

dCODE Dextramer® (RiO) staining protocol

Introduction

This protocol describes staining of hPBMC samples with dCODE Dextramer® (RiO), BD® AbSeq antibodies, BD® Single-Cell Multiplexing Kit (SMK), and fluorochrome labeled FACS antibodies and subsequent FACS sorting to enrich for the desired antigen specific immune cells.

It is recommended to include relevant BD® AbSeq assay antibodies for more accurate phenotyping, and detection of antigen specific cells in subsequent data analysis.

BD Rhapsody™ System Workflow with dCODE Dextramer®

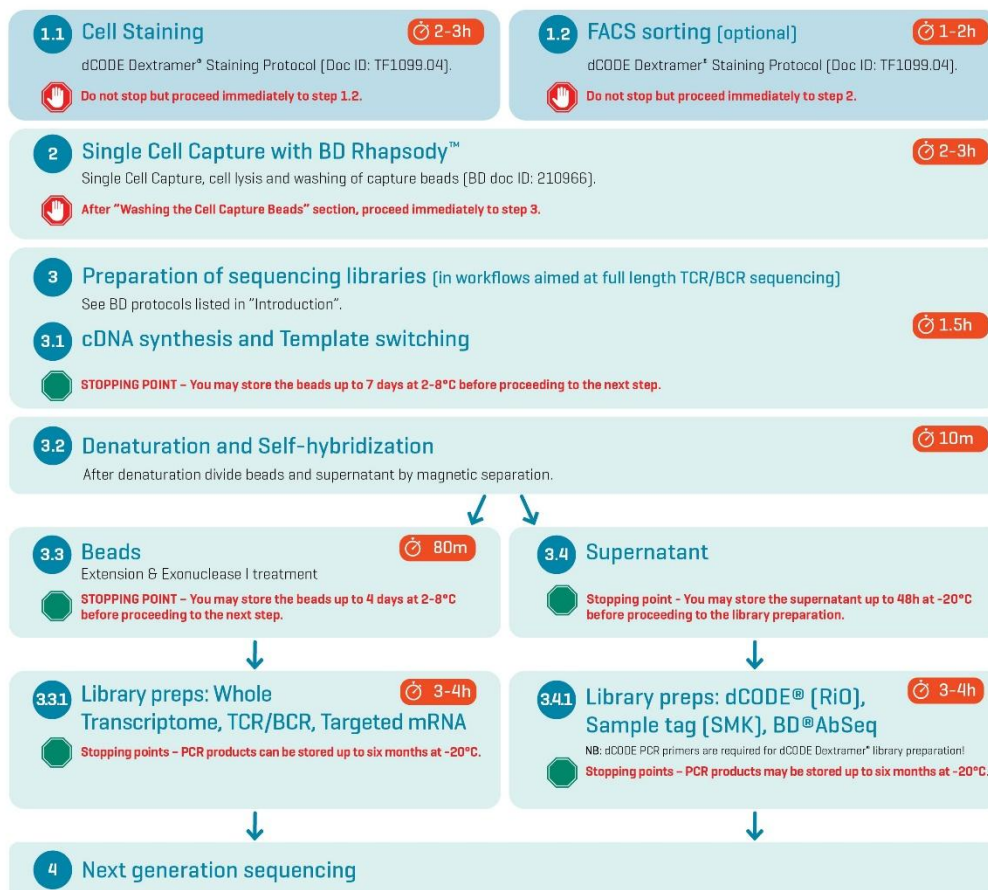
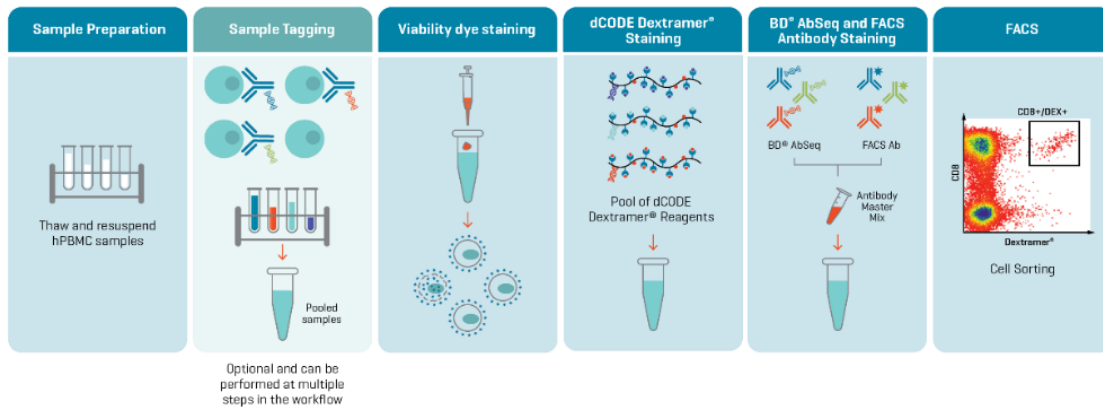


Fig. 1: Workflow describing single cell analysis of antigen specific cells with dCODE Dextramer® and the BD Rhapsody™ System. This protocol involves steps 1.1 and 1.2.

Staining Procedure at a Glance

dCODE Dextramer® RiO WORKFLOW – Staining workflow overview



Materials provided:

- MHC dCODE Dextramer® reagents and/or CD1d dCODE Dextramer® / MR1 dCODE Dextramer® / HLA-G dCODE Dextramer® and/or U-Load dCODE Dextramer®.

Not provided Required Materials:

- 5 mL Falcon polystyrene disposable 12 x 75-mm test tubes or equivalent.
- 1,5 mL LoBind® Eppendorf tubes or equivalent.
- Cell labeling buffer: PBS with 5% FCS and 0,1 g/L sheared herring sperm DNA (Sigma-Aldrich D3159).
- Wash buffer: PBS, pH 7.4 containing 1-5% FCS.
- 100 µM d-Biotin (e.g., Avidity, cat# BIO200 or Thermo-Fisher cat# B20656 or similar) diluted in PBS, pH 7.4.
- LIVE/DEAD Fixable Viability Stain.
- Fluorescent FACS Antibodies (with labels compatible with PE on the dCODE Dextramer®). See appendix for choice of antibodies. If applied.
- Oligo conjugated BD® AbSeq assay, and if applied.
- BD® Single Cell Sample Multiplexing Kit (referred to as Sample tagging), if applied.
- BD Rhapsody™ Single-Cell Multiomics System.
- BD Rhapsody™ Cartridge Reagent Kit.
- All required consumable components for the BD Rhapsody™ Single-Cell Multiomics System for Universal 5' Gene Expression. Please consult the BD Biosciences website: [Single-Cell Multiomics Reagents | BD Biosciences](#)

The subsequent preparation of the dCODE® DNA libraries, with the Rhapsody™ library preparation kits, requires dCODE® specific primers for PCR1 and PCR2:

- PCR1: 5'- GGAGGGAGGTTAGCGAAGGT
- PCR2: 5'- CAGACGTGTGCTCTCCGATCTGGAGGGAGGTTAGCGAAGGT

These are not provided with the dCODE Dextramer® reagents, or with the Rhapsody™ reagents kits, and must be provided by the user.

Critical: The cell labeling buffer must contain sheared DNA and it is important that the size of the DNA fragments is <50 bp!

1.1 Staining Protocol

This protocol is optimized for hPBMC (human peripheral blood mononuclear cells) samples. When using other types of samples, please contact customer@immudex.com.

Please see Appendix B for important notes on the staining protocols including recommendations when analyzing clonal cell lines.

Sample preparation

1. Thaw PBMC cells and immediately resuspend them in 10 mL wash buffer.
2. Centrifuge 300x g for 10 min and remove supernatant to remove DMSO from the cells

Viability dye staining

1. Resuspend up to 3×10^6 washed PBMCs in 1 mL wash buffer.
2. Add recommended volume of viability stain (follow manufactures protocol).
3. Incubate for 15 min. at room temperature (all incubations must be performed shielded from light).
4. Add 9 mL Wash buffer, centrifuge 300 x g for 10 min, resuspend cells in 50 μ L cell labeling buffer.

dCODE Dextramer® Staining

1. To prepare a pool of multiple dCODE Dextramer® reagents, mix the following reagents in a 1.5 mL LoBind® Eppendorf tube:
 - a. First Add 0.2 μ L of 100 μ M d-Biotin per dCODE Dextramer® into an empty tube.
 - b. Add 2 μ L of each dCODE Dextramer® reagents
 - c. Add 5 μ L of each peptide loaded U-Load dCODE Dextramer® reagents
2. Vortex the dCODE Dextramer® pool briefly. **The pool must be used shortly after preparation and cannot be stored!**
3. Centrifuge the pool at 10,000 x g for 1 min to avoid transferring any potential precipitate
4. Add the pool (supernatant) to the cell sample and vortex briefly
5. Incubate at room temperature in the dark for minimum 30 min
 - a. For MHC I, CD1d, MR1, and HLA-G dCODE Dextramer®, **allow the Dextramer reagents to stain for a minimum of 10 min before addition of the antibody pool.**
 - b. MHCII dCODE Dextramer® can be incubated simultaneously with the antibody pool
 - c. When working with a total volume of dCODE® reagents exceeding 150 μ l see appendix B

Antibody staining

1. Centrifuge BD® AbSeq antibody tubes at 400 × g for 30 sec and place on ice.
2. Pipet the BD® AbSeq and FACS antibodies into a new 1.5 mL LoBind® Eppendorf Tube on ice:

Antibody labeling master mix

Component	1 sample	For samples >1, add 30% overage volume
Per BD® AbSeq Ab	2 µL	2.6 µL
Per FACS Ab	Recommend volume*	Recommended vol* x 1.3
Cell labelling buffer	Add to final volume of 100µL	Add to final volume of 130 µL

*Vendor recommendation.

1. Pipet-mix the labeling master mix and place on ice, shielded from light, until used.
2. Add the labeling master mix to the dCODE Dextramer® stained sample(s) and mix by pipetting.
3. Incubate at room temperature for 30 min., shielded from light – **see appendix B!**
4. To the stained sample, add 2 mL wash buffer.
5. Centrifuge at 300-600 x g for 5 min. and remove the supernatant.
For highest cell retention, invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from tube rim.
6. Repeat for a total of 3 washes and resuspend cells in remaining buffer.
7. Add adequate volume of Wash buffer and store sample on ice.
 - a. For subsequent cell sorting, cell concentration / resuspension volume depends on the Sorter.
 - b. For resuspension volume when continuing directly to the BD Rhapsody™ system please refer to BD Rhapsody™ user guides - see step 2-4 below.
8. Proceed to cell sorting or continue with partitioning of single cells on the BD Rhapsody™ system.

Critical: do not pause the procedure here!

1.2 Fluorescence-activated cell sorting (FACS)

FACS enrichment of the dCODE® positive cells is recommended

1. Sort the antibody and dCODE Dextramer® positive gated cells
- follow the guidelines and practices of your sorting facility.
The recommended sort-mode is "Yield"
2. Collect sorted cells into a tube containing 500 µL FCS or a suitable buffer at 4°C to improve viability.
(Keep the unsorted and sorted cells at 4°C while performing the cell sorting).
3. Centrifuge the sorted cell sample 300-600 x g for 5-10 min. (depending on the sorting volume), invert to decant supernatant into biohazardous waste. Keep the tube inverted and gently blot on a lint-free wiper to remove residual supernatant from tube rim.
4. Go immediately to Single-Cell capture with the BD Rhapsody™ system.

Critical: do not pause the procedure here!

2. Single cell capture with BD Rhapsody™

Perform single cell capture and cDNA synthesis, following the BD protocols for “Single Cell Capture and cDNA Synthesis” that can be found here:

<https://scomix.bd.com/hc/en-us/articles/360023293851-Targeted-Gene-Expression-Analysis-Protocols>

Barcode oligonucleotides attached to dCODE Dextramer® (RiO) and BD® AbSeq antibodies have a 3' polyA sequence that is captured by hybridization to the 3' terminal complementary polyT sequence of the barcode on the bead inside the microwell of the Rhapsody cartridge. After cDNA synthesis the duplexes are denatured and the supernatant is used for construction of the dCODE®, and BD® AbSeq libraries containing information about pMHC/TCR specificity and cell surface proteins from the captured cells.

3. dCODE Dextramer® (RiO) library preparation

To finish the workflow to first stopping point, please follow the “dCODE Dextramer® (RiO) Library Preparation Protocol” available at <https://www.immudex.com/resources/protocols/> together with the relevant BD Rhapsody™ System Library Preparation Protocols: <https://scomix.bd.com/hc/en-us/categories/360000838932-User-Guides-and-Protocols> (see Fig. 1).

Above protocols provide instructions on cartridge loading and creating the dCODE Dextramer® DNA library in combination with BD® AbSeq, sample tag (SMK), mRNA, and TCR/BCR Full length(V(D)J) DNA libraries.

Appendix A:

dCODE Dextramer® (10x) reagents consist of a dextran polymer backbone carrying multiple MHC, CD1d, MR1, or HLA-G-antigen complexes, and R-phycoerythrin (PE) for sorting of dCODE Dextramer® (10x) positive cells before loading them on the 10x Chromium platform. In addition, each dCODE Dextramer® has a DNA oligonucleotide attached with a DNA barcode sequence that defines the monomeric MHC-antigen complexes on the Dextramer®.

The DNA Barcode oligo comprises:

- dCODE® (RiO) specific PCR handle, compatible with BD Rhapsody™ library preparation and PCR amplification
- A Unique Molecule Identifier (UMI) sequence.
- ID sequence (barcode) that specifies the MHC-peptide specificity (compatible with BD® Abseq barcodes)
- A poly A sequence that is captured by a complimentary poly T sequence of the BD Rhapsody™ bead.



Critical:

Our DNA barcodes are **not unique** to a given dCODE Dextramer® specificity but is assigned randomly during production. It is crucial, during planning of the experiment and ordering of the products, to ascertain that their barcode sequences are different if used together with another order of dCODE® reagents.

Appendix B

Notes for dCODE Dextramer® staining

- These protocols are optimized for hPBMCs. In the case of clonal cell lines, it is important to use fewer cells when preparing the sample: We recommend a maximum of 5×10^4 cells.
- d-biotin is required to avoid artefacts during staining when multiple MHC-Dextramer® reagents are pooled.
- To mitigate lower staining efficiency at larger staining reaction volumes, the incubation time may be increased. If the total volume of dCODE® reagents exceed 150µl we recommend 30 min incubation before addition of antibodies (total incubation time with dCODE® reagents of 60 min!)
- Negative controls are important in single cell experiments involving barcoded dCODE Dextramer® reagents to define thresholds for identification of antigen-specific cells in downstream data analysis. Background staining can be both allele-specific and donor-dependent. We therefore recommend staining all samples with allele matched antigen presenting dCODE Dextramer® and negative control dCODE Dextramer®. More information about Dextramer controls can be found here: <https://immudex.com/resources/dextramer-controls/>
- With experimental setups that involve positive controls (e.g. virus specific T cells) it is important to ensure that the control cells are not so prevalent that they prevent detection of rare target T cells of interest. A simple solution to this problem is to spike the sample with a known number of positive control cells that are absent from the sample.

Notes for antibody staining

- Staining with FACS and DNA-barcoded antibodies for CITE-seq please follow vendor recommendations.
With the exception: staining specificity of dCODE Dextramer® is optimal at room temperature. It is therefore recommended that the full length of incubation being at Room temperature
- Staining with CD8, and to some degree CD3, before or simultaneously with the MHC I dCODE Dextramer® has a negative impact on the staining intensity of the MHC I Dextramer® positive cells. This effect depends on the antibody clones used. Therefore, MHC I, CD1d, MR1, and HLA-G dCODE Dextramer® should be allowed to stain for a minimum of 10 min. before staining with the CD3 and CD8 antibodies.
- Staining with MHC II dCODE Dextramer® is not affected by staining with CD4 antibody.
- If using FACS and BD® AbSeq antibodies with the same specificity, and especially if they are from the same clone, it is important to stain simultaneously with these. If not staining with FACS-antibodies may abrogate staining with the BD® AbSeq of the same specificity or vice versa. The staining "intensity" of these will be reduced, but this is not an issue for high intensity markers, such as CD8, CD3 and CD19, CD14.

- Sample tagging (BD® Single-Cell Multiplexing Kits). Processing multiple samples simultaneously to minimize handling and reduce costs is possible with this workflow. It can be achieved by tagging each sample with an oligo-barcode conjugated antibody and subsequent pooling of samples. Using the BD® Single-Cell Multiplexing Kit up to 12 samples can be labelled and pooled prior to single cell capture with the BD Rhapsody™ Single-Cell Analysis system. For instructions about how to perform sample tagging please refer to the relevant BD protocol for Single Cell Labelling - cell surface protein with sample multiplexing (SMK): [BD® AbSeq Protocols \(cell-surface/intracellular protein\) – BD Biosciences](#).
- Sample tagging with the BD® Single-Cell Multiplexing Kit antibodies does not interfere with dCODE Dextramer® staining.
- Sample tagging can be performed at multiple steps. Prior to all other staining's, together with other antibodies or after FACS sorting of different population of cells.
- Working with a pool of multiple samples comes with a cost of reduced sensitivity. A maximum 40.000 cells per lane may be loaded on the Rhapsody cartridge. Consequently, loading a lane with a pool of tagged samples will result in analysis with proportionally less cells/sample and rare cell types may go undetected.

Notes for Cell sorting

- Enrichment of rare antigen specific target cells (ex. if the Dextramer® positive population is <1% of total cells) by FACS is highly recommended to ensure detection of the cells in downstream analysis of DNA sequencing data.
- Gating parameters:
It is recommended to negatively gate away dead cells e.g. by fixable viability staining, and positive gating of relevant canonical cell markers:
 - a. T cells; positive gating for CD3, CD4 and/or CD8, and negative gating of monocytes, by CD14, and B cells by CD19.
 - b. B-cells; positive gating for CD19, and negative gating for T cell markers CD3, and monocytes CD14.

Patents

The dCODE® technology is disclosed in granted and pending patents within the WO 2015/185067 and WO 2015/188839 patent families including US11402373, US11585806, US11668705, EP3155426, EP3628684, HK40026921, AU2015271324, AU2019264685, AU2021204496, CA2951325, SG11201610177, JP6956632 and JP7271465.

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), +1 (215) 931-9627 (US).

Note:

Immudex® is the sole manufacturer and provider of dCODE Dextramer® (10x) reagents, and support related to these products is through Immudex.