BD Rhapsody™ System

Whole transcriptome analysis alpha protocol

12/2018

Overview

This protocol is intended to provide a method to screen RNA expression of single cells using a 3' whole transcriptome analysis (WTA) approach using both BD-orderable reagents and third-party reagents as outlined in this document. The data set generated from this protocol can be input to generate a custom panel for subsequent 3' targeted mRNA-seq. Specifically, the protocol outlines how to generate whole transcriptome libraries from BD Rhapsody™ Cell Capture Beads for 5,000-10,000 single cells per sample. The procedure described herein is not currently compatible with the BD™ AbSeq assay and the BD™ Single Cell Multiplexing Kit.

IMPORTANT: The protocol described herein has not been thoroughly tested or optimized and is provided as is and without warranty. Any use of this protocol is at the risk of the user. BD reserves the right to change specifications at any time. Information in the protocol is subject to change without notice. BD assumes no responsibility for any errors or omissions. In no event shall BD be liable for any damages in connection with or arising from the use of this protocol.

References

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* - Doc ID 214062 Rev.1.0.
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* - Doc ID 214063 Rev.1.0.
- *Single Cell Targeted Library Preparation with the BD Rhapsody™ Single-Cell Analysis System User Guide* - Doc ID 47395 Rev.3.0.

Required and recommended materials

Store the reagents at the storage temperature specified on the label.

Required Reagents

Material	Supplier	Catalog No.
BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit	BD Biosciences	633774
SPRIselect reagent	Beckman Coulter Life Sciences	B23318
100% ethyl alcohol	Major supplier	-
10 mM Tris-HCl with 0.05% Tween-20, pH 8.0 (Tris-Tween20)	Teknova	T1485
DNA Suspension Buffer	Teknova	T0223
Nuclease-Free Water	Major supplier	-
Randomer (5' TCA GAC GTG TGC TCT TCC GAT CTNNNNNNNNN 3') Stock concentration of Randomer is 100 µM in 10 mM Tris, 0.1 mM EDTA, pH 8.0. Mixed bases achieved by handmixing of 25% per nucleotide content	Major supplier	-
Klenow Fragment (3' -> 5' exo-) (includes NEBuffer 2)	New England Biolabs	M0212L
10 mM dNTP	New England Biolabs	N0447L
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
• Agilent DNA High Sensitivity Kit	Agilent Technologies	5067-4626
OR		
• Agilent High Sensitivity D5000 ScreenTape	Agilent Technologies	5067-5592
• Agilent High Sensitivity D5000 Reagents	Agilent Technologies	5067-5593

Recommended Consumables

Material	Supplier	Catalog No.
Pipettes (P10, P20, P200, P1000)	Major supplier	-
Low-retention filtered pipette tips	Major supplier	-
0.2 mL PCR strip tubes	Major supplier	-
15 mL conical tube	Major supplier	-
1.5 mL microcentrifuge tubes	Eppendorf	022431021
DNA LoBind Tubes, 1.5 mL	Eppendorf	0030108051

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	-
Pipet-Aid	Major supplier	-
Digital timer	Major supplier	-
Multi-channel pipettes (P20, P200)	Major supplier	-
Heat block	Major supplier	-
Eppendorf ThermoMixer® C	Eppendorf	5382000023
SmartBlock Thermoblock 1.5 mL to fit on the ThermoMixer® C	Eppendorf	5360000038
6-tube magnetic separation rack for 1.5 mL tubes	New England Biolabs	S1506S
Low-profile magnetic separation stand for 0.2 mL, 8-strip tubes	V&P Scientific, Inc. OR Clontech	VP772F4-1 635011
Qubit™ 3.0 Fluorometer	Thermo Fisher Scientific	Q33216
• Agilent 2100 Bioanalyzer OR	Agilent Technologies	G2940CA
• Agilent 4200 TapeStation Instrument	Agilent Technologies	G2991AA

Procedure

Perform the experiment on the BD Rhapsody™ Single-Cell Analysis system following either the *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide* (Doc ID: 214062) or the *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide* (Doc ID: 214063) for cell capture, reverse transcription, and Exonuclease treatment. Please ensure that the intended total cell load is between 5000-10000 single cells for this protocol. Cell load below or above this recommendation may not be suitable for current protocol configuration. Then proceed as described in the following procedure.

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

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

Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 20% overage (µL)
Nuclease-free water	134	160.8	294.8
NEBuffer 2	20	24	44
Randomer	20	24	44
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 Exonuclease I-treated beads, skip to step 6. If using a subsample, proceed to step 5.

5 Subsample the Exonclease I-treated BD Rhapsody 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 subsample for sequencing.
- Completely resuspend the beads by pipet-mixing and 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 Tris-Tween20 buffer.

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

6 Resuspend beads with a pipette. Place the tube with beads in a 95°C heat block for 2 minutes (no shaking).

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

8 Remove the tube 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.

9 Incubate the tube in the following order:

- Heat block with no shaking and at 95°C for 2 minutes
- Thermomixer at 1,200 rpm and at 37°C for 5 minutes
- Thermomixer at 1,200 rpm and at 25°C for 5 minutes

10 Briefly centrifuge the tube and keep at room temperature.

11 In a new 1.5 mL LoBind tube, pipet the following reagents:

Primer Extension Enzyme Mix

Component	For 1 library (µL)	For 1 library with 50% overage (µL)	For 2 libraries with 30% overage (µL)
dNTP	8	12	20
Bead RT/PCR Enhancer	12	18	31
Klenow Fragment (3' -> 5' exo-)	6	9	16
Total	**26**	**39**	**67**

12 Pipet-mix the **Primer Extension Enzyme Mix.**

13 Pipet 26 µL of the **Primer Extension Enzyme Mix** into the sample tube containing the beads (for a total volume of 200 µL).

14 Place the tube in the thermomixer at 1,200 rpm and 37°C for 30 minutes. Remove the tube, but keep the thermomixer shaking at 1,200 rpm and at 37°C to use later in step 17.

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

16 Remove the tube from the magnet and resuspend the beads in 200 µL of Tris-Tween20 buffer.

17 To denature the random priming products off the beads, incubate the tube in the following order:

- Ensure that the beads are resuspended. Pipet to resuspend, if needed.
- Incubate the sample at 95°C in a heat block (no shaking) for 2 minutes.
- Place the tube in the shaking thermomixer (from step 14) and continue to shake for 10 seconds to resuspend beads.

18 Place the tube in a 1.5 mL tube magnet. Immediately transfer the **supernatant** containing the Random Primer Extension Product (RPE Product) to a new 1.5 mL LoBind tube and keep at room temperature.

(**Optional**) 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.

Purifying RPE product

NOTE **This section should be performed 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. Vortex the tube for 10 seconds.

NOTE **Make fresh 80% ethyl alcohol, and use in <24 hours.**

2 Vortex the SPRIselect beads at high speed for 1 minute until beads are fully resuspended.

3 Pipet **320 µL** of SPRIselect beads into the tube containing the **200 µL** of **RPE Product** supernatant. Pipet-mix at least 10 times and 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 supernatant.

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

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

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 **30 µL** of Elution Buffer into the tube. Vigorously pipet-mix until the beads are uniformly dispersed. Small clumps do not affect performance.

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 (~30 µL) to a new PCR tube. This is the **purified RPE Product**.

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

1 In the pre-amplification workspace, in a new 1.5 mL LoBind tube, pipet the following components:

RPE PCR Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 10% overage (µL)
PCR MasterMix	50	60	110
Universal Oligo	10	12	22
BD™ AbSeq Primer	10	12	22
Total	**70**	**84**	**154**

2 Add 70 µL of the **RPE PCR Mix** to the tube with the 30 µL of **Purified RPE product**. Pipet-mix 10 times.

3 Split the **RPE PCR Reaction** into 2 PCR tubes with 50 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		95°C	30 s
Annealing	13	60°C	1 min
Extension		72°C	1 min
Final extension	1	72°C	2 min
Hold	1	4°C	∞

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

Purification of the RPE PCR amplification product (single-sided cleanup)

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

1. Combine the two RPE PCR reactions into a new 0.2 mL PCR tube.
2. Briefly centrifuge the tubes with the RPE PCR product.
3. Vortex the SPRIselect beads at high speed for 1 minute until the beads are fully resuspended.
4. Pipet **100 µL** of SPRIselect beads into the tube containing 100 µL of RPE PCR product. Pipet-mix at least 10 times and 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 3-5 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 **30 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 (~30 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 ≤ 6 months or 4°C for ≤ 6 weeks.

16. Quantify and perform quality control of the RPE PCR products with a Qubit Fluorometer using the Qubit dsDNA HS Assay and an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit or an Agilent 4200 TapeStation system using the Agilent High Sensitivity D5000 ScreenTape Assay.

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

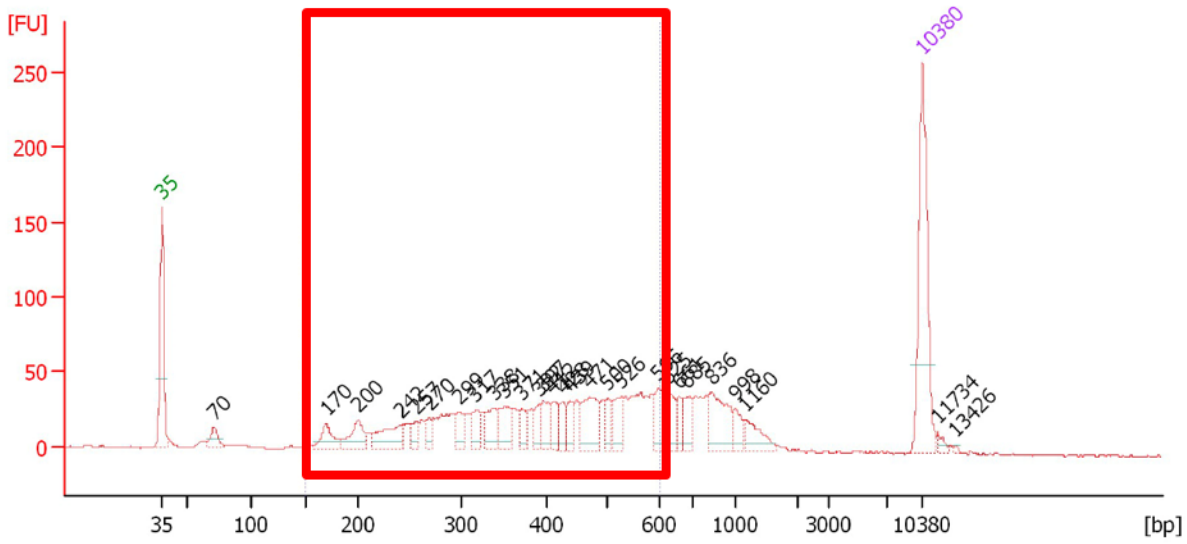
    b. The Bioanalyzer/TapeStation trace should show a broad peak from ~200 to 2000 bp. **Use the concentration from 150 to 600 bp** to calculate how much template to add into Index PCR. Refer to the red-boxed regions in the sample trace images on page 9.

NOTES

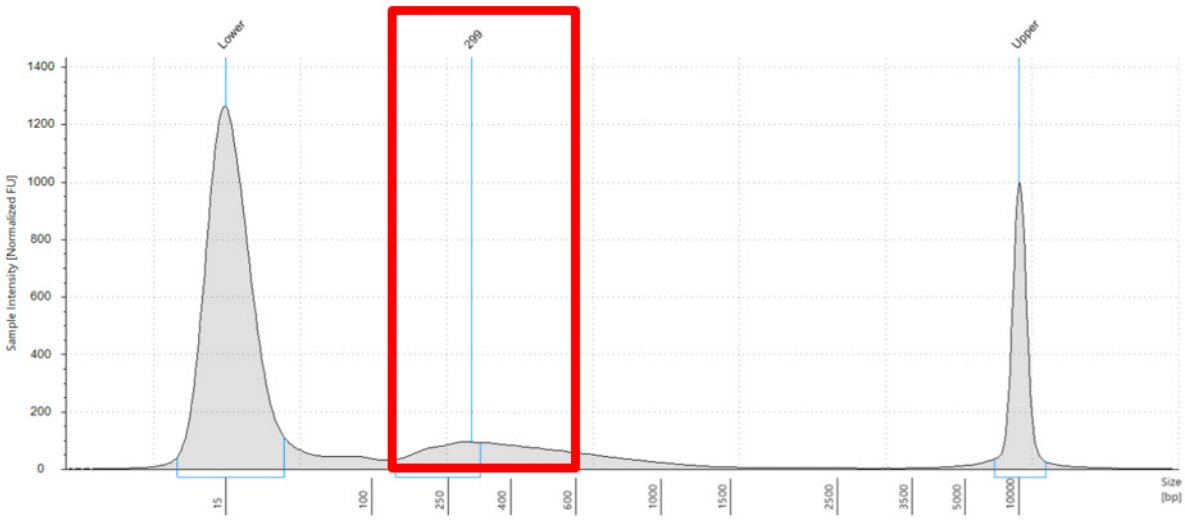
1 **Although there are products > 600 bp, these products should be removed in the double-sided cleanup after the next PCR.**

2 **Peaks around 170 and 200 bp have also been observed in samples with PBMCs. These peaks should not negatively impact assay sensitivity.**

Sample Bioanalyzer High Sensitivity DNA trace:



Sample TapeStation High Sensitivity D5000 trace:



Performing WTA Index PCR

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

1 Dilute the RPE PCR products in Teknova DNA Suspension Buffer such that the concentration of the 150-600 bp peak is 2 nM. If 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 three-fold with Teknova DNA Suspension Buffer to 2 nM.

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

WTA Index PCR Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)	For 2 libraries with 10% overage (µL)
PCR MasterMix	25	30	55
Library Forward Primer	5	6	11
*Library Reverse Primer (1-4)	5	6	-
Nuclease-free water	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 Table 1	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	∞

Table 1

Concentration of diluted RPE PCR products	Recommended no. of PCR cycles
1–2 nM	9
> 2 nM	8

7 When the WTA Index PCR is complete, briefly centrifuge to collect contents at the bottom of the tubes.

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

NOTE This section should be performed 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 Vortex the SPRIselect beads at high speed for 1 minute. The beads should appear homogeneous and uniform in color.

4 Add **60 µL** of SPRIselect beads to the 0.2 mL PCR tube from step 2.

5 Pipet-mix at least 10 times and then briefly centrifuge the samples.

6 Incubate the suspensions at room temperature for 5 minutes, and then place on the 0.2 mL strip tube magnet for 2 minutes.

7 Pipet **10 µL** of SPRIselect 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 SPRIselect beads (from step 7) and pipet-mix 10 times.

9 Incubate the suspension at room temperature for 5 minutes, and 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 SPRIselect beads.

11 Keeping the tubes on the magnet, gently pipet 200 uL of fresh 80% ethyl alcohol.

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 SPRIselect beads.

14 Repeat the 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 SPRIselect beads at room temperature for ~2 minutes or until the beads no longer look glossy.

17 Pipet 30 µL of Elution Buffer into the tubes and pulse vortex to completely resuspend the SPRIselect 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.

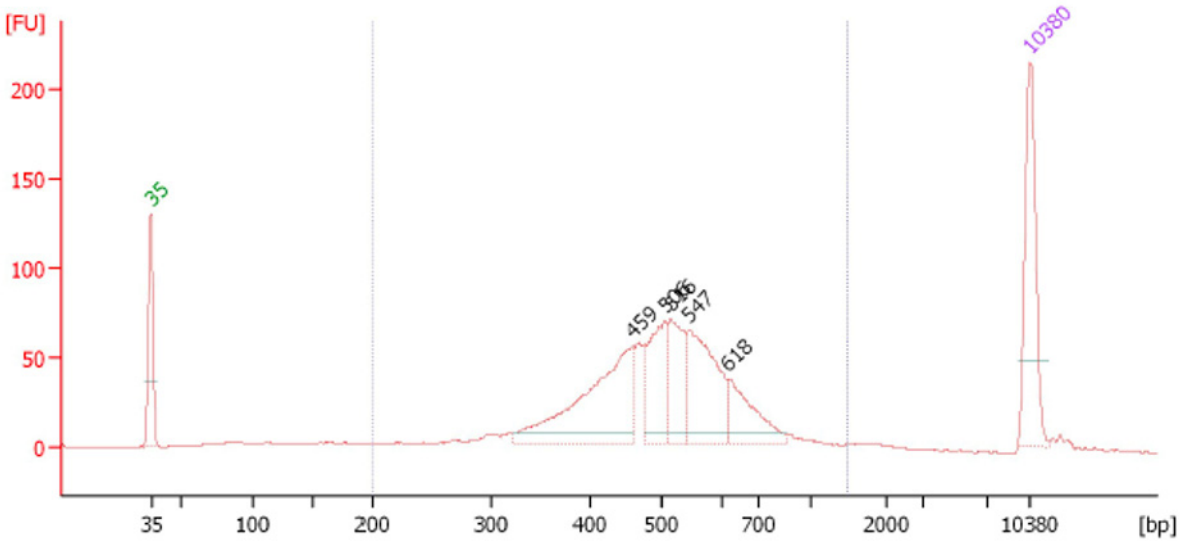
Stopping point: The Index PCR libraries can be stored at –20°C for ≤ 6 months until sequencing.

22 Quantify and perform quality control of the Index PCR libraries with a Qubit Fluorometer using the Qubit dsDNA HS Assay and an Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit or an Agilent 4200 TapeStation system using the Agilent High Sensitivity D1000 or D5000 ScreenTape Assay.

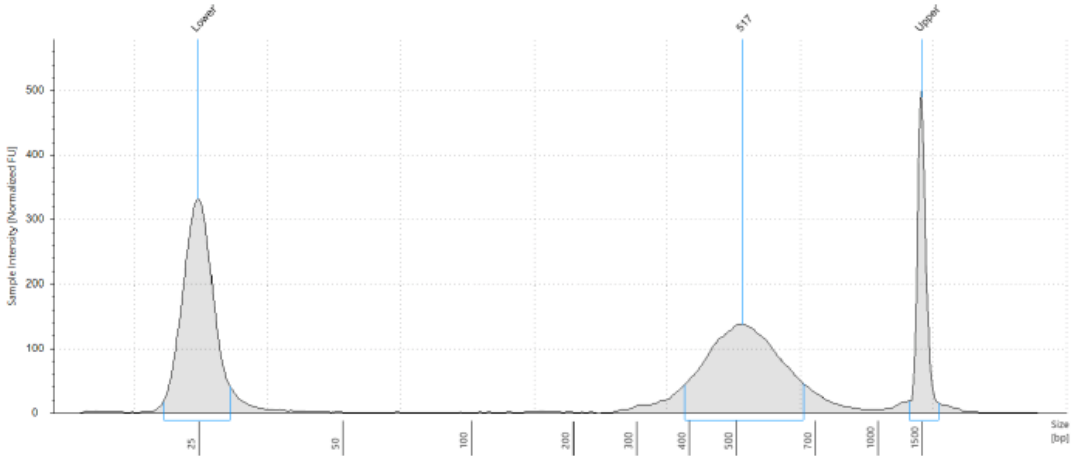
a The expected concentration from the Qubit Fluorometer is > 1 ng/µL.

b The Bioanalyzer/TapeStation trace should show a peak from ~ 300–700 bp. Refer to the sample trace images on page 13.

Sample Bioanalyzer High Sensitivity DNA trace:



Sample TapeStation High Sensitivity D1000 trace:



Sequencing Recommendations

- For a NextSeq High or Mid Output and MiniSeq High or Mid Output runs, load the flow cell at a concentration between 1–1.2 pM with 20% 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 customer support at SCOMIX@bd.com for access to the latest whole transcriptome sequencing analysis pipeline.

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Regulatory Information

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History

Revision	Date	Changes Made
23-21179-00	12/2018	Initial release

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