BD Rhapsody™ System

mRNA Whole Transcriptome Analysis (WTA) and Sample Tag 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
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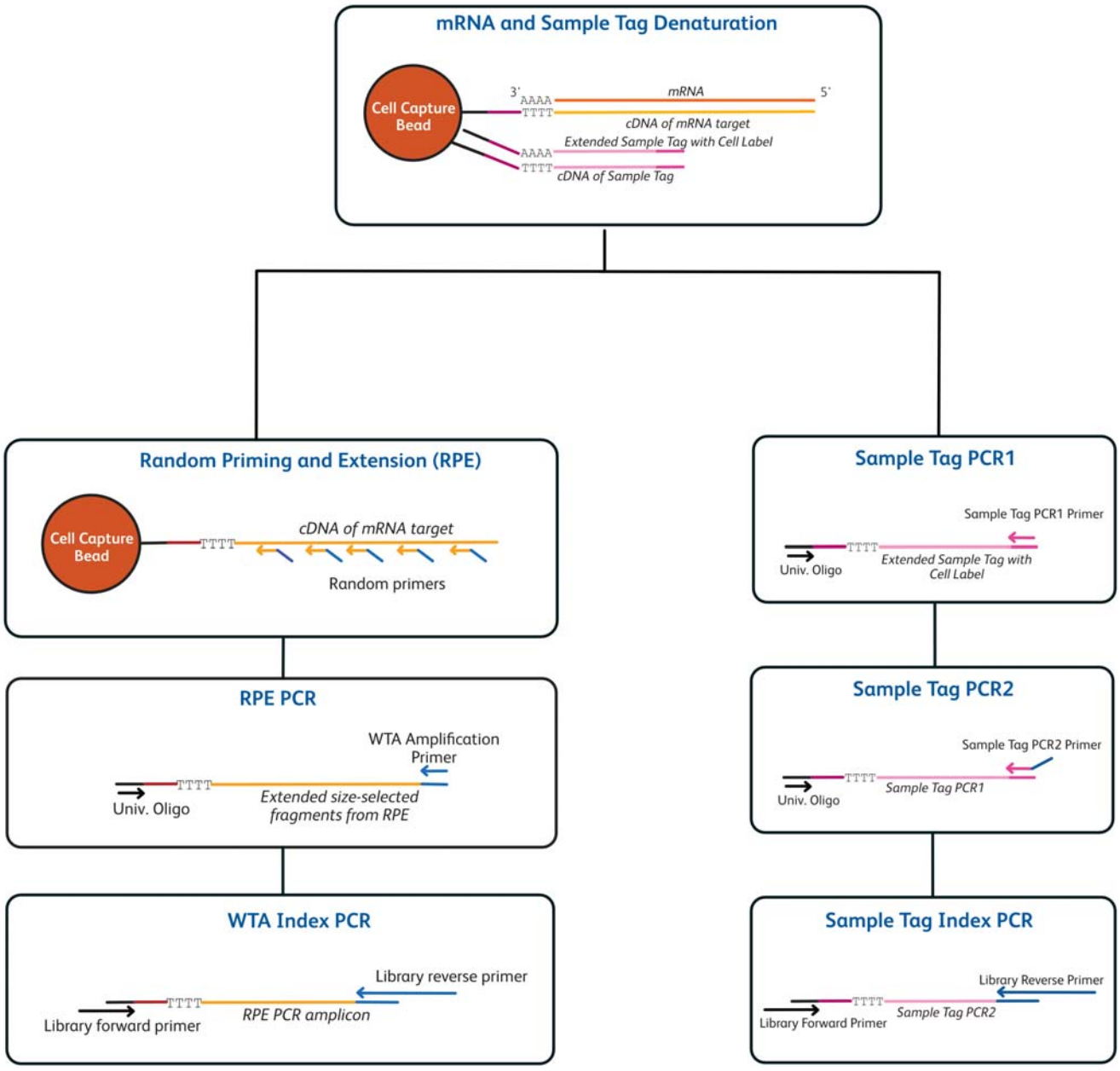
Introduction

This protocol provides instructions on creating single cell whole transcriptome mRNA and Sample Tag 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 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 Sample Tags during reverse transcription, which enables amplification of Sample Tags in solution. To generate the Sample Tag sequencing libraries, the extended Sample Tags are first denatured from the BD Rhapsody Cell Capture Beads, which 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 Sample Tag 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 been labeled using the BD® Single Cell Multiplexing Kit. The data set generated from this protocol can be used to generate a custom panel for subsequent 3' targeted mRNA sequencing. Specifically, the 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 21. For cell types other than resting PBMCs, protocol optimization might be required by the user.

Workflow


mRNA and Sample Tag Denaturation
Cell Capture Bead
3'
AAAA
TTTT
mRNA
5'
cDNA of mRNA target
Extended Sample Tag with Cell Label
AAAA
TTTT
cDNA of Sample Tag
Random Priming and Extension (RPE)
Cell Capture Bead
TTTT
cDNA of mRNA target
Random primers
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
Sample Tag PCR1
Sample Tag PCR1 Primer
TTTT
Univ. Oligo
Extended Sample Tag with Cell Label
Sample Tag PCR2
Sample Tag PCR2 Primer
TTTT
Univ. Oligo
Sample Tag PCR1
Sample Tag Index PCR
Library Reverse Primer
TTTT
Library Forward Primer
Sample Tag PCR2

Required materials

- Exonuclease I-treated 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

- BD® Human Sample Multiplexing Kit (Cat No. 633781)
- 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 low-retention 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 before use.
- Remove supernatants without disturbing AMPure XP magnetic beads.

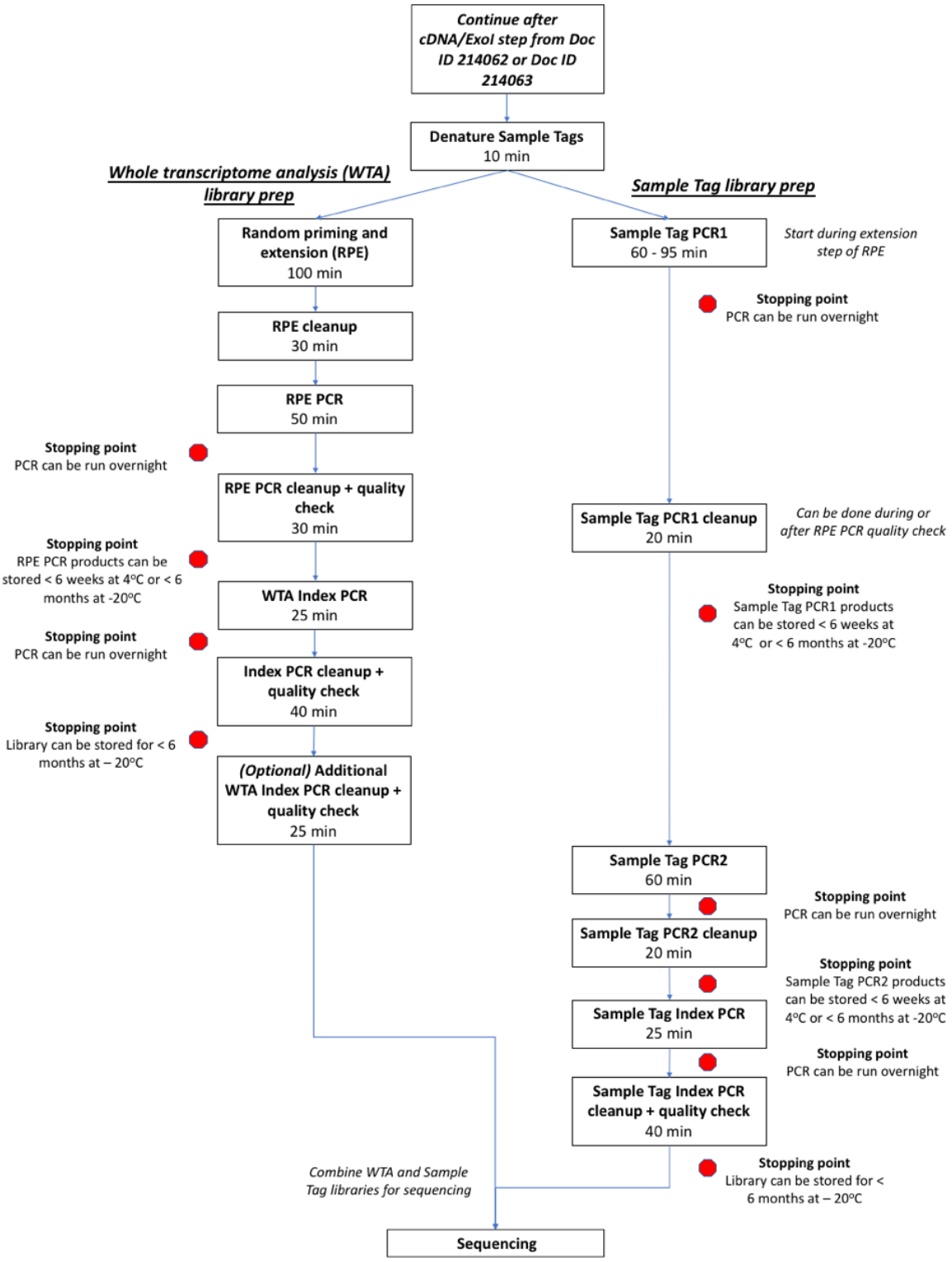
Additional documentation

- *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



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

This section describes how to generate random priming products. First, Sample Tags with barcode information from BD Rhapsody Cell Capture Beads are denatured off of the beads and saved for Sample Tag amplification. Then, random primers are hybridized to the cDNA on the BD Rhapsody Cell Capture Beads, followed by extension with an enzyme.

NOTE This section should be performed 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 Place the tube of Exonuclease I-treated beads in Bead Resuspension buffer on the 1.5-mL magnet for <2 minutes. Remove the supernatant.

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

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

7 Label a new 1.5-mL tube as *Sample Tag products*.

8 Briefly centrifuge the tube, then immediately place the tube on 1.5-mL magnet for <2 minutes. Remove the supernatant and transfer to the *Sample Tag products* tube. Keep the tube at 4°C for <24 hours until ready to proceed to Performing Sample Tag PCR1 on page 12. Immediately, proceed to step 9. Avoid drying out the beads.

9 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 beads.

10 Incubate the tube in the following order:

   a 95° 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

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

12 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**

13 Pipet-mix the Extension Enzyme Mix.

14 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.

15 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.

16 Place the tube from step 14 in the thermomixer. Remove the tube after the program is finished.

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

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

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

19 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.

20 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.

21 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 Sample Tag PCR1

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

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

Sample Tag 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)	20	24
Bead RT/PCR Enhancer (Cat. No. 91-1082)	12	14.4
Sample Tag PCR1 Primer (Cat. No. 91-1088)	1	1.2
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 Sample Tag PCR1 reaction mix. Add 67 µL of the Sample Tag product from step 8 from Performing random priming and extension (RPE) on BD Rhapsody Cell Capture Beads with cDNA on page 10. Pipet-mix 10 times. Do not vortex.

4 Pipet 50 µL Sample Tag reaction 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	3 min
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	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 17 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.

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, e.g. 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.

NOTE 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, 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	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 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 tubes 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 120 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 tubes on the magnet, gently pipet 200 µL of fresh 80% ethyl alcohol to the tube.

8 Incubate the samples 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 tubes 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 samples at room temperature for 2 minutes. Briefly centrifuge the tubes to collect the contents at the bottom.

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

15 Pipet the eluate (~40 µL) into new 1.5-mL LoBind tubes. 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 D5000 ScreenTape Assay

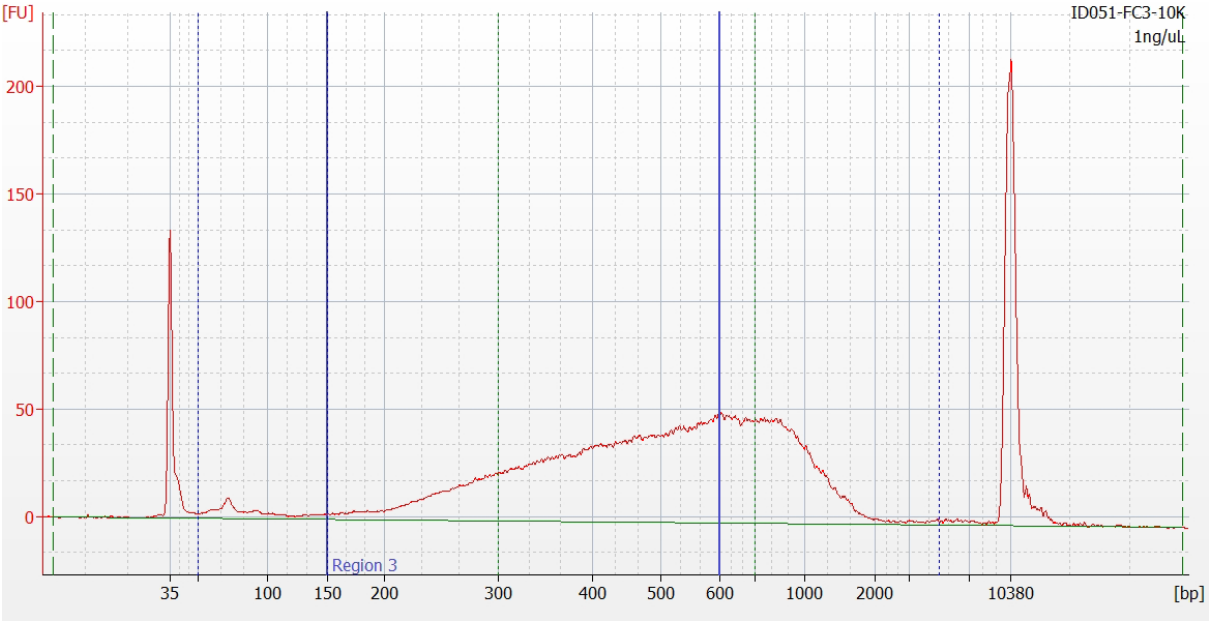
a The expected concentration from the Qubit Fluorometer is ~0.5 to 10 ng/µL.

b The Bioanalyzer/TapeStation trace should show a broad peak from ~200 to 2,000 bp. Use the concentration from 150 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.

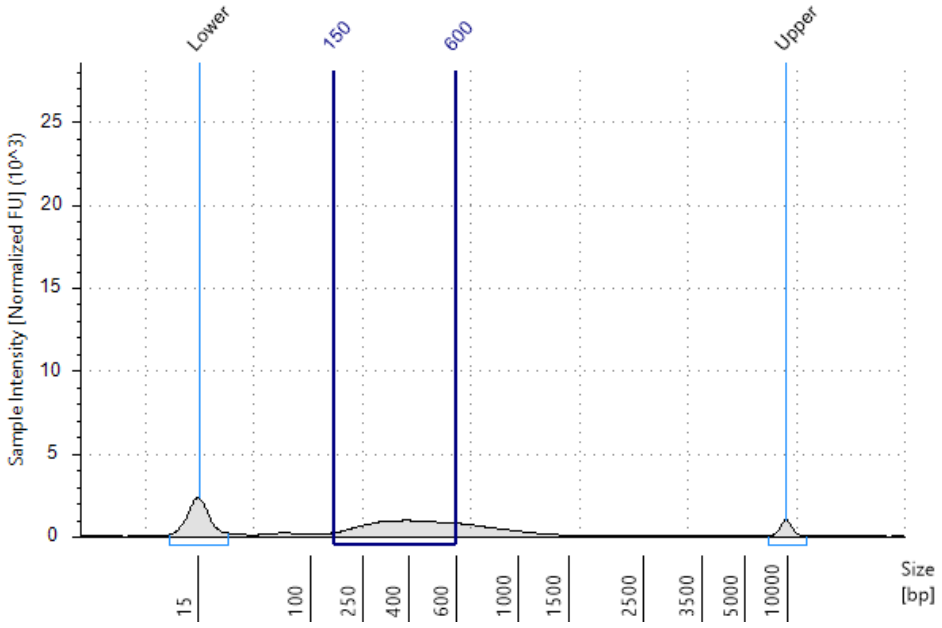
NOTE Although there are products >600 bp, these products should be removed in the double-sided cleanup after the next PCR.

Figure 1 RPE PCR product traces

A. Sample Bioanalyzer High Sensitivity DNA trace



B. Sample TapeStation High Sensitivity D5000 trace



Purifying Sample Tag PCR1 products

This section describes how to perform a single-sided AMPure cleanup to remove primer dimers from the Sample Tag PCR1 products. The final product is purified double-stranded DNA.

NOTE Perform the purification in the post-amplification workspace.

1 In a new 5.0-mL LoBind tube, prepare 5 mL 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 in ≤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 360 µL AMPure XP beads into a tube with 200 µL Sample Tag PCR1. 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 tube on the magnet, gently add 600 µL of fresh 80% ethyl alcohol, and incubate for 30 seconds. Remove the supernatant.

7 Repeat steps 5 once for two washes.

8 Keeping tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from 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 Sample Tag PCR1 products).

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

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 This section should be performed in the post-amplification workspace.

1 Dilute the RPE PCR products with Elution Buffer such that the concentration of the 150–600 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 150–600 bp peak is 2 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 1 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, 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	∞

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.

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

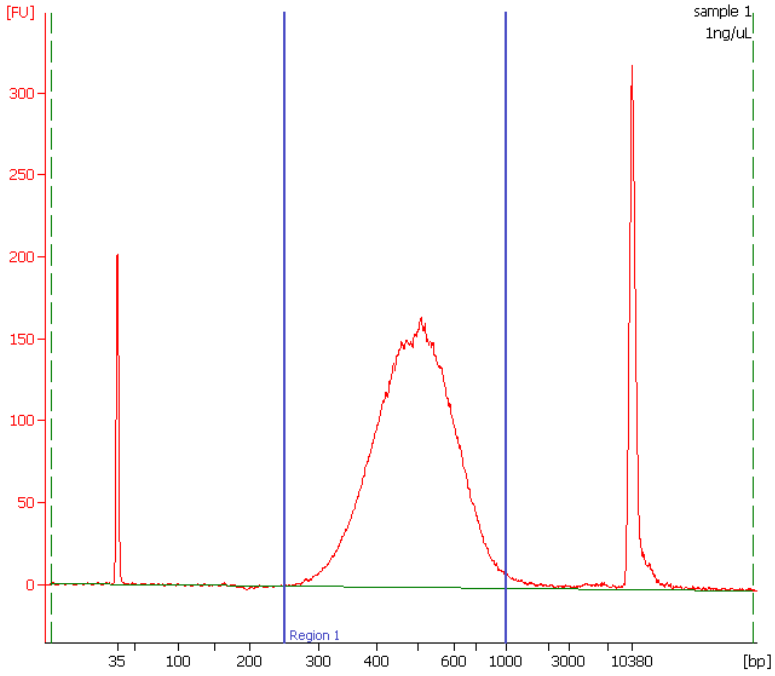
- Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape Assay.

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

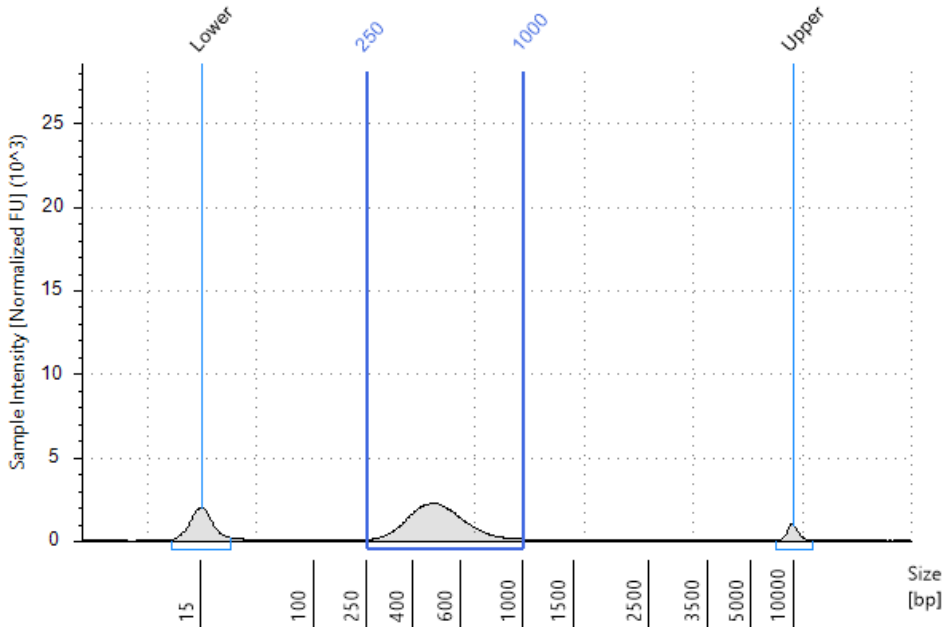
b The Bioanalyzer/TapeStation trace should show a peak from ~ 250–1,000 bp. Refer to the sample trace images on page 27.

Figure 2 WTA Index PCR product

A. Sample Bioanalyzer High Sensitivity DNA trace

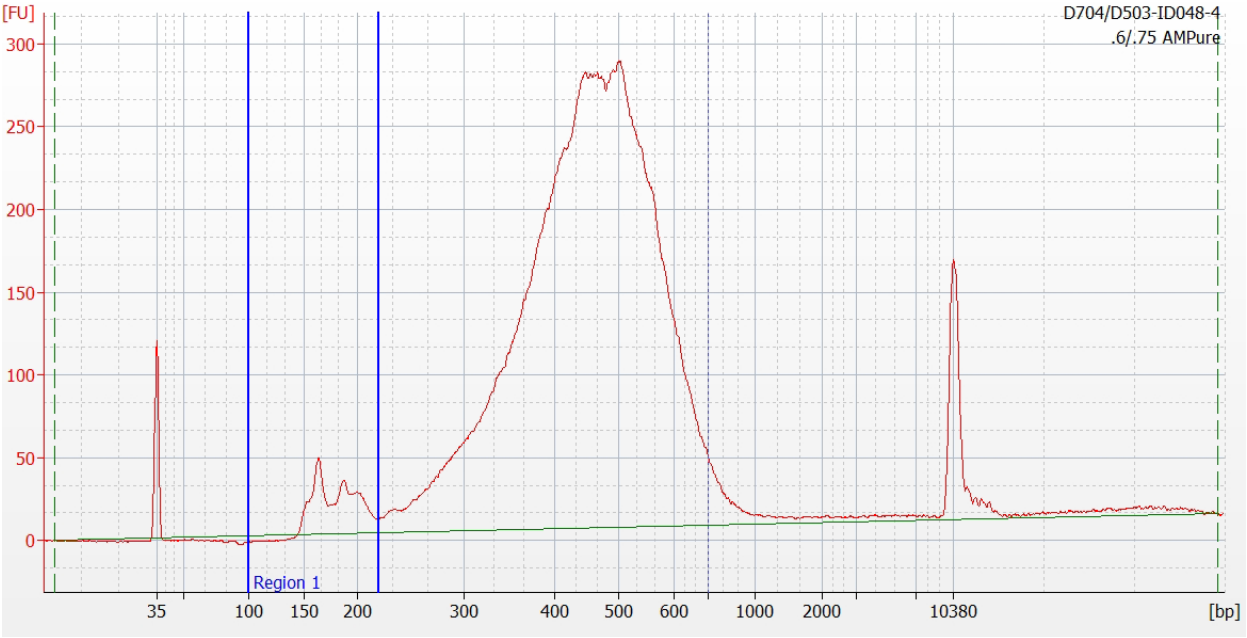


B. Sample TapeStation High Sensitivity D5000 trace



NOTE If a ~165 bp peak is observed in Figure 2, such as the peak shown in Figure 3, a second round of AMPure XP magnetic purification is recommended. See Additional WTA Index PCR purification steps in the following section.

Figure 3 Sample Bioanalyzer High-Sensitivity DNA trace for an Index PCR product with an observable peak at ~165 bp



Additional WTA Index PCR purification steps

If a ~165 bp peak is observed from Figure 2, 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 21 on page 22 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 22).

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

Performing Sample Tag PCR2 on the Sample Tag PCR1 products

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

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

Sample Tag PCR2 Reaction Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. No. 91-1118)	25	30
Universal Oligo (Cat. No. 650000074)	2	2.4
Sample Tag PCR2 Primer (Cat. No. 91-1089)	3	3.6
Nuclease-free Water (Cat. No. 650000076)	15	18
Total	**45**	**54**

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

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

4 Pipet 5.0 µL of PCR1 products into 45 µL Sample Tag PCR2 reaction 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	3 min
Denaturation	10*	95°C	30 s
Annealing		60°C	3 min
Extension		72°C	1 min
Final extension	1	72°C	5 min
Hold	1	4°C	∞

* Cycle number might require optimization according to cell number and type.

STOPPING POINT: The PCR can run overnight.

Purifying Sample Tag PCR2 products

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

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. To 50.0 µL of PCR2 products, pipet 60 µL of AMPure beads.
3. Pipet-mix 10 times and incubate at room temperature (15°C to 25°C) for 5 minutes.
4. Place each tube on the strip tube magnet for 3 minutes. Remove the supernatant.
5. Keeping the tubes on magnet, gently add 200 µL of fresh 80% ethyl alcohol into each tube and incubate for 30 seconds. Remove the supernatant.
6. Repeat step 5 for a total of two washes.
7. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
8. Air-dry the beads at room temperature (15°C to 25°C) for 3 minutes.
9. 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.
10. Incubate at room temperature (15°C to 25°C) for 2 minutes and briefly centrifuge.
11. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
12. Pipet the entire eluate (~30 µL) to new 1.5-mL LoBind tubes (purified Sample Tag PCR2 products).

    STOPPING POINT: Store at 2°C to 8°C before proceeding on the same day or at –25°C to –15°C for up to 6 months.
13. Estimate the concentration with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.
14. Dilute an aliquot of the products with Elution Buffer to 0.1-1.1 ng/µL.

Performing Sample Tag Index PCR

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

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

NOTE For a single cartridge or sample, consider using the same index for both the WTA and Sample Tag Index PCR products for that cartridge or sample. Otherwise, different library reverse primers can be used for WTA and Sample Tag Index PCR products.

Sample Tag Index PCR mix

Kit component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix (Cat. No. 91-1083)	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 Sample Tag library.

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

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

4 Pipet 3.0 µL of 0.1-1.1 ng/µL products into 47.0 µL Sample Tag 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, 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 products.

Recommended number of PCR cycles

Conc. index PCR input for Samples Tag libraries (ng/µL)	Recommended number of PCR cycles
0.5–1.1	6
0.25–0.5	7
0.1–0.25	8

STOPPING POINT: The PCR can run overnight.

Purifying Sample Tag Index PCR products

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

2 Briefly centrifuge the Sample Tag Index PCR products.

3 To 50.0 µL of the Sample Tag Index PCR products add 40 µL AMPure beads.

4 Pipet-mix 10 times, and incubate at room temperature (15°C to 25°C) 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 the residual 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 the 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) into new 1.5 mL LoBind Tubes (final sequencing libraries).

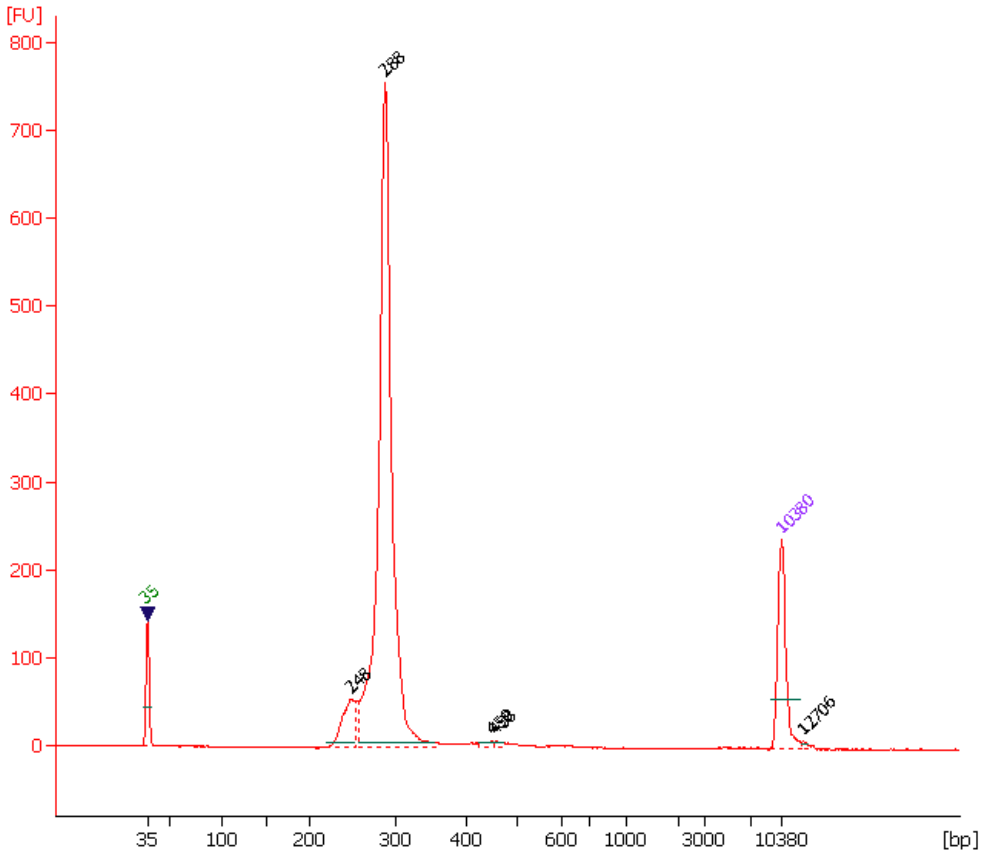
STOPPING POINT. Store at –25°C to –15°C for up to 6 months until sequencing.

14 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 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.

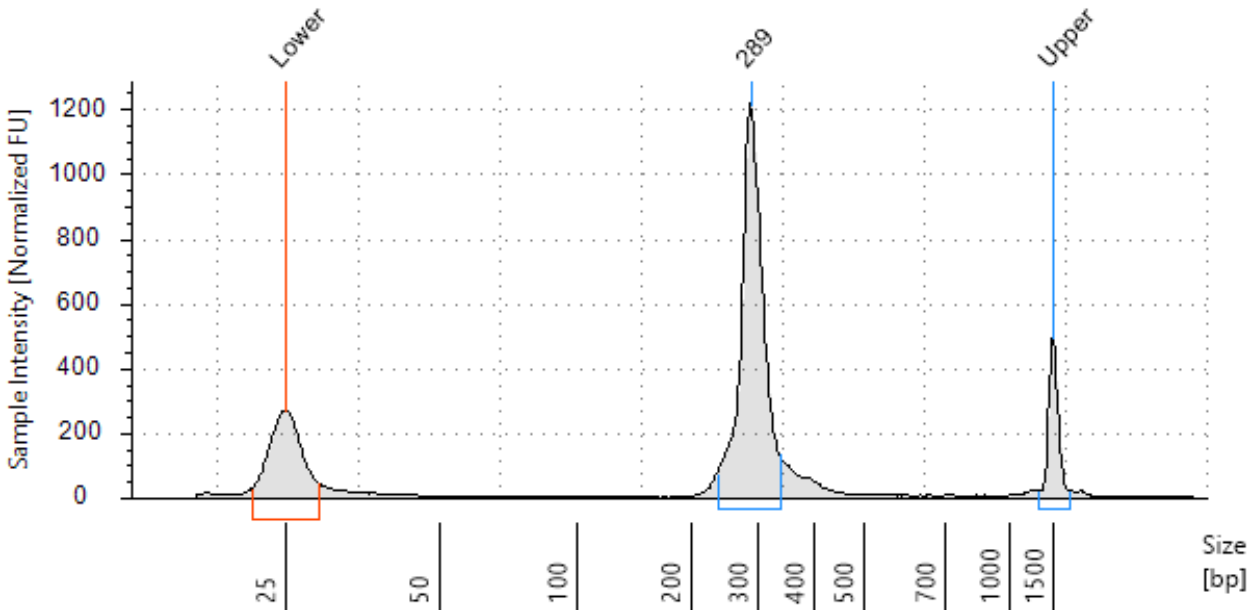
The Sample Tag library should show a peak of ~290 bp.

Figure 4 Sample Tag Index PCR product

A. Sample Bioanalyzer High Sensitivity DNA trace



B. Sample 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 Sample Tag libraries:
  - Pooling samples of the same type: ≥120 reads/cell. For example, combining different donor PBMCs.
  - Pooling different sample types: ≥600 reads/cell. For example, combining Jurkat cells with PBMCs.

NOTE BD Biosciences recommends sequencing WTA mRNA libraries with Sample Tag libraries. Only a limited number of reads are required to accurately assign the Sample Tags.

NOTE To determine the ratio of BD Rhapsody WTA mRNA library to Sample Tag 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.