BD Rhapsody™ System

mRNA Whole Transcriptome Analysis (WTA)

Library Preparation Protocol

23-24117(02)
2022-11

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For US patents that may apply, see bd.com/patents.

Regulatory information

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

History

Revision	Date	Change made
23-24117(01)	2021-12	Initial release.
23-24117(02)	2022-11	Updated for BD Rhapsody™ Enhanced Cell Capture Beads version 2.0.

Contents

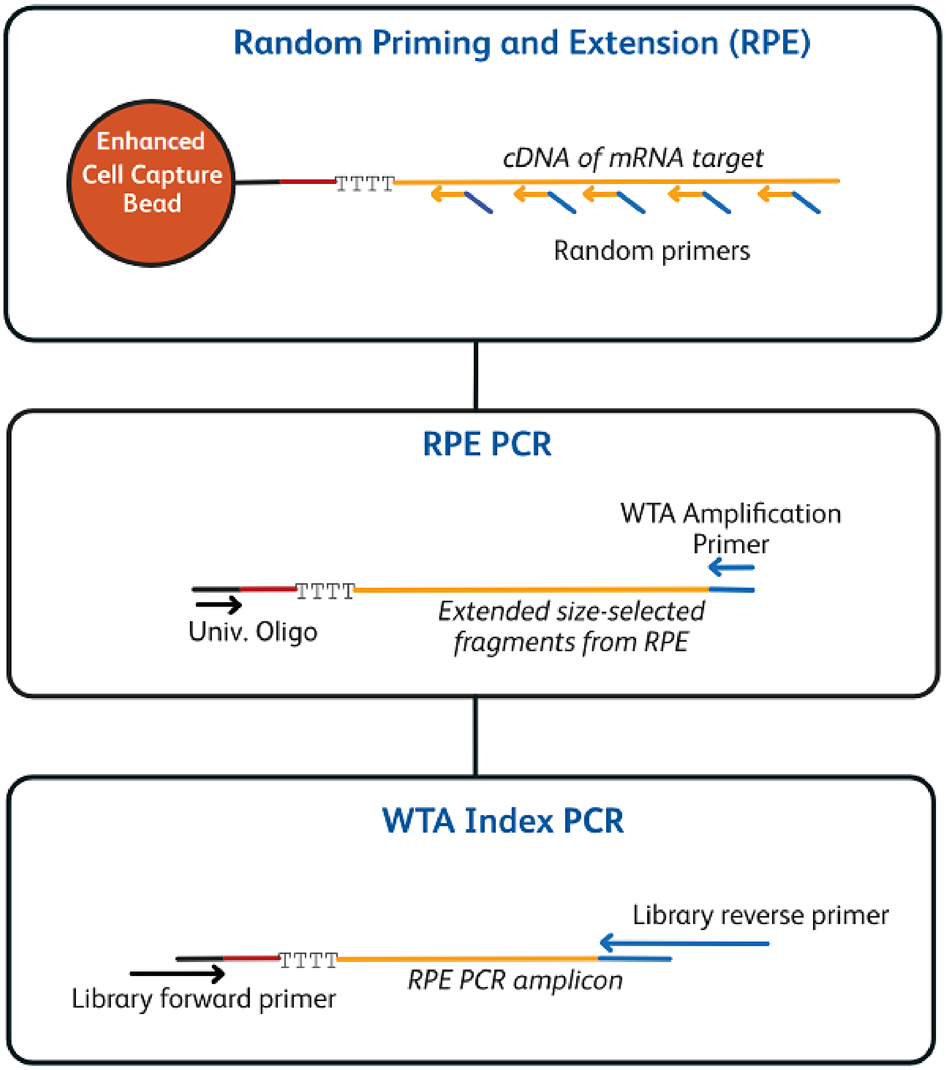
Introduction

This protocol provides instructions on creating a single cell whole transcriptome mRNA library 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* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*.

The cDNA of mRNA targets is first encoded on the BD Rhapsody™ Enhanced Cell Capture Beads as described in the instrument user guides. This protocol then continues from that point to generate a whole transcriptome amplification library by employing a random priming approach, followed by an index PCR step. The generated library can be sequenced 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. 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™ Enhanced Cell Capture Beads inputs between 1,000 to 20,000 resting PBMCs per sample for library generation. For BD Rhapsody™ Enhanced 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 12 and Purifying WTA index PCR product (dual-sided cleanup) on page 18. For cell types other than resting PBMCs, protocol optimization might be required by the user.

Workflow


Random Priming and Extension (RPE)
Enhanced 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

Required and recommended materials

Required reagents

Material	Supplier	Catalog no.
BD Rhapsody™ WTA Amplification Kit	BD Biosciences	633801
Agencourt® AMPure® XP magnetic beads	Beckman Coulter	A63880
100% ethyl alcohol	Major supplier	–
Nuclease-free water	Major supplier	–
Refer to the Technical Bulletin *Ordering Additional Indexes for the BD Rhapsody™ Library Reagent Kits* to order additional indexing primers for high throughput library preparation workflows.		

Recommended consumables

Material	Supplier	Catalog no.
Pipettes (P10, P20, P200, P1000)	Major supplier	–
Low-retention, filtered pipette tips	Major supplier	–
0.2-mL PCR 8-strip tubes	Major supplier	–
Axygen™ 96-Well PCR Microplates[a] Or, MicroAmp Optical 96-Well Reaction Plate[a]	Corning Thermo Fisher Scientific	PCR96HSC N8010560
MicroAmp Clear Adhesive Film[a]	Thermo Fisher Scientific	4306311
15-mL conical tube	Major supplier	–
DNA LoBind® tubes, 1.5-mL	Eppendorf	0030108051
DNA LoBind® tubes, 5.0-mL	Eppendorf	0030108310
Qubit™ Assay Tubes	Thermo Fisher Scientific	Q32856
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
Agilent High Sensitivity DNA Kit Or, Agilent High Sensitivity D1000 ScreenTape Agilent High Sensitivity D1000 Reagents Or, Agilent High Sensitivity D5000 ScreenTape Agilent High Sensitivity D5000 Reagents	Agilent Agilent Agilent Agilent Agilent	5067-4626 5067-5584 5067-5585 5067-5592 5067-5593

[a] Recommended for processing high throughput library preparation workflows.

Equipment

Material	Supplier	Catalog no.
Microcentrifuge for 1.5–2.0-mL tubes	Major supplier	–
Microcentrifuge for 0.2-mL tubes	Major supplier	–
Vortexer	Major supplier	–
Digital timer	Major supplier	–
Eppendorf ThermoMixer® C	Eppendorf	5382000023
6-tube magnetic separation rack for 1.5-mL tubes Or, 12-Tube Magnetic Separation Rack[a] Or, Invitrogen™ DynaMag™-2 Magnet[a]	New England Biolabs New England Biolabs Thermo Fisher Scientific	S1506S S1509S 12321D
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc.	VP772F4-1
Magnetic Stand-96[b]	Thermo Fisher Scientific	AM10027
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
Agilent® 2100 Bioanalyzer Or, Agilent® 4200 TapeStation System	Agilent Technologies Agilent Technologies	G2940CAG G2991AA

[a] Recommended for processing greater than 6 samples.

[b] Recommended for processing high throughput library preparation workflows.

Before you begin

- Obtain Exonuclease I-treated and inactivated BD Rhapsody™ Enhanced Cell Capture Beads.
- Thaw reagents in the BD Rhapsody™ WTA Amplification Kit at room temperature (15–25 °C), then immediately place on ice.

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 AMPure XP magnetic beads to room temperature before use.
- Remove supernatants without disturbing AMPure XP magnetic beads.

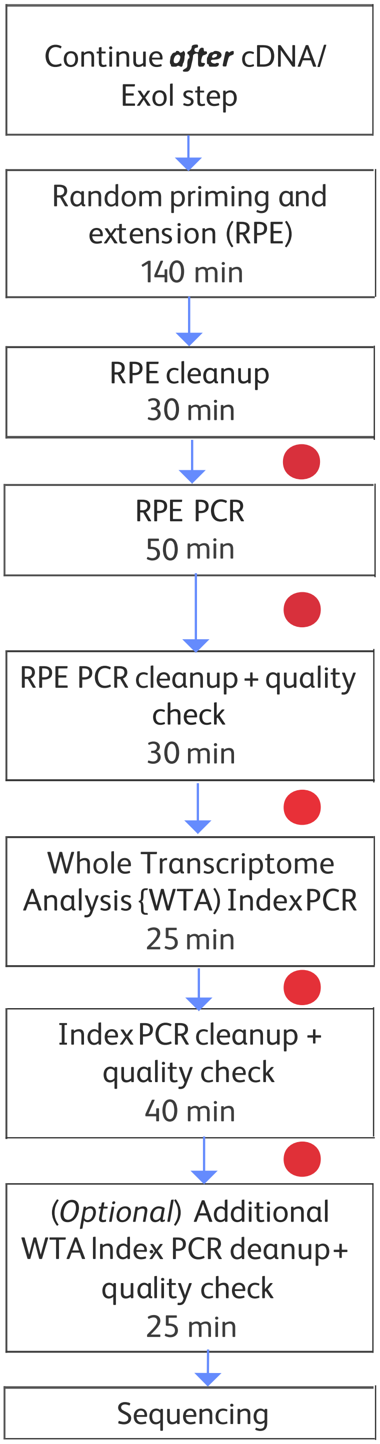
Additional documentation

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*
- *BD® Single-Cell Multiomics Bioinformatics Handbook*

Safety information

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

Time considerations



Stopping point
Store the RPE product in a LoBind tube on ice or at 4 ˚C for up to 24 hours until PCR

Stopping point
PCR can be run overnight

Stopping point
RPE PCR products can be stored < 6 weeks at 4 ˚C or < 6 months at -20 ˚C

Stopping point
PCR can be run overnight

Stopping point
Library can be stored for < 6 months at -20 ˚c

Procedure

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system following either the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* for cell capture, reverse transcription, and Exonuclease treatment.

This protocol is intended for the whole transcriptome amplification library generation of cell inputs between 1,000 to 20,000 single cells, specifically resting PBMCs. There are additional sections within the protocol for cell inputs between 1,000 to 5,000 single cells. For this cell input range, follow the additional steps outlined in both Purifying RPE product on page 12 and Purifying WTA index PCR product (dual-sided cleanup) on page 18.

Please ensure that the intended total cell load is between 1,000 to 20,000 single cells for this protocol. Cell load below or above this recommendation might not be suitable for the current protocol configuration. Then proceed as described in the following procedure.

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, then extended with an enzyme. This random primers hybridization and extension is repeated a second time to increase assay sensitivity.

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 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
WTA Extension Buffer	20	24	96	192
WTA Extension Primers	20	24	96	192
Nuclease-free water	134	160.8	643.2	1,286.4
Total	**174**	**208.8**	**835.2**	**1,670.4**

3. Pipet-mix the Random Primer Mix and keep at room temperature.
4. Choose between using the entire sample or a sub-sample of the Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads. If using the entire sample of Exonuclease I-treated beads, skip to **step 6**. If using a sub-sample, proceed to **step 5**.
5. (Optional) Sub-sample the Exonuclease I-treated BD Rhapsody™ Enhanced Cell Capture Beads:
   - Based on the expected number of viable cells captured on beads in the final bead-resuspension volume, determine the volume of beads to sub-sample for sequencing.
   - Completely resuspend the beads by pipet-mixing, then pipet the calculated volume of 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 beads with a pipette.

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

8 Afterwards, briefly centrifuge the tube, then immediately place the tube in the 1.5-mL magnetic separation rack. Remove and discard the supernatant. Avoid drying out the BD Rhapsody™ Enhanced Cell Capture Beads.

9 Remove the tube from the magnet and use a low-retention tip to pipet 87 µL of Random Primer Mix into the tube. Pipet-mix 10 times to resuspend the beads. Save the remaining volume of Random Primer Mix for a second RPE. Keep Random Primer Mix at room temperature.

10 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 5 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.

Primer extension enzyme mix

Kit component	**For 1 library (µL)**	**For 1 library with 20% overage (µL)**	**For 4 libraries with 20% overage (µL)**	**For 8 libraries with 20% overage (µL)**
10 mM dNTP	8	9.6	38.4	76.8
Bead RT/PCR Enhancer	12	14.4	57.6	115.2
WTA Extension Enzyme	6	7.2	28.8	57.6
Total	**26**	**31.2**	**124.8**	**249.6**

13 Pipet-mix the Primer Extension Enzyme Mix.

14 Pipet 13 µL of the Primer Extension Enzyme Mix into the sample tube containing the beads (for a total volume of 100 µL) and keep at room temperature until ready. Save the remaining volume of Primer Extension Enzyme Mix for a second RPE. Keep Primer Extension Enzyme Mix on ice.

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 Confirm “Time Mode” is set to “Time Control” before the program begins.

16 Place the tube from **step 14** in the thermomixer. Start the program set in **step 15**.

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

21 Go to **step 9** on page 11 and repeat **steps 9 to 20** for a second RPE. Store supernatant containing RPE product on ice.

22 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 for up to 3 months in the pre-amplification workspace. Immediately proceed to Purifying RPE product on page 12.

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 this procedure in the pre-amplification workspace.

1 In a new 15-mL conical tube, prepare 10 mL of fresh 80% (v/v) ethyl alcohol by pipetting 8.0 mL of absolute ethyl alcohol to 2.0 mL of nuclease-free water (from major supplier). Vortex the tube for 10 seconds.

Note: Make fresh 80% ethyl alcohol and use within 24 hours. The 80% ethyl alcohol volume should be adjusted depending on the number of libraries.

2 Bring 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 If RPE sample volume is <400 µL, bring volume to 400 µL with Elution Buffer. Pipet 720 µL of AMPure XP magnetic beads into the tube containing the 400 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 and discard the supernatant.

6 Keeping the tube on the magnet, gently add 1 mL of fresh 80% ethyl alcohol into the tube.

7 Incubate the sample on the magnet for 30 seconds. Remove and discard 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 For best results, briefly centrifuge the AMPure beads while still wet and place the tube back on the magnet. Remove and discard any excess ethanol that may collect at the bottom. Air dry the beads at room temperature until no longer glossy (~15-20 minutes).

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, for example, starting with fewer than 5,000 PBMCs, proceed to **step 1** 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 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
PCR MasterMix	60	72	288	576
Universal Oligo	10	12	48	96
WTA Amplification Primer	10	12	48	96
Total	**80**	**96**	**384**	**768**

2. Add 80 µL of the RPE PCR Mix to the tube with the 40 µL of Purified RPE product. Pipet-mix 10 times.
3. Split the RPE PCR reaction mix into two PCR tubes with 60 µL of reaction mix per tube.
4. Bring the reaction to the post-amplification workspace and run the following PCR program.

PCR program

Step	Cycles	Temperature	Time
Hot start	1	95 °C	3 min
Denaturation	Refer to the following table, **Recommended number of PCR cycles.**[a,b]	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	∞

a. Suggested PCR cycles might need to be optimized for different cell types and cell number.
b. Recommended number of PCR cycles is based on resting PBMCs only.

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
20,000	11

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

STOPPING POINT: The PCR can run overnight.

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. The final product is purified double-stranded DNA.

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.

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. If the volume is <120 µL, bring the volume to 120 µL with Elution Buffer.

3 Bring AMPure XP magnetic beads to room temperature (15–25 °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. Use care to avoid getting AMPure on the lid of the tube, as residual AMPure and PCR mix buffer can negatively impact downstream results.

5 Incubate the suspension at room temperature for 5 minutes.

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

7 Keeping the tube on the magnet, gently pipet 500 µL of fresh 80% ethyl alcohol to the tube.

8 Incubate the samples for 30 seconds on the magnet. Remove and discard 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 and discard 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.

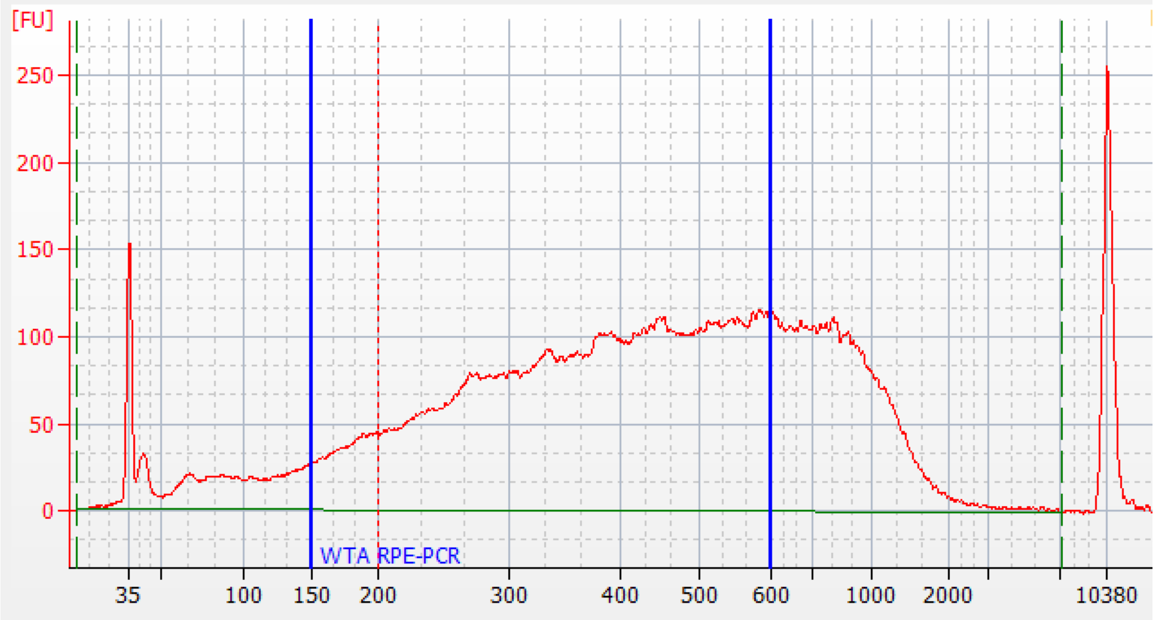
  b The Bioanalyzer or TapeStation trace should show a broad peak from ~150 to 2,000 bp. Use the concentration from 150 to 600 bp to calculate how much template to add into Index PCR. Refer to the blue-boxed regions in the sample trace images on page 16.

  The Bioanalyzer or TapeStation are used to calculate molarity for the WTA library because of the distribution in fragment sizes for this library type.

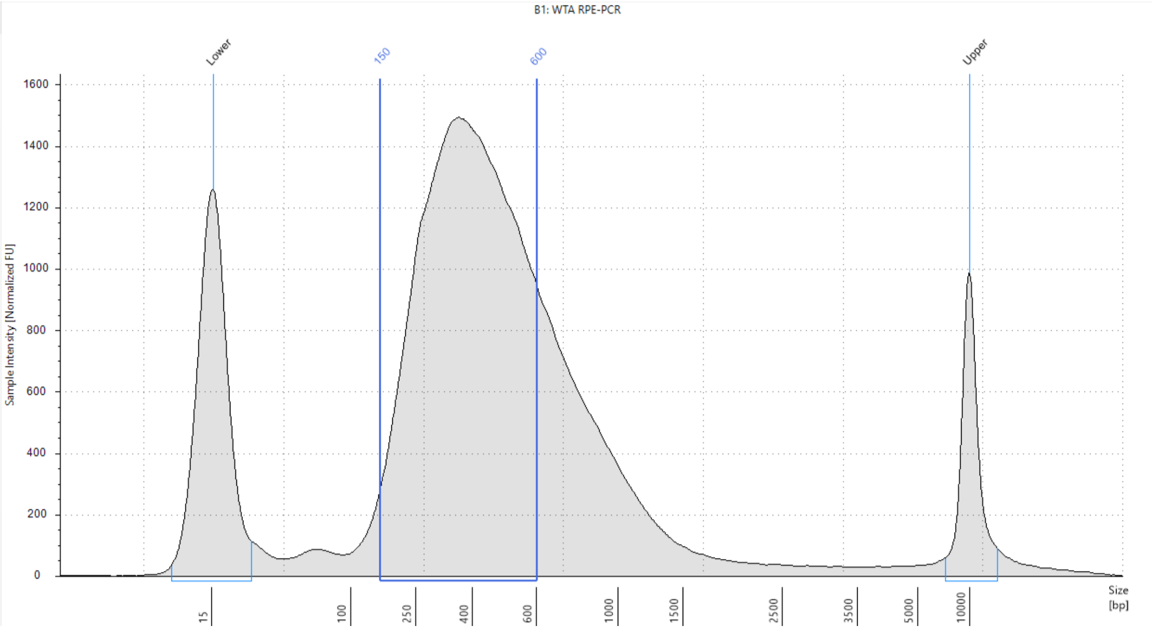
Note: Although there are products <150 bp and >600 bp, these products should be removed in the double-sided cleanup after the index PCR.

Figure 1 RPE PCR product traces

A. Sample Bioanalyzer high-sensitivity DNA trace



B. Sample TapeStation high-sensitivity D5000 trace



Performing WTA index PCR

This section describes how to generate 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 14 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 6 nM, then dilute the sample threefold 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 4 libraries with 20% overage (µL)	For 8 libraries with 20% overage (µL)
PCR MasterMix	25	30	120	240
Library Forward Primer	5	6	24	48
Library Reverse Primer (1-4)[a]	5	6	–	–
Nuclease-free water	5	6	24	48
Total	40	48	168	336

[a] For more than one WTA 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 If working with multiple libraries, in separate tubes for each sample, combine 35 µL of WTA index PCR mix with 5 µL of the corresponding Library Reverse Primer and 10 µL of 2 nM 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	∞

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 (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. 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 and discard 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. Remove tube from magnet and pipette 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:

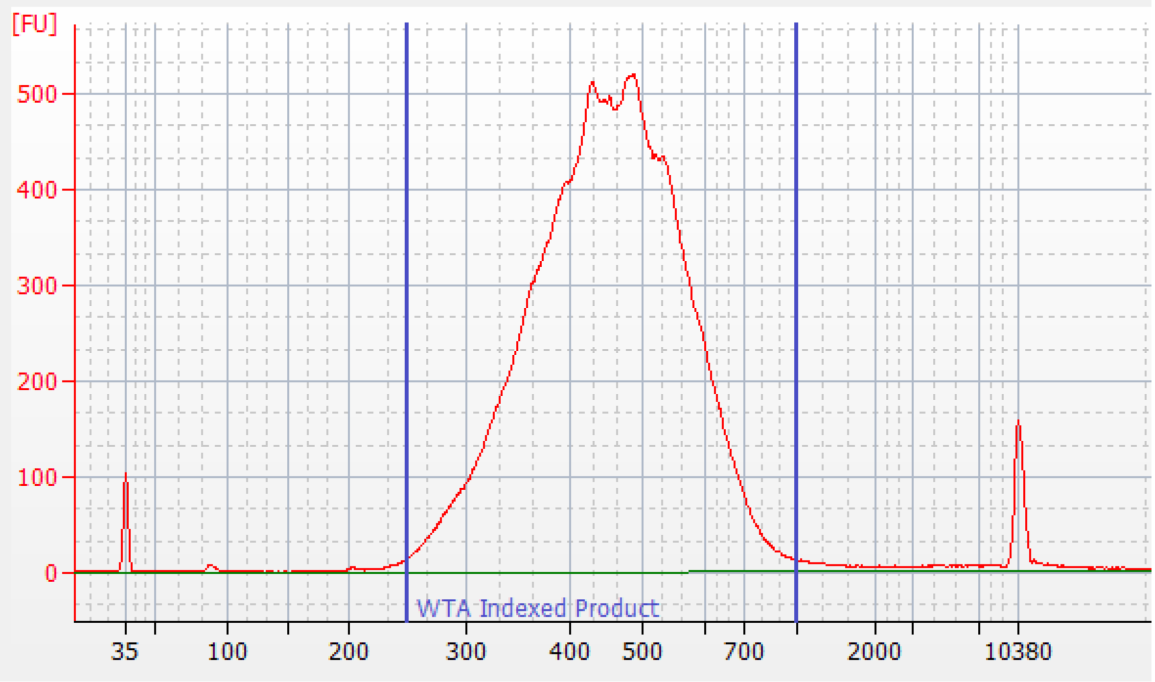
- Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit
- Agilent 4200 TapeStation system using the Agilent High Sensitivity D100 or D5000 ScreenTape Assay

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

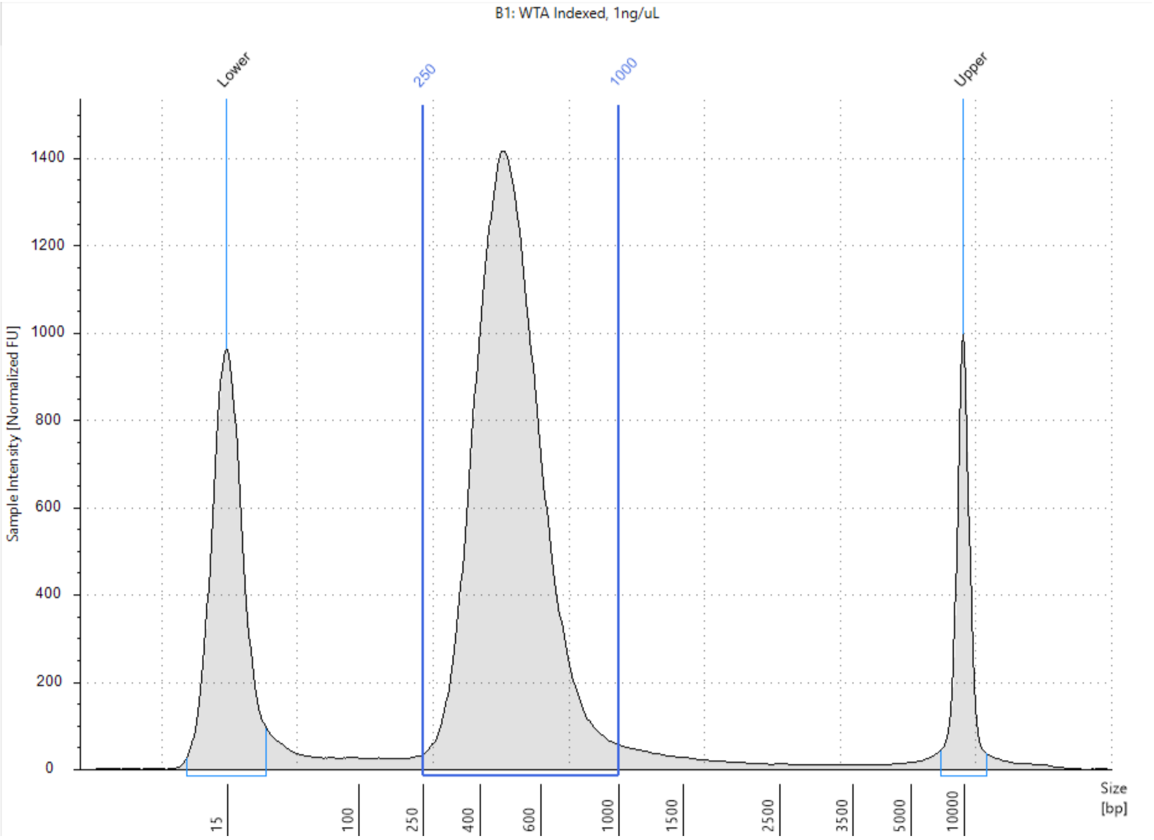
b The Bioanalyzer and TapeStation traces should show a peak from ~250 to 1,000 bp. Refer to the sample trace images in **Figure 2**.

Figure 2 WTA index PCR product

A. Sample Bioanalyzer high-sensitivity DNA trace

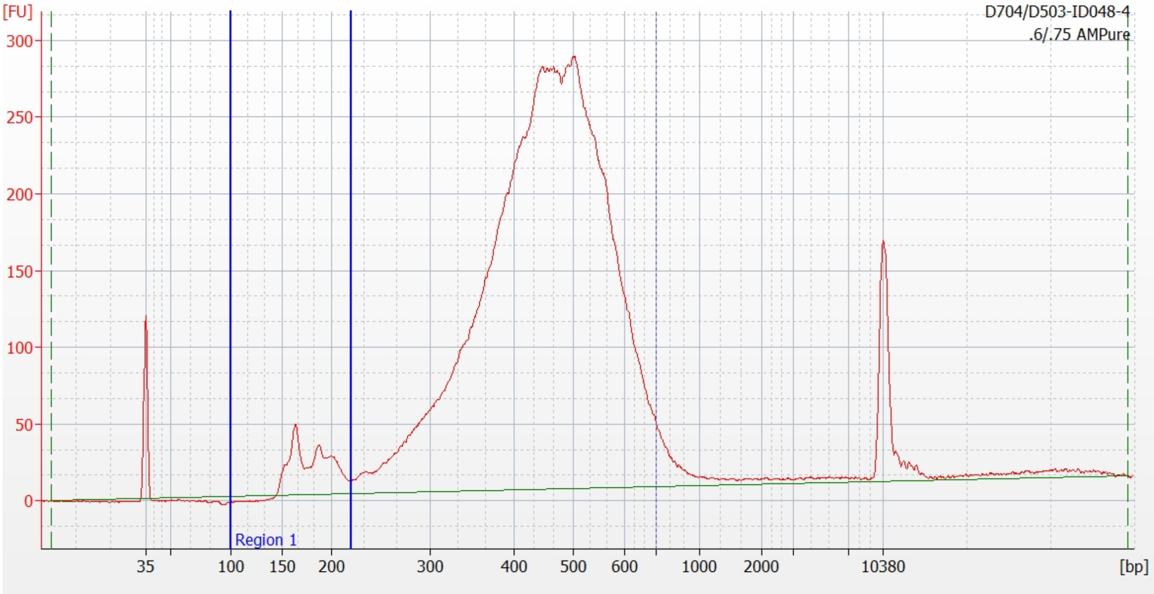


B. Sample TapeStation high-sensitivity D5000 trace



Note: If a <270 bp peak is observed from **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 on page 21.

Figure 3 Sample Bioanalyzer high-sensitivity DNA trace for an index PCR product with observable peaks <270 bp



Additional WTA index PCR purification steps

If a <270 bp peak is observed from the **Figure 2** Bioanalyzer or TapeStation traces, we recommend a second round of AMPure XP magnetic purification.

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 WTA index PCR product from the first round of purification.

4 Pipet-mix 10 times, then briefly centrifuge.

5 Repeat **step 9** through **step 21** in Purifying WTA index PCR product (dual-sided cleanup) on page 18 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 22 on page 18.

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

Sequencing

Requirements

Run setup for Illumina® BaseSpace and sample sheet sequencing. Enter the pooled libraries as one sample if both libraries were made with the same Library Reverse primer or if both libraries share the same i7 index.

Required parameters

Parameter	Requirement
Platform	Illumina[a]
Paired-end reads	Recommend Read 1: 51 cycles; Read 2: 71 cycles
PhiX	1% recommended
Analysis	See the *BD® Single-Cell Multiomics Bioinformatics Handbook*

[a] To review Illumina Index 1 (i7) sequences, see Appendix on page 24.

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 1.5-1.8 pM with 1% PhiX for a sequencing run.
- 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 deep sequencing to harvest the majority of UMIs in the library

Sequencing analysis pipeline

Contact your local Field Application Specialist (FAS) or 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. • Alternatively, repeat PCR using the RPE PCR product for additional cycles. • Alternatively, increase index PCR cycles.
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.
Index 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 with diluted RPE-PCR product.
Final sequencing product size too large.	Upper and lower markers on the Agilent Bioanalyzer or TapeStation are incorrectly called.	• Ensure that markers are correct. Follow manufacturer's instructions.
	Incorrect volume of Agencourt AMPure XP magnetic beads used.	• Use volume specified in protocol.

Sequencing

Observation	Possible causes	Recommended solutions
Over-clustering on the Illumina flow cell due to under-estimation of the library.	Inaccurate measurement of the library concentration.	• Quantitate library according to instructions in protocol.
Low sequencing quality.	Suboptimal cluster density, or library denaturation, or both.	• See troubleshooting in Illumina documentation.

Appendix

Illumina index 1 (i7) sequences

Library reverse primer	Sequence
1	GCTACGCT
2	CGAGGCTG
3	AAGAGGCA
4	GTAGAGGA

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