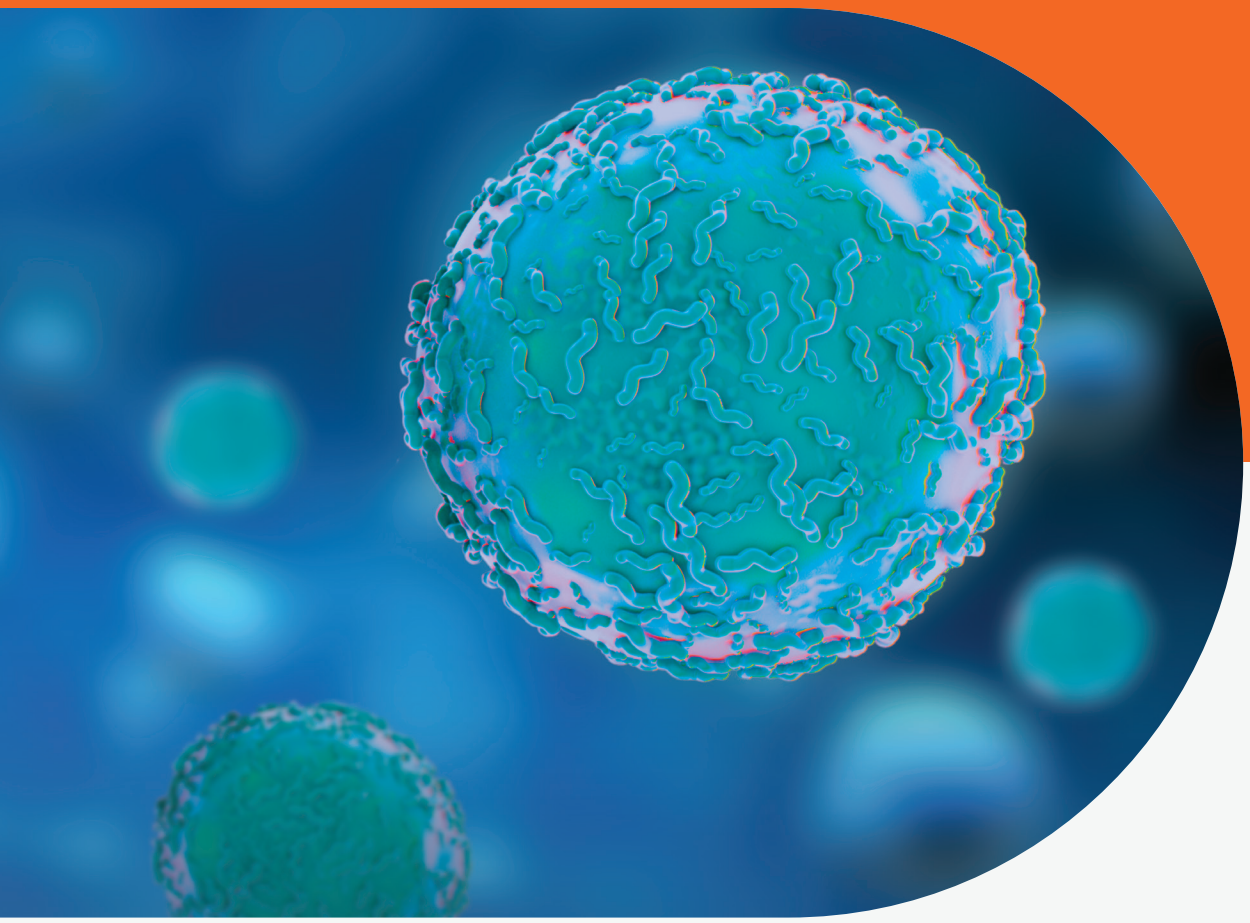


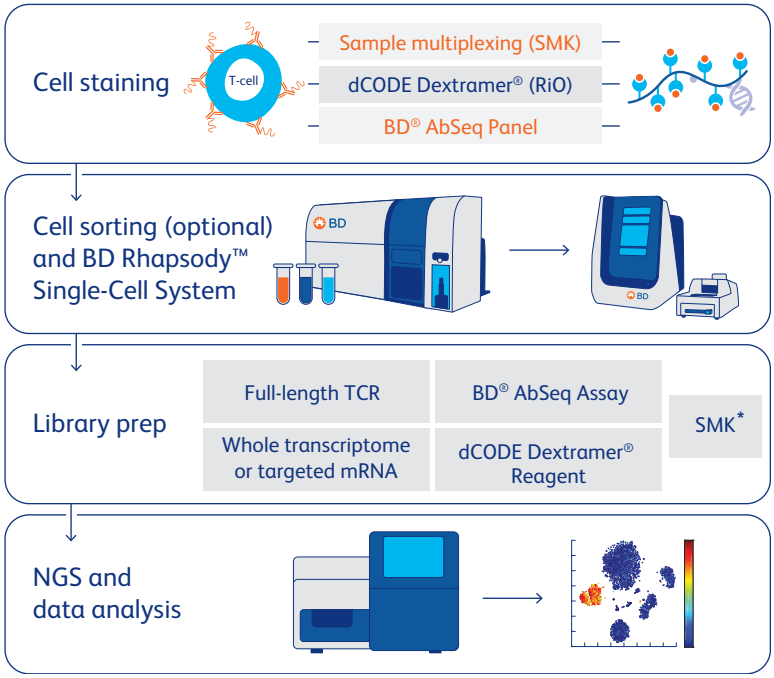
Multiomic profiling of antigen specific T-cells using BD Rhapsody™ Single-Cell Analysis System

Overview

Deep characterization of antigen-specific T-cells can provide essential insight needed to advance the field of immunology. Here, we offer a multiomic approach for profiling T-cells at the single-cell level using two powerful technologies: Immudex® dCODE Dextramer® (RiO) Reagents and the BD Rhapsody™ Single-Cell Analysis System. This approach allows users to detect and characterize low-frequency antigen-specific T-cells while simultaneously obtaining the full sequences of the V(D)J gene segments of T-cell receptors, along with transcriptome and cell surface protein expression information within a single streamlined workflow (Figure 1).

immudex®

BD



*BD® Flex SMK Assay may not be compatible with this assay. The standard BD® SMK Assay is recommended instead.

Highlights

- Compatible with BD Rhapsody™ TCR Full Length Assay using targeted mRNA or whole transcriptome approaches
- Cell surface protein profiling with BD® AbSeq Antibody-Oligonucleotide Conjugates
- Flexibility for high-throughput sample processing using the BD® Single-Cell Multiplexing Kit
- Controlled sequencing depth for dCODE Dextramer® (RiO) libraries allowing increased sensitivity
- Reliable dCODE Dextramer® (RiO) performance: highly concordant single-cell and flow cytometry performance

Figure 1. Workflow overview.

Multiple samples can be combined after staining with the BD® Single-Cell Multiplexing Kit (SMK) to increase throughput and assay efficiency. Following SMK staining, the combined samples can be stained with a panel of dCODE Dextramer® (RiO) Reagents followed by BD® AbSeq Antibody-Oligonucleotide Conjugates. Using the PE fluorophore on dCODE Dextramer® (RiO), dCODE Dextramer® (RiO)+ cells can be enriched using FACS followed by loading into the BD Rhapsody™ Single-Cell Analysis System. The sample can then be processed using the BD Rhapsody™ Full Length TCR Assay, generating sequencing-ready libraries, which will provide full-length TCR sequences alongside the gene and protein expression information of individual cells.
This may not be compatible with BD® Flex SMK Single-Cell Multiplexing Kits.

Controlled sequencing depth for dCODE Dextramer® libraries allows increased sensitivity

This assay allows users to control sequencing depths for the dCODE Dextramer® library separately from all other libraries, including AbSeq. Using certain AbSeq panels and cell types, AbSeq molecules can be higher in abundance compared to the dCODE Dextramer® molecules that were captured on TCR, which would make it harder to detect dCODE Dextramer® when the AbSeq and dCODE Dextramer® library are combined. To detect dCODE Dextramer®, the library needs to be sequenced with higher reads per cell to obtain higher sequencing saturation (Figure 2). It is imperative, therefore, that these libraries are generated separately to avoid deep sequencing that would otherwise be required to achieve dCODE Dextramer® molecule detection if these libraries were to be combined. This key feature can save money by avoiding extraneous sequencing expenses.

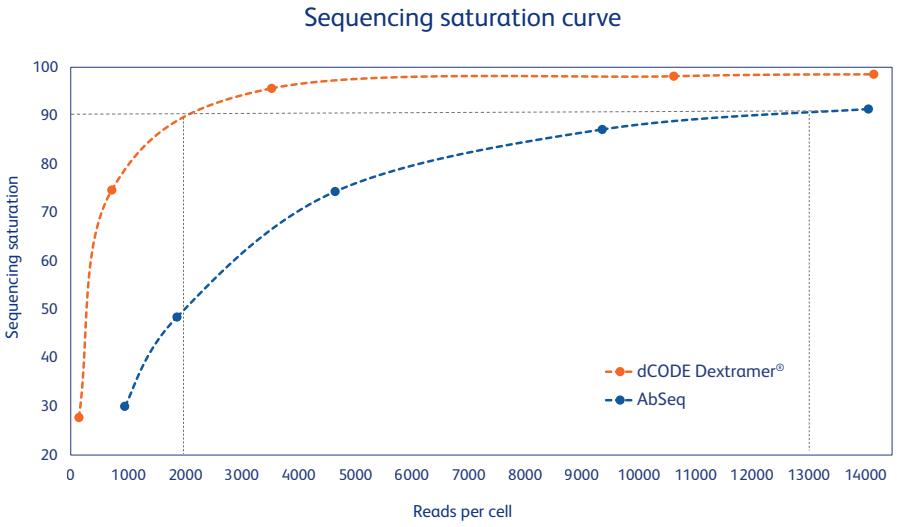


Figure 2: A split library approach can result in lowering sequencing costs.

Sequencing saturation curves for an 8-plex dCODE Dextramer® panel (orange) and a 15-plex AbSeq panel (blue) are shown from an example experiment. At 2,000 reads per cell, only a minority of AbSeq molecules (sequencing saturation >50%) have been sequenced since the AbSeq molecules for this experiment are in such high abundance. However, at that same read depth, the majority of dCODE Dextramer® molecules (sequencing saturation ~90%) were detected since there were fewer molecules of dCODE Dextramer®. This underscores the importance of being able to separately control read depths for AbSeq as compared to dCODE Dextramer®, which, as shown in this example, may require less read depth than AbSeq for complete molecule detection. Gray lines show reads per cell needed to get ~90% sequencing saturation for dCODE Dextramer® (2,000 reads per cell) versus AbSeq libraries, which need 13,000 reads per cell.

Reliable dCODE Dextramer® performance: highly concordant single-cell and flow cytometry performance

The BD Rhapsody™ System effectively identifies dCODE Dextramer®-positive populations with similar cell counts as compared to positive dCODE Dextramer® cells identified by flow cytometry (Figure 3). Cell aliquots were individually stained with eight dCODE Dextramer® reagents by flow cytometry using a BD FACSCanto™ Analyzer. These cell counts were then compared to dCODE Dextramer®-positive cell populations identified using the BD Rhapsody™ System, which used the same cell type and dCODE Dextramer® panel. The results show that the frequencies of positive cells found from single-cell sequencing using the BD Rhapsody™ System are highly concordant with those found by flow cytometry.

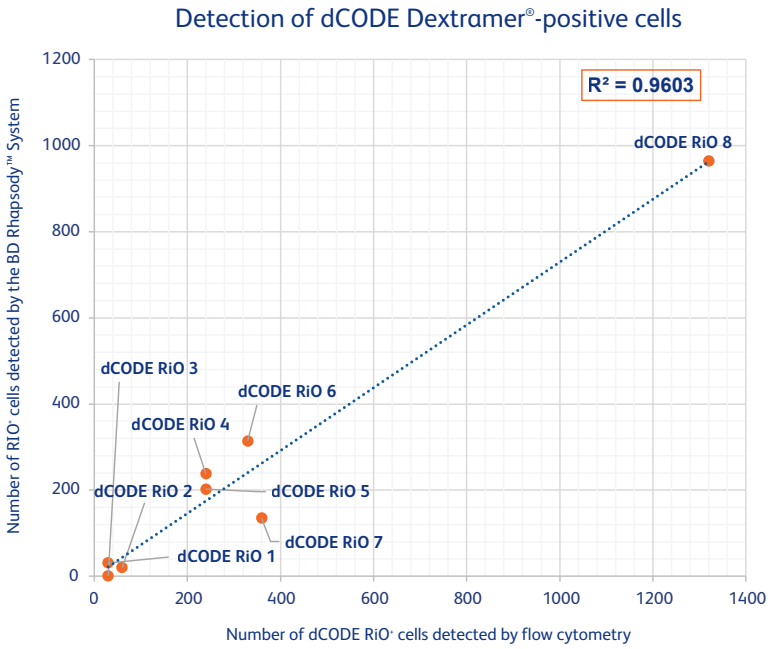


Figure 3: High correlation between dCODE Dextramer®-positive cell detection using two different approaches: Flow cytometry versus the BD Rhapsody™ Single-Cell Analysis System.

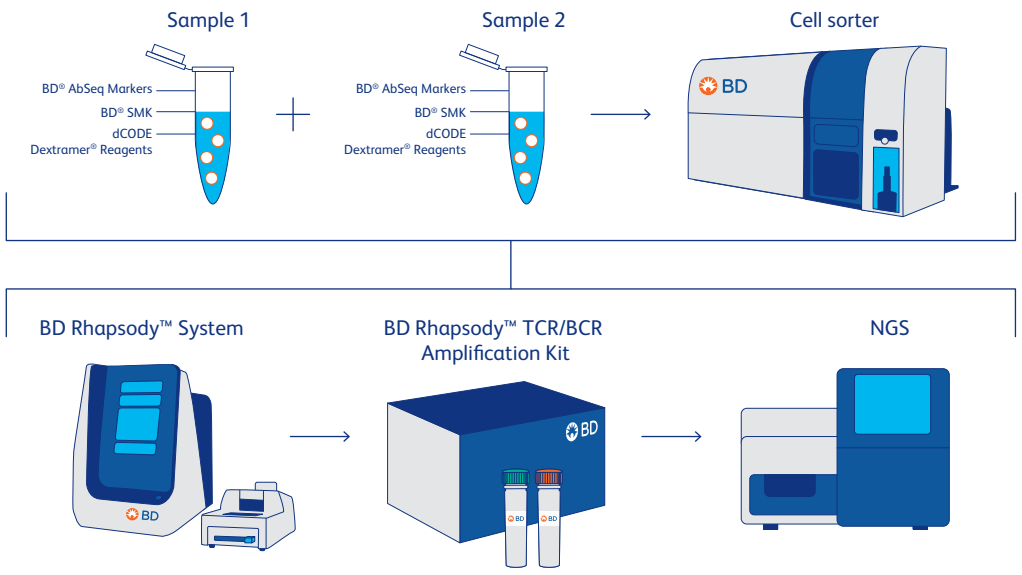
dCODE Dextramer®-positive cells were identified by either flow cytometry or by the BD Rhapsody™ Single-Cell Analysis System from a cell sample stained with eight antigen-specific dCODE Dextramer® (RiO) reagents, listed here as dCODE RiO 1 to dCODE RiO 8.

Experimental data

Demonstrating the compatibility of Immudex® dCODE Dextramer® (RiO) Reagents with the BD Rhapsody™ TCR Full Length Assay

To demonstrate the integration of Immudex® dCODE Dextramer® dCODE reagent profiling into the TCR full-length, targeted mRNA, BD® AbSeq Assay and sample tag workflow, we conducted a study using resting hPBMCs from the same donor that were independently stained with an identical panel of dCODE Dextramer® reagents followed by co-staining of the same BD® AbSeq Reagents and different sample tags. Independently staining each cell sample allowed us to evaluate the reproducibility and reliability of multiplexing, dCODE Dextramer® reagent and AbSeq marker performance. The cell samples were sorted for dCODE Dextramer®+ and live CD8+ cells using a BD FACSAria™ Cell Sorter by additional staining with anti-CD8 APC-H7 antibody and 7-AAD dye for live/dead cell detection. After sorting, the cells were combined and loaded into a single BD Rhapsody™ Cartridge whereby single cells were partitioned into microwells. Beads were then loaded prior to cell lysis enabling the capture of polyadenylated transcripts and oligonucleotide tags from the cells. Following capture, cDNA and library preparations were completed for next generation sequencing (NGS) and data analysis (Figure 4).

A.



B.

dCODE® RiO Panels		
dCODE ID number	Specificity	MHC allele/peptide
RiO_5036	EBV 1	B3501_EPLPQGQLTAY
RiO_5037	EBV 2	B3501_HPVGEADYFEY
RiO_5030	EBV 3	A0201_GLCTLVAML
RiO_5031	EBV 4	A0201_FLYALALLL
RiO_5038	CMV 1	B3501_IPSINVHHY
RiO_5032	CMV 2	A0201_NLVPMVATV
RiO_5035	CMV 3	A0201_VLEETSVML
RiO_5029	FLU	A0201_GILGFVFTL
RiO_5024	Neg.Ctrl 1	A0101_SLEGGGLGY
RiO_5025	Neg.Ctrl 2	A0201_ALIAPVHAV
RiO_5026	Neg.Ctrl 3	A2402_AYSSAGASI
RiO_5027	Neg.Ctrl 4	B0702_GPAESAAGL
RiO_5028	Neg.Ctrl 5	General NC_NC

BD® AbSeq Panel		
AbSeq specificity	Clone	Product number
CD11b	M1/70	940008
CD45RA	HI100	940011
CD69	FN50	940019
CD279	EH12.1	940015
TCRgd	B1	940057
TIM-3	7D3	940066
LAG-3	T47-530	940080
CD56	NCAM16.2	940007
CD19	SJ25C1	940004
CD4	SK3	940001
CD3	SK7	940000
HLA-DR	G46-6	940010
CD45RO	UCHL1	940022
CD62L	DREG-56	940041
TCRab	IP26	940074

Figure 4. Experimental workflow.

(A) A schematic of the experimental workflow showing two samples independently stained with SMK, AbSeq and dCODE Dextramer® reagents, which were then combined and sorted using flow cytometry to enrich for CD8+ dCODE Dextramer®-positive cells. The multiplexed samples were then loaded onto a single cartridge and processed through the BD Rhapsody™ System followed by library preparation and NGS. **(B)** Listed are the dCODE Dextramer® and BD® AbSeq Panels used to stain cells in this experiment. Both samples were stained with the same dCODE Dextramer® and BD® AbSeq Panels.

Results

Since Samples 1 and 2 were replicates of each other, data from these samples were combined for further analysis. Together these samples yielded approximately 4,000 CD8+ dCODE Dextramer®+ sorted cells and showed distinct clustering that corresponded to one of eight antigen-positive dCODE reagents as shown in the tSNE plots below (Figure 5). Importantly, cells were also stained with five negative dCODE Dextramer® reagents (non-antigen reagents) used to evaluate the background level of the dCODE Dextramer® signal. The sum of all five negative dCODE Dextramer® reagents yielded little to no background of nonspecific binding as shown in the tSNE plot below, suggesting high specificity of antigen-positive dCODE Dextramer® binding.

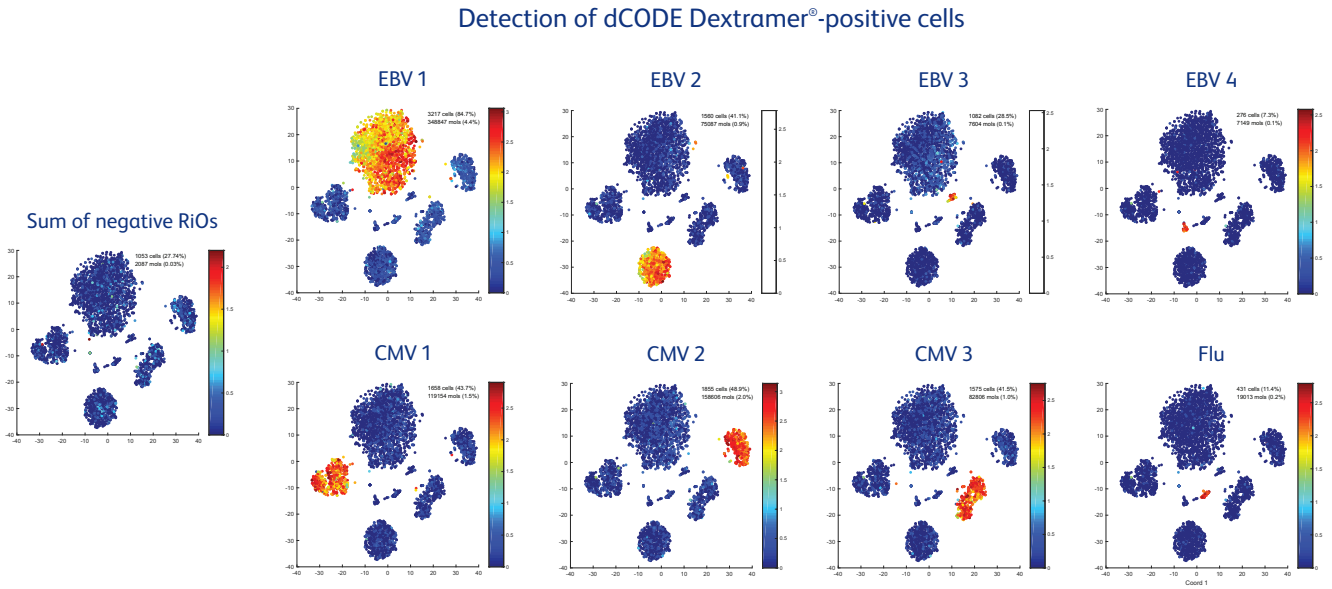
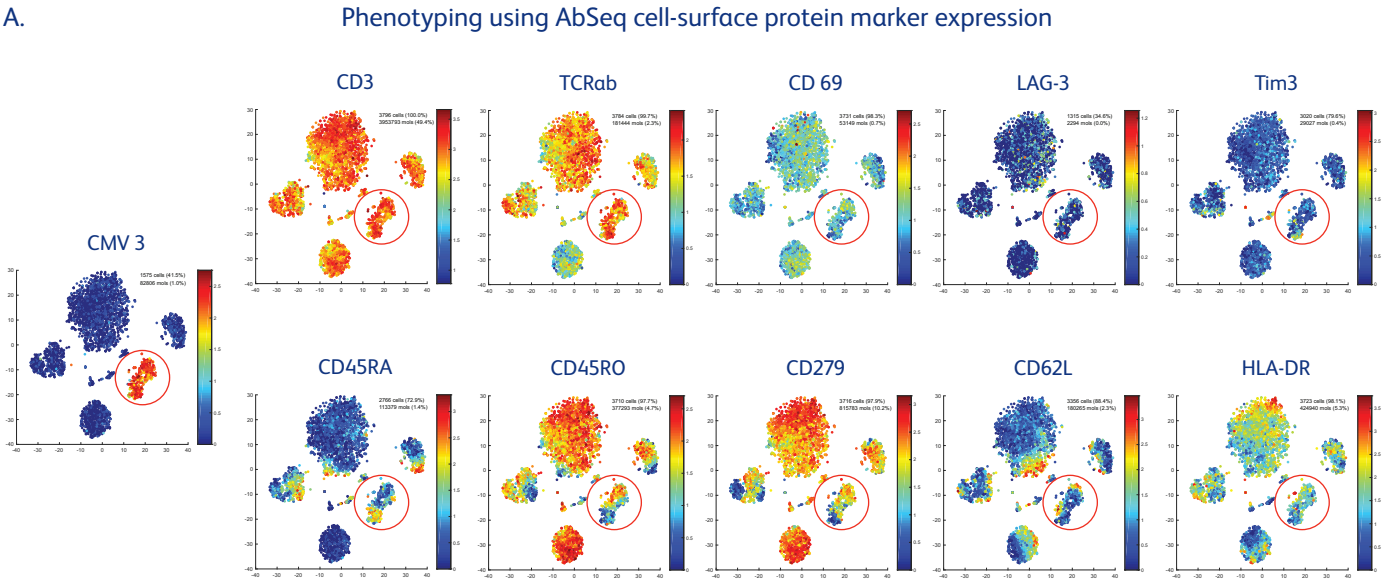


Figure 5. Detection of dCODE Dextramer®-positive cells.

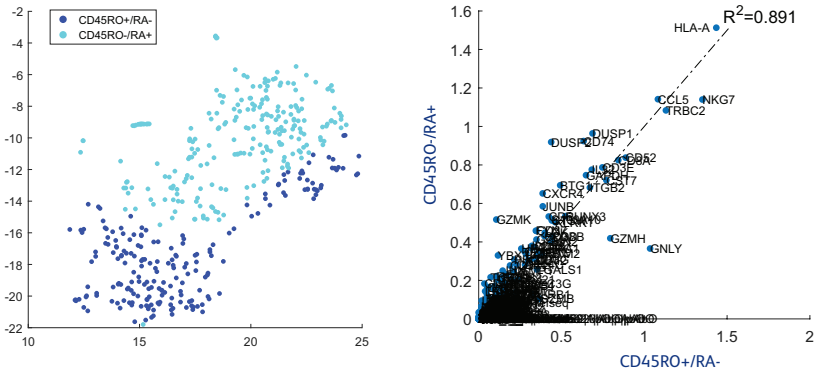
Shown are tSNE plots highlighting the molecules per cell detected for each of the eight antigen-specific dCODE Dextramer® reagents used in this experiment. Molecules per cell detected for the five negative dCODE Dextramer® (non-antigen) reagents were combined and displayed in a single tSNE plot and show little to no background noise.

To demonstrate as an example of how the data could be analyzed more deeply, we choose to focus on one cluster corresponding to cells showing high CMV 3 dCODE signal (a similar approach could be used to further analyze other clusters or dCODE Dextramer®+ cells). Using AbSeq markers to profile cell surface protein expression, distinct sub-populations were identified (Figure 6A). Two discrete sub-populations of cells were distinguished as either CD45RO+/CD45RA- or CD45RO-/CD45RA+. Using the targeted mRNA gene expression data (containing approximately 400 gene targets), a differential gene expression plot was generated revealing certain genes in these sub-populations that were significantly upregulated (Figure 6B).

A.



B. Sub-populations found in a cluster with high CMV 3 dCODE Dextramer® detection



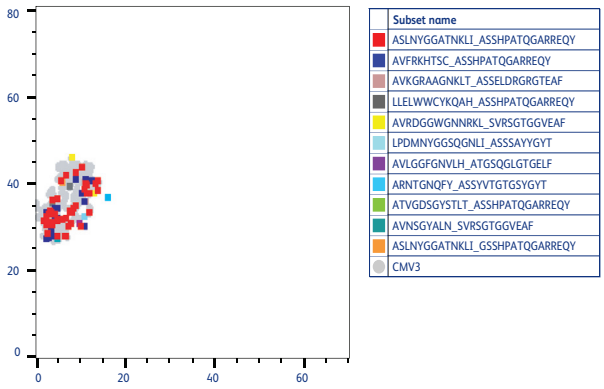
Group name	Gene	Fold change	p-value
CD45RO-/RA+	ZNF683	7.65	4.36E-16
CD45RO-/RA+	GZMB	4.07	8.40E-27
CD45RO-/RA+	GNLY	3.3	2.10E-66
CD45RO-/RA+	KLRB1	2.93	5.22E-20
CD45RO-/RA+	GZMH	1.96	7.59E-19
CD45RO-/RA+	NKG7	1.47	3.76E-28
CD45RO+/RA-	GZMK	5.88	2.58E-33
CD45RO+/RA-	DUSP2	3.17	3.48E-33
CD45RO+/RA-	CD74	2.33	5.95E-14
CD45RO+/RA-	DUSP1	1.97	8.91E-14

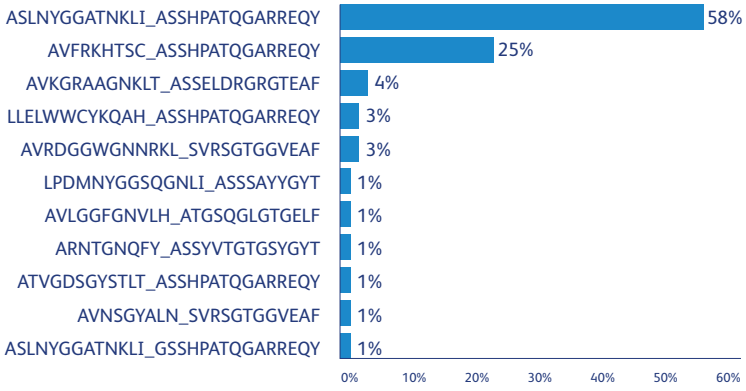
Figure 6. Profiling sub-populations within a cluster using cell surface protein and mRNA gene expression.

(A) tSNE plots showing AbSeq expression from 10 relevant markers. Circled is the cluster of cells that shows a high detection of CMV 3 dCODE Dextramer®. **(B)** Differential gene expression plots of two sub-populations; CD45RO-/CD45RA+ (naïve) and CD45RO+/CD45RA- (effector) showing several genes that are significantly upregulated.

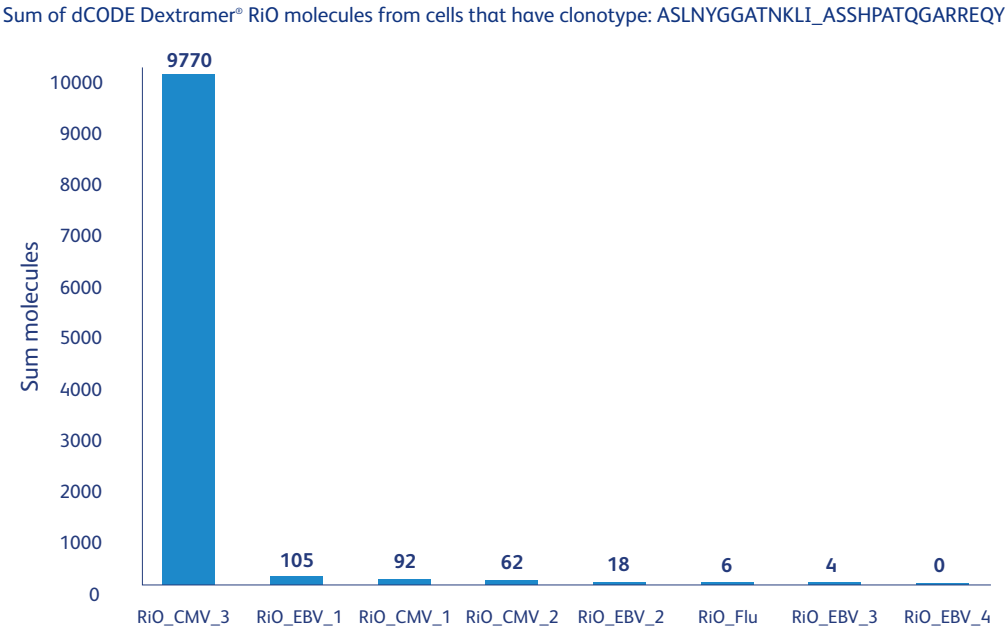
Next, we examined the CDR3 clonotypes identified in the cluster of cells that had a high detection of CMV 3 dCODE Dextramer® and overlayed these clonotypes on a newly generated tSNE plot (note that the tSNE plot in Figure 7A is slightly different than that in Figure 6, but the cells are the same). The percentage of cells with these clonotypes are shown alongside the tSNE plot below (Figure 7A). The amino acid translated sequence for the most frequent alpha/beta paired CDR3 clonotype found was "ASLNYGGATNKLI/ASSHPATQGARREQY." Since these cells were identified in a cluster that had high detection of CMV 3 dCODE Dextramer®, it is likely that this clonotype may have high affinity to the antigen epitope (VLEETSVML) associated with this CMV 3 dCODE Dextramer®. However, to further support this, we identified the paired CDR3 clonotypes using all the cells in the experiment and assessed the number of molecules associated with each dCODE Dextramer®. The results showed that there was an overwhelming number of molecules for CMV 3 dCODE Dextramer® from all CD8+ cells harboring the "ASLNYGGATNKLI/ASSHPATQGARREQY" clonotype (Figure 7B). Finally, using the cells having the "ASLNYGGATNKLI/ASSHPATQGARREQY" CDR3 clonotype, we identified the full-length alpha/beta paired TCR sequences. There were a variety of full-length sequences; however one sequence was most prevalent (Figure 7C).

A. TCR alpha/beta chain clonotypes found in cluster of cells that had high detection of CMV3 dCODE Dextramer®





B. TCR alpha/beta chain clonotypes found in cluster of cells that had high detection of CMV3 dCODE Dextramer® RiO



C.

Frequency alpha/beta paired sequence	TCR chain	FR1_Translation_Dominant	CDR1_ Translation_ Dominant	FR2_Translation_ Dominant	CDR2_ Translation_ Dominant	FR3_Translation_Dominant	CDR3_Translation_ Dominant	FR4_ Translation_ Dominant
71%	alpha	ILNVEQSPQSLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	GSLRRRYGESLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	ILNVEQSPQSLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRIRATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	ILNVEQSPQSLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSSLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	ILNVEQSPQSLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	DAVVAQSPRYMITEKSPAVAFWCDPF	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	ILNVEQSPQSLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVTFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	LINKRRRYGGLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	LLMLSTCGESLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLY	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYRYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	PDMRIMYGESLHVQEGDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT
3%	alpha	QIAVTLLVLRDSTNFTCSFP	SSNFYA	LHWYRWETAKSPEALFV	MTLNGDE	KKKGRISATLNTKEGYSYLYIKGSQPEDSATYLC	ASLNYGGATNKLI	FGTGTLLAVQP
	beta	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT	LYWYRQILGQGPELLVQ	FQDESV	VDDSQLPKDRFSAERLKGVDSTLKIQPAELGDSAMYLC	ASSHPATQGARREQY	FGPGTRLTVT

Figure 7. TCR alpha/beta clonotype analysis.

(A) A tSNE plot of cells with high detection of CMV 3 dCODE Dextramer® with an overlay of the CDR3 clonotypes found in this population. Alongside the tSNE is a plot showing the frequencies of these clonotypes. **(B)** Using all the CD8+ cells in the experiment that harbored the clonotype "ASLNYGGATNKLI/ ASSHPATQGARREQY," the sum of dCODE Dextramer® molecules was reported for each dCODE Dextramer®. **(C)** Full-length TCR alpha/beta chain sequences are shown for a subset of cells that share the same CDR3 clonotype (ASLNYGGATNKLI/ASSHPATQGARREQY).

Demonstrating the reproducibility and reliability of sample multiplexing in the Immudex® dCODE Dextramer® dCODE (RiO) plus BD Rhapsody™ TCR Full Length Assay workflow

We next evaluated the compatibility of using the BD® Single-Cell Multiplexing Kit (SMK) with the dCODE Dextramer® technology in the TCR full length + targeted mRNA, BD® AbSeq Assay and sample tag workflow by examining dCODE Dextramer® reagent and AbSeq marker performance. As previously mentioned, the two samples in this study were independently stained with an identical panel of dCODE Dextramer® reagents followed by co-staining of the same BD® AbSeq Reagents. A tSNE plot was generated as seen below in Figure 8A, which shows an overlay of the two samples with no batch effect (samples were stained with Sample tag 5 and Sample tag 6). An evaluation of AbSeq marker expression and dCODE Dextramer® detection was conducted showing very similar profiles for AbSeq expression and a high correlation of dCODE Dextramer® molecules for each sample (Figure 8B and 8C).

A. tSNE plot showing no batch effects for multiplexed samples

B. Median number of molecules per cell, log 10

C. Correlation: mean(log10 (molecules per cell per gene))

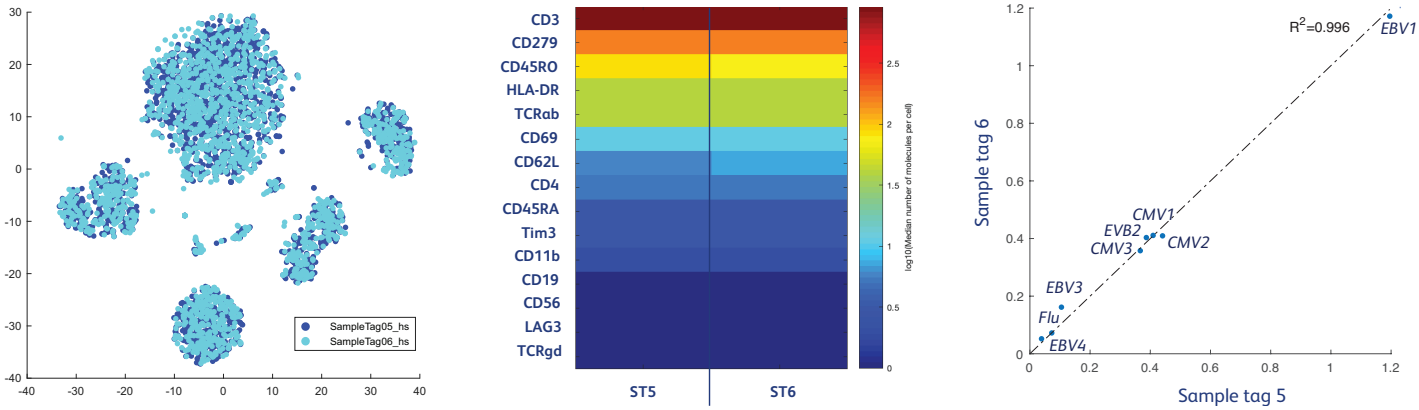


Figure 8. Evaluation of sample multiplexing.

(A) tSNE plot of two multiplexed samples showing no batch effect. **(B)** 15-plex AbSeq marker expression for two samples that were independently stained with the same AbSeq panel (ST5 = Sample tag 5, ST6 = Sample tag 6). **(C)** Correlation plot of eight antigen-positive dCODE Dextramer® for both samples (Sample tag 5 and Sample tag 6) that were independently stained with the same dCODE Dextramer® panel.

Conclusions

- dCODE Dextramer® (RiO) Reagents can be seamlessly integrated into a targeted mRNA and TCR full-length workflow to profile hPBMCs, while being compatible with BD® AbSeq Assays and the BD® Single-Cell Multiplexing Kit.
- Antigen-specific T-cells along with their corresponding full-length TCR sequences were identified by viral-specific HLA-peptide complexes displayed on dCODE Dextramer® (RiO) Reagents used in combination with TCR full-length assays.
- Sample multiplexing of three different samples showed reproducibility of BD® AbSeq Assays and dCODE Dextramer® performance as well as a sensitive detection of dCODE Dextramer® reagents from different cell types (CD4+ and CD8+ cells).

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