dCODE Dextramer® (10x) staining protocol

Introduction

This protocol describes staining of hPBMC samples with dCODE Dextramer® (10x), BioLegend TotalSeq™-C surface antigen and hashtag antibodies, 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 10x Chromium Single Cell Analysis Workflow illustrated below for characterization of antigen specific T cells.

It is recommended to include relevant BioLegend TotalSeq™-C surface protein antibodies for more accurate phenotyping, and detection of antigen specific cells in subsequent data analysis.

10x Chromium Workflow with dCODE Dextramer®

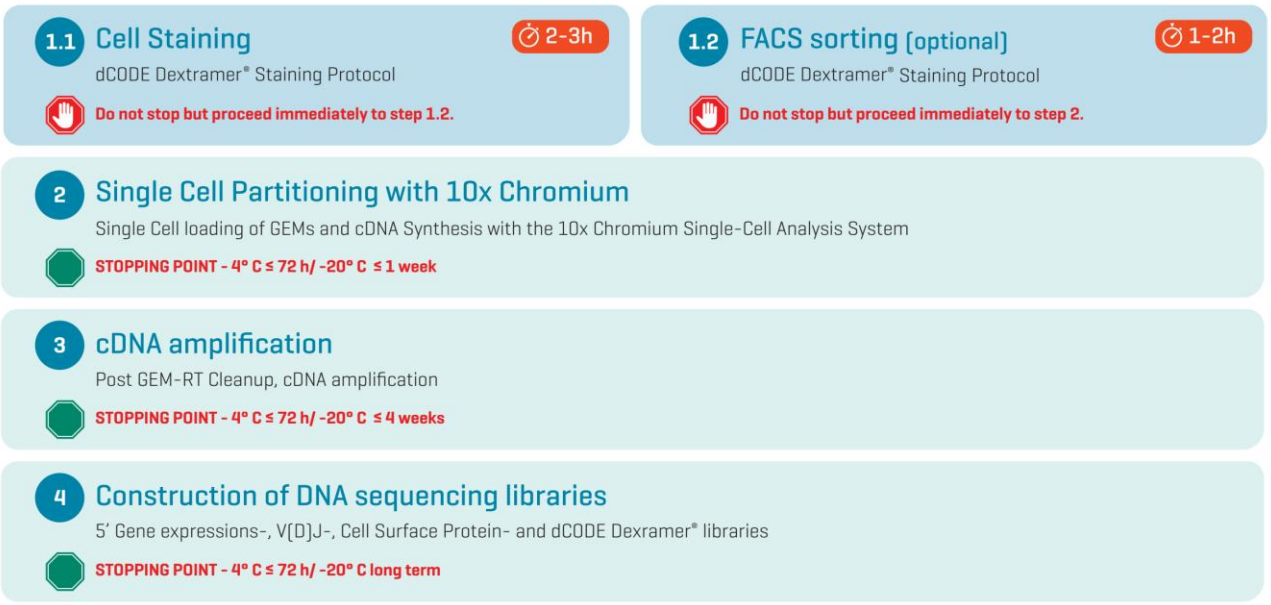
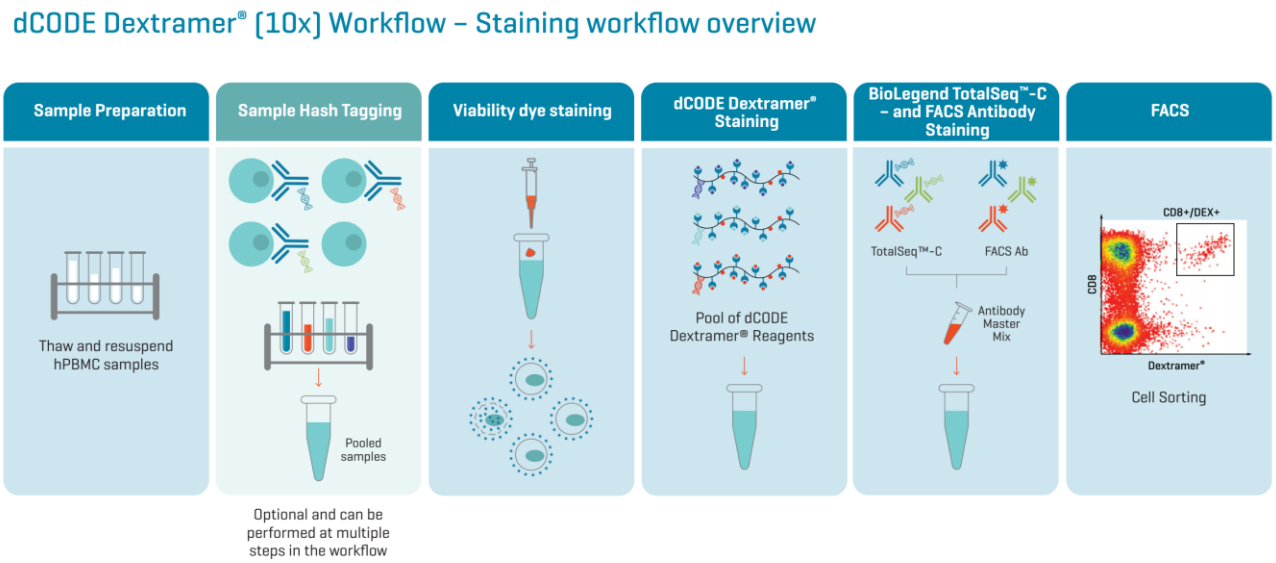


Fig. 1: Workflow describing single cell analysis of antigen specific cells with dCODE Dextramer® and the 10x Chromium Single-Cell Platform. This protocol involves steps 1.1 and 1.2 only. Please consult the 10x Genomics webpage for more information about single cell partitioning, cDNA amplification and construction of DNA sequencing libraries.

Staining Procedure at a Glance



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 Themo-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 BioLegend TotalSeq™-C surface antigen antibodies. (If applied)
- BioLegend TotalSeq™-C Hashtag antibodies, if applied
- 10x Genomics Chromium X or iX instrument for single cell partitioning
- All required 10x single cell consumable components for Universal 5’ Gene Expression. Please consult the 10x Genomics website for more information: https://www.10xgenomics.com/products/universal-five-prime-gene-expression

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 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. Add 2 µL of each dCODE Dextramer® reagents
    c. Add 5 µL 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 150µl see appendix B

Antibody staining

1. Prepare the pool of FACS, and TotalSeq™-C antibodies and Hashtag antibodies (if applied) according to vendors protocols. Technical protocols for DNA-barcoded TotalSeq™-C antibodies can be found here: https://www.biolegend.com/en-us/protocols/totalseq-b-or-c-with-10x-feature-barcoding-technology
2. Add the antibody pool to the cell sample and vortex briefly.
3. Incubate minimum 30 min, ***at room temperature - see appendix B!***
4. Add 2 mL wash buffer, to the stained sample.
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 wash buffer and store the 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 10x chromium platform please refer to 10x Chromium user guides - see step 2-4 below.
8. Proceed to cell sorting or continue with partitioning of single cells on the 10x chromium platform

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 10x Genomics Chromium single cell analysis platform.

Critical: do not pause the procedure here!

2-4. Single cell analysis with the 10x Chromium platform

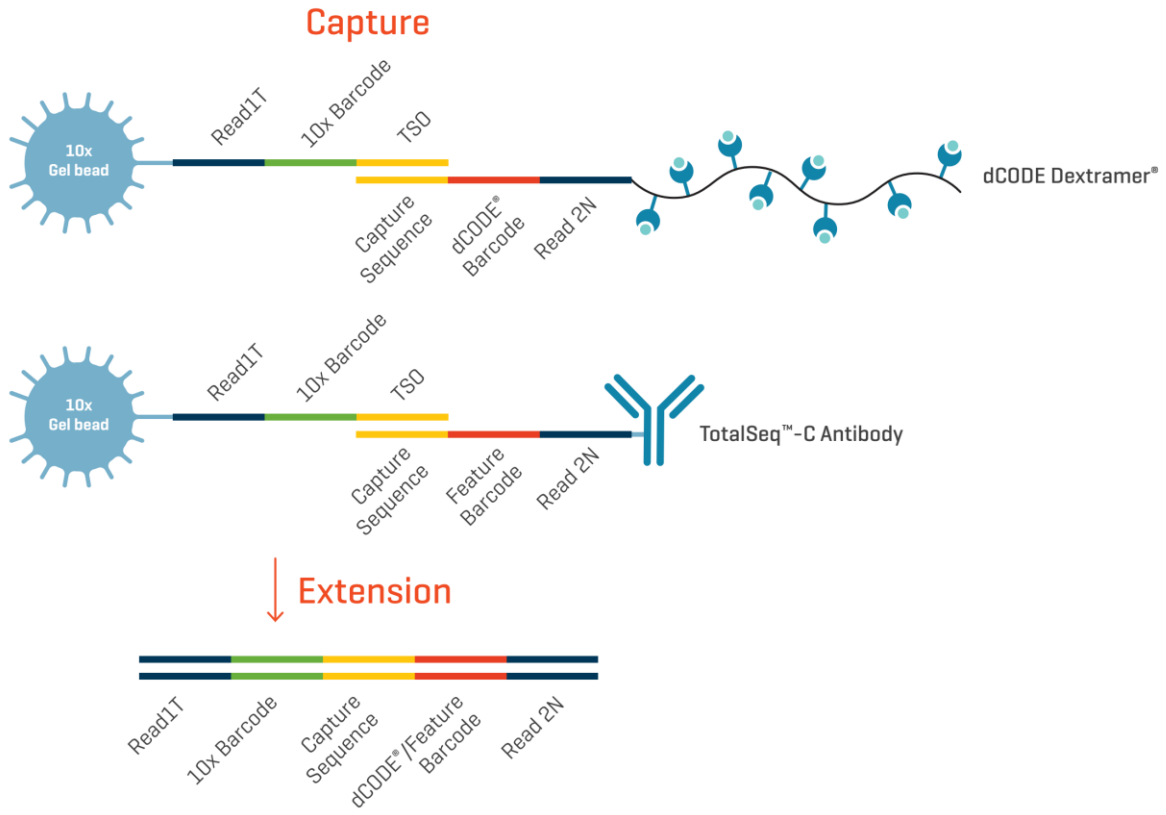
User guides for partition of single cells in GEMs, cDNA synthesis & amplification and construction of DNA sequencing libraries (dCODE® and Feature Barcode library, 5' Gene Expression and TCR/BCR Full length(V(D)J) libraries) can be found here:

https://www.10xgenomics.com/support/user-guides

Select "Chromium 5' Gene Expression", then filter for "Cell Surface Protein" and choose the appropriate user guide. Follow instructions for "Cell Surface Protein Library Construction" to create pMHC (dCODE® barcode) libraries.

Barcode oligonucleotides attached to dCODE Dextramer® and TotalSeq™-C antibodies are directly captured by the Gel Bead inside a GEM during GEM generation and amplified with the primers of the 10x 5' Feature Barcode kit. The amplified DNA is then used for construction of a DNA sequencing library containing information about pMHC/TCR specificity and cell surface proteins. Therefore, dCODE® barcodes and Feature barcodes must be treated together in the data analysis pipeline. Go to our website for an example of a reference file that includes both dCODE Dextramer® and TotalSeq™-C antibodies:

https://immudex.com/resources/protocols/



cDNA from dCODE® and Surface Protein Feature Barcodes:
The barcode sequences of dCODE Dextramer® (10x) reagents are designed to be compatible with 10x Genomics Single Cell 5' Chip Kits!

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.

Note:
Ensure that the cell load on the Chromium machine does not exceed the recommended cells/sample and cells/chip specified by 10x Genomics.

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 comprises:

- Primer sequence compatible with Illumina® Sequencers (Nextera pR2)
- Unique molecule identifier (UMI)
- ID sequence (barcode) that specify the MHC-peptide specificity
- Capture sequence for 10x Chromium single cell immune profiling solution.

5’	-Nextera Read 2 -	- UMI -	- ID -	- UMI -	- Capture seq -	3’
CGGAGATGTGTATAAGAGACAG	NNNNNNNNNN	XXXXXXXXXXXXXXX	NNNNNNNNN	CCCATATAAGAAA	3’	

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 2-5x10^4 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 simultaneous 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 TotalSeq™-C antibodies with the same specificity, and especially if they are from the same clone, it is important to stain simultaneous with these, if not staining with FACS-antibodies may abrogate staining with the TotalSeq™-C 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.

- Labeling with Hashtag antibodies does not interfere with dCODE Dextramer® staining.

- Hashtagging / 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 20.000 cells may be loaded on the 10x Genomics GEM chip. Consequently, loading the Chip with a pool of tagged samples will result in analysis with proportionally less cells/sample and rare cell types may go undetected.
-

- For instructions about how to perform TotalSeq™ and sample hashtagging, please refer to the BioLegend technical protocol for TotalSeq™ staining protocols: TotalSeq™-B or -C with 10x Feature Barcoding Technology

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.