BD Rhapsody™ System

Immudex dCODE Dextramer® (RiO)

Library Preparation Protocol

For use with BD Rhapsody™ System TCR/BCR Full Length Library Preparation Protocols

- *BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA* 23-24013
- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, and BD® AbSeq* 23-24015
- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, BD® AbSeq, and Sample Tag* 23-24016
- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, and Sample Tag* 23-24014
- *BD Rhapsody™ System TCR/BCR Full Length and mRNA WTA* 23-24017
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, and BD® AbSeq* 23-24019
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, BD® AbSeq, and Sample Tag* 23-24020
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, and Sample Tag* 23-24018

23-24230(01)
2023-06

For Research Use Only

Regulatory information

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

History

Revision	Date	Change Made
23-24230(01)	2023-06	Initial version.

Contents

Introduction

This protocol describes the preparation of the Immudex dCODE Dextramer® (RiO) library with or without the addition of BD® AbSeq and with or without Sample Tag. It is intended to be used in combination with any BD Rhapsody™ System TCR/BCR Full Length Protocol listed in the following table.

Desired workflow	Required additional BD TCR full length protocol	Part number
Targeted mRNA		
dCODE Dextramer® + TCR + Targeted	TCR/BCR Full Length and Targeted	23-24013
dCODE Dextramer® + TCR + BD® AbSeq + Targeted	TCR/BCR Full Length, Targeted, and BD® AbSeq	23-24015
dCODE Dextramer® + TCR + BD® AbSeq + Sample Tag + Targeted	TCR/BCR Full Length, Targeted, BD® AbSeq, and Sample Tag	23-24016
dCODE Dextramer® + TCR + Sample Tag + Targeted	TCR/BCR Full Length, Targeted, and Sample Tag	23-24014
WTA		
dCODE Dextramer® + TCR + WTA	TCR/BCR Full Length and WTA	23-24017
dCODE Dextramer® + TCR + BD® AbSeq + WTA	TCR/BCR Full Length, WTA, and BD® AbSeq	23-24019
dCODE Dextramer® + TCR + BD® AbSeq + Sample Tag + WTA	TCR/BCR Full Length, WTA, BD® AbSeq, and Sample Tag	23-24020
dCODE Dextramer® + TCR + Sample Tag + WTA	TCR/BCR Full Length, WTA, and Sample Tag	23-24018

Additional reagents required for dCODE Dextramer® library preparation

dCODE Dextramer®-specific amplification primers:

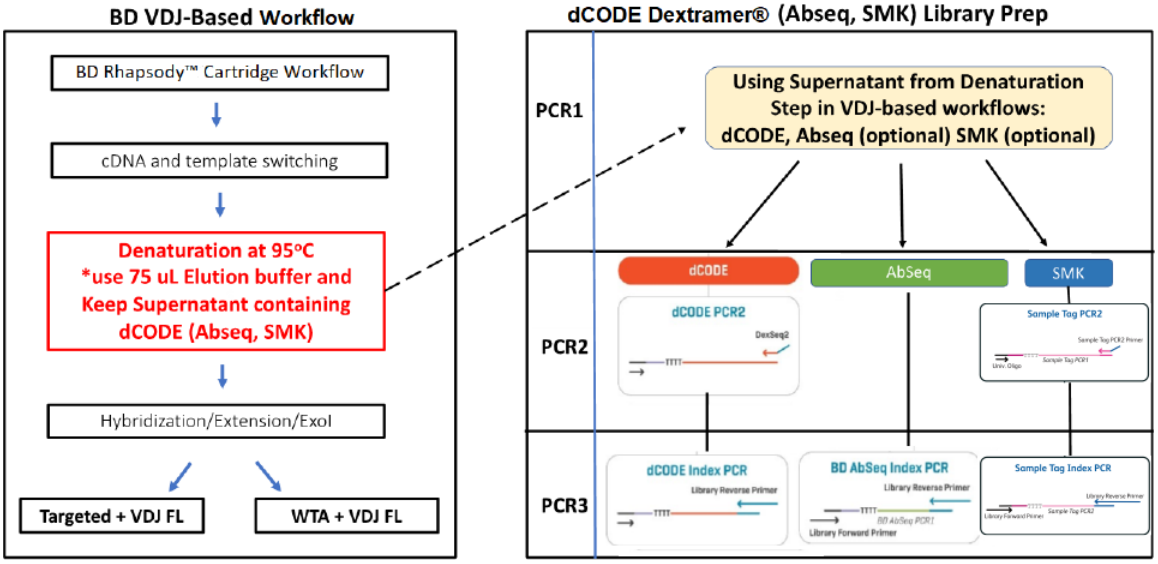
- dCODE Dextramer® PCR1 primer: 5'-GGAGGGAGGTTAGCGAAGGT-3'
- dCODE Dextramer® PCR2 primer: 5'-CAGACGTGTGCTCTTCCGATCTGGAGGGAGGTTAGCGAAGGT-3'
- dCODE Dextramer®-specific primers can be ordered from a preferred DNA oligo provider and should be used at 10 µM working concentration.

The BD Rhapsody™ kits include reagents for four reactions. If preparing more than four libraries, additional reactions may be required.

Additional Index Primers may also need to be ordered.

Refer to the Ordering Additional Indexes for the BD Rhapsody™ Library Reagent Kits Technical Bulletin (23-22747).

Workflow for dCODE Dextramer®, Sample Tag, and BD® AbSeq amplification



For cartridge workflow refer to the following documents:

- *BD Rhapsody™ Single-Cell Analysis System Instrument User Guide*
- *BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide*

For mRNA and TCR workflow, refer to the protocols listed in Introduction, on page 4.

dCODE Dextramer® library preparation without BD® AbSeq (with or without Sample Tag)

This section describes how to prepare dCODE Dextramer® libraries when using a workflow with or without Sample Tag. If the workflow also includes BD® AbSeq, see dCODE Dextramer® library preparation with BD® AbSeq or dCODE Dextramer® with BD® AbSeq and Sample Tag, on page 19.

Required protocol changes

To prepare dCODE Dextramer® libraries without Sample Tag (and no BD® AbSeq), modifications need to be made to the “Denaturation and self-hybridization” section in the following protocols:

- *BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA Library Preparation Protocol* 23-24013
- *BD Rhapsody™ System TCR/BCR Full Length and mRNA WTA Library Preparation Protocol* 23-24017

Protocol modifications in the Denaturation and self-hybridization section

In the protocols listed in the previous section, the denaturation step requires 200 µL of elution buffer that is then discarded. However, for use with dCODE Dextramer® reagents, the denaturation will use only 75 µL of elution buffer and the supernatant will be kept and used for dCODE Dextramer® PCR1 amplification. Keep the tube with the 75 µL supernatant at 4 °C until it is ready to be processed on the same day as collection. If it is not processed on the same day, store the supernatant at –20 °C for up to 6 weeks.

See the following tables for further details of the required changes to the corresponding protocols.

A. Changes to TCR/BCR Full Length and Targeted mRNA 23-24013

Section	Step	Change
"Denaturation and self-hybridization"	3	Pipet **75 µL** elution buffer instead of 200 µL.
	6	Keep the supernatant, and do not discard. The supernatant contains the dCODE Dextramer® templates needed for PCR1 amplification.

B. Changes to TCR/BCR Full Length and WTA 23-24017

Section	Step	Change
"Denaturation and self-hybridization"	3	Pipet **75 µL** elution buffer instead of 200 µL.
	6	Keep the supernatant, and do not discard. The supernatant contains the dCODE Dextramer® templates needed for PCR1 amplification.

Use the 75 µL supernatant described in these protocol changes to prepare dCODE Dextramer® libraries following the steps Performing dCODE Dextramer® PCR1 (with or without Sample Tag), on page 8.

Important: When using dCODE Dextramer® + TCR/BCR Full length workflows, use the entire sample of AMPure beads to prepare the mRNA and TCR libraries. Sub-sampling AMPure beads is not recommended for dCODE Dextramer® + TCR/BCR Full length combination assays.

To prepare dCODE Dextramer® libraries with Sample Tag (and no BD® AbSeq), no modifications are needed to the “Denaturation and self-hybridization” section of the following protocols:

- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, and Sample Tag Library Preparation Protocol* 23-24014
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, and Sample Tag Library Preparation Protocol* 23-24018

The 75 µL supernatant captured in the “Denaturation and self-hybridization” section will be used to prepare dCODE Dextramer® and Sample Tag libraries following the steps in Performing dCODE Dextramer® PCR1 (with or without Sample Tag), on page 8. Sample Tag library preparations are also described in this protocol.

Performing dCODE Dextramer® PCR1 (with or without Sample Tag)

1 In the pre-amplification workspace, pipet the following reagents into a new 1.5-mL LoBind® tube. Choose between either dCODE Dextramer®-only or dCODE Dextramer® with Sample Tag PCR1 reaction mix steps in the following tables.

dCODE Dextramer®-only PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
dCODE Dextramer® PCR1 Primer (10 µM)	12	14.4
Nuclease-free water	10	12
Total	**132**	**158.4**

OR

dCODE Dextramer® and Sample Tag PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
dCODE Dextramer® PCR1 Primer (10 µM)	12	14.4
Sample Tag PCR1 primers	1	1.2
Nuclease-free water	9	10.8
Total	**132**	**158.4**

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

3 In a new 1.5-mL tube, pipet 132 µL of the dCODE Dextramer® PCR1 reaction mix (with or without Sample Tag). Add 68 µL of the dCODE Dextramer® (with or without Sample Tag) template captured during the Denaturation and self-hybridization step described previously. Pipet-mix 10 times.

4 Divide the PCR1 reaction mix with template into four 0.2-mL PCR tubes by transferring 50 µL into each tube. Transfer any residual mix to one of the tubes.

5 Bring the reaction mix to the post-amplification workspace.

6 Program the thermal cycler.

dCODE Dextramer® PCR1 conditions (with or without Sample Tag)

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	10-12*	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 numbers.			

STOPPING POINT: The PCR can run overnight.

Suggested number of PCR cycles

Number of cells in PCR1	Recommended PCR cycles
5,000-7,500*	12
7,500-10,000	11
10,000-20,000	10
*Note: if cell numbers are 5,000 to 7,500 also use 12 cycles of PCR1 for Targeted mRNA and TCR amplification.	

7 After PCR, briefly centrifuge the tubes.

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

Purifying dCODE Dextramer® PCR1 products

Note: Perform the purification in the post-amplification workspace.

1. In a new 5.0-mL LoBind® tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

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

2. Bring AMPure beads to room temperature. Vortex on high speed for 1 minute until the AMPure beads are fully resuspended.
3. Briefly centrifuge PCR1 products and adjust volume if needed to bring up to 200 µL using nuclease-free water.
4. To 200 µL of dCODE Dextramer® PCR1 amplification products, add 280 µL AMPure beads to obtain a 1.4x AMPure bead ratio.
5. Incubate at room temperature for 5 minutes.
6. Place tubes on strip tube magnet for 5 minutes. Discard the supernatant.
7. Keeping the tube on the magnet, gently add 500 µL fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
8. Repeat step 7 once for a total of two washes.
9. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
10. Air-dry the AMPure beads at room temperature for 3 minutes.
11. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.
12. Incubate at room temperature for 2 minutes and briefly centrifuge.
13. Place the tube on the magnet until the solution is clear, usually within 30 seconds.
14. Pipet entire eluate (~50 µL) into separate new 1.5-mL LoBind® tube. (Purified dCODE Dextramer® with or without Sample Tag PCR1 products)

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

Performing dCODE Dextramer® and Sample Tag PCR2 (if applicable)

Note: dCODE Dextramer® and Sample Tag PCR1 products are amplified separately in PCR2. Skip Sample Tag instructions if not relevant to your workflow.

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

dCODE Dextramer® PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
dCODE Dextramer® PCR2 primers (10 µM)	10	12
Nuclease-free water	8	9.6
Total	**45**	**54**

OR

Sample Tag PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PC MasterMix	25	30
Universal Oligo	2	2.4
Sample Tag PCR2 primers	3	3.6
Nuclease-free water	15	18
Total	**45**	**54**

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

3 Bring the PCR2 mix without template into the post-amplification workspace.

4 In a new 0.2-mL PCR tube, pipet 5.0 µL of purified PCR1 products into 45 µL of PCR2 reaction mixtures.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler as follows.

dCODE Dextramer® PCR2 conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	13*	95 °C	30 s
Annealing		66 °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 dCODE Dextramer® panels, cells types, and cell numbers.			

Sample Tag PCR2 conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	10	95 °C	30 s
Annealing		66 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

STOPPING POINT: The PCR can run overnight.

Purifying dCODE Dextramer® and Sample Tag PCR2 products (if applicable)

Note: Perform the purification in the post-amplification workspace. dCODE Dextramer® and Sample Tag PCR2 products can be purified in parallel with this protocol.

1 In a new 5.0-mL LoBind® tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

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

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

3 Briefly centrifuge the PCR2 products.

4 To 50 µL PCR2 products pipet:

   - For dCODE Dextramer® PCR2 products: 60 µL AMPure beads to get a 1.2x AMPure bead ratio
   - For Sample Tag PCR2 products: 60 µL AMPure beads to get a 1.2x AMPure bead ratio

5 Pipet-mix 10 times and incubate at room temperature for 5 minutes.

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

7 Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.

8 Repeat step 7 once for a total of two washes.

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

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

11 Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.

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

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

14 Pipet the entire eluate (~50 µL) into a new 1.5-mL LoBind® tube (purified dCODE Dextramer® or Sample Tag product).

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

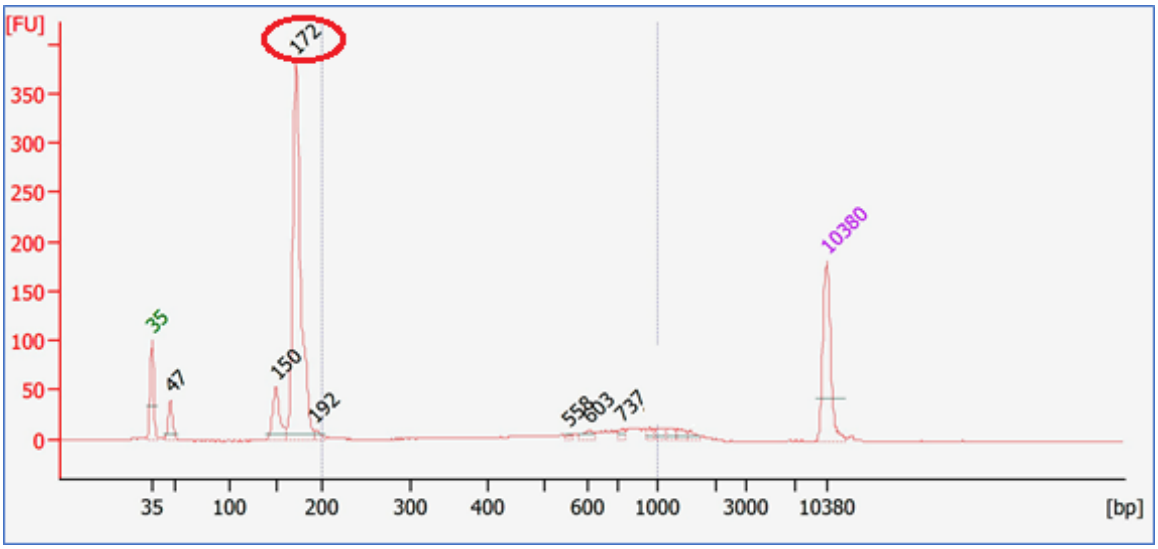
Quantifying dCODE Dextramer® and Sample Tag PCR2 products

For Quantifying dCODE Dextramer® PCR2 products

Measure the yield of the dCODE Dextramer® PCR2 product which will be ~170 bp by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.

Based on the corresponding concentration of the 170 bp peak, dilute an aliquot of dCODE Dextramer® PCR2 product to 0.1–1.1 ng/µL with nuclease-free water to use for index PCR of dCODE Dextramer® libraries. It is important to index dCODE Dextramer® libraries differently from all others libraries since bioinformatic trimming of the FASTQs from these libraries will be needed. See Trimming dCODE Dextramer® sequencing reads, on page 34 for more details.

Figure 1 Sample Bioanalyzer High Sensitivity DNA Trace - dCODE Dextramer® PCR2 product



Peak		Size [bp]	Conc. [pg/µl]	Molarity	Observations
1		35	125	5,411.3	Lower Marker
2		47	48.45	1,555.0	
3		150	77.91	787.5	
4		172	571.61	5,045.3	
5		192	9.59	75.6	

For Quantifying Sample Tag PCR2 products

Estimate the concentration by quantifying 2 µL of the PCR2 products with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

Performing Index PCR to prepare final dCODE Dextramer® libraries

This section describes how to generate 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: dCODE Dextramer® libraries are required to be indexed with a different index primer from other libraries for downstream bioinformatic processing. See Trimming dCODE Dextramer® sequencing reads, on page 34.

dCODE Dextramer® and Sample Tag Index PCR mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	2	2.4
Library Reverse Primer 1-4	2	2.4
Nuclease-free water	18	21.6
Total	**47**	**56.4**

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

3 Bring index PCR mixes to the post-amplification workspace.

4 In new 0.2-mL PCR tubes:

- For dCODE Dextramer® libraries: Pipet 3.0 µL of 0.1–1.1 ng/µL RiO PCR2 product into 47 µL dCODE Dextramer® Index PCR mix. (From For Quantifying dCODE Dextramer® PCR2 products, on page 14)
- For Sample Tag libraries: Pipet 3.0 µL of 0.1–1.1 ng/µL Sample Tag PCR2 product into 47 µL Sample Tag Index PCR mix. (From For Quantifying Sample Tag PCR2 products, on page 14)

5 Gently vortex, and briefly centrifuge.

6 Program the thermal cycler.

dCODE Dextramer® and Sample Tag Index PCR conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	See the following table: Recommended number of PCR cycles for dCODE Dextramer® libraries.*	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	30 min
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞

*Cycle number varies based on the concentration of the dCODE Dextramer® PCR2 product.

Recommended number of PCR cycles for dCODE Dextramer® libraries

Concentrated Index PCR input for dCODE Dextramer® and Sample 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 Index PCR products

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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

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

2. Bring AMPure beads to room temperature and vortex at high speed for 1 minute until the AMPure beads are fully resuspended.
3. Briefly centrifuge all the index PCR products.
4. To 50.0 µL of the index PCR products, pipet:
   a. For dCODE Dextramer® library: 40 µL AMPure beads to get a 0.8x AMPure bead ratio
   b. For Sample Tag library: 40 µL AMPure beads to get a 0.8x AMPure bead ratio
5. Incubate at room temperature for 5 minutes.
6. Place tubes on the strip tube magnet for 3 minutes. Discard the supernatant.
7. Keeping the tube on the magnet, for each tube, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
8. Repeat step 7 for a total of two washes.
9. Keeping the tube on the magnet, use a small-volume pipette to remove and discard the residual supernatant from the tube.
10. Air-dry the AMPure beads at room temperature for 3 minutes.
11. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.
12. Incubate at room temperature for 2 minutes, and briefly centrifuge.
13. Place the tube on the magnet until the solution is clear, usually ≤30 seconds.
14. For each tube, pipet the entire eluates (~50 µL) into separate new 1.5-mL LoBind® tubes (final sequencing libraries).
15. Perform quality control before freezing samples. See Performing quality control on the final sequencing libraries, on page 18.

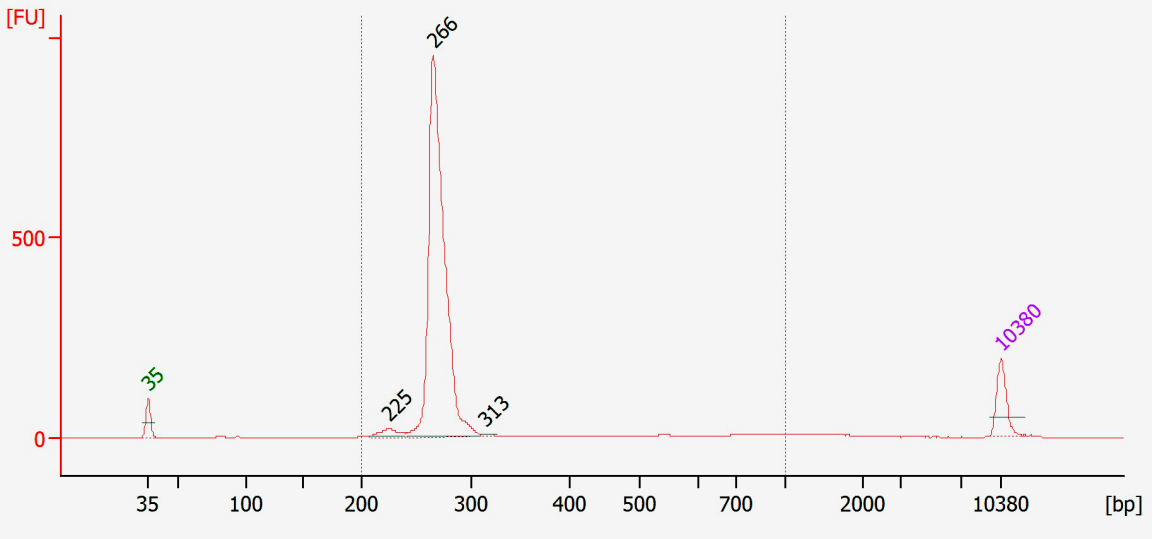
STOPPING POINT: Store at –25 °C to –15 °C for up to 6 months until sequencing.

Performing quality control on the final sequencing libraries

1 Estimate the concentration of each sample by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer. Follow the manufacturer's instructions.

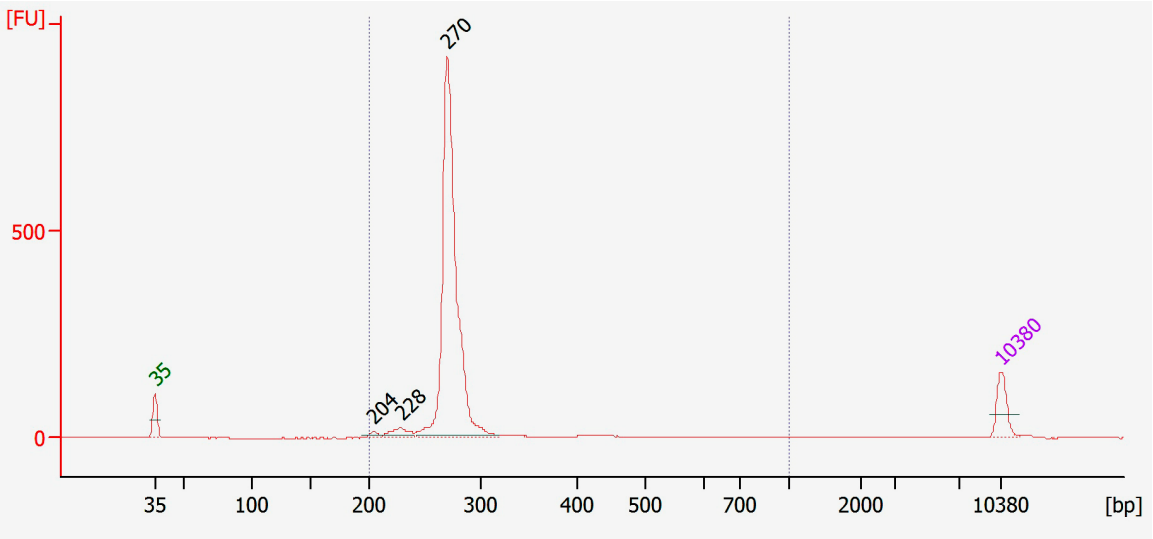
2 Measure the average fragment size of the targeted mRNA library within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626) for 50–7,000 bp, 5–1,000 pg/µL. Follow the manufacturer's instructions.

Figure 2 Sample Bioanalyzer High Sensitivity DNA Trace – dCODE Dextramer® Index PCR product (~270 bp)



For dCODE Dextramer® Sequencing Recommendations and Processing dCODE Dextramer® Sequencing Reads, see dCODE Dextramer® sequencing recommendations, on page 33 and Trimming dCODE Dextramer® sequencing reads, on page 34, respectively.

Figure 3 Sample Bioanalyzer High Sensitivity DNA Trace – Sample Tag Index PCR product (~270 bp)



dCODE Dextramer® library preparation with BD® AbSeq or dCODE Dextramer® with BD® AbSeq and Sample Tag

This section describes how to prepare dCODE Dextramer® libraries when using a workflow with BD® AbSeq or with BD® AbSeq and Sample Tag. Use one of the following protocols in combination with the protocol described in this section.

- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, and BD® AbSeq Library Preparation Protocol* 23-24015
- *BD Rhapsody™ System TCR/BCR Full Length, Targeted mRNA, BD® AbSeq, and Sample Tag Library Preparation Protocol* 23-24016
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, and BD® AbSeq Library Preparation Protocol* 23-24019
- *BD Rhapsody™ System TCR/BCR Full Length, mRNA WTA, BD® AbSeq, and Sample Tag Library Preparation Protocol* 23-24020

The 75 µL supernatant captured in the section Introduction on page 4 will be used to prepare dCODE Dextramer®, BD® AbSeq, and Sample Tag libraries beginning from Performing dCODE Dextramer®, BD® AbSeq, and Sample Tag PCR1, on page 20.

Performing dCODE Dextramer®, BD® AbSeq, and Sample Tag PCR1

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

dCODE Dextramer® and BD® AbSeq PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
dCODE Dextramer® PCR1 primers (10 µM)	12	14.4
BD® AbSeq primers	Add later*	N/A
Total	**122**	**146.4**
*2.5 µL of BD® AbSeq Primers will be spiked into each PCR reaction tube after the first part of the PCR1 program is complete and before the second part of PCR1 begins (see the PCR1 conditions in the following table).		

OR

dCODE Dextramer®, BD® AbSeq, and Sample Tag PCR1 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	100	120
Universal Oligo	10	12
dCODE Dextramer® PCR1 primers (10 µM)	12	14.4
Sample Tag PCR1 primers	1	1.2
BD® AbSeq primers	Add later*	N/A
Total	**123**	**147.6**
*2.5 µL of BD® AbSeq Primers will be spiked into each PCR reaction tube after the first part of the PCR1 program is complete and before the second part of PCR1 begins (see the PCR1 conditions in the following table).		

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

3 For dCODE Dextramer® and BD® AbSeq workflow, pipet 122 µL of the PCR1 reaction mix into a new 1.5-mL tube. Add 68 µL of the dCODE Dextramer® and BD® AbSeq captured during the "Denaturation and self-hybridization", on page 6. Pipet-mix 10 times.

OR

For dCODE Dextramer®, BD® AbSeq, and Sample Tag workflow, pipet 123 µL of the PCR1 reaction mix into a new 1.5-mL tube. Add 67 µL of the dCODE Dextramer®, BD® AbSeq, and Sample Tag product captured during the "Denaturation and self-hybridization", on page 6. Pipet-mix 10 times.

4 Divide the PCR1 reaction mix into four 0.2-mL PCR tubes by transferring 47.5 µL into each tube.

Transfer any residual mix to one of the tubes.

5 Bring the reaction mix to the post-amplification workspace.

6 Program the thermal cycler.

Note: PCR1 conditions are split into 2 parts. After part 1 is complete, add 2.5 µL of BD® AbSeq primer into each PCR tube and then continue to part 2. These PCR conditions have been optimized to enrich the dCODE Dextramer® amplicons.

dCODE Dextramer®, BD® AbSeq, and Sample Tag PCR1 conditions

Part	Step	Cycles	Temp	Time
Part 1	Hot start	1	95 °C	3 min
	Denature	6*	95 °C	30 s
	Annealing		60 °C	30 s
	Extension		72 °C	1 min
	Hold	1	4 °C	∞
	Add: 2.5 µL BD® AbSeq Primer to each of the 4 PCR tubes. Restart program by "skip to next step" and start with "Denature" step.			
Part 2	Denature	5*	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 dCODE Dextramer® panels, cell types, and cell numbers.				

STOPPING POINT: The PCR can run overnight.

Suggested number of PCR cycles

Number of cells in PCR1	Recommended PCR1 cycles for Part 1	Recommended PCR1 cycles for Part 2	Total PCR1 cycles
5,000-7,500*	6	6	12
7,500-10,000	6	5	11
10,000-20,000	5	5	10
*Note: If cell numbers are 5,000 to 7,500, also use 12 cycles of PCR1 for Targeted mRNA and TCR amplification.			

7 After PCR, briefly centrifuge the tubes.

8 Pipet-mix and combine the four reactions into a new 1.5-mL LoBind® tube labeled dCODE Dextramer® PCR1. Keep the tube on ice and proceed with purifying as described in the next section.

Purifying dCODE Dextramer®, BD® AbSeq, and Sample Tag PCR1 products

Note: Perform the purification in the post-amplification workspace.

1. In a new 5.0-mL LoBind® tube, prepare 5 mL of fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL nuclease-free water. Vortex the tube for 10 seconds to mix.

   Note: Make fresh 80% ethyl alcohol and use it within 24 hours.
2. Bring AMPure beads to room temperature. Vortex on high speed for 1 minute until the AMPure beads are fully resuspended.
3. Briefly centrifuge PCR1 products and adjust volume if needed to bring up to 200 µL using nuclease-free water.
4. To the PCR1 products, add 280 µL AMPure beads to get a 1.4x AMPure bead ratio.
5. Incubate at room temperature for 5 minutes.
6. Place tubes on strip tube magnet for 5 minutes. Discard supernatant.
7. Keeping the tube on the magnet, gently add 500 µL fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.
8. Repeat step 7 once for a total of two washes.
9. Keeping the tube on the magnet, use a small-volume pipette to remove and discard any residual supernatant from the tube.
10. Air-dry the AMPure beads at room temperature for 3 minutes.
11. Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.
12. Incubate at room temperature for 2 minutes and briefly centrifuge.
13. Place the tube on the magnet until the solution is clear, usually within 30 seconds.
14. Pipet entire eluate (~50 µL) into separate new 1.5-mL LoBind® tube. (Purified PCR1 products)

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

Quantifying dCODE Dextramer® and BD® AbSeq with or without Sample Tag PCR1 products

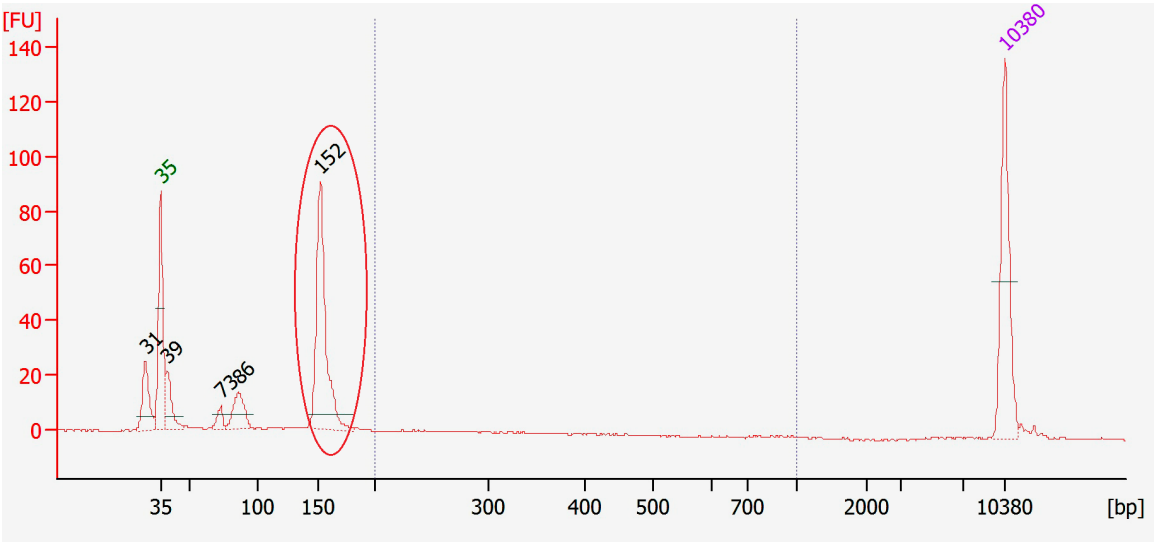
For Quantifying dCODE Dextramer® PCR1 products

Measure the yield of the dCODE Dextramer® and BD® AbSeq with or without Sample Tag PCR1 products, which should be ~150 bp long by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.

For Quantifying BD® AbSeq PCR1 products

Based on the corresponding concentration of the 150 bp peak, dilute an aliquot of PCR1 products to 0.1–1.1 ng/µL with nuclease-free water to use for index PCR of BD® AbSeq libraries.

Figure 4 Sample Bioanalyzer High Sensitivity DNA Trace – BD® AbSeq PCR1 product



Peak		Size [bp]	Conc. [pg/µl]	Molarity	Observations
1		35	125	5,411.3	Lower Marker
2		39	47.52	1,855.0	
3		73	16.35	338.4	
4		86	46.57	820.5	
5		152	216.28	2,151.2	
6		10,380	75.00	10.9	Upper Marker

Performing dCODE Dextramer® and Sample Tag PCR2

Note: dCODE Dextramer® and Sample Tag PCR1 products are amplified separately in PCR2. Skip Sample Tag instructions if not relevant to your workflow. BD® AbSeq does not require PCR2 amplificaiton.

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

dCODE Dextramer® PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
dCODE Dextramer® PCR2 primers (10 µM)	10	12
Nuclease-free water	8	9.6
Total	**45**	**54**

Sample Tag PCR2 reaction mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Universal Oligo	2	2.4
Sample Tag PCR2 primers	3	3.6
Nuclease-free water	15	18
Total	**45**	**54**

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

3 Bring the PCR2 mix into the post-amplification workspace.

4 In new 0.2-mL PCR tubes, pipet 5.0 µL of purified dCODE Dextramer® PCR1 products into 45 µL of dCODE Dextramer® and Sample Tag PCR2 reaction mixtures.

5 Gently vortex and briefly centrifuge.

6 Program the thermal cycler as follows.

dCODE Dextramer® PCR2 conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	13*	95 °C	30 s
Annealing		66 °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 dCODE Dextramer® panels, cell types, and cell numbers.			

Sample Tag PCR2 conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	10	95 °C	30 s
Annealing		66 °C	30 s
Extension		72 °C	1 min
Final extension	1	72 °C	5 min
Hold	1	4 °C	∞

STOPPING POINT: The PCR can run overnight.

Purifying dCODE Dextramer® and Sample Tag PCR2 products

Note: Perform the purification in the post-amplification workspace. dCODE Dextramer® and Sample Tag PCR2 products can be purified in parallel with this protocol.

1 In a new 5.0-mL LoBind® tube, prepare 5 mL fresh 80% (v/v) ethyl alcohol by combining 4 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

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

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

3 Briefly centrifuge the PCR2 products.

4 To 50 µL PCR2 products pipet:

   - For dCODE Dextramer® PCR2 products: 60 µL AMPure beads to get 1.2x AMPure bead ratio
   - For Sample Tag PCR2 products: 60 µL AMPure beads to get 1.2x AMPure bead ratio

5 Pipet-mix 10 times and incubate at room temperature for 5 minutes.

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

7 Keeping the tube on the magnet, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.

8 Repeat step 7 once for a total of two washes.

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

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

11 Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.

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

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

14 Pipet the entire eluate (~50 µL) into a new 1.5-mL LoBind® tube (purified dCODE Dextramer® or Sample Tag PCR2 product).

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

Quantifying dCODE Dextramer® and Sample Tag PCR2 products

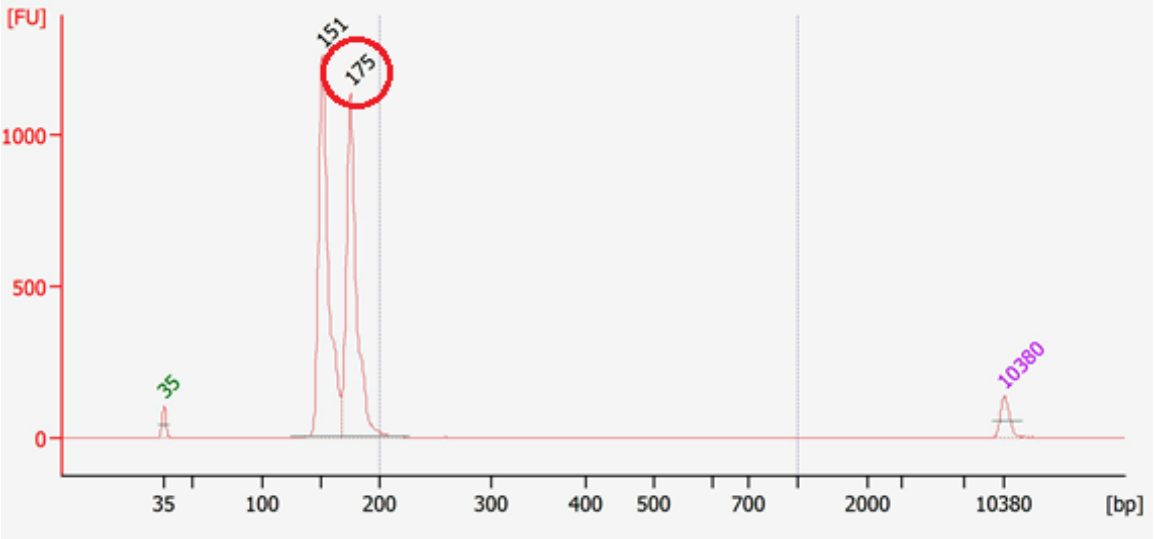
For Quantifying dCODE Dextramer® PCR2 products

Measure the yield of the dCODE Dextramer® PCR2 product, which will be ~170 bp, by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.

Based on the corresponding concentration of the 170 bp peak, dilute an aliquot of dCODE Dextramer® PCR2 product to 0.1–1.1 ng/µL with nuclease-free water to use for index PCR of dCODE Dextramer® libraries. It is important to index dCODE Dextramer® libraries differently from all others libraries since bioinformatic trimming of the FASTQs from these libraries will be needed. See Trimming dCODE Dextramer® sequencing reads, on page 34 for more details.

Note: There may be a ~150 bp peak in the dCODE Dextramer® PCR2 product Bioanalyzer trace (see Figure 5). This 150 bp peak can be ignored and will not be indexed. It will be resolved and eliminated in the final indexed dCODE Dextramer® library. Do not include this 150 bp peak for quantification of the dCODE Dextramer® PCR2 library.

Figure 5 Sample Bioanalyzer High Sensitivity DNA Trace – dCODE Dextramer® PCR2 product



Peak		Size [bp]	Conc. [pg/µl]	Molarity	Observations
1		35	125	5,411.3	Lower Marker
2		151	2,964.08	29, 694.1	
3		175	2,472.67	21,355.2	
6		10,380	75.00	10.9	Upper Marker

For Quantifying Sample Tag PCR2 products

Estimate the concentration by quantifying 2 µL of the PCR2 products with a Qubit Fluorometer using the Qubit dsDNA HS Assay Kit. Follow the manufacturer's instructions.

Performing Index PCR to prepare final dCODE Dextramer®, BD® AbSeq, and Sample Tag libraries

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: dCODE Dextramer® libraries are required to be indexed with a different index primer from other libraries for downstream bioinformatic processing. See Trimming dCODE Dextramer® sequencing reads, on page 34.

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

dCODE Dextramer®, BD® AbSeq, and Sample Tag Index PCR Mix

Component	For 1 library (µL)	For 1 library with 20% overage (µL)
PCR MasterMix	25	30
Library Forward Primer	2	2.4
Library Reverse Primer 1-4	2	2.4
Nuclease-free water	18	21.6
Total	**47**	**56.4**

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

3 Bring index PCR mixes to post-amplification workspace.

4 In new 0.2-mL PCR tubes,

- For dCODE Dextramer® libraries: Pipet 3.0 µL of 0.1–1.1 ng/µL dCODE Dextramer® PCR2 product into 47 µL dCODE Dextramer® Index PCR mix. (From For Quantifying dCODE Dextramer® PCR2 products, on page 27.)
- For Sample Tag libraries: Pipet 3.0 µL of 0.1–1.1 ng/µL Sample Tag PCR2 product into 47 µL Sample Tag Index PCR mix. (From For Quantifying Sample Tag PCR2 products, on page 27.)
- For BD® AbSeq libraries: Pipet 3.0 µL of 0.1–1.1 ng/µL BD® AbSeq PCR1 product into 47 µL BD® AbSeq Index PCR mix. (From For Quantifying BD® AbSeq PCR1 products, on page 23.)

5 Gently vortex, and briefly centrifuge.

6 Program the thermal cycler.

dCODE Dextramer®, BD® AbSeq, and Sample Tag Index conditions

Step	Cycles	Temp	Time
Hot start	1	95 °C	3 min
Denature	See the following table: Recommended number of PCR cycles for dCODE Dextramer® libraries.*	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	30 min
Final extension	1	72 °C	1 min
Hold	1	4 °C	∞
*Cycle number varies based on the concentration of the dCODE Dextramer® PCR2 product.			

Recommended number of PCR cycles for dCODE Dextramer® libraries

Concentrated Index PCR input for dCODE Dextramer® and Sample Tag libraries (ng/µL)	Recommended number of PCR cycles for BD® AbSeq	Recommended number of PCR cycles for dCODE Dextramer® and Sample Tag
0.5-1.1	7	6
0.25-0.5	8	7
0.1-0.25	9	8

STOPPING POINT: The PCR can run overnight.

Purifying Index PCR products

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 mL absolute ethyl alcohol, molecular biology grade, with 1 mL of nuclease-free water. Vortex the tube for 10 seconds to mix.

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

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

3 Briefly centrifuge all the index PCR products.

4 To 50.0 µL of the index PCR products, pipet:

- For dCODE Dextramer® library: 40 µL AMPure beads to get a 0.8x AMPure bead ratio
- For Sample Tag library: 40 µL AMPure beads to get a 0.8x AMPure bead ratio
- For BD® AbSeq library: 40 µL AMPure beads to get a 0.8x AMPure bead ratio

5 Incubate at room temperature for 5 minutes.

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

7 Keeping the tube on the magnet, for each tube, gently add 200 µL of fresh 80% ethyl alcohol into the tube and incubate for 30 seconds. Discard the supernatant.

8 Repeat step 7 for a total of two washes.

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

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

11 Remove the tube from the magnet and resuspend the bead pellet in 50 µL of elution buffer. Pipet-mix until the AMPure beads are fully resuspended.

12 Incubate at room temperature for 2 minutes, and briefly centrifuge.

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

14 For each tube, pipet the entire eluates (~50 µL) into separate new 1.5-mL LoBind® tubes (final sequencing libraries).

15 Perform quality control before freezing samples. See Performing quality control on the final sequencing libraries, on page 31.

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

Performing quality control on the final sequencing libraries

1 Estimate the concentration of each sample by quantifying 2 µL of the final sequencing library with a Qubit Fluorometer using the Qubit dsDNA HS Kit to obtain an approximate concentration of PCR products to dilute for quantification on an Agilent 2100 Bioanalyzer. Follow the manufacturer's instructions.

2 Measure the average fragment size of the libraries within the size range of 200–1,000 bp by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626). Follow the manufacturer's instructions.

Figure 6 Sample Bioanalyzer High Sensitivity DNA Trace – dCODE Dextramer® Index PCR product (~270 bp)

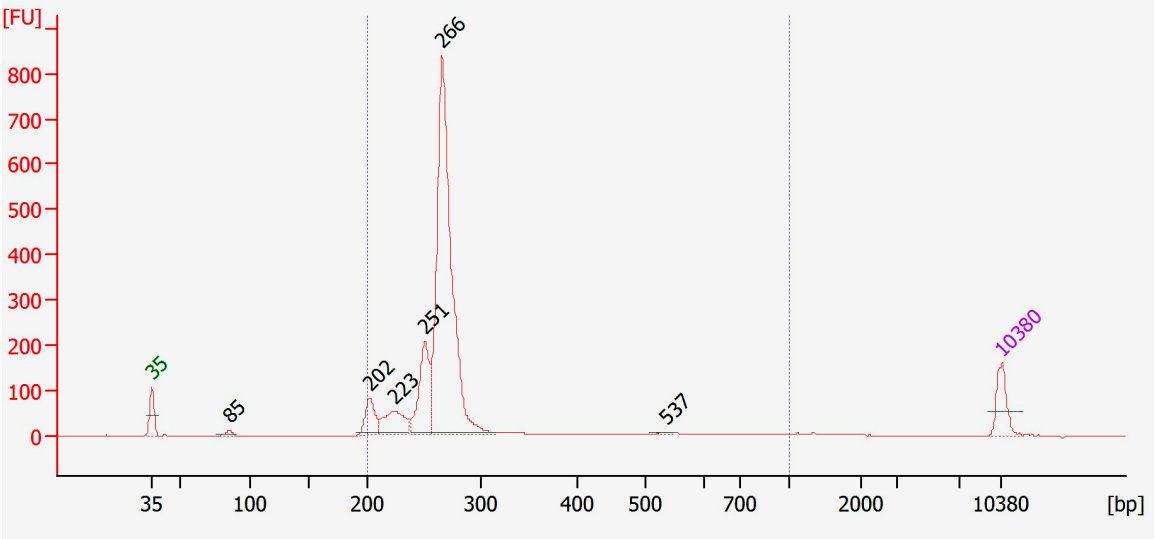


Figure 7 Sample Bioanalyzer High Sensitivity DNA Trace – BD® AbSeq Index PCR product (~250 bp)

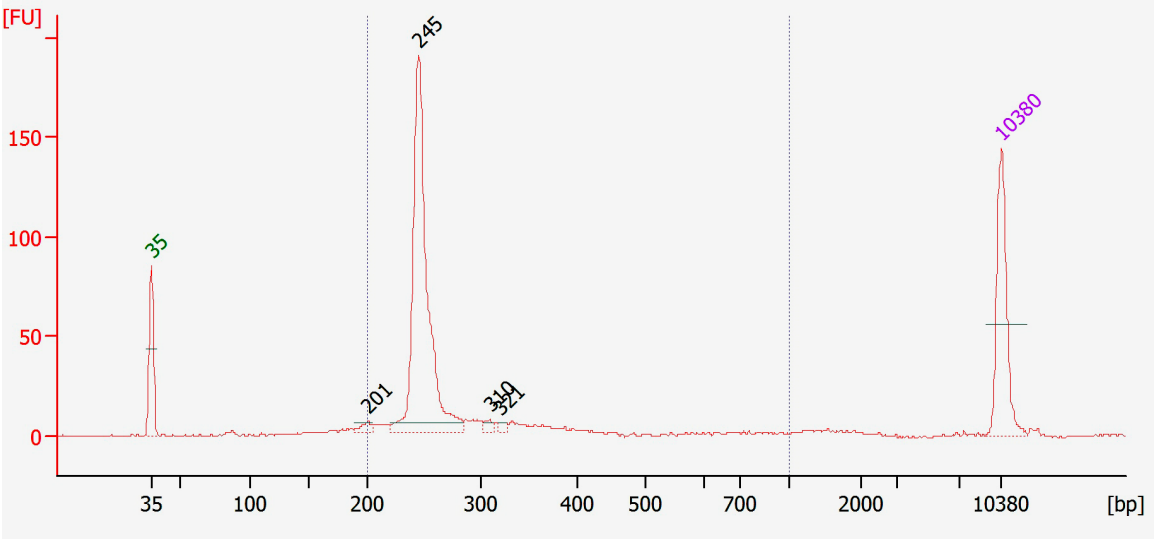
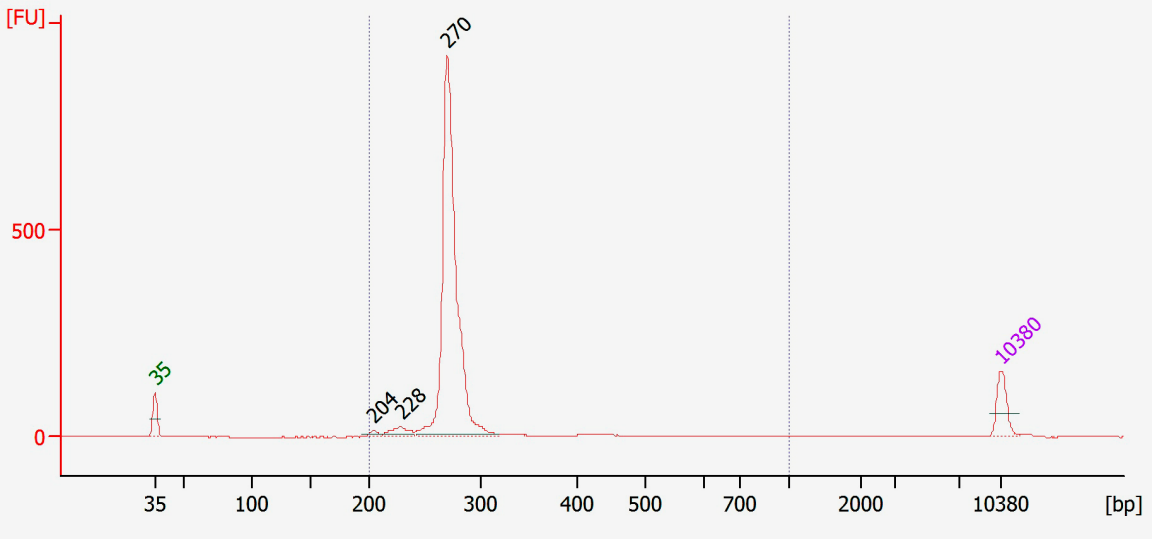


Figure 8 Sample Bioanalyzer High Sensitivity DNA Trace – Sample Tag Index PCR product (~270 bp)



dCODE Dextramer® sequencing recommendations

The amount of sequencing needed for dCODE Dextramer® libraries will vary depending on the application, dCODE Dextramer® panel, and cell type. In general, 500 reads per cell per dCODE Dextramer® will provide sufficient depth for most applications.

To quantify libraries for sequencing, calculate the molar concentration of the dCODE Dextramer® libraries using Qubit quantitation concentration (ng/µL) and average Bioanalyzer size (200 bp - 1000 bp). Use the calculated molar concentrations to pool libraries.

dCODE Dextramer® libraries can be sequenced using the following parameters:

Parameter	Requirement
Platform	Illumina: 150 cycle kit
Paired-end reads	Minimum of 51 x 75 paired read length
PhiX	Required (1% minimum)

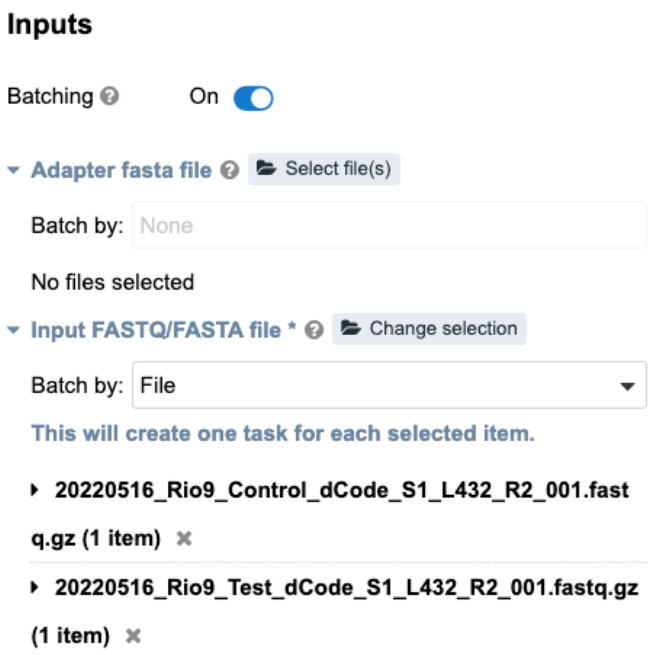
Note: Refer to BD Rhapsody™ TCR/BCR Full Length Protocols for sequencing recommendations for non-dCODE Dextramer® libraries.

Trimming dCODE Dextramer® sequencing reads

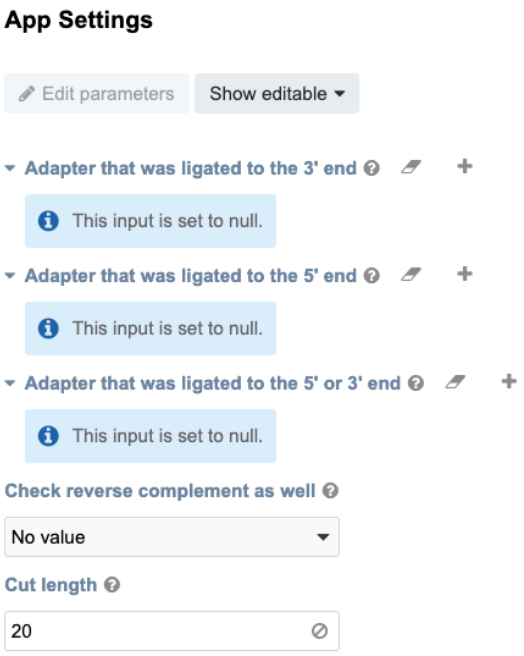
BD Rhapsody™ Analysis Pipelines are available on the Seven Bridges Genomics platform at sevenbridges.com/bdgenomics/ and can also be run using a local installation following the instructions in the *BD® Single-Cell Multiomics Analysis Setup User Guide*. Importantly, prior to loading the dCODE Dextramer® sequencing reads into the pipeline run, Read 2 of the dCODE Dextramer® reads will need to be trimmed. Note that separate indexing of the dCODE Dextramer® library will produce a separate file for the dCODE Dextramer® libraries, allowing the user to trim only the dCODE Dextramer® FASTQ reads.

To trim Read 2 of the dCODE Dextramer® sequencing reads, use the following steps:

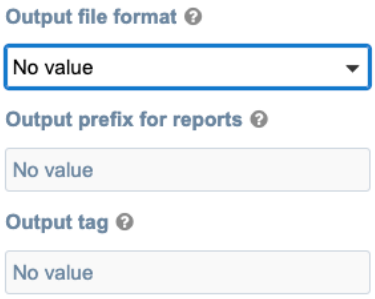
1 Copy the Cutadapt (Cutadapt 2.9) app from the Seven Bridges public apps on your project and run it with the following settings:
   - *Input FASTQ/FASTA file*: Input a single fastq file corresponding to the Read 2 (R2) of the dCODE Dextramer® library. Note that only one fastq file can be processed at a single time.
     - You can run multiple fastq files by selecting the “Batch” options



- App Settings Cut length: set to 20



- *Output file format*: Keep default as “no value”. This will generate the desired output file format, which will be “<sample>.cutadapted.fastq.gz”. Note: It is critical to have a .gz file in order to successfully run in the BD pipeline.



- Click the **Run** button.

2 The trimmed `fastq.gz` files generated by Cutadapt for Read 2 (R2) [“<sample>.cutadapted.fastq.gz”] can now be used as input files for a regular BD Rhapsody™ analysis pipeline run (refer to the *BD® Single-Cell Multiomics Analysis Setup User Guide*). All libraries from the same cartridge are analyzed in a single pipeline run, including Targeted mRNA with WTA, BD® AbSeq, Sample Tag, and dCODE Dextramer® libraries.

3 Before running the BD Rhapsody™ analysis pipeline, obtain an appropriate `.fasta` reference file for the dCODE Dextramer® panel. The dCODE Dextramer® reference `.fasta` file is order-specific and can be provided by Immudex customer support, if it is not already provided along with the purchase.

Note: If BD® AbSeq was also part of the workflow, you can generate a BD® AbSeq `.fasta` reference file using this link: abseq-ref-gen.genomics.bd.com/.

4 Upload the dCODE Dextramer® reference `.fasta` file and the BD® AbSeq `.fasta` file, if applicable to the "AbSeq Reference" section in Seven Bridges, as separate files.

Note: You need the dCODE Dextramer® reference file to deconvolute the dCODE Dextramer® library. The dCODE Dextramer®-specific barcodes are order-specific and can be found in the delivery note, or can be obtained through Immudex customer support (customer@immudex.com).

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