Redefining Cellular Phenotyping: Comprehensive Characterization and Resolution of the Antigen-Specific T Cell Response

Introduction

Adaptive immunity requires the ability to respond to different types of antigens, activate pathways that eliminate specific pathogens, and develop immunological memory so that subsequent infections or disease can be quickly identified and removed. This complex process is mediated through the specific recognition of antigens by T and B cells, and the dynamics of the immune response are fueled by the genetic diversity of T- and B-cell receptors.

To orchestrate the immune response, T cells are activated when the T-cell receptor (TCR) recognizes an antigen, a peptide complexed with a major histocompatibility complex (pMHC) molecule displayed on antigen-presenting cells. Due to the complexity of the adaptive immune response, a comprehensive understanding requires cutting edge tools and technologies. In order to fully characterize T cells involved in immunological responses to infections, autoimmunity, cancer, and other pathologies, it is necessary to understand and phenotype the T cells in the context of their cell surface protein markers, antigen specificity (TCR-pMHC interaction), as well as their transcriptional state, to link all of these phenotypes to the expressed TCR clonotype.

Highlights

The Single Cell Immune Profiling Solution with Feature Barcoding technology enables:

- Comprehensive cellular phenotyping with simultaneous characterization of gene expression, cell surface protein expression, paired T-Cell receptor (TCR) sequences, and TCR antigen specificity in the same single cell
- Resolution of TCR peptide-antigen interactions in the context of cellular phenotype
- Discovery of novel TCR:antigen binding relationships
- Characterization of the adaptive immune response at unprecedented scale and resolution

Figure 1. High throughput approaches for multi-dimensional cellular phenotyping using the Single Cell Immune Profiling Solution from 10x Genomics. This schematic shows an immune cell suspension that is labeled with both a fluorophore and a Feature Barcode conjugated pMHC-multimer and then flow sorted to collect multimer-bound T Cells. Using different library preparation protocols, the cells are then processed and analyzed to simultaneously characterize gene expression, paired TCR sequences, and TCR antigen specificity in each single cell.
Analysis of antigen specificity in T cell populations has typically been accomplished by attaching a fluorophore to pMHC multimers and analyzing antigen-specific T cells using flow cytometry, or linking to metal ions for mass cytometry. Both flow and mass cytometry enable the phenotypic characterization of cells with the addition of labeled antibodies (1). These approaches allow the antigen binding specificity and limited phenotypic characteristics of a T cell to be obtained (based on cell surface markers) but do not provide the more detailed phenotyping that gene expression profiling allows. Furthermore, due to the spectral overlap of fluorophores and the limited number of unique heavy metals available for flow and mass cytometry, respectively, these experiments are restricted in throughput to ~100 pMHC specificities (2).

Approaches for the simultaneous measurement of cell surface proteins and transcriptome expression at single cell resolution have been reported (3, 4), providing comprehensive, high throughput characterization of cell types utilizing next generation sequencing (NGS) methods. The development of precise, high throughput NGS-analysis techniques provides opportunities for a much higher resolution view of the behavior of adaptive immune cells than has previously been possible.

A recent report demonstrated multiplexing of >1000 DNA barcoded pMHCs in a single reaction (5), where DNA barcodes were amplified and sequenced to identify bound molecules. However, this was done in bulk and not at the single cell level. The Chromium Single Cell Immune Profiling Solution with Feature Barcoding technology (Figure 1) is the first demonstration of the combination of comprehensive NGS immune profiling with labeled pMHC technologies in a single assay. Using this workflow, one can simultaneously classify cell types at high resolution using transcriptome profiling, cell surface protein expression, and characterization of the immune cell receptor repertoire and cognate antigen on the same single cell, all at high throughput.

Here, we used the 10x Genomics Chromium Single Cell Immune Profiling Solution with Feature Barcoding technology to characterize peripheral blood mononuclear cells (PBMCs) from cytomegalovirus (CMV) seronegative and seropositive donors using both gene expression and cell surface protein expression, revealing diverse cell types and allowing T cell maturation stage determination. We then examined the repertoire and antigen binding specificities of the T cells using a panel of pMHC multimers (dCODE Dextramer reagents) presenting peptide antigens from CMV, Epstein Barr Virus (EBV), and Influenza (Flu), in the context of MHC I. In the CMV seropositive donor, we were able to identify small clonal expansions with specificity for the CMV antigens. In the CMV seronegative donor, we identified a large clonal expansion of EBV-specific T cells but no reactivity towards CMV epitopes. In both cases, we were able to obtain the antigen-receptor sequences and characterize the cell types. Comparison of the identified CMV binding clonotypes, identified in the seropositive donor with publicly available sequences, revealed both previously identified CMV-specific CDR3 amino acid sequences and novel sequences.

**Methods**

**Chromium Single Cell Immune Profiling Workflow and Cell Ranger Pipeline**

The Single Cell Immune Profiling Solution with Feature Barcoding technology allows multiomic characterization of cells. In the following experiment, PBMCs from HLA-typed CMV positive (Immudex) and seronegative (Astarte) donors were used (HLA types listed in Table 1).

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**Table 1. HLA types of donors.** The CMV+ donor was identified by flow cytometry using CMV-derived pMHC dextramers. The CMV- donor was identified by a serum test.

We created two panels. The first was made up of 14 TotalSeq-C oligonucleotide conjugated antibodies and 3 isotype controls (BioLegend, Resource Table 2), while the second panel included 12 MHC dCODE Dextramer reagents displaying epitopes derived from CMV, EBV, and Flu viruses as well as 2 negative controls with HLA matching to at least one of the donors (Immudex, Resource Table 3).
Barcoding technology for Cell Surface Protein protocol (CG000186 Rev A). Three sequencing libraries were generated: a gene expression library, a protein expression library (containing the antibody and Dextramer barcodes) and a TCR repertoire library (Figure 2).

Gene expression libraries were sequenced to a minimum targeted depth of ~50,000 read pairs per cell (rpc); actual read depths varied (for more information on choosing an appropriate read depth for your experiment, see User Guide CG000186 Rev A. The cell surface protein (antibody and Dextramer) libraries were sequenced at target depths of ~10,000 rpc, and the V(D)J enriched (TCR) library at ~5,000 rpc. The sequencing data combined with the description of the sequences identifying the antibodies and Dextramer multimer were analyzed with the Cell Ranger analysis pipeline (see What is Cell Ranger?). The results were then visualized using Loupe Cell Browser (see What is Loupe Cell Browser?). We performed additional custom analyses to determine antigen binding specificity and used the TCRdist algorithm to examine epitope-specific TCR recognition motifs within our cell population (6).

**Results**

**High resolution cell type classification using combined gene and protein expression data**

To obtain a holistic view of the nature of the cells within each sample, we informatically combined the unsorted PBMCs and CD4-/CD8a+/Dextramer+ sorted cells (using cellranger aggr). Using Loupe Cell Browser, we were able to visualize the tSNE projections and color the plots by sample, revealing an expected cluster of sorted cells within the whole cell population (Figure 3 i). Cells were clustered using Graph-Based clustering based on gene expression data, and cell clusters were manually assigned cell types based on both their gene expression and cell surface protein expression profiles (Figure 3 ii). Cell type classification can be performed based on gene or protein expression alone, each with its own benefits: Gene expression profiling allows for a more comprehensive view of the cell, with no prior knowledge of the expressed transcripts. Interrogation of cell surface protein markers allows the researcher to compare the transcriptional state of the cell with canonical cell type markers. The combination of both gene and

![Feature Barcoding technology workflow](image)

Figure 2. Feature Barcoding technology workflow for the multiomic characterization of single cells. A. Gating strategy used in flow cytometry to isolate CD4-/CD8a+/Dextramer+ cells. B. Single Cell Immune Profiling with Feature Barcoding technology workflow where gene expression and immune repertoire libraries are generated alongside libraries from Feature Barcode conjugated TotalSeq-C antibodies or dCODE Dextramer reagents, allowing quantification of cell surface proteins and identification of TCR specificities. Representative Bioanalyzer traces revealing average size distribution for each of the generated libraries are shown to the right.
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cell surface protein expression provides power in allowing a clearer, more confident identity call. In Figure 3A ii, two subsets of natural killer (NK) cells could be identified (CD16 low/CD56 high and CD16 high/CD56 low) based on the cell surface protein expression but not gene expression. Conversely, gene expression data could identify additional cell types which were not targeted by the antibody panel we used.

**Naive and memory T cells can be discerned by CD45 protein isoform characterization**

Surface protein expression analysis allows identification of protein isoforms where mRNA analysis cannot. The CD45 mRNA transcript has many isoforms that are not easily detectable with RNA-seq unless bespoke analysis is performed. The different isoforms are expressed at various differentiation stages and thus provide a useful marker for assessment of naive (CD45RA) and experienced/memory (CD45RO) cells. Examining the surface protein expression of these isoforms allows for robust characterization of the cell maturity state, clearly distinguishing the naïve and memory CD8+ T cell populations in both donors (Figure 3A, B ii and Figure 4A, B ii).

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**A. CMV Seropositive DONOR**

i. Unsorted PBMCs and CD4-/CD8a+/Dextramer+ sorted cells were aggregated. tSNE projections were generated by Cell Ranger and visualized in Loupe Cell Browser. Cells were clustered on gene expression data with graph-based clustering. Each dot represents a single cell. A i. CMV seropositive donor cells colored based on sample. Unsorted PBMCs = grey (6506 cells), sorted cells = green (2043 cells). B i. CMV sero-negative donor cells colored based on sample. Unsorted PBMCs = grey (5799 cells), sorted cells = green (1547 cells). In ii. cell type classification was performed using both gene expression data and surface protein expression profiles. (MT= mitochondrial, NK= natural killer)

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**B. CMV SeroNEGATIVE DONOR**

i. Unsorted Cells

ii. Sorted Cells

Figure 3. The combination of gene expression and cell surface protein expression using labeled barcoded antibodies provides increased resolution of cell type characterization. Unsorted PBMCs and CD4-/CD8a+/Dextramer+ sorted cells were aggregated. ISNE projections were generated by Cell Ranger and visualized in Loupe Cell Browser. Cells were clustered on gene expression data with graph-based clustering. Each dot represents a single cell. A i. CMV seropositive donor cells colored based on sample. Unsorted PBMCs = grey (6506 cells), sorted cells = green (2043 cells). B i. CMV sero-negative donor cells colored based on sample. Unsorted PBMCs = grey (5799 cells), sorted cells = green (1547 cells). In ii. cell type classification was performed using both gene expression data and surface protein expression profiles. (MT= mitochondrial, NK= natural killer)
Toward a more complete cellular read-out: multiomic analyses allow cell type classification, repertoire profiling, and determination of antigen specificity of the same single T cells

The combination of TCR-specificity and cell phenotyping with cellular gene expression and TCR sequence information provides a complete view of cell type, state, and functionality. Analysis of the CD4-/CD8+/Dextramer+ sorted cell populations from both donors revealed clean sorts, indicated by the high fraction of CD8 T cells in these populations (Figure 4 i) that can be separated into memory and naïve T cells based on CD45RA/RO expression (Figure 4 ii). Clusters of cells that show significantly enriched antigen binding (based on dCODE Dextramer reagent binding) when compared to the rest of the population (p<0.001) can be seen in Figure 4 iii. As expected, the majority of antigen binding cells are classified as memory T cells. In the CMV seropositive donor, binding specificity for 3 CMV epitopes can clearly be seen, in addition to small clusters of cells with specificity for EBV and Flu (Figure 4A iii). While the identification of the CMV clusters is expected, the EBV and Flu serostatus of this patient was not known. The small clusters of antigen-specific memory T cell clonotypes identified here are suggestive of a past infection. Interestingly, we identified a large cluster of cells with specificity for the HLA-A*02:01 restricted EBV epitope (GLCLTVAML) in the CMV seronegative donor (Figure 4B iii), allowing us to infer a past EBV infection in a case where we had no prior knowledge of the donor outside of CMV status.

Characterizing antigenicity of T cell clonal expansions using dCODE Dextramer reagents

Next, we examined the top ten TCR clonotypes in the largest clusters of antigen binding cells for each donor. For the CMV seropositive donor, no single clonotype dominated the population. A HLA-A*01:01 restricted CMV epitope (VTEHDTLLY) was recognized by specific T cells at the highest frequency. The most prevalent single clonotype for this epitope had only limited expansion, with 12 cells detected (0.8% of all clonotypes) (Figure 4A iii). Examination of the other CMV binding T cells in this donor revealed a similar level of expansion, with

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A. CMV Seropositive DONOR

i. **Gene Expression**

Monocytes (CD14+)

Dying Cells (MT genes)

NK Cells (FCGR3A+ GZMB+)

CD8 T Cells (CD16+ CD56+ CD3D/E+ CD8A/B+)

Naïve CD8 T Cells (CD8A+ CD45RA+)

B Cells (CD19+ CD79A+)

Dying Cells (Non-specific Ab binding)

**TCR Clonotypes**

CMV - B*07:02 RPHENGFVTL

CMV - A*01:01 VTEHDTLLY

Flu - A*02:01 GILGIFVFL

CMV - B*07:02 TPRVTGGGAM

EBV - B*08:01 RAKFKQKL

EBV - A*02:01 CLGGLLTMV

EBV - A*02:01 GLCLTVAML

ii. **Cell Surface Protein Expression**

Memory CDB T Cells (CD8A+ CD45RO+)

NK Cells (CD8A+ CD45RA+)

B Cells (CD19+)

CD8 T Cells (CD8A+ CD45RA+)

Naïve CD8 T Cells (CD8A+ CD45RO+)

Dying Cells (Non-specific Ab binding)

**TCR Clonotypes**

CMV - B*07:02 RPHENGFVTL

CMV - A*01:01 VTEHDTLLY

Flu - A*02:01 GILGIFVFL

CMV - B*07:02 TPRVTGGGAM

EBV - B*08:01 RAKFKQKL

EBV - A*02:01 CLGGLLTMV

EBV - A*02:01 GLCLTVAML

iii. **Antigen Binding Specificity**

B. CMV SeroNEGATIVE DONOR

i. **Gene Expression**

Monocytes (CD14+)

Dying Cells (MT genes)

NK Cells (FCGR3A+ GZMB+)

CD8 T Cells (CD16+ CD56+ CD3D/E+ CD8A/B+)

Naïve CD8 T Cells (CD8A+ CD45RA+)

B Cells (CD19+ CD79A+)

Dying Cells (Non-specific Ab binding)

**TCR Clonotypes**

CMV - A*02:01 RAKFKQKL

CMV - B*07:02 TPRVTGGGAM

Flu - A*02:01 GILGIFVFL

CMV - B*07:02 TPRVTGGGAM

EBV - A*02:01 CLGGLLTMV

EBV - A*02:01 GLCLTVAML

EBV - A*02:01 GLCLTVAML

ii. **Cell Surface Protein Expression**

Memory CDB T Cells (CD8A+ CD45RO+)

NK Cells (CD8A+ CD45RA+)

B Cells (CD19+)

CD8 T Cells (CD8A+ CD45RO+)

Naïve CD8 T Cells (CD8A+ CD45RO+)

Dying Cells (Non-specific Ab binding)

**TCR Clonotypes**

CMV - A*02:01 RAKFKQKL

CMV - B*07:02 TPRVTGGGAM

Flu - A*02:01 GILGIFVFL

CMV - B*07:02 TPRVTGGGAM

EBV - B*08:01 RAKFKQKL

EBV - A*02:01 CLGGLLTMV

EBV - A*02:01 GLCLTVAML

EBV - A*02:01 GLCLTVAML

iii. **Antigen Binding Specificity**

**Figure 4.** Analysis of TCR repertoires and their binding specificity, in combination with gene and cell surface protein expression, provides a high resolution view of T cell activity at the single cell level. Cell clustering and classification of the sorted cell populations from CMV seropositive (A) and seronegative (B) donors was based on: i. Gene expression. ii. Cell surface protein expression based on binding of BioLegend TotalSeq-C antibodies. iii. TCR specificity based on dCODE Dextramer reagent binding. Highlighted clusters show significantly enriched binding over the rest of the cell population, p<0.001. MHC allele and peptide antigen amino acid sequence are shown. The paired TCR clonotype gene calls and frequency for the top 10 clonotypes in the dominant cluster are shown. (MT= mitochondrial, NK = natural killer)
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16 cells binding CMV B*07:01/RPHERNGFTVL (1.1% of all clonotypes, data not shown) and 8 cells binding CMV B*07:02/TPRVTGGGAM (0.6% of all clonotypes, data not shown). By contrast, when we performed the same analysis of the large EBV cluster in the CMV seronegative donor, a very large clonal expansion was identified, with the top clonotype having 487 cells in the cluster (504 cells in the total population, 37.5% of all clonotypes) (Figure 4B i). A clonal expansion of this magnitude indicates an active T cell response to a current EBV infection. These findings highlight the power of combining TCR clone frequency with antigen specificity to gain insight into the status of the adaptive immune response.

Advantages of flow sorting for the identification of antigen-specific T cell populations

The statistically enriched and under-represented clonotypes identified in the populations of each sample are presented in the heatmaps in Figure 5. The high specificity and sensitivity of the Dextramer reagents is highlighted by the detection of several unique clonotypes that show specific binding for multiple cognate antigens (Figure 5A i). Due to the low frequency of TCR:antigen binding events, none of the CMV-specific TCRs were identified with high statistical significance in the unsorted cell population despite the large number of cells analyzed (Figure 5A ii). The highly expanded EBV-specific clonotype in the CMV seronegative donor can be identified in both the sorted and unsorted cells, however its frequency in the unsorted sample is much lower (Figure 5B). While the importance of enriching for low frequency binding events is well recognized, these data clearly illustrate the potential advantages of sorting T cells prior to attempting analysis of antigen-specific cell populations.

Interestingly, this analysis allows for the identification of under-represented clonotypes (Figure 5B i, red bars); under-represented clonotypes are present at a high level in the donor sample but are not enriched by the Dextramer reagent sorting. This observation would not have been possible using flow cytometry alone, highlighting the additional knowledge that can be gained by performing multiomic assays and analyses. Across all of the specificities in our dCODE Dextramer panel, with donors possessing distinct HLA haplotypes, we did not see any cross-reactivity where there is an allele mismatch with the donor (Figure 5 and Table 3).

**Figure 5. Identification of antigen-specific T cell clonotypes in sorted cell populations.** Heatmaps show the significantly enriched (blue) or significantly under-represented (red) TCR clonotypes. Each column represents a TCR clonotype. The number of clones specifically binding a particular dCODE Dextramer receptor (specific binders) and the frequency of the clonotype in the whole population (Clonotype count) are indicated below each column. For all samples, the top cells ranked by magnitude of their log10 p-values are shown, and at least ten cells were included for each heatmap. The raw unadjusted p-value (computed by taking 0.001 and dividing it by the product of the number of non-singleton clonotypes multiplied by the number of Feature Barcode sequences considered) is shown on each chart.
Feature Barcoding technology and flow cytometry analysis yield comparable data

In order to confirm the Feature Barcoding technology assay robustness, we compared our data with data generated from the same cells, using three of the same CMV-specific Dextramer reagents, by flow cytometry. Using the CMV seropositive sorted CD4-/CD8+/Dextramer+ population, we assessed the fraction of positive binding cells to these CMV epitopes. For the Feature Barcoding technology, positive binders were classified as those cells with specific dCODE Dextramer UMI counts enriched over the background (negative controls) with an associated statistical significance of p<0.001 based on the corresponding null model (indicated by the upper dashed blue diagonal line in Figure 6A). Moreover, cells called as positive binders were also required to have a minimum total Dextramer UMI count exceeding the predicted 95% quantile level for background counts according to a Poisson model of the negative controls (indicated by the dashed red horizontal line in Figure 6A). The fraction of cells (out of the total number of CD8+ cells) identified as positive binders using the Feature Barcoding approach or flow cytometry analysis was calculated and found to be comparable (Figure 6B). The flow cytometry plots show the cells that were gated to count positive binding cells using this approach (Figure 6C).

Novel and previously reported CMV-specific clonotypes are identified

Clustering analysis of the paired TCR sequences from clonotypes specific for two of the CMV-derived dCODE Dextramer reagents allowed us to look for relationships between the different TCR sequences that bound specifically to the same CMV epitope (Figure 7). We compared CDR3 amino acid sequences of the CMV-specific TCR clonotypes with sequences with the same binding specificities in VDJdb (7, https://vdjdb.cdr3.net/), an online database of published TCR specificities. For the CMV-specific Dextramer (A*01:01/ VTEHDTLLY), one of the TCRB amino acid sequences was identified in VDJdb (Figure 7A, green box). No TCRA associated with this TCRB was reported in VDJdb, due to a lack of paired TCR data. Other CDR3 sequences for TCRB in the same clade (bottom portion of Figure 7A) differed only very slightly from the identified match, some with only one amino acid mismatch. For the CMV-specific Dextramer B*07:02/ RPHERNGFTVL, one paired TCR clone was reported in VDJdb with the same binding specificity (Figure 7B, green box). These data highlight the robustness of the Feature Barcoding technology, allowing the identification of previously reported binding specificities while also providing a platform for the discovery of novel TCRs in a manner with greater throughput than was previously possible.

![Figure 6. Feature Barcoding technology and flow cytometry identify similar frequencies of CMV positive cells. A. Feature Barcoding technology analysis with a CMV-specific dCODE Dextramer panel. Positive cells are above the dashed lines in the region indicated in pink. B. Quantification of the fraction of positive binding cells as a percentage of the total number of CD8+ cells identified by Feature Barcoding technology and by flow cytometry. C. Flow cytometric analysis of the same CMV seropositive sample with the same dCODE Dextramer panel. Positive cells are inside the dashed box with pink background. Based on analysis of the CMV seropositive donor CD4-/CD8+Dextramer+ sorted cells. NC = Negative Control.](image-url)
**Conclusions**

Identifying discrete cellular phenotypes that underlie immune receptor specificity and antigen binding capabilities is critical for developing a better understanding of the adaptive immune response and its relation to disease. Achieving a detailed understanding of the immune response requires tools that will enable simultaneous analysis of multiple cellular phenotypes at single cell resolution. The Single Cell Immune Profiling Solution with Feature Barcoding technology from 10x Genomics is the first commercially available solution that can simultaneously link gene expression, cell-surface protein expression, paired adaptive immune receptor sequences, and TCR antigen binding specificity in the same single cell at high throughput.

We used this solution to perform multiomic single cell phenotyping of PBMCs from CMV seronegative and seropositive donors. Combining the analysis of gene expression, protein expression, paired TCR sequences, and antigen specificity at single cell resolution allowed us to phenotype the adaptive immune response at unprecedented resolution. Specifically, we show that: (i) simultaneous analysis of gene expression and cell surface protein expression provides increased phenotypic specificity and better distinction between cell types compared to either method alone; (ii) examining the sequences and antigen specificities of TCR clonotypes can identify active adaptive immune responses from previously unknown infections; and (iii) analysis of paired TCR sequences from sorted seroreactive clonotypes can identify both novel and previously reported antigen binding specificities.

Previously, up to 82 antibody-oligonucleotide conjugates have been described to reliably detect cell surface markers (3), and -1000 DNA oligo conjugated Dextramer reagents (5) in NGS assays. In contrast, the more widely used flow cytometry techniques can only evaluate known cell surface proteins and yield limited information about cell behavior and activity, which diminishes the ability to identify new biomarkers or targets. Here we demonstrate multiomic single cell phenotyping at small scale, but the Feature Barcoding technology allows for the easy scaling of experiments to potentially hundreds of barcoded antigen reagents, vastly increasing the number of analyses conducted and leading to clearer and more comprehensive insights from a single experiment.

Leveraging this understanding of the immune response using NGS-based tools, such as the 10x Genomics Single Cell Immune Profiling Solution, will be key in the development of successful cellular and transgenic immunotherapies and will...
be particularly important for monitoring therapeutic interventions. As more and more data are generated that allow the TCR:antigen binding relationship to be understood, generalizable models of TCR specificity can start to be established. While much work is still needed to generate these models, the power of a better understanding of the TCR:antigen binding relationship holds great promise for the development of therapeutics, for identifying novel targets, and for building a clearer and more actionable understanding of adaptive immunity.

Table 2. BioLegend TotalSeq-C antibody panel.

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Table 3. Immudex dCODE Dextramer panel.

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References

Resources from 10x Genomics

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