dCODE Dextramer® (RiO)
Library Preparation Protocol

dCODE® specific library preparation from BD Rhapsody™ cDNA capture beads

Overview

This protocol provides instructions on creating dCODE Dextramer® with or without BD® AbSeq Assay and targeted mRNA single cell libraries with the BD Rhapsody™ Express Single-Cell Analysis System and BD Rhapsody™ Single-Cell Analysis System, followed by sequencing on Illumina sequencers.

This protocol is an addendum to the BD™ protocol (mRNA Targeted and BD® AbSeq Assay library preparation with the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit, see required protocols below) and is used to obtain dCODE Dextramer® libraries alongside targeted mRNA and optional BD® AbSeq Assay libraries.

cDNA encoded on the single-cell capture beads (see required protocols, below) is amplified in PCR1, generating a universal cDNA library that contains the cell barcoded dCODE®, targeted mRNA, and optional AbSeq libraries.

The dCODE (and optional AbSeq) PCR1 products are separated form the longer targeted mRNA library by double-sided size selection, using Agencourt® AMPure® XP magnetic beads.

To increase the sensitivity of the dCODE® library, a dCODE®-specific PCR2 reaction is introduced using a dCODE® specifid library primer (PCR2, see "Schematic Workflow"). Separation of the two libraries allows the user to adjust the ratio of the dCODE® library in the final sequencing pool alongside mRNA and optional AbSeq® libraries.

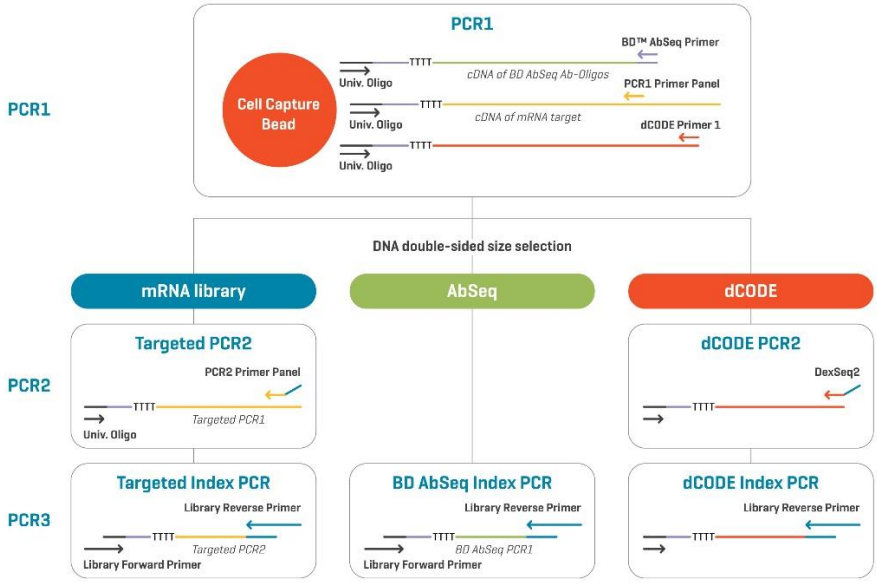
Note: The dCODE® and BD Rhapsody™ mRNA targeted PCR1 products undergo PCR2 amplification. The BD® AbSeq Assay library will go directly from PCR1 into indexing PCR3. After index PCR3, the dCODE®, BD Rhapsody™ mRNA, and BD® AbSeq Assay libraries can be combined for sequencing.

Note that the dCODE® products should be indexed uniquely from mRNA or BD® AbSeq products for successful preparation of dCODE® targeted mRNA and AbSeq™ libraries for sequencing.

- The dCODE® and BD Rhapsody™ mRNA targeted PCR1 products undergo PCR2 amplification.
- The prepered libraries undergo index PCR3 with seperate index primers (if the libraries are to be pooled before Illumine sequencing)
- After index PCR3, the dCODE®, BD Rhapsody™ mRNA, and BD® AbSeq Assay libraries can be combined for sequencing.

Note: dCODE Dextramer® can be used in combination with the BD® AbSeq Assay Library Preparation with or without AbSeq™ antibody staining.

Schematic workflow



PCR1: Universal oligo (black), AbSeq™(Purple), mRNA target (Yellow), and dCODE® (Orange), represent the primers used in PCR1 for amplification of the cDNA bound to the capture beads.

PCR2: Selective amplification of the mRNA targeted library and the dCODE® library from PCR1 products.

PCR3: Index primers compatible with Illumina Sequencing systems are used to prepare libraries for Illumina sequencing.

Required materials (not provided)

Amplification primers for dCODE Dextramer® specific library preparation (see below).
Agencourt® AMPure® XP magnetic beads (Beckman Coulter Cat. No. A63880).
Absolute ethyl alcohol, molecular biology grade (major supplier).
Nuclease-free water (major supplier).
Magnetic Separation Rack for 1.5 ml tubes (major supplier).
Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Cat. No. Q32851).

BD Rhapsody™ specific reagents:
BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Cat. No. 633774)
For a complete list of materials, see BD Rhapsody™ protocol.

dCODE® specific Amplification primers

dCODE PCR1 primer: 5'-GGAGGGAGGTTAGCGAAGGT-3'
dCODE PCR2 primer: 5'-CAGACGTGTGCTCTTCCGATCTGGAGGGAGGTTAGCGAAGGT-3'

dCODE® specific primers can be ordered from a preferred DNA oligo provider and should be used at 10µM.

Required protocols

This protocol:

- "dCODE Dextramer® (RhaSeq) library preparation protocol (TF1196)" is an addendum to the BD™ protocol:
- "mRNA Targeted and BD® AbSeq Library Preparation with the BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Doc ID: 214293)".

Before beginning this protocol, the user should have an on-bead cDNA library obtained by following the protocols:

- "dCODE® Staining procedure for BD Rhapsody™ package insert (TF1099)".
- "Single Cell Capture and cDNA Synthesis with the BD Rhapsody™ (Doc ID: 210966)".

Collectively, these protocols provide instructions on creating dCODE Dextramer® DNA library, targeted mRNA, and optional BD® AbSeq Assay single cell barcoded libraries with the BD Rhapsody™ Express Single-Cell Analysis System and BD Rhapsody™ Single-Cell Analysis System.

Performing PCR 1 amplification of captured dCODE®, BD® AbSeq oligos and targeted mRNA

PCR 1

Follow the protocol:

- mRNA Targeted and BD® AbSeq Library Preparation with the BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Doc ID: 214293)

with the modifications and additions described here:

In the Step "Performing PCR1", use the following tables to make the PCR reaction mix for:

A: dCODE®, AbSeq, and mRNA targeted libraries
B: dCODE® and mRNA targeted libraries, only

(use 20% overage if multiple samples are amplified)

A: PCR1 reaction mix for dCODE®, AbSeq, and targeted mRNA

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

A: Kit component name		
	1 sample (µL)	**2 samples +20% overage (µL)**
Nuclease-Free Water[a)]	4,0	9,6
PCR MasterMix [a)]	100,0	240,0
PCR1 primer panel (mRNA targeted primers)[b)]	40,0	96,0
AbSeq PCR1 Primer [a)]	12,0	28,8
dCODE primer 1 (10µM) [c)]	12,0	28,8
Universal Oligo [a)]	20,0	48,0
Bead RT/PCR Enhancer [a)]	12,0	28,8
Total volume	**200**	**480**

B: PCR1 reaction mix for dCODE® and targeted mRNA, only

B: Kit component name		
	1 sample (µL)	2 samples +20% overage (µL)
Nuclease-Free Water[a)]	16,0	38,4
PCR MasterMix [a)]	100,0	240,0
PCR1 primer panel (mRNA targeted primers)[b)]	40,0	96,0
dCODE primer 1 (10µM) [c)]	12,0	28,8
Universal Oligo [a)]	20,0	48,0
Bead RT/PCR Enhancer [a)]	12,0	28,8
Total volume	**200**	**480**

a) Reagents provided in "BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Cat. No. 633774)"
b) Order from BD Biosciences
c) Primers not provided, see required materials

2. In the section "Performing PCR1" in the BD Rhapsody™ protocol(1,required protocols) perform steps 2 through 14.

Purifying PCR1 products by double-sided size selection

Perform double-sided AMPure size selection to separate the shorter dCODE® and BD® AbSeq Assay PCR1 products (~170 bp) from the longer mRNA targeted PCR1 products (350–800 bp).

Note: Perform selection in the post-amplification workspace

Proceed with the following sections of the Rhapsody protocol(1,required protocols):

- Separating BD® AbSeq PCR1 products from mRNA targeted PCR1 products, step 1 through step 7.
  Note: Keep both the supernatants (contain the dCODE® and BD® AbSeq Assay amplification products) and the AMPure beads bound fraction (mRNA targeted amplification products) for purification.
- Purifying mRNA targeted PCR1 products, step 1 through 8
- Purifying BD® AbSeq Assay PCR1 products, step 1 through 12
  Note: This fraction contains both the dCODE® and BD® AbSeq Assay libraries ("dCODE/AbSeq library").

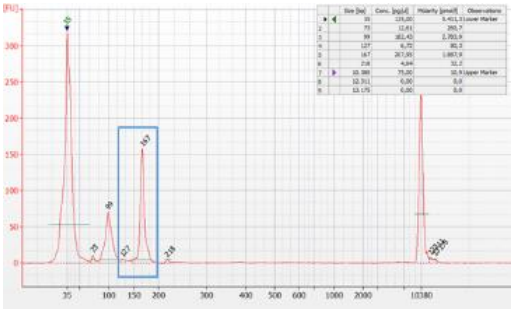
Stopping point: Store at 2°C to 8°C before proceeding in ≤24 hours or at –25°C to –15°C for ≤6 months.

Quantifying BD® AbSeq PCR1 products

The purified eluate from PCR1 containing "dCODE/AbSeq library" libraries

Proceed with step 1 through 2 in this section of the BD Rhapsody™ protocol

dCODE® and AbSeq PCR1 amplicon is ~ 170bp



Both the BD® AbSeq and dCODE® amplicons are ~170bp and can not be quantified seperatly at this step.
Note: Smaller contaminating products may be present after PCR1.

Performing PCR 2 to amplify dCODE® library from the purified dCODE®/AbSeq fraction from PCR 1
Note: PCR2 using PCR1 product is an additional section that is not described in the BD Rhapsody™ protocol(1,required protocols).

dCODE® specific PCR2

The dCODE®/AbSeq purified PCR1 product is used for amplifying the dCODE® library, using unique dCODE® library primers (see required matrials).

1. In pre-amplification workspace, pipet reagents into a new 1.5 ml LoBind Tube on ice *(if more than one sample, make 20% overage of the reaction mix)*

dCODE PCR 2 reaction mix:

Kit component name	1 sample (µL)	2 samples +20% overage (µL)
Nuclease-Free Water	8	**19,2**
PCR MasterMix	25	**60,0**
Universal Oligo	2	**4,8**
dCODEprimer 2[b)]	10	**24,0**
Total	45	**108**
PCR1 purified AbSeq and dCODE library (UNDILUTED)	**5 µL/sample**	**5 µl/sample**

a) Reagents for PCR2 amplification is included in the "BD Rhapsody™ Targeted mRNAand BD® AbSeq Amplification Kit (Cat. No. 633774)"

b) dCODE specific primer 2 is not provided (see required reagents).

2. Gently vortex mix, briefly centrifuge, and place back on ice.
3. Bring PCR2 mix into post-amplification workspace.
4. In a new 0.2 ml PCR tube pipet 5.0 µL PCR1, purified dCODE®/AbSeq product into 45.0 µL dCODE® PCR2 reaction mix. Gently vortex and briefly centrifuge.
5. For dCODE® library PCR2, program the thermal cycler. *(Do not use fast cycling mode)*

Step	Cycles	Temp	time
Hot start	1	95°C	3 min
Denature	10*	95°C	30 sec
Annealing		66°C	30 sec
Extension		72°C	1 min
Final extention	1	72°C	5 min
Hold	1	4°C	∞

* Cycle number might require optimization if cell number is low, or dCODE specific cells are of low frequeny.

Purifying the PCR2 dCODE® product

1. Bring AMPure XP beads to room temperature (15°C to 25°C), and vortex at high speed 1 min until beads are fully resuspended.
2. Briefly centrifuge PCR2 products.
3. To 50.0 µL PCR2 products, pipet 60 µL AMPure beads.
4. Pipet-mix 10 times and incubate at room temperature (15°C to 25°C) for 5 min.
5. Place tube on strip tube magnet for 3 min. Remove supernatant.
6. Keeping tube on magnet, gently add 200 µL fresh 80% ethyl alcohol into tube and incubate 30 sec. Remove supernatant.
7. Repeat step 6 one time.
8. Keeping tube on magnet, use a small-volume pipette to remove residual supernatant from tube and discard.
9. Air-dry beads at room temperature (15°C to 25°C) for 3 min.
10. Remove tubes from magnet and resuspend bead pellet in 30 µL Elution Buffer (Cat. No. 91-1084). Pipet-mix until beads are fully resuspended.
11. Incubate at room temperature (15°C to 25°C) for 2 min and briefly centrifuge.
12. Place tubes on magnet until solution is clear, usually ≤30 seconds.
13. Pipet entire eluate (~30 µL) of the sample into a new 1.5 ml LoBind Tubes (purified dCODE PCR2 products).
14. Estimate the concentration of the sample by quantifying 2 µL of the PCR2 products with a Qubit™ Fluorometer, using the Qubit dsDNA HS Assay Kit.

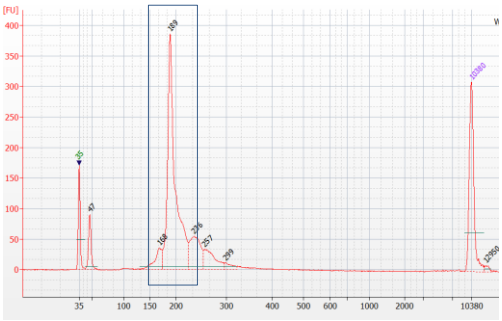
Stopping point: Store at 2°C to 8°C before proceeding on the same day or at –25°C to –15°C for ≤6 months.

Quantify the dCODE® library

Measure yield of the dCODE® PCR2 product (amplicon is app. 190 bp) by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626)
Follow the manufacturer's instructions.

Note:The dCODE® PCR2 product can be distinguished from the BD® AbSeq PCR1 product by app. 20 bp difference in amplicon size. The BD® AbSeq amplicon form PCR1 may be visul as a 170bp trace, but is not amplifyed in the PCR2 dCODE® specific reaction.

dCODE PCR2 product app.190 bp



Performing PCR2 on the mRNA targeted PCR1 products

The mRNA targeted PCR1 products are further amplifyed in this PCR2.
Follow step 1 through 6 in this secction of the BD Rhapsody™ protocol(1,required protocols).

Purifying mRNA targeted PCR2 products

Purification of the targeted mRNA product from PCR2.
Follow step 1 through 15 in this section of the BD Rhapsody™ protocol(1,required protocols)

Index PCR3

Performing index PCR to prepare final libraries

Index PCR3 is performed seperately for each of the purified PCR1 AbSeq, PCR2 dCODE, and PCR2 mRNA products.
If the libraries will be pooled for sequencing, it is required to use different reverse index primers for each library (4 different reverse index primers are provided in the "BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit (Cat. No. 633774)).

In the section of the BD Rhapsody™ protocol "Performing index PCR to prepare final libraries", proceed with the following:.

- For mRNA libraries, follow all steps pertaining to mRNA in this section.
- For optional BD® AbSeq Assay libraries, follow all steps pertaining for BD® AbSeq in this section.
- For the dCODE® libraries follow all steps pertaining for BD® AbSeq Assay in this section, **but use the dCODE® PCR2 product as input.**
- For dCODE® index amplifiation use the same suggested PCR cycles as for the BD® AbSeq amplification.

Purifying index PCR products

Proceed with the following:

- For mRNA libraries, follow all steps pertaining to mRNA.
- For dCODE® and BD® AbSeq libraries, follow all steps pertaining to BD™ AbSeq.
- For dCODE® use the same ratio of AmPure® beads as is used for the BD® AbSeq purification.

Proceed to the section "Performing quality control on the final sequencing libraries".

Performing quality control on the final sequencing libraries

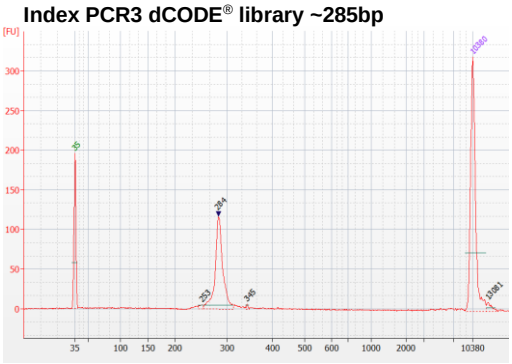
In this section of the BD Rhapsody™ protocol proceed with the following:.

- For the dCODE® PCR3 and the BD® AbSeq PCR3 library, follow all steps pertaining to BD® AbSeq Assay in this section.
- For mRNA libraries, follow all steps pertaining to mRNA in this section.

Measure the yield of the index PCR3 products by using the Agilent Bioanalyzer with the High Sensitivity Kit (Agilent Cat. No. 5067-4626). Follow the manufacturer's instructions.

Note:The dCODE® PCR3 final library can be distinguished from the AbSeq™ library by the ~20 bp difference in amplicon size.

Index PCR3 dCODE® library ~285bp



Sequencing requirements

For sequencing of the dCODE® library, follow the requirement and recommendations for BD® AbSeq Assay in the "mRNA Targeted and BD® AbSeq Library Preparation with the BD Rhapsody™ Targeted mRNA and BD® AbSeq Amplification Kit, protocol (Doc ID: 214293).

Required protocols

[1] mRNA Targeted and BD® AbSeq Library Preparation with the BD Rhapsody™ Targeted mRNA and AbSeq Amplification Kit (Doc ID: 214293).

[2] Single Cell Capture and cDNA Synthesis with the BD Rhapsody™ system (Doc ID: 210966).

[3] dCODE_Staining_procedure_for_BD-Rhapsody_package_insert_(TF1099.01).

Note: Doc ID refers to BD protocol documentation number.

Tech support

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