


dCODE Dextramer® (RiO) Staining protocol

Products	dCODE Dextramer® (RiO) (Gold / Explore), cat# WBxxxxxdRG / FBxxxxxdRG CD1d dCODE Dextramer® (RiO) (Gold / Explore), cat# XDxxxxxdRG / YDxxxxxdRG MR1 dCODE Dextramer® (RiO) (Gold / Explore), cat# ZAxxxxxdRG Collectively denominated dCODE Dextramer® reagents in the following.
Recommended use	Single-cell characterization of antigen-specific T-, iNKT-, or MAIT cells in cell samples, using the BD Rhapsody™ Single-Cell Analysis System. For research use only. Not for use in diagnostic or therapeutic procedures.
Description	<p>dCODE Dextramer® reagents consist of a dextran backbone, carrying multiple MHC-, CD1d-, or MR1-antigen complexes (monomers) and R-phycoerythrin (PE) for FACS sorting of dCODE Dextramer® positive cells before loading the sample into the BD Rhapsody™ system. 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®.</p> <ul style="list-style-type: none"> • dCODE Dextramer® reagents are provided at a concentration of 160 nM in PBS buffer, containing 1% bovine serum albumin (BSA) and 15 mM NaN₃, pH 7.2. 2 µL (one test) is recommended for staining of 1-3 x 10⁶ PBMCs. <p>The DNA Barcode oligo comprises:</p> <ul style="list-style-type: none"> • BD Rhapsody™ compatible PCR handle sequences for library preparation and PCR amplification • A Unique Molecule Identifier (UMI) sequence • A DNA Barcode sequence that defines the monomeric MHC-antigen complex of the Dextramer® and which is compatible with BD® AbSeq Assay. • 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.
Sizes	Dextramer® (RiO) reagents - Gold: Single reagents of 25 tests (50 µL), 50 tests (100 µL), or 150 tests (300 µL) each. Dextramer® (RiO) reagents - Explore: Panels of 16, 32, 48, 64, 80, or 96 dCODE Dextramer® (RiO) reagents for 10 tests (20 µL), 25 tests (50 µL), or 50 tests (100 µL) each.
Storage	Dextramer® reagents should be stored at 2-8°C in the dark – the plastic vial only partially protects the reagents against light.
Precautions	Contains sodium azide (NaN ₃), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
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, EP3152232, AU2015271324, AU2019264685, SG11201610177U and JP6956632.

Symbols	See www.immudex.com/symbols
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Manufacturer	Immudex, Bredevej 2A, DK-2830 Virum, Denmark

Introduction

This protocol describes staining of hPBMC samples with dCODE Dextramer®, 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. These are the first two steps of the Single Cell Analysis Workflow illustrated below for characterization of antigen specific T 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®

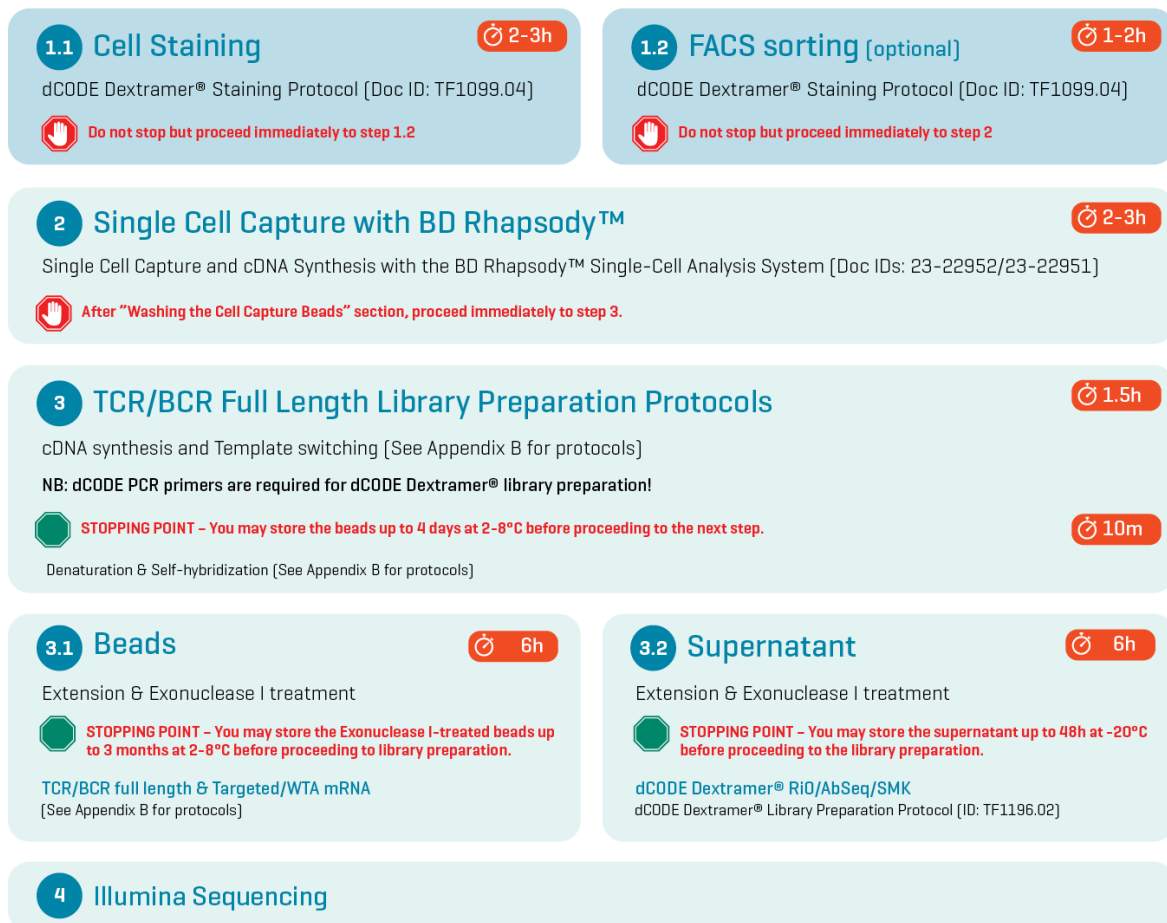
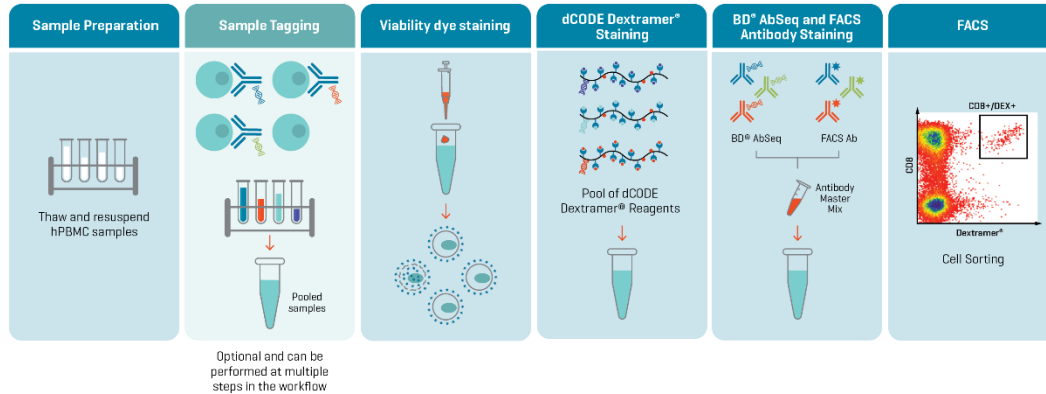


Fig. 1: Workflow describing single cell analysis of antigen specific cells with dCODE Dextramer® and the BD Rhapsody™ System. This protocol involves steps 1.1 and 1.2.

Staining procedure at a glance

dCODE Dextramer® RiO WORKFLOW – Staining workflow overview



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.

Materials Provided MHC dCODE Dextramer® reagents
and/or CD1d dCODE Dextramer®
and/or MR1 dCODE Dextramer®

Other Required Materials (not provided)

- 1) 5 mL Falcon polystyrene disposable 12 x 75-mm test tubes or equivalent.
- 2) 1,5 mL LoBind® Eppendorf tubes or equivalent.
- 3) LIVE/DEAD Fixable Viability Stain.
- 4) Fluorescent FACS Antibodies (with labels compatible with PE on the dCODE Dextramer®) if applied.
- 5) Oligo conjugated BD® AbSeq assay, if applied.
- 6) BD™ Hu Single Cell Sample Multiplexing Kit (Cat No: 633781) (referred to as Sample tagging), if applied.
- 7) Cell labeling buffer: BD Pharmingen™ Stain Buffer (FBS).
- 8) 0,1 g/L herring sperm DNA (shirred DNA. Sigma-Aldrich D3159).
- 9) Wash buffer: PBS, pH 7.4 containing 1-5% FCS.
- 10) 100 µM d-Biotin (e.g., Avidity, cat# BIO200) diluted in PBS, pH 7.4.
- 11) BD Rhapsody™ Single-Cell Analysis System.
- 12) BD Rhapsody™ Cartridge Reagent Kit (Cat. No. 633731).

Note:

Reagents for use with the BD Rhapsody™ Single-Cell Analysis System must be ordered from BD Biosciences (Information can be found in the below required BD protocols).

Sample preparation

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

Sample tagging (BD® Single-Cell Multiplexing Kits) - Optional

Processing of multiple samples simultaneously to minimize handling and reduce cost 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 BD protocol: [BD-AbSeq-Ab-Oligos-Single-Cell-Multiplexing-Kit-User-Guide.pdf](#)

Note:

1. Working with multiplexed samples comes with a cost of reduced sensitivity. A maximum of 20,000 cells should be loaded into the Rhapsody™ cartridge to ensure that only a low number of wells contains more than one cell. Consequently, loading the cartridge with pools of tagged samples will result in analysis with proportionally less cells/sample and rare cell types may go undetected.
2. Sample tagging can be performed at multiple steps. Prior to all other stainings, together with other antibodies or after FACS sorting of different population of cells. Sample tag antibody can be added to the labelling master mix together with other antibodies. In this case use incubation temperature and time indicated in the section below about Antibody staining”.

Viability dye staining

1. Resuspend 1-3 x 10⁶ washed PBMCs in 1 mL wash buffer (azide free) and add recommended volume of viability stain.
 - a. Incubate for 15 min. at room temperature (all incubations must be performed shielded from light).
2. Add 9 mL Wash buffer, centrifuge 300 x g for 10 min., resuspend cells in 50 µL 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 µL of 100 µM d-Biotin per dCODE Dextramer® into an empty tube.
 - b. Then add 2 µL of each dCODE Dextramer® (if staining more than one sample, use 20% overage to compensate for pipetting error, see Appendix A).
2. Vortex the dCODE Dextramer® pool briefly. *The pool must be used directly 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.
 - a. MHC I, MR1, or CD1d dCODE Dextramer® pool: 10 min. incubation
 - b. MHC II dCODE Dextramer® pool: 30 min. incubation
 - c. dCODE Dextramer® pool comprised of a and b: 30 min. incubation.

While incubating, prepare BD® AbSeq™ assay and FACS antibody master mix as described in Step 4.

Critical:

Staining with antibodies against CD3 and CD8 have a negative impact on simultaneous or subsequent staining with MHC I Dextramer®. In most cases it is therefore highly recommended to stain with MHC I-, MR1- and CD1d-Dextramer® **before** staining with CD3 and CD8 antibodies. Simultaneous staining will reduce the Dextramer® staining intensity significantly. Labeling of cells with sample tag antibodies however does not interfere with dCODE Dextramer® staining.

Antibody staining (FACS- and AbSeq antibodies)

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

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
BD Stain Buffer (FBS) (Cat. No. 554656)	Add to final volume of 100µL	Add to final volume of 130 µL

*Vendor recommendation

3. Pipet-mix the labeling master mix and place on ice, shielded from light, until used.
4. Add the labeling master mix to the dCODE Dextramer® stained sample(s) and mix by pipetting.
5. Incubate at 4°C for 30-60 min., shielded from light.
6. To the stained sample, add 2 mL wash buffer.
7. 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.
8. Repeat for a total of 3 washes and resuspend cells in remaining buffer.

9. Add adequate volume of Wash buffer and store sample on ice until sorting is performed.

Note:

We recommend using freshly prepared antibody pools before each experiment.

If cell sorting is not performed, then continue with step 2 in the outlined workflow in Fig.1 and go directly to 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> .

1.2 Fluorescence-activated cell sorting (FACS)

1. Initial enrichment for T-cells is performed by positive gating of cells stained with fluorescent antibodies against characteristic T-cell markers (e.g., CD3, CD4, and CD8) and negative gating of cells that stain with Live/Dead fixable viability dye (to get rid of dead cells) and other fluorescent antibodies (e.g. CD14 to get rid of monocytes).
2. dCODE Dextramer® positive cells are then sorted by flow cytometry, using the PE-fluorescence of the dCODE® (RiO) reagent following the guidelines and practices of your sorting facility.
The recommended sort mode is "Yield". If "Purity" mode is used, fewer positive cells will be sorted.
3. Collect sorted cells directly into a tube containing 500 µL FCS or a suitable buffer at 4°C to improve viability.
Viability is increased if the final sorted cell is in a buffer containing >10% FCS. Keep the unsorted and sorted cells at 4°C while performing the cell sorting.
4. 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.
5. Go immediately to Single-Cell capture with the BD Rhapsody™ Express Single-Cell Analysis System.

Critical: do not pause the procedure here!

Note:

Enrichment of rare antigen specific target cells (<1% of total cells) by FACS is highly recommended to ensure that they are captured in the BD Rhapsody™ Cartridge in numbers sufficient to give statistical significance in the downstream data analysis.

Notes to the staining and FACS protocols

1. These protocols are optimized for hPBMCs. In the case of clonal cell lines, it is important to use fewer cells when preparing the sample: Resuspend $2,5 \times 10^4$ cells in 1 mL wash buffer (azide free) and continue with viability staining.
2. d-biotin is required to avoid artefacts during staining when multiple MHC-Dextramer[®] reagents are pooled.
3. Other cell labeling buffers may be used: PBS, pH 7.4 containing 1-5% FCS, BD Horizon™ Brilliant Stain Buffer (cat.no. 563794) or equivalent should be used with antibodies conjugated with BD Horizon Brilliant™ dyes.

Critical: 0.1 g/L herring sperm DNA (shirred. Sigma-Aldrich D3159) must be added cell labelling buffers!

4. Incubation time should be increased for larger pools of MHC I dCODE Dextramer[®]. For more than 25 specificities, use 20-30 min incubation.
5. If using FACS and BD[®] AbSeq™ antibodies with the same specificity, and especially if they are from the same clone, then the signal from these will be reduced compared to staining with only one of them. This is not an issue for high intensity markers, such as CD8, CD3 and CD19.
6. Ensure that the intended total cell load after sorting is between 7,500–20,000 single cells. Cell loads below or above this recommended range may not be suitable for this protocol configuration.

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>

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 BD Rhapsody™ System TCR/BCR Library Preparation Protocols (see appendix B, and Fig. 1).

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

Note:

For preparation of the dCODE Dextramer® DNA library, dCODE® (RiO) specific PCR primers are required:

dCODE® PCR1 primer:

5'-GGAGGGAGGTTAGCGAAGGT-3'

dCODE® PCR2 primer:

5'-CAGACGTGTGCTCTTCCGATCTGGAGGGAGGTTAGCGAAGGT-3'

dCODE® (RiO) specific primers can be ordered from a preferred DNA oligo provider and are used at a concentration of 10 µM. It is recommended to order them as HPLC purified.

Critical:

Establish and maintain a designated area for PCR setup that is separate from areas where PCR amplification and PCR product handling are performed and where dCODE Dextramer® and barcoded antibodies are pipetted. This will reduce the risk of contaminating NGS libraries with amplicons from previous PCR reactions and DNA barcode templates.

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.

Appendix A:

Preparing pool of dCODE® (RiO) reagents for staining 1 sample:

Number of dCODE Dextramer® per sample	2	5	10
d-Biotin 100 µM (1/10 x Volume of dCODE Dextramer®)	0.4 µL	1 µL	2 µL
dCODE Dextramer®	4 µL	10 µL	20 µL
Total volume added / sample	4.4 µL	11 µL	22 µL

When preparing a pool for more than 1 sample, we recommend preparing 20 % overage of the reagents to compensate for pipetting errors.

Appendix B:

1. Desired workflows for TCR/BCR Full Length Library Preparation Protocols in combination with dCODE Dextramer® reagents		
Assay Type	Assay dCODE Dextramer® (RiO) +	Required additional BD® TCR/BCR Full Length Protocol
Surface marker libraries	Optional: - BD® AbSeq assay - BD® Single-Cell Multiplexing Kit (SMK)	dCODE Dextramer® (RiO) Library Preparation Protocol (TF1196)
Targeted mRNA	TCR/BCR + BD® AbSeq + Sample Tag + Targeted mRNA	TCR/BCR Full Length, Targeted mRNA, BD® AbSeq, and Sample Tag (Doc ID: 23-24016)
	TCR/BCR + Targeted mRNA	TCR/BCR Full Length and Targeted mRNA (Doc ID: 23-24013)
	TCR/BCR + BD® AbSeq + Targeted mRNA	TCR/BCR Full Length, Targeted mRNA and BD® AbSeq (Doc ID: 23-24015)
	TCR/BCR + Sample Tag + Targeted mRNA	TCR/BCR Full Length, Targeted mRNA and Sample Tag (Doc ID: 23-24014)
WTA	TCR/BCR + BD® AbSeq + Sample Tag + WTA	TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA), BD® AbSeq, and Sample Tag (Doc ID: 23-24020)
	TCR/BCR + WTA	TCR/BCR Full Length and mRNA Whole Transcriptome Analysis (WTA) (Doc ID: 23-24017)
	TCR/BCR + BD® AbSeq + WTA	TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA) and BD® AbSeq (Doc ID: 23-24019)
	TCR/BCR + Sample Tag + WTA	TCR/BCR Full Length, mRNA Whole Transcriptome Analysis (WTA), and Sample Tag (Doc ID: 23-24018)