dCODE Dextramer® (RiO) Library Preparation Protocol

For use with BD Rhapsody™ Single Cell Analysis System and BD protocols for TCR/BCR Full Length Library Preparation

- BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA (23-24013)
- BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA and BD® Abseq (23-24015)
- BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA, BD® Abseq, and Sample Tag (23-24016)
- BD Rhapsody™ System TCR/BCR Full Length and 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 and mRNA WTA, BD® AbSeq, and Sample Tag (23-24020)
- BD Rhapsody™ System TCR/BCR Full Length and mRNA WTA and Sample Tag (23-24018)

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Introduction

This protocol describes the preparation of Immudex (MHC I, MHC II, MR1 and CD1d) dCODE Dextramer® (RiO) - and dCODE Klickmer® (RiO) barcode libraries with or without additional preparation of BD® AbSeq Assay and Sample Tag libraries. It is intended to be used in combination with any BD Rhapsody™ System TCR/BCR Full Length Protocol listed in the table below. In the following we use the term "dCODE® (RiO)" to refer to barcodes of dCODE Dextramer® (RiO) and dCODE Klickmer® (RiO) reagents.

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

The above BD protocols are available at:
https://scomix.bd.com/hc/en-us/articles/9285990045197-VDJ-full-length-TCR-BCR-assay-protocols-Human

Preceding protocols; required for staining, and cell capture.

Cell staining and sorting

- Immudex dCODE Dextramer® (RiO) staining protocol (TF1099)

For Rhapsody™ Cartridge Kit workflow please refer to the following documents:

- Single Cell Capture and cDNA Synthesis with the BD Rhapsody™ Single-Cell Analysis System (Doc. ID:210966)
- Single Cell Analysis Workflow with BD Rhapsody™ Systems (doc.ID:220524)
- BD Rhapsody™ Single-Cell Analysis System Instrument User Guide (Doc ID: 214062)

- BD Rhapsody™ Express Single-Cell Analysis System Instrument User Guide (Doc ID: 214063)

Important Note:
First **stopping point** in this workflow is after "cDNA synthesis and Template switching". See figure 1 page 5.

Additional reagents required for dCODE Dextramer® (RiO) library preparation

dCODE® (RiO) specific amplification primers

- dCODE® (RiO) PCR1 primer: 5'-GGAGGGAGGTTAGCGAAGGT-3'
- dCODE® (RiO) PCR2 primer: 5'-CAGACGTGTGCTCTTCCGATCTGGAGGGAGGTTAGCGAAGGT-3'

dCODE® (RiO) specific primers should be HPLC purified and can be ordered from any preferred DNA oligo provider. Oligos are used at a 10µM working concentration.

Additional reagents needed for generation of more than 4 final indexed libraries

- The BD-Rhapsody™ kits, include reagents for 4 reactions. If preparing more than four libraries, additional BD® Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kits (cat. No. 633774) are required.
- Additional Index library primers are also required. Please refer to: Ordering Additional Indexes for Rhapsody

Required reagents provided by BD Biosciences

- Please refer to the selected BD TCR/BCR Full Length Protocols listed in the above table.

Laboratory setup

- It is critical to establish and maintain a designated area for PCR setup that is separate from areas where PCR amplification and PCR product handling are performed. In addition, use a dedicated set of pipettes that are used only for setting up PCR reaction mix and always use filter tips. This will reduce the risk of contaminating NGS libraries with amplicons from previous PCR reactions.

Workflow overview

Workflow from cell staining to library preparation and sequencing.

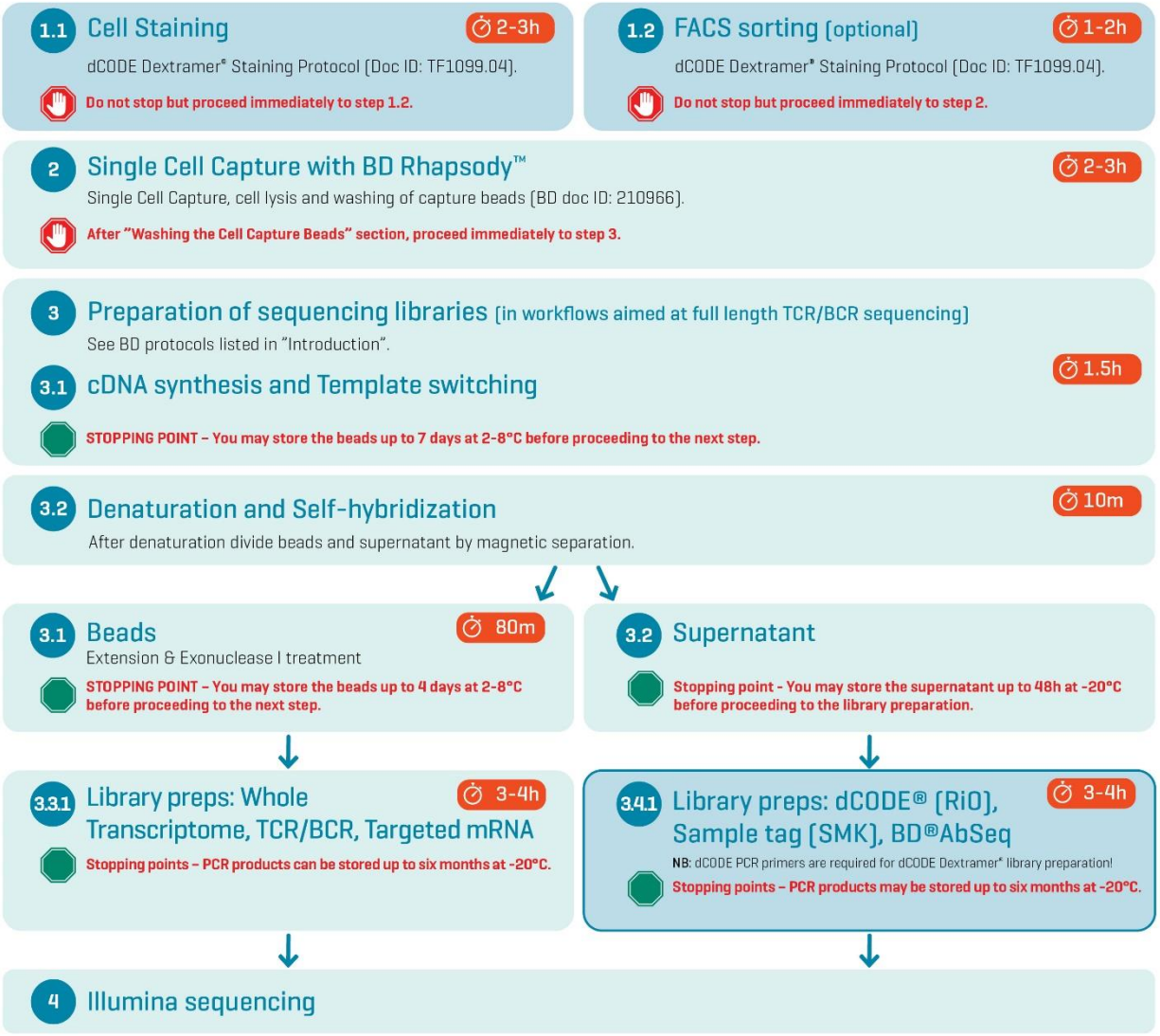


Figure 1. Workflow describing single cell analysis of antigen specific cells with dCODE Dextramer® and the BD lic™ System.
The subject of this protocol is preparation of dCODE® (RiO), BD® Abseq and Sample Tag libraries (3.4.1). It is possible to conduct the different library preparations described in step 3.3.1 and 3.4.1 in parallel, but for most practical purposes it is more convenient to perform them separately.
Note: This workflow is meant for experiments aimed at determining full length TCR/BCR sequences and includes a special template switching step during cDNA synthesis (3.1).

Preparation of dCODE® (RiO), BD®Abseq and Sample Tag Sequencing Libraries

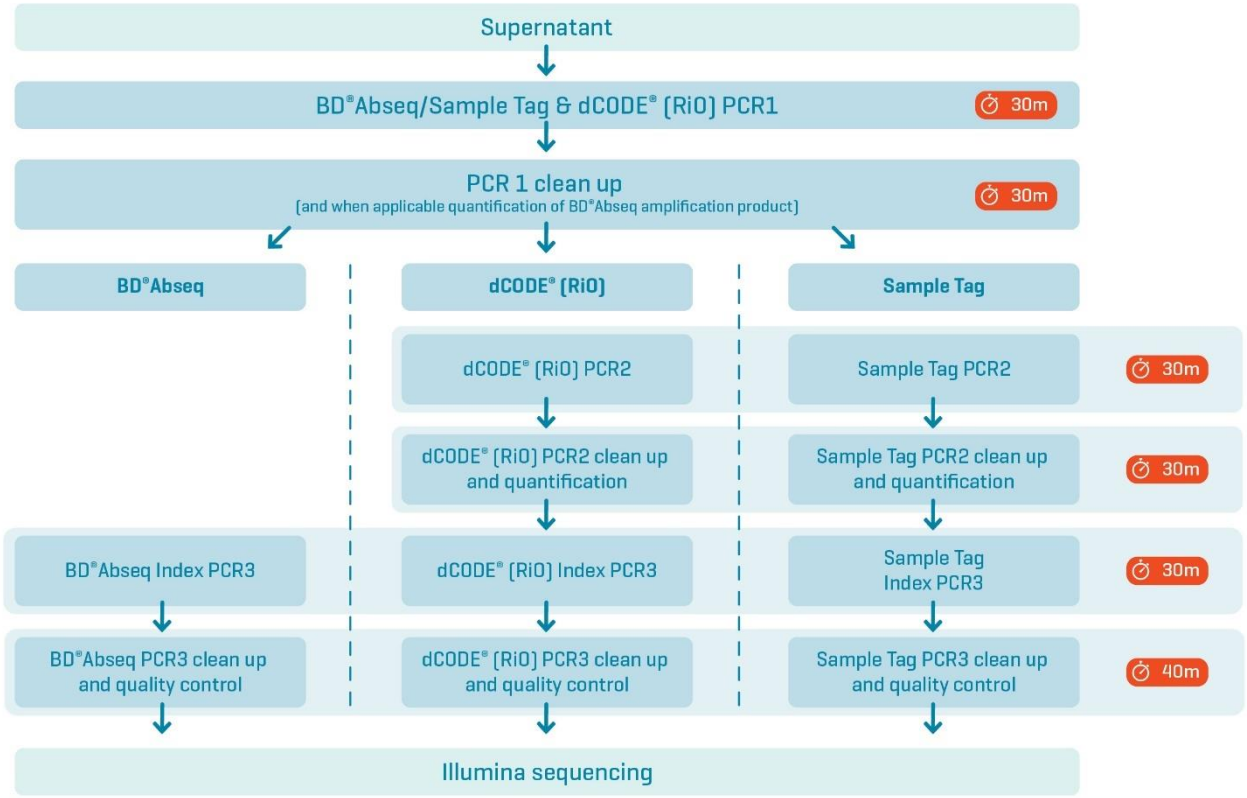


Figure 2: Workflow describing preparation of BD®Abseq, dCODE® (RiO) and Sample Tag NGS libraries.
The protocol starts with the supernatant from the upstream denaturation and self-hybridization step (see fig. 1), containing antibody and dCODE Dextramer® (RiO) DNA barcodes. After a common PCR1 step the three different libraries are constructed in three separate workflows that can be performed in parallel.
Stopping points: PCR reactions can be run over night and purified PCR products can be stored up to 24 hrs at +4°C and 6 months at -20°C.

Preparation of dCODE® (RiO) and Sample Tag libraries

This section describes how to prepare dCODE® (RiO) libraries when using workflows where cells are either co-stained or not stained with Sample Tag antibodies. Use one of the following protocols in combination with the protocol described in this section.

- *BD Rhapsody™ System TCR/BCR Full Length and Targeted mRNA Library Preparation Protocol (23-24013)*
- *BD Rhapsody™ System TCR/BCR Full Length and WTA Library Preparation Protocol (23-24017)*
- *BD Rhapsody™ System Library Preparation Protocol TCR/BCR Full Length, Targeted mRNA, and Sample Tag (23-24014)*
- *BD Rhapsody™ System Library Preparation Protocol TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA), and Sample Tag (23-24018)*

The above BD protocols are available at:
https://scomix.bd.com/hc/en-us/articles/9285990045197-VDJ-full-length-TCR-BCR-assay-protocols-Human

If the workflow also includes BD® AbSeq staining, please see section “Preparation of dCODE® (RiO), BD® AbSeq and Sample Tag Libraries” on page 16.

Performing dCODE® (RiO) and Sample Tag PCR1

The 75 µL supernatant captured in the "Denaturation and self-hybridization" sections of the BD® protocols listed above, will be used as template in the preparation of dCODE® (RiO), BD® AbSeq and Sample Tag libraries beginning from the "Performing dCODE® (RiO), BD® AbSeq, and Sample Tag PCR1" section of this protocol described below.

1 In the PCR setup area, pipet the following reagents into a new 1.5-mL LoBind® tube. Please choose between either dCODE® (RiO) only or dCODE® (RiO) and Sample Tag PCR1 Reaction Mix steps in tables below.

dCODE® (RiO) 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® primer 1 (10 uM)	12	14.4
Nuclease-free water	10	12
Total	**132**	**158.4**

* To compensate for pipetting errors

OR

dCODE® (RiO) 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® PCR 1 primer (10 uM)	12	14.4
Sample Tag PCR1 Primer	1	1.2
Nuclease-free water	9	10.8
Total	**132**	**158.4**

* To compensate for pipetting errors

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® (RiO) and Sample Tag PCR1 Reaction Mix. Add 68 µL of the dCODE® (RiO)/Sample Tag product captured during the Denaturation and self-hybridization described above. Pipet-mix 10 times.

4 Pipet 50 µL dCODE® (RiO) and Sample Tag PCR1 Reaction Mix into each of four 0.2-mL PCR tubes.

5 Bring the reaction mix to the PCR amplification area.

6 Program the thermal cycler as follows.

dCODE® (RiO)and Sample Tag PCR1 conditions

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® (RiO) PCR1. Keep the tube on ice.

Purifying dCODE® (RiO)and Sample Tag PCR1 products

Note: Do not perform the purification in the PCR setup area!

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 XP magnetic beads to room temperature. Vortex on high speed for 1 minute until the 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 PCR1 product, add 280 µL AMPure beads to obtain a 1.4x AMPure bead/PCR1 product (V/V) 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 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 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 within30 seconds.
14 Pipet entire eluate (~50 µL) into separate new 1.5 mL LoBind® tube. (Purified dCODE® (RiO) / Sample Tag PCR1 products)
*__NB: It is important to respect the indicated AMPure bead/PCR1 product (V/V) ratio!__

STOPPING POINT: Store at 2–8 °C if proceeding with the protocol within 24 hours or store at –25 °C to –15 °C for up to 6 months.

Performing dCODE® (RiO) and Sample Tag PCR2

Note: dCODE® (RiO) and Sample Tag PCR1 products are amplified separately in PCR2. Ignore Sample Tag instructions if not relevant to your workflow. Use only filter tips for pipetting.

1 In the PCR setup area pipet reagents into a new 1.5-mL LoBind® tube.

dCODE® (RiO) 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® PCR2 primers (10 uM)	10	12
Nuclease-free water	8	9.6
Total	**45**	**54**

* To compensate for pipetting errors

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**

* To compensate for pipetting errors

2 Gently vortex mix, briefly centrifuge, and place back on ice.
3 Move the PCR2 mix outside the PCR setup area.
4 In a new 0.2-mL PCR tube, pipet 5.0 µL of purified PCR1 products into 45 µL of PCR2 reaction mixtures.
NB: If the experiment involves parallel processing of multiple samples stained independently with dCODE Dextramer®, then care must be taken to avoid cross-contamination of PCR1 products in the PCR2 reaction mix. This can be achieved by pipetting the PCR1 products from different samples in separate lab spaces or by pipetting in a laminar flow hood equipped with UV light.
5 Gently vortex and briefly centrifuge.
6 Program the thermal cycler as follows.

dCODE® (RiO) 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	∞
* Optimization of PCR cycles may be needed according to the experimental setup in terms of: number of different dCODE Dextramer® used in staining of cells, 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® (RiO) and Sample Tag PCR2 products

Note: Do not perform purification of PCR products in the PCR setup area!

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 XP beads to room temperature and vortex at high speed for 1 minute until beads are fully resuspended.
3 Briefly centrifuge the PCR2 products.
4 Pipet 50 µL dCODE® (RiO) and Sample Tag PCR2 products in two separate tubes. Add :
- 60 µL AMPure beads to get a 1.2x AMPure bead/PCR2 product (V/V)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 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 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 separately (purified dCODE® (RiO) PCR2 and Sample Tag products).
*** NB: It is important to respect the indicated AMPure bead/PRC2 product (V/V) ratio!**

STOPPING POINT: Store at 2–8 °C if proceeding with the protocol within 24 hours, or store at –25 °C to –15 °C for up to 6 months.

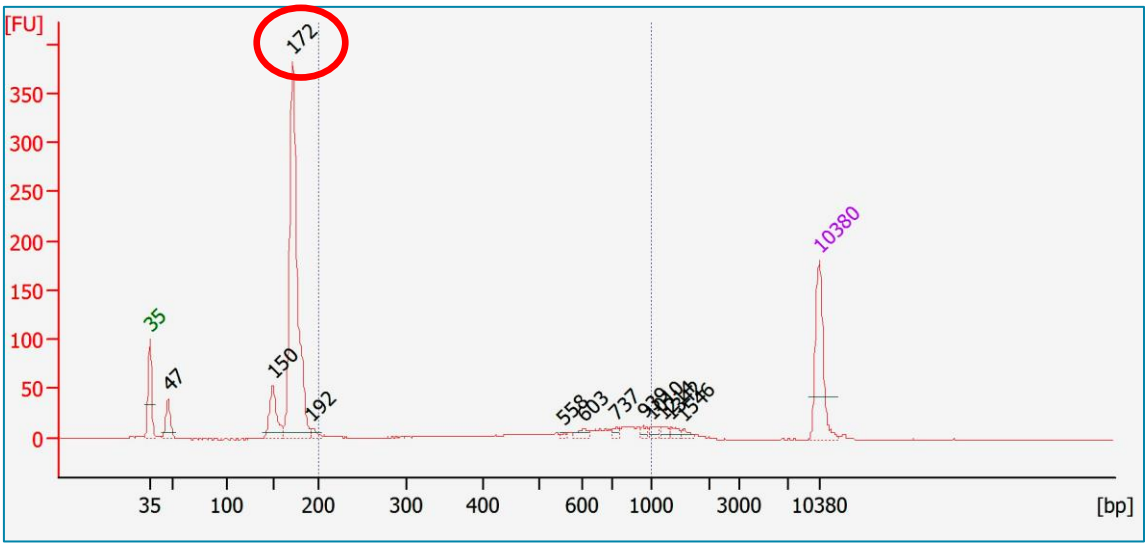
Quantifying dCODE® (RiO) and Sample Tag PCR2 products

Quantification of dCODE® (RiO) PCR2 product
Measure the yield of the dCODE® (RiO) PCR2 product which will be ~170 bp by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay (see fig. 3). Follow the manufacturer's instructions.

Based on the corresponding concentration of the 170 bp peak, dilute an aliquot of dCODE® (RiO) PCR2 product to 0.1–1.1 ng/µL with Nuclease-Free water to use for index PCR of dCODE® (RiO) libraries.

Note: To perform index PCR for the final sequencing libraries, go to page 26.

Figure 3 Sample Bioanalyzer High Sensitivity DNA Trace - dCODE® (RiO) 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	

Quantification of Sample Tag PCR2 products:
Measure the yield of the Sample tag product which will be ~170 bp by using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.
The Bioanalyzer A DNA trace for the Sample Tag PCR2 product is identical to the dCODE Dextramer® (RiO) PCR2 trace shown in figure 3.

Based on the corresponding concentration of the 170 bp peak, dilute an aliquot of the sample tag PCR2 product to 0.1–1.1 ng/µL with Nuclease-Free water to use for index PCR of dCODE® (RiO) libraries.

Note: To perform index PCR for the final sequencing libraries, go to page 26.

Preparation of dCODE® (RiO), BD® AbSeq and Sample Tag Libraries

This section describes how to prepare dCODE® (RiO) libraries when using workflows in which cells are co-stained with BD® AbSeq and Sample Tag antibodies. Use one of the following protocols in combination with the one described here.

- TCR/BCR Full Length and Targeted mRNA and BD® AbSeq (23-24015)
- TCR/BCR Full Length and Targeted mRNA, BD® AbSeq, and Sample Tag (23-24016)
- TCR/BCR Full Length and WTA and BD® AbSeq (23-24019)
- TCR/BCR Full Length and WTA, BD® AbSeq and Sample Tag (23-24020)

The above BD protocols are available at:
https://scomix.bd.com/hc/en-us/articles/9285990045197-VDJ-full-length-TCR-BCR-assay-protocols-Human

The 75 µL supernatant captured in the "Denaturation and self-hybridization" sections of the BD® protocols listed above, will be used as template in the preparation of dCODE® (RiO), BD® AbSeq and Sample Tag libraries beginning from the "Performing dCODE® (RiO), BD® AbSeq, and Sample Tag PCR1" section of this protocol described below.

Performing dCODE® (RiO), BD® AbSeq, and Sample Tag PCR1

1 In the PCR setup area, pipet the following reagents into a new 1.5-mL LoBind® tube.

dCODE® (RiO) and BD® AbSeq Reaction Mix

Component	For 1 Library (µL)	For 1 Library with 20% overage (µL)*
PCR MasterMix	100	120
Universal Oligo	10	12
dCODE®primer 1 (10 uM)	12	14.4
BD® AbSeq Primer	add later**	N/A
Total	**122**	**146.4**

* To compensate for pipetting errors
** 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 PCR1 conditions below).

OR

dCODE® (RiO), 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®primer 1 (10 uM)	12	14.4
Sample Tag PCR1 Primer	1	1.2
BD® AbSeq Primer	add later**	N/A
Total	**123**	**147.6**

* To compensate for pipetting errors
** 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 PCR1 conditions below).

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

3 a. For dCODE® (RiO) and BD® Abseq workflows, pipet 122 µL of the PCR1 reaction mix into a new 1.5-mL tube. Add 68 µL of the dCODE® (RiO) and BD® AbSeq products captured during the "Denaturation and self hybridization" - see page 17.
Pipet-mix 10 times
OR

b. For dCODE® (RiO) and BD® AbSeq and Sample tag workflow, pipet 123 µL of the PCR reaction into a new 1.5-mL tube. Add 67 µL of the dCODE® (RiO), BD® AbSeq and Sample Tag products captured during the "Denaturation and self-hybridization" – see page 17. 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.

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® (RiO) amplicon.

dCODE® (RiO) and BD® AbSeq with or without Sample Tag PCR1 conditions

Part	Step	Cycles	Temp	Time
Part 1	Hot start	1	95°C	3 min
	Denature	6*	95°C	30 sec
	Annealing		60°C	30 sec
	Extension		72°C	1 min
	Hold	1	4°c	∞
	Add: 2.5µl 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 sec
	Annealing		60°C	30 sec
	Extension		72°C	1 min
	Final extension	1	72°C	5 min
	Hold	1	4°C	∞

*Suggested PCR cycles might need to be optimized according to the number of different dCODE Dextramer® used, cell types and cell numbers.

STOPPING POINT: The PCR can run overnight.

Suggested number of PCR cycles

Number of cells in sample	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

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® (RiO) PCR1. Keep the tube on ice and proceed with purification as described below.

Purifying dCODE® (RiO),BD® AbSeq, and Sample Tag PCR1 products

Note: Do not perform purification of PCR products in the PCR setup area.

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 XP magnetic beads to room temperature. Vortex on high speed for 1 minute until the 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 Add 280 µL AMPure beads to theo the PCR1 products, to get a 1.4x AMPure bead/PCR1 (volume) 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 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)
* **NB: It is important to respect the indicated AMPure bead/PRC1 product (V/V) ratio!**

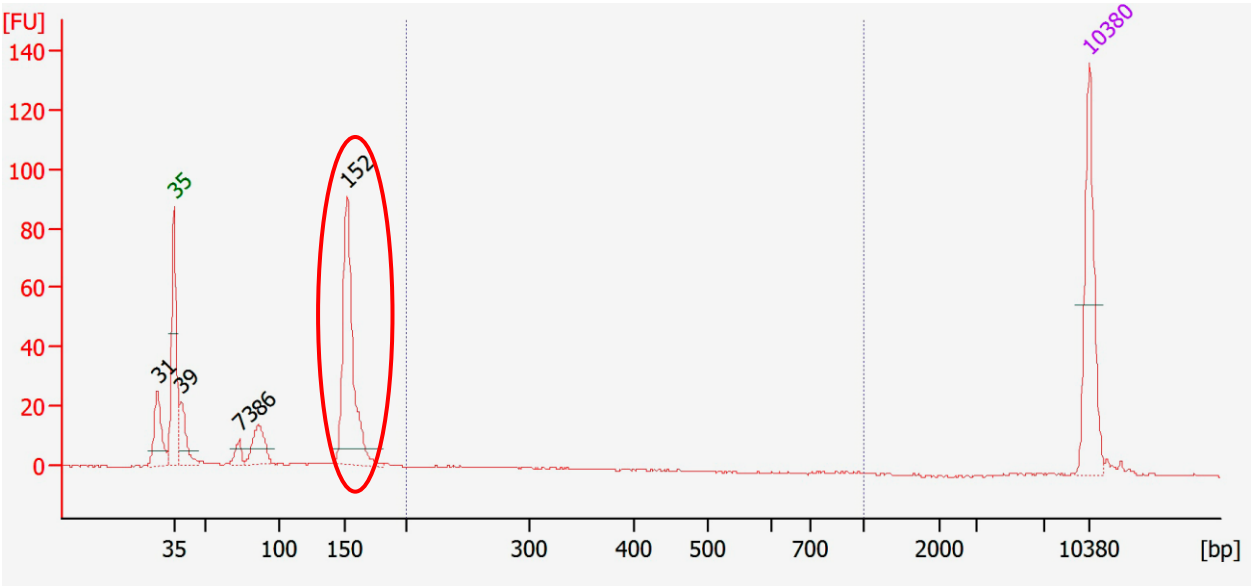
STOPPING POINT: Store at 2–8 °C if proceeding with the protocol within 24 hours or at –25 °C to –15 °C for up to 6 months.

Quantifying BD® AbSeq PCR1 products:

dCODE® (RiO), BD® AbSeq and Sample Tag PCR1 products are all ~150 bp and the amplicons are predominantly BD® AbSeq derived. Measure the yield of the BD® AbSeq amplification using the Agilent 2100 Bioanalyzer with the High Sensitivity Kit assay. Follow the manufacturer's instructions.

Based on the corresponding concentration of the 150 bp peak, dilute an aliquot of the PCR1 product to 0.1–1.1 ng/µL with Nuclease-Free water to use for index PCR of BD® AbSeq libraries. Keep the remaining PCR1 product for the PCR2 amplification of dCODE® (RiO)and Sample Tag barcodes.

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® (RiO) and Sample Tag PCR2

Note: dCODE® (RiO) 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 amplification. BD® AbSeq is not amplified in PCR2.

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

dCODE® (RiO) 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® PCR2 primer (10 uM)	10	12
Nuclease-free water	8	9.6
Total	**45**	**54**

*To compensate for pipetting error

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 Primer	3	3.6
Nuclease-free water	15	18
Total	**45**	**54**

*To compensate for pipetting error

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

3 Move the PCR2 mix outside the PCR setup area.In a new 0.2-mL PCR tube, pipet 5.0 µL of purified PCR1 products into 45 µL of dCODE® (RiO) and Sample Tag PCR2 reaction mixtures. NB: If the experiment involves parallel processing of multiple samples stained independently with dCODE Dextramer®, then care must be taken to avoid cross-contamination of PCR1 products in the PCR2 reaction mix. This can be achieved by pipetting the PCR1 products from different samples in separate lab spaces or by pipetting in a laminar flow hood equipped with UV light.

4 Gently vortex and briefly centrifuge.

5 Program the thermal cycler as follows.

dCODE® (RiO) 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	∞
*Optimization of PCR cycles may be needed according to the experimental setup in terms of: number of different dCODE Dextramer® used in staining of cells, 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® (RiO) and Sample Tag PCR2 products

Note: Do not perform purification of PCR products in the PCR setup area.

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 beads are fully resuspended.
3 Briefly centrifuge the PCR2 products.
4 Pipet 50 µL of dCODE® (RiO) and Sample Tag PCR2 products in separate tubes. Add60 µL AMPure beads to get 1.2x AMPure bead/PCR2 (volume) 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® (RiO) or Sample Tag PCR2 products).
* **NB: It is important to respect the indicated AMPure bead/PRC2 product (V/V) ratio!**

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

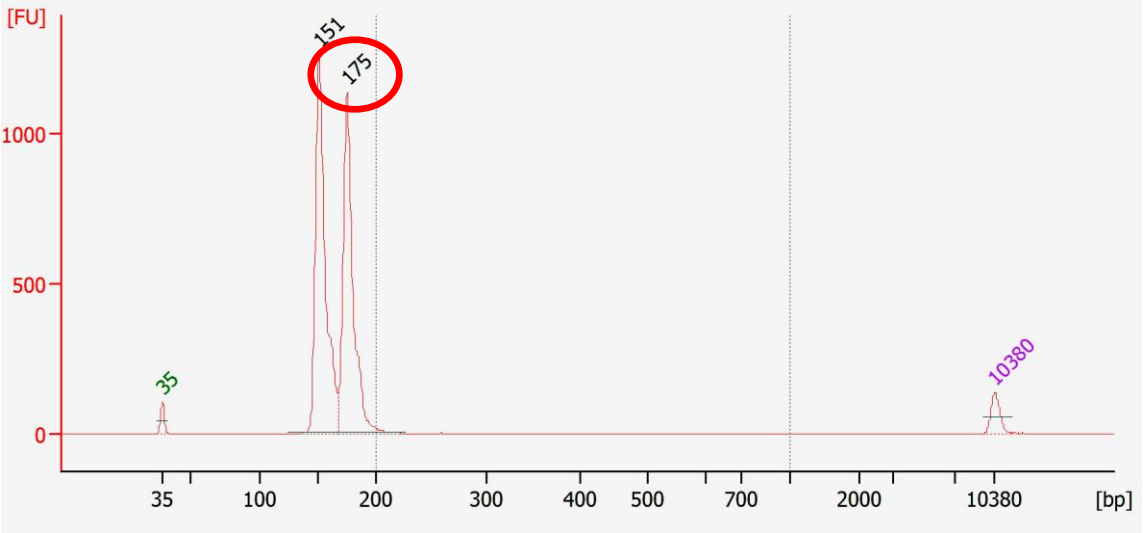
Quantifying dCODE® (RiO) and Sample Tag PCR2 products

Quantification of dCODE® (RiO) PCR2 products:
Measure the yield of the dCODE® (RiO) PCR2 product which is ~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® (RiO) PCR2 product to 0.1–1.1 ng/µL with Nuclease-Free water to use for index PCR of dCODE® (RiO) libraries.

Note: Occasionally there is a ~150 bp peak in the Bioanalyzer trace (see Figure 5). This is an illegitimate PCR2 product that can be ignored, since it is not amplified in the final index PCR reaction. Thus, it will not be part of the final indexed dCODE® (RiO) library.

Figure 5 Sample Bioanalyzer High Sensitivity DNA Trace - dCODE® (RiO) PCR2 product



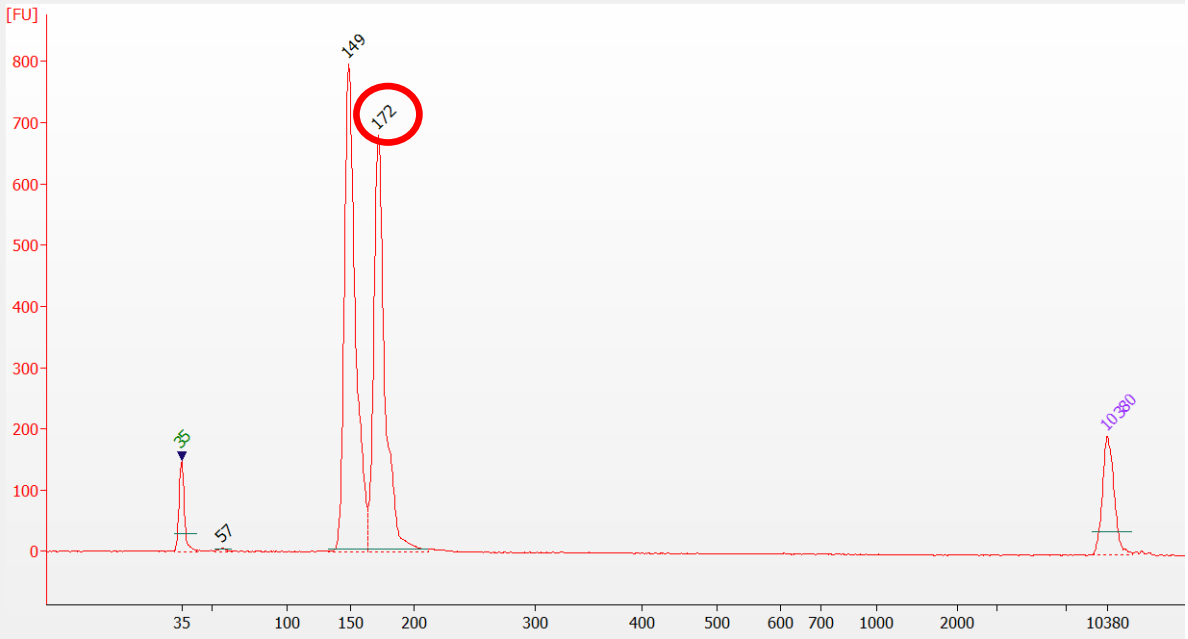
Peak		Size [bp]	Conc. [pg/µl]	Molarity	Observations
1		35	125	5,411.3	Lower Marker
2		151	2,964.08	29, 694.1	
3		175	2,472.67	21,355.2	
6		10,380	75.00	10.9	Upper Marker

Quantification of Sample Tag PCR2 products:
Measure the yield of the Sample Tag 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 PCR2 product to 0.1–1.1 ng/µL with Nuclease-Free water to use for index PCR of dCODE® (RiO) libraries.

Note: Occasionally there is a ~150 bp peak in the Bioanalyzer trace (see figure 6). This is an illegitimate PCR2 product that can be ignored since it is not amplified in the final index PCR reaction. Thus, it will not be part of the final indexed dCODE® (RiO) library.

Figure 6 Sample Bioanalyzer High Sensitivity DNA Trace – Sample tag PCR2 product



		Size [bp]	Conc. [pg/µl]	Molarity [pmol/l]	Observations
1	◀	35	125.00	5	Lower Marker
2		57	7.41	198.1	
3		149	1	12	
4		172	925.92	8	
▶	▶	10	75.00	10.9	Upper Marker

Performing Index PCR to prepare final dCODE® (RiO), 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® (RiO) libraries **must** be indexed with a different index primer from other libraries for downstream bioinformatic processing. See Trimming dCODE® (RiO) sequencing reads, on page 32.
If more than 4 different libraries are produced, additional index PCR primers must be ordered (not provided in the Kits) and can be found in "Ordering Additional Indexes for Rhapsody".

1 In the pre-amplification workspace, pipet reagents into a new 1.5-mL LoBind® tube on ice. Prepare one tube per library. Each library must be individually indexed in separate PCR reactions, using different combinations of Library Forward and Reverse Primers.

dCODE® (RiO), 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**

*To compensate for pipetting errors.
**If more than 4 libraries are generated additional index primers are needed (see note above).

2 Gently vortex mix, briefly centrifuge, and place back on ice.
3 Move index PCR mixes out of the PCR setup area.
4 In new 0.2 mL PCR tubes,
    a. For dCODE® (RiO) libraries, pipet 3.0 µL of 0.1–1.1 ng/µL dCODE® (RiO) PCR2 product into 47 µL dCODE® (RiO) Index PCR mix. (From Quantification of dCODE® (RiO) PCR2 products, on page 24).
    b. 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 Quantification of Sample Tag PCR2 library products, on page 25).
    c. For BD® AbSeq library form purified PCR1 product, pipet 3.0 µL of 0.1–1.1 ng/µL BD® AbSeq PCR2 product into 47 µL BD® AbSeq Index PCR mix. (From Quantifying BD® AbSeq library PCR1 products, on page 20).

5 Gently vortex, and briefly centrifuge.
6 Program the thermal cycler.

dCODE® (RiO), 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.*	95 °C	30 s
Annealing		60 °C	30 s
Extension		72 °C	30 s
Final Extension	1	72 °C	1 min
Hold	1	4 °C	∞

* Cycle number varies based on the concentration of the dCODE® (RiO) and Sample Tag PCR2 products respectively – see table below.

Recommended number of PCR cycles for BD® AbSeq, dCODE® (RiO) and Sample Tag libraries

Concentration of PCR2 template input in Index PCR reaction mix (ng/µL)	Recommended number of PCR cycles for BD® AbSeq	Recommended number of PCR cycles for dCODE® (RiO)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: Do not perform purification of PCR products in the PCR setup area.

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 XP beads to room temperature and vortex at high speed for 1 minute until the beads are fully resuspended.
3 Briefly centrifuge all the index PCR products.
4 Pipet 50.0 µL of each of the index PCR products in separate tubes. Add 40 µL AMPure beads to get a 0.8x AMPure bead/index PCR (V/V) 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).
Perform quality control before freezing samples. See Performing quality control on the final sequencing libraries, on page 29.
* **NB: It is important to respect the indicated AMPure bead/Index PCR product (V/V) ratio!**

STOPPING POINT: Store at –25 °C to –15 °C for ≤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® (RiO) Index PCR product (~270 bp)

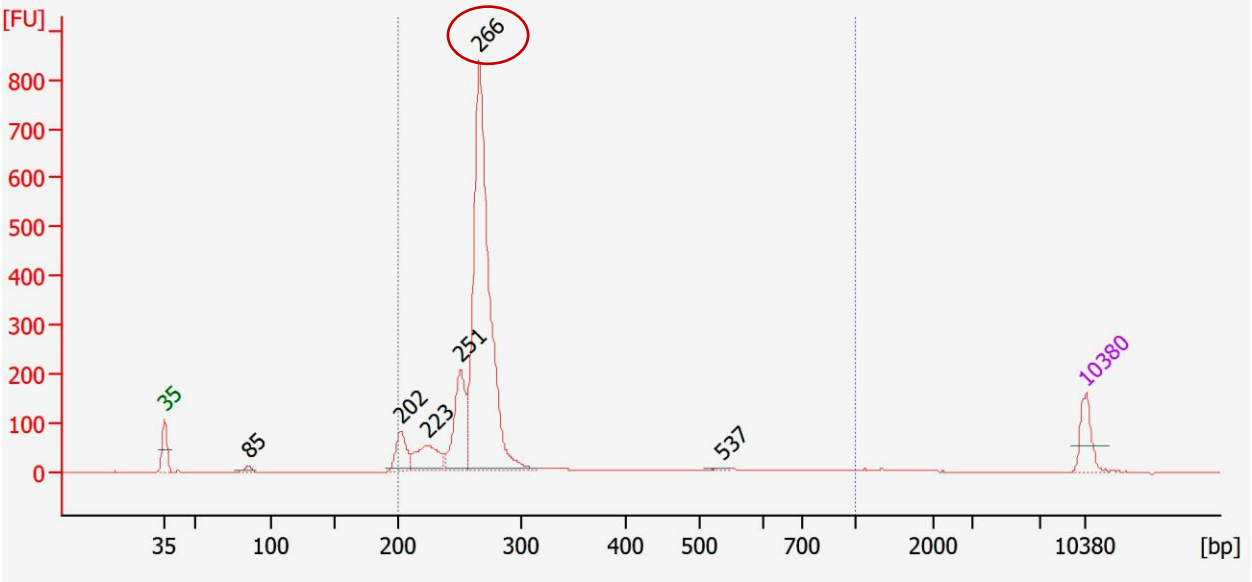


Figure 7 Sample Bioanalyzer High Sensitivity DNA Trace – BD® AbSeq Index PCR product (~250 bp)

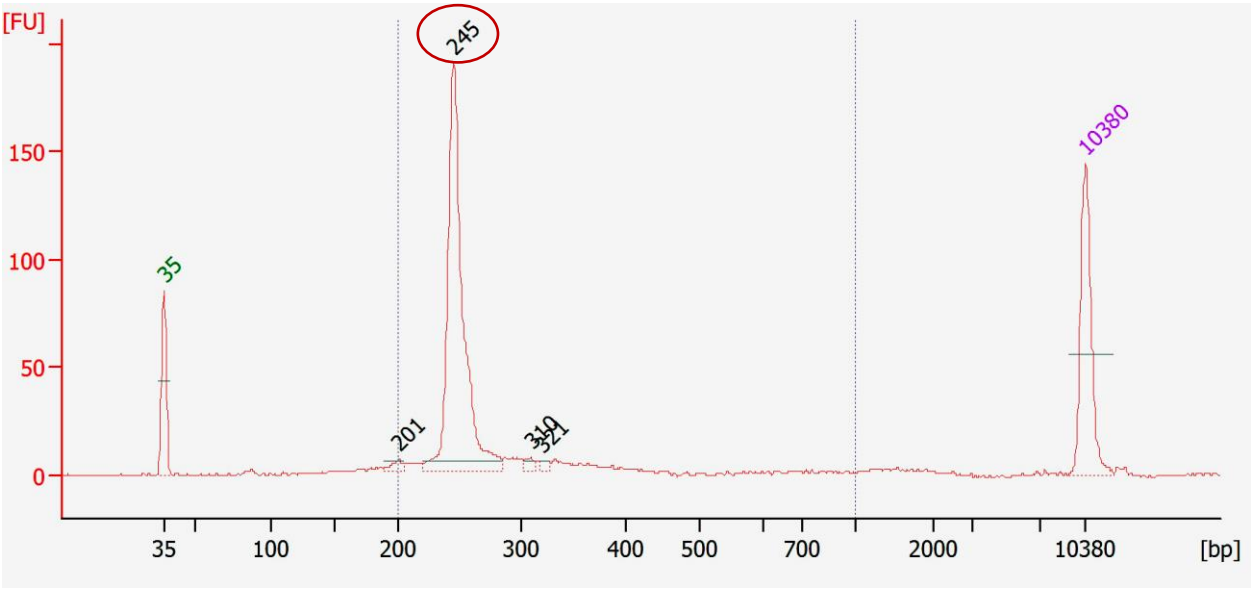
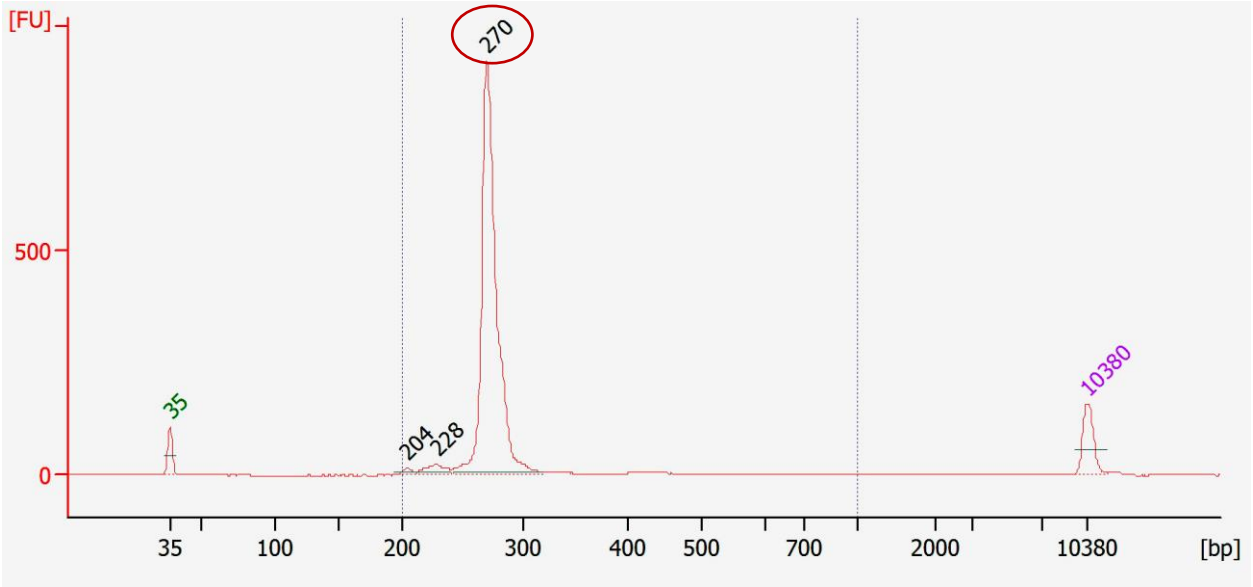


Figure 8 Sample Bioanalyzer High Sensitivity DNA Trace – Sample Tag Index PCR product (~270 bp)



dCODE® (RiO) Sequencing Recommendations

The amount of sequencing needed for dCODE® (RiO) libraries will vary depending on the experimental setup in terms of number of different dCODE Dextramer® used for staining of cells, cell types and cell numbers. 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® (RiO) libraries using Qubit quantitation concentration (ng/µL) and average Bioanalyzer size (200 bp - 1000 bp). Use the calculated molar concentrations to pool libraries.

dCODE® (RiO) 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: A dCODE® (RiO) reference file is needed to deconvolute the dCODE® library. The dCODE® (RiO) specific barcodes are order specific, and can be found in the delivery note, or can be obtained through Immudex customer support. (customer@immudex.com)

Note: see BD TCR/BCR Full Length Protocols for sequencing recommendations for non-dCODE® (RiO) libraries.

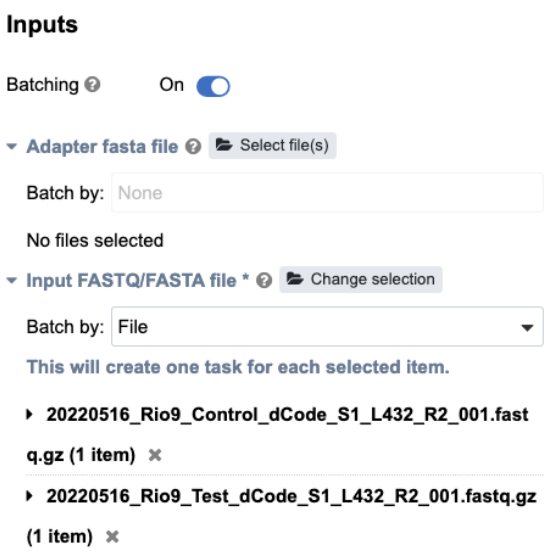
Note: Please see example pool calculation, or download excel document "dCODE® (RiO) pool calculation".

Trimming dCODE® (RiO) Sequencing Reads

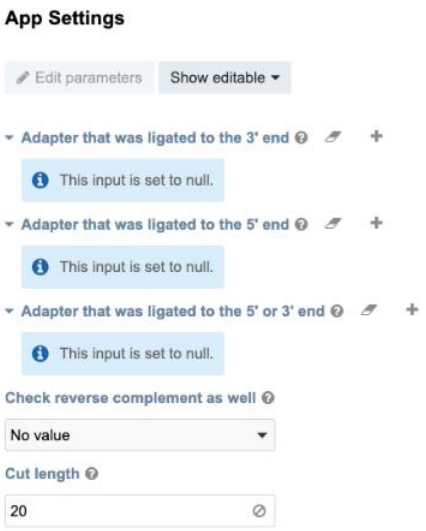
BD Rhapsody™ Analysis Pipelines are available on the Seven Bridges Genomics platform at https://www.sevenbridges.com/bdgenomics/ and can also be run using a local installation following the instructions in the *BD Single Cell Genomics Analysis Setup User Guide* (Doc. ID 47383). Importantly, prior to loading the dCODE® (RiO) sequencing reads into the pipeline run, Read 2 of the dCODE® (RiO) reads will need to be trimmed. Note that separate indexing of the dCODE® (RiO) library will produce a separate file for the dCODE® (RiO) libraries, allowing the user to trim only the dCODE® (RiO) FASTQ reads.

To trim the Read 2 of the dCODE® (RiO) 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 Read2 (R2) of the dCODE® (RiO) library. Note only one FASTQ file can be processed at a single time.
  - You can run multiple FASTQ files by selecting the "Batch" options.

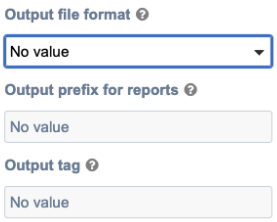


- *App Settings Cut length:* set to 20.



- *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 (follow the instructions on the *BD Single Cell Genomics Analysis Setup User Guide*, Doc. ID 47383)
Note: the trimmed R2 file must have the same <sample> name as the original untrimmed <sample> name.
All libraries from the same cartridge are analyzed in a single pipeline run, including Targeted mRNA with WTA, BD® AbSeq, Sample Tag, dCODE® (RiO) libraries.

3 Before running the BD Rhapsody™ analysis pipeline, obtain an appropriate ".fasta" reference file for the dCODE Dextramer® panel. The dCODE Dextramer® (RiO) 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 an BD® AbSeq .fasta file reference file using this link: http://abseq-ref-gen.genomics.bd.com/

4 Upload the dCODE Dextramer® (RiO) 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® (RiO) 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).

Technical support
For additional Tips & Tricks, FAQs and protocols, please visit
https://www.immudex.com/resources/
or contact our support team at customer@immudex.com
Telephone: +45 3110 9292 (Denmark)

Note: Immudex® is the sole manufacturer and provider of dCODE Dextramer® (RiO) reagents, and support related to these products is through Immudex.