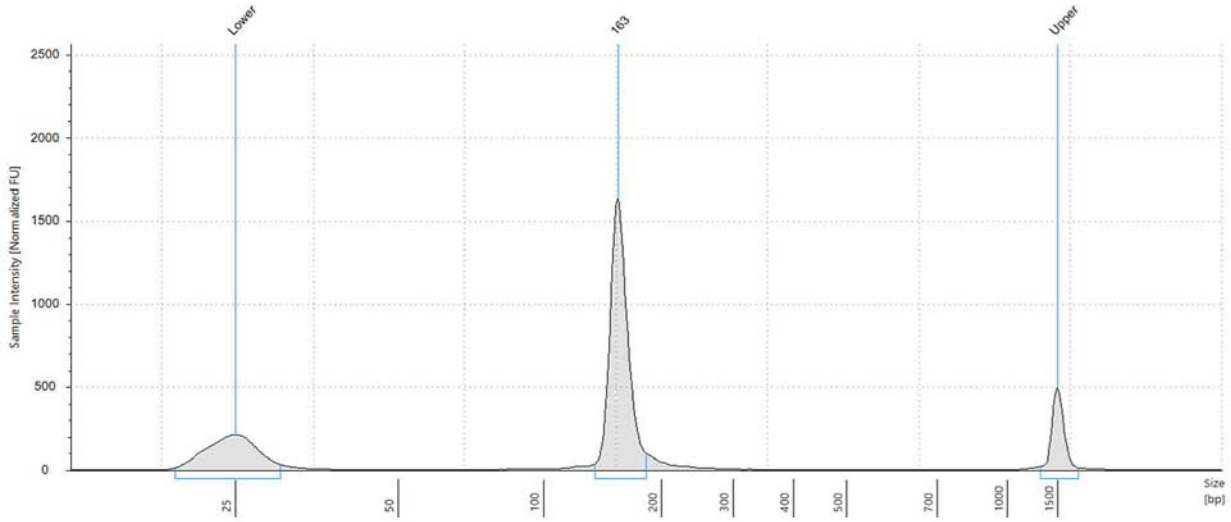
2 Dilute an aliquot of BD AbSeq PCR1 products to 0.1–1.1 ng/µL with Elution Buffer (Cat. No. 91-1084) before index PCR of BD AbSeq PCR1 products. See Performing AbSeq Index PCR on page 27.

Figure 2 AbSeq PCR1

A. Sample Bioanalyzer High Sensitivity DNA trace



B. Sample TapeStation High Sensitivity D1000 trace



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 Purification of the RPE PCR amplification product (single-sided cleanup) on page 16 with Elution Buffer 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 Elution Buffer 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)	For 2 libraries with 10% overage (µL)
PCR MasterMix (Cat. No. 91-1118)	25	30	55
Library Forward Primer (Cat. No. 91-1085)	5	6	11
Library Reverse Primer (1-4)* (Cat. Nos. 650000080, 650000091-93)	5	6	–
Nuclease-free water (Cat. No. 650000076)	5	6	11
Total	**40**	**48**	**77**

* 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 products.

   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:

Step	Cycles	Temperature	Time
Hot start	1	95°C	3 min
Denaturation	Refer to the following table, Suggested 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	∞

Suggested number of PCR cycles

Concentration of diluted RPE PCR products	Suggested 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.

Purification of the WTA Index PCR product (dual-sided cleanup)

This section describes how to perform a double-sided AMPure cleanup to ensure that the library is at a proper size (~250–1,000 bp) 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. Bring AMPure XP magnetic beads to room temperature (15°C to 25°C). Vortex the AMPure XP magnetic beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.
4. Add 60 µL of AMPure XP magnetic beads to the 0.2-mL PCR tube from step 2.
5. Pipet-mix at least 10 times, then briefly centrifuge the samples.
6. Incubate the suspensions at room temperature for 5 minutes, then place on the 0.2-mL strip tube magnet for 2 minutes.
7. Pipet 15 µL of AMPure XP magnetic beads into a different strip tube.
8. While the strip tube in step 6 is still on the magnet, carefully, without disturbing the beads, remove and transfer the 160 µL of supernatant into the 0.2-mL strip tube with AMPure XP magnetic beads (from step 7) and pipet-mix 10 times.
9. Incubate the suspension at room temperature for 5 minutes, then place the new tube on a 0.2-mL tube magnet for 1 minute.
10. While on the magnet, carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP magnetic beads.
11. Keeping the tubes on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol into the tubes.
12. Incubate the samples for 30 seconds on the magnet.
13. While on the magnet, carefully remove and appropriately discard only the supernatant without disturbing the AMPure XP magnetic beads.
14. Repeat the 200 µL of fresh 80% ethyl alcohol wash for a total of two washes.
15. Keeping the tubes on the magnet, use a small-volume pipette to remove any residual supernatant from the tube.
16. 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.

17 Pipet 30 µL of Elution Buffer into the tubes and pipet-mix to completely resuspend the AMPure XP magnetic beads.

18 Incubate the samples at room temperature for 2 minutes.

19 Briefly centrifuge the tubes to collect the contents at the bottom.

20 Place the tubes on the magnet until the solution is clear, usually ~30 seconds.

21 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.

22 Quantify and perform quality control of the Index PCR libraries with a Qubit Fluorometer using the Qubit dsDNA HS Assay and either of the following systems:

- Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit

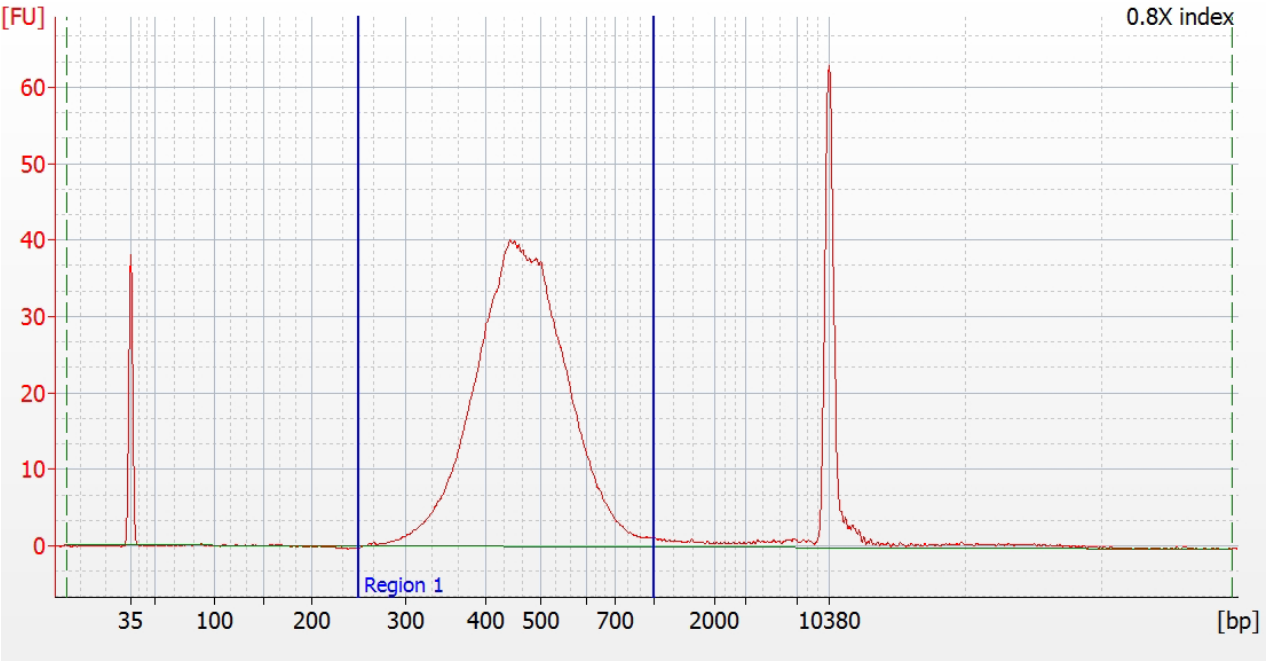
- Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay.

a The expected concentration from the Qubit Fluorometer is >1 ng/µL.

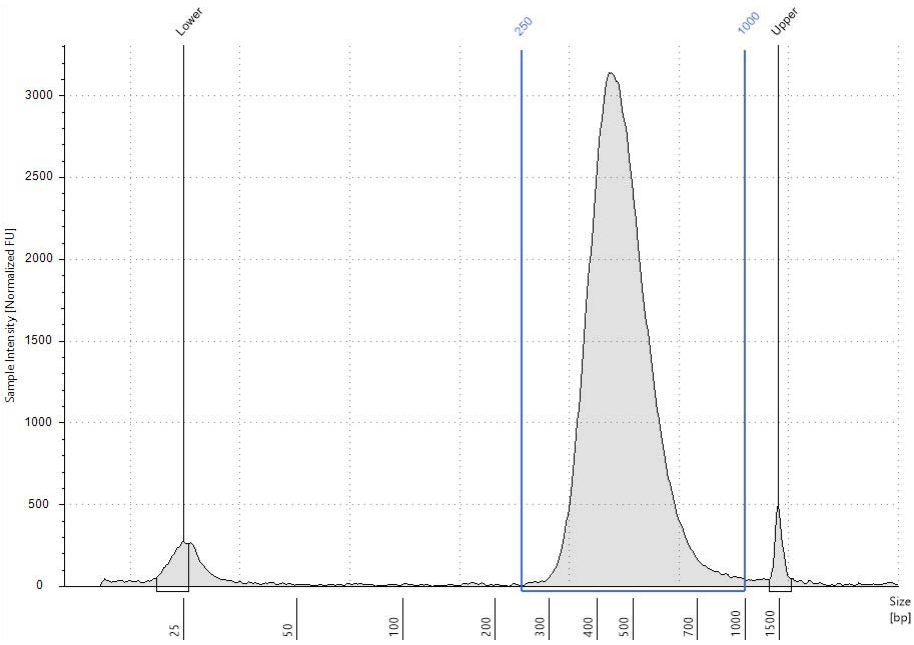
b The Bioanalyzer/TapeStation trace should show a peak from ~250–1,000 bp. Refer to the sample trace images on page 24.

Figure 3 WTA Index PCR product

A. Sample Bioanalyzer High Sensitivity DNA trace



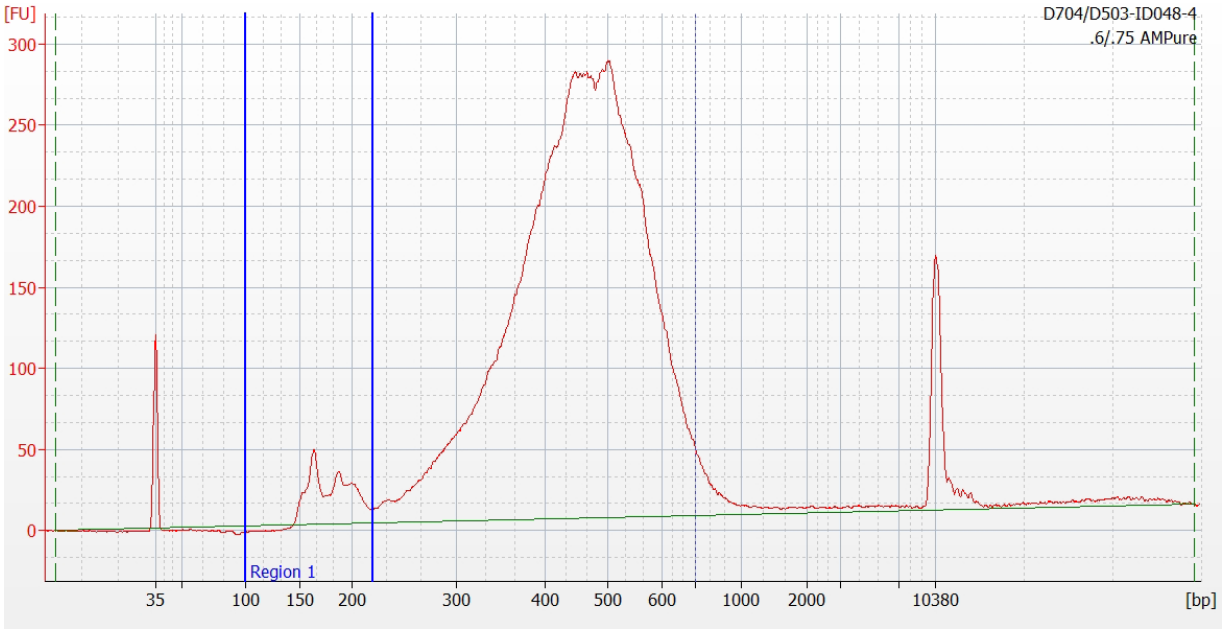
B. Sample TapeStation High Sensitivity D1000 trace



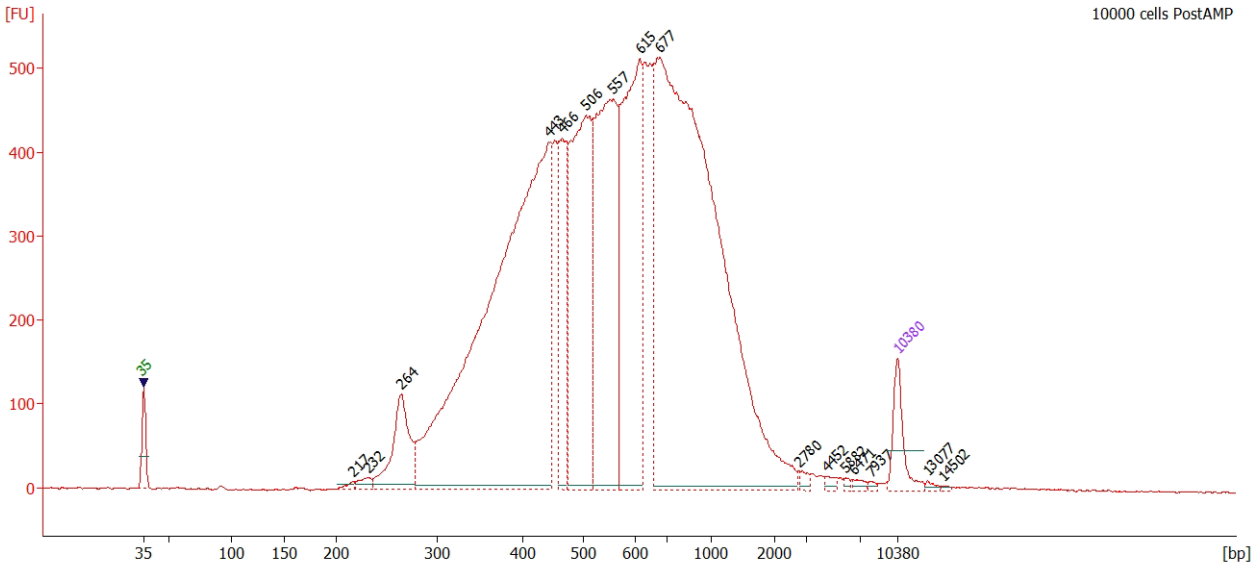
NOTE If smaller products (~165 bp or ~270 bp) are observed in Figure 3, such as the peaks shown in Figure 4, a second round of AMPure XP magnetic purification is recommended. See Additional WTA Index PCR purification steps in the following section.

Figure 4 Sample traces of noise peaks in WTA Index PCR product

A. Sample Bioanalyzer High Sensitivity DNA trace for an Index PCR product with an observable peak at ~165 bp



B. Sample Bioanalyzer High Sensitivity DNA trace for an Index PCR product with an observable peak at ~270 bp



Additional WTA Index PCR purification steps

If peaks at ~160–170 bp are observed from Figure 3, Bioanalyzer /TapeStation traces, a second round of AMPure XP magnetic purification is recommended.

1 To the tube from step 21, bring the total purified WTA Index PCR elute volume up to 100 µL with nuclease-free water.

NOTE It is critical for the final volume to be exactly 100 µL to achieve the desired size selection of the purified WTA Index PCR library.

2 Pipet-mix 10 times, then briefly centrifuge.

3 Pipet 75 µL of AMPure XP magnetic beads into the tube containing 100 µL of eluted RPE product from the first round of purification.

4 Pipet-mix 10 times, then briefly centrifuge.

5 Repeat step 9 through step 20 beginning on page 23 once more, resulting in a total of two rounds of purification.

6 Collect the elute (~30 µL) to a new PCR tube.

7 Repeat the quality control step (step 22 on page 24).

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

Performing AbSeq Index PCR

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

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

AbSeq Index PCR mix

Kit Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. No. 91-1118)	25	30
Library Forward Primer (Cat. No. 91-1085)	2	2.4
Library Reverse Primer (1-4)* (Cat. Nos. 650000080, 650000091-93)	2	2.4
Nuclease-free water (Cat. No. 650000076)	18	21.6
Total	**47**	**56.4**

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

2 Gently vortex mix and briefly centrifuge.

3 Bring the AbSeq Index PCR mix to the post-amplification workspace.

4 Pipet 3.0 µL of 0.1–1.1 ng/µL AbSeq PCR1 products into 47 µL AbSeq Index PCR mix.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler. Do not use fast cycling mode:

Step	Cycles	Temperature	Time
Hot start	1	95°C	5 min
Denaturation	Refer to the following table, Suggested 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 products.

Suggested number of PCR cycles

Conc. index PCR input for AbSeq libraries (ng/µL)	Suggested 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 AbSeq Index PCR products

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

NOTE Perform the purification in the post-amplification workspace.

1. Bring the AMPure XP beads to room temperature (15°C to 25°C) and vortex at high speed for 1 minute until the beads are fully resuspended.
2. Briefly centrifuge the AbSeq Index PCR products.
3. To 50 µL of AbSeq Index PCR products, pipet 40 µL of AMPure beads.
4. Pipet-mix 10 times and incubate at room temperature (15°C to 25°C) for 5 minutes.
5. Place each tube on the strip tube magnet for 3 minutes. Remove the supernatant.
6. Keeping the tubes on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into each 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 the residual ethyl alcohol supernatant from the tube.
9. Air-dry the beads at room temperature (15°C to 25°C) for 3 minutes.
10. Remove the tube from the magnet and resuspend each bead pellet in 30 µL of Elution Buffer. Pipet-mix until the beads are fully resuspended.
11. Incubate at room temperature (15°C to 25°C) 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 (~30 µL) to new 1.5-mL LoBind tubes. These are the final sequencing libraries.

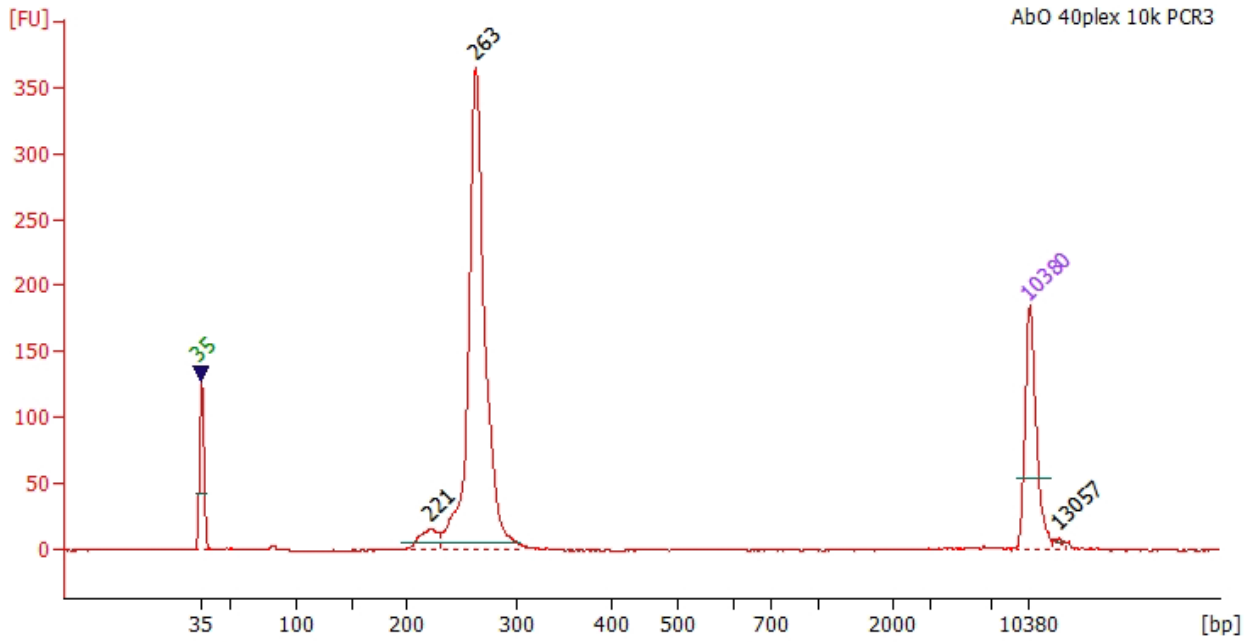
    STOPPING POINT: Store at –25°C to –15°C for up to 6 months until final sequencing.

14 Estimate the library 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 the library for quantification on an Agilent 2100 Bioanalyzer or an Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape assay. Follow the manufacturer's instructions. The expected concentration of the libraries is >1.5 ng/µL. The concentration derived from the bioanalyzer is recommended for dilution calculations for best sequencing results.

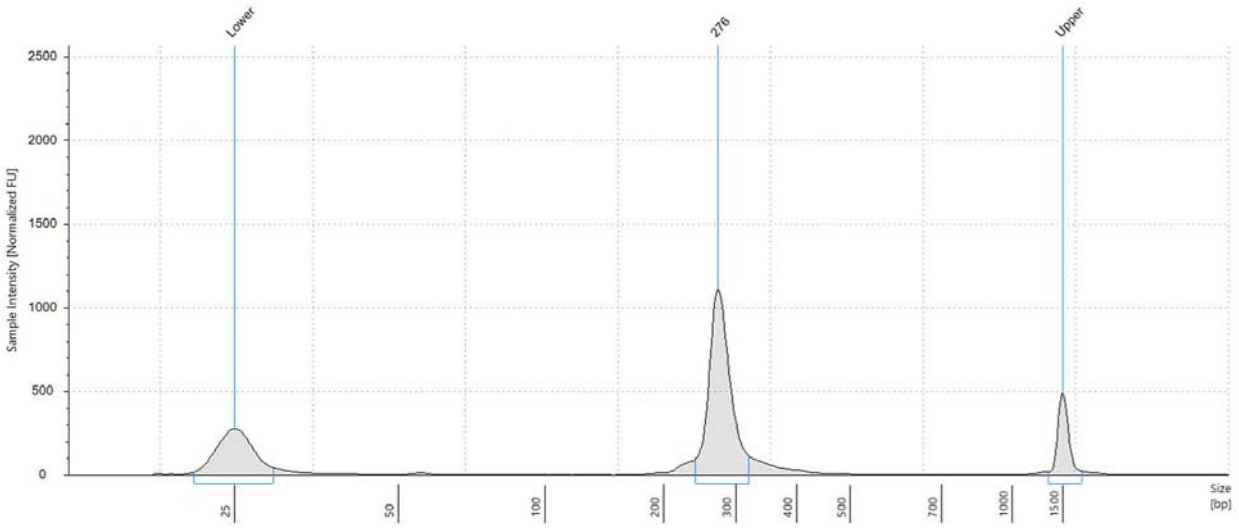
The AbSeq library should show a peak of ~270 bp.

Figure 5 AbSeq Index PCR product

A. Sample Bioanalyzer High Sensitivity DNA trace



B. AbSeq TapeStation High Sensitivity D1000 trace



Sequencing Recommendations

- For a NextSeq High or Mid Output run and MiniSeq High or Mid Output run, load the flow cell at a concentration between 1–1.2 pM with 20% PhiX for a sequencing run.
- Sequencing depth of the WTA mRNA libraries 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 deep sequencing to harvest the majority of UMIs in the library
- Sequencing amount for AbSeq libraries:
  - The amount of sequencing needed for BD AbSeq libraries will vary depending on application, BD AbSeq panel plexy, and cell type. BD Biosciences has 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 To determine the ratio of BD Rhapsody WTA mRNA library to AbSeq library to pool for sequencing, use the sequencing calculator available by contacting BD Biosciences technical support at scomix@bdscomix.bd.com.

Sequencing Analysis Pipeline

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

Troubleshooting Library Preparation

Observation	Possible causes	Recommended solutions
No RPE-PCR product	RPE step failed due to lack of addition of all required components	Repeat RPE from beads again.
Low yield of RPE-PCR	Cell number lower than expected	Repeat RPE from beads again and increase PCR cycle number.
Indexing PCR bioanalyzer trace of WTA library has 270 bp peak	AbSeq library contamination in mRNA library	If peak takes up high percentage of sequencing reads (manifests as lower reads/cell than expected for WTA library, alongside higher reads/cell than expected for AbSeq) perform a second round of AMPure purification according to Additional WTA Index PCR purification steps on page 26.
Low yield of indexing PCR	Input DNA not high enough or cycle number too low	• Repeat indexing PCR with higher cycle number. • Alternatively, if RPE-PCR product was diluted before adding to indexing PCR, repeat indexing PCR with less or no dilution.
Indexing PCR bioanalyzer trace of WTA library shows large amount of product larger than 600 bp	Over-amplification during indexing PCR	• Repeat indexing PCR with lower cycle number. • Alternatively, repeat indexing PCR with diluted RPE-PCR product.
Lower number of reads/cell than expected from mRNA	270 bp or 160 bp products taking reads from mRNA library	If noise peak is seen in the 270 bp or 160 bp range, perform a second round of AMPure purification according to Additional WTA Index PCR purification steps on page 26.