A high-throughput flow cytometry assay to assess PD-1 receptor occupancy

Drug-discovery applications for the BD FACSCelesta™ flow cytometer with the High Throughput Sampler (HTS) option

Features

- Assess drug-target interactions in a cell-based screening assay
- Measure binding of anti-PD-1 therapeutic antibodies to T-cell subsets
- Automate sample acquisition from a 96-well plate on a High-Throughput Sampler

Multicolor flow cytometry has emerged as a useful platform in drug discovery and development, especially to monitor drug-target cell interactions and responses in cellular assays. Because these assays often involve a large number of drug compounds, dosages or test samples, they require automated high-throughput microplate-based sampling to make them feasible.

In this data sheet, we describe a flow-cytometry-based receptor-occupancy assay to evaluate therapeutic antibodies that target programmed cell death protein 1 (PD-1 or CD279), an immune checkpoint inhibitor expressed on the surface of T cells. The BD FACSCelesta™ flow cytometer equipped with the High Throughput Sampler (HTS) option is ideally suited for investigating the interactions between these antibodies and their target.

PD-1 is a member of the CD28 superfamily of cell-surface receptors that delivers co-inhibitory signals upon interaction with its ligands, PD-L1 and PD-L2. PD-1 signaling inhibits T-cell proliferation, cytokine production and cytotoxic activity, and thus plays an important role

in the control of immune responses and the maintenance of T-cell homeostasis. In cancer, PD-1 signaling may go awry, resulting in a progressive loss of T-cell effector activity and ultimately cancer-cell immune escape and tumor progression.

Thus, PD-1 is an important target for research on new cancer therapeutics. For example, pembrolizumab and nivolumab are clinically approved monoclonal antibodies that target PD-1. These therapeutic antibodies block the interaction of PD-1 and its ligands, restoring T-cell immunity against cancer cells. In addition to their use as monotherapy, both the antibodies are being tested in combination with other anti-cancer therapies.[1,2]

We used a receptor-occupancy approach to determine the binding of pembrolizumab and nivolumab to the target T cells. Because the anti-PD-1 therapeutic drugs block receptor-ligand interactions, the approach we adopted measures the expression of free PD-1 receptors that are available for ligand binding (free receptor assay). Other receptor-occupancy strategies include directly determining the percentage of drug-occupied receptors (bound receptor assay) or total receptors (total receptor assay).

The plate design (Figure 1) shows why the HTS option was essential to streamline the experimental workflow: it enabled the simultaneous analysis of cells from multiple donors subjected to different in vitro cell culture conditions and a range of antibody dilutions. In this particular example, cells from one donor were tested with several dilutions of therapeutics. Multicolor flow cytometry with the BD FACSCelesta BVUV configuration also enabled the analysis of the interactions between anti-PD-1 therapeutic drugs and PD-1 on both $CD4^+$ and $CD8^+$ T-cell subsets.

Figure 1

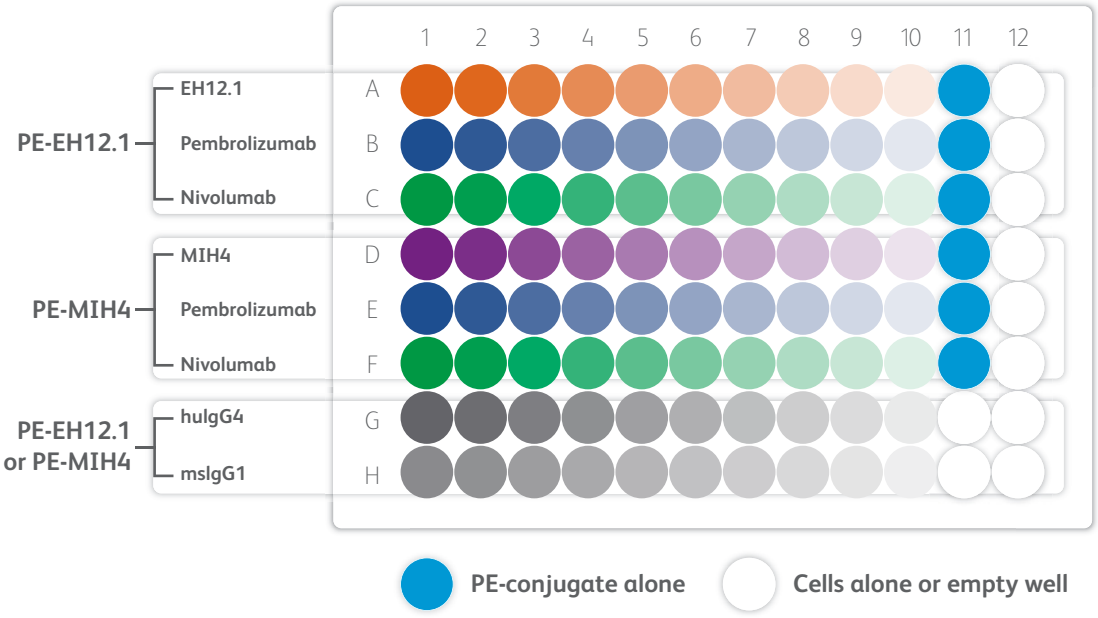

Figure 1. Example layout for 96-well microplate sample acquisition on HTS
The color scheme represents serial dilutions of anti-PD-1 antibodies, with a fixed amount of competitive PE conjugate anti-PD-1 antibodies.

Our first step was to identify and validate BD Pharmingen™ PE Mouse Anti-Human CD279 (PD-1), clone EH12.1 (PE-EH12.1), as a detection reagent for the analyses of free PD-1 receptors. As shown in Figure 2, PE-EH12.1 detected PD-1 expression in both unstimulated and anti-CD3- stimulated peripheral blood mononuclear cells (PBMCs). The percentages of PD-1$^+$ T cells were higher in the anti-CD3-stimulated cultures from all six donors investigated (Figures 2A and 2B). Analysis of PD-1 expression on CD4$^+$ and CD8$^+$ T cells showed that the percentages of PD-1$^+$ T cells increased after stimulation in both cell subsets (Figure 2B).

The increase in PD-1$^+$ cells in anti-CD3 stimulated cultures prompted us to titrate PE-EH12.1 in different cell culture conditions to determine the optimal saturating concentration of the reagent for cell analyses. The titration curves showed that at the optimal saturating concentration of 1.25 µg/mL, PE-EH12.1 yielded maximum stain index in both unstimulated and anti-CD3 stimulated cells (Figure 2C). Although unstimulated and anti-CD3 stimulated cells expressed similar levels of PD-1, the stain index values showed that CD8$^+$ (CD3$^+$CD4$^-$) T cells expressed higher levels than CD4$^+$ (CD3$^+$CD4$^+$) T cells.

Figure 2A

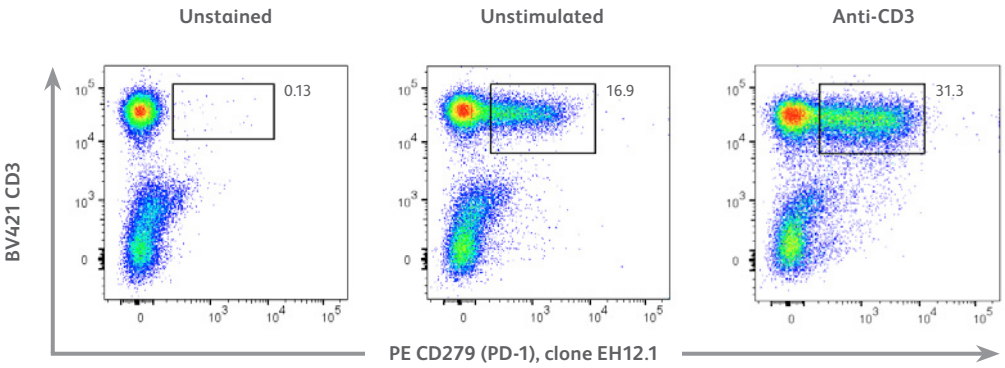

Figure 2B

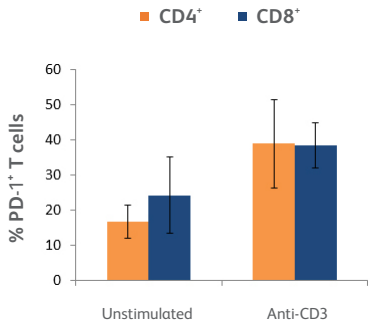

Figure 2C

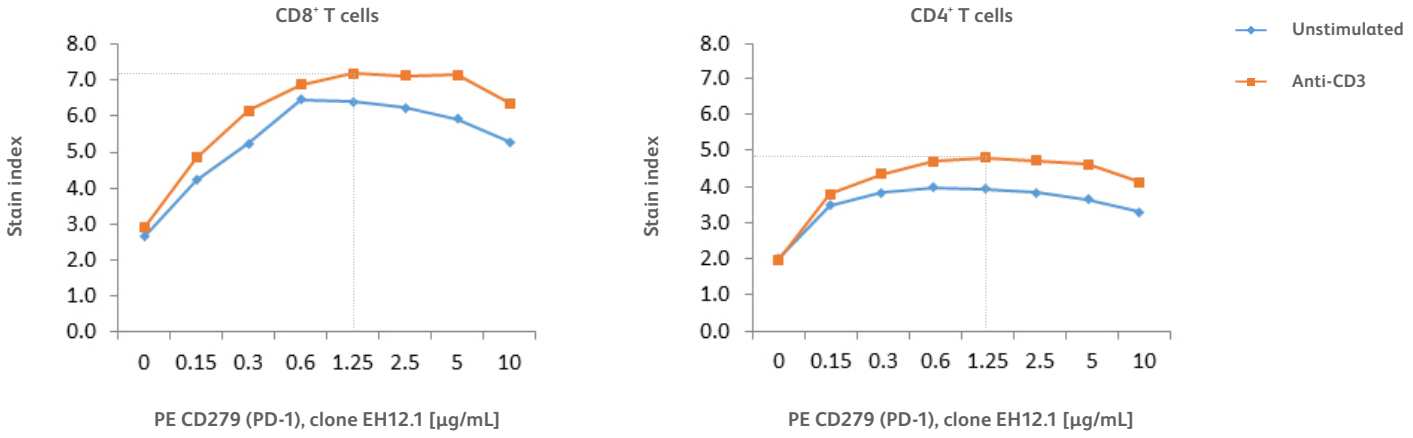

Figure 2. Analysis of PD-1 expression on activated T cells using PE-EH12.1 as detection reagent

PMBCs were stimulated overnight with 10 µg/mL of immobilized BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD3 or cultured in media alone (unstimulated cells). Unstimulated and anti-CD3 stimulated cells were pre-incubated with BD Pharmingen™ Human BD Fc Block™ and stained with BD Horizon™ BV421 Anti-Human CD3, BD Horizon™ BUV395 Anti-Human CD4 and serial two-fold dilutions (ranging from 0.15 µg/mL to 10 µg/mL) of BD Pharmingen™ PE Mouse Anti-Human CD279 (PD-1), clone EH12.1 (PE-EH12.1). **Results: A.** Representative data from one donor showing expression of PD-1 and CD3 on lymphocytes (gated using forward and side scatter, not shown). **B.** Bar graph represents percentages of PD-1$^+$ T cells among CD4$^+$ (orange) and CD8$^+$ (blue) T-cell subsets in samples from six donors that were stimulated with anti-CD3 or left unstimulated. **C.** Titration curves show the optimal concentration of PE-EH12.1 (1.25 µg/mL) yielding the highest stain index on CD4$^+$ and CD8$^+$ T-cell subsets in the different culture conditions. Overall, results show that anti-CD3 stimulation increased the percentages of T cells expressing PD-1 among both CD4$^+$ T and CD8$^+$ T cell subsets, with PD-1 expression higher on CD8$^+$ T cells.

Next, we designed a competition assay between PE-EH12.1 and the therapeutic antibodies to compare their binding strength or affinity. Our fixed optimal concentration of PE-EH12.1 (1.25 µg/mL) was mixed with serial dilutions of the purified anti-PD-1 antibodies. Conjugated antibodies anti-CD3 and anti-CD4 were also added to the mixture, and anti-CD3 stimulated PBMCs from one donor were stained with the antibody cocktail in triplicate. The percentages of PE-EH12.1-labeled cells within the $CD4^+$ or $CD8^+$ T-cell subsets were calculated and graphed in Figure 3 as a percentage of the maximum, corresponding to cells stained with PE-EH12.1 without purified antibodies.

Figure 3A

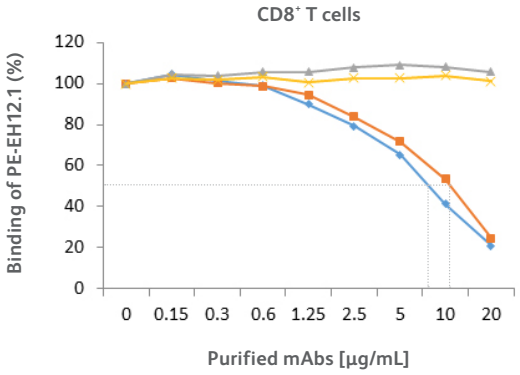

Figure 3B

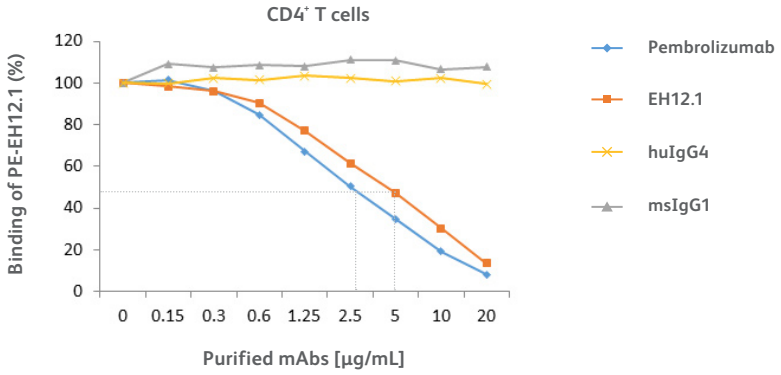

Figure 3. PE-EH12.1 competes with pembrolizumab for binding to PD-1 on activated T cells

PBMCs were stimulated overnight with 10 µg/mL of immobilized BD Pharmingen™ Purified NA/LE Anti-Human CD3. The cells were pre-incubated with BD Pharmingen™ Human BD Fc Block and stained as follows in triplicate. To determine whether clone EH12.1 competes with pembrolizumab for binding to PD-1, the cells were stained with BD Pharmingen™ PE Mouse Anti-Human CD279 (PD-1), clone EH12.1 (PE-EH12.1) at a fixed saturating concentration of 1.25 µg/mL and with serial two-fold dilutions (ranging from 0.15 µg/mL to 20 µg/mL) of the purified antibodies BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD279 (PD-1) or pembrolizumab (BioVision). The cells were also co-stained with BD Horizon™ BUV395 Mouse Anti-Human CD3 and BD Horizon™ BV510 Mouse Anti-Human CD4 to allow discrimination of $CD8^+$ **(A)** and $CD4^+$ **(B)** T-cell subsets. **Results:** Reference binding curves (orange) consist of cells that were stained with PE-EH12.1 and serial dilutions of Purified NA/LE anti-PD-1, clone EH12.1. PE-EH12.1 staining alone in the absence of purified antibodies represents the maximum binding of PE-EH12.1 (100%). As negative controls, the cells were incubated with PE-EH12.1 and serial dilutions of purified isotype controls, BD Pharmingen™ Purified NA/LE Mouse IgG1, κ Isotype control (msIgG1) or human monoclonal IgG4 (huIgG4, BioLegend®), which do not react with PD-1 (yellow and gray curves). The results show that EH12.1 (orange) and pembrolizumab (light blue) compete for binding to PD-1 on both $CD8^+$ and $CD4^+$ T cells with relatively similar affinity.

The binding curve (orange) generated from the competition between PE-EH12.1 and purified EH12.1 was used as reference, since these antibodies bind to the same epitope. Interestingly, the PE-EH12.1/pembrolizumab binding curve (light blue) was almost identical to the reference binding curve for both $CD4^+$ and $CD8^+$ T-cell subsets, suggesting that clone EH12.1 competes with pembrolizumab for binding to PD-1 with similar affinity.

PE-EH12.1 overall binding also decreased in the presence of nivolumab (Figure 4, dark blue curve). In fact, for the single donor tested, it took lower doses of nivolumab (for example 0.3 µg/mL) as compared to purified EH12.1 (2.5 µg/mL) or pembrolizumab (1.25 µg/mL) to reduce PE-EH12.1 binding in 50% of cells. This indicates that nivolumab might compete with EH12.1 with even stronger affinity for the PD-1 receptor.

Figure 4

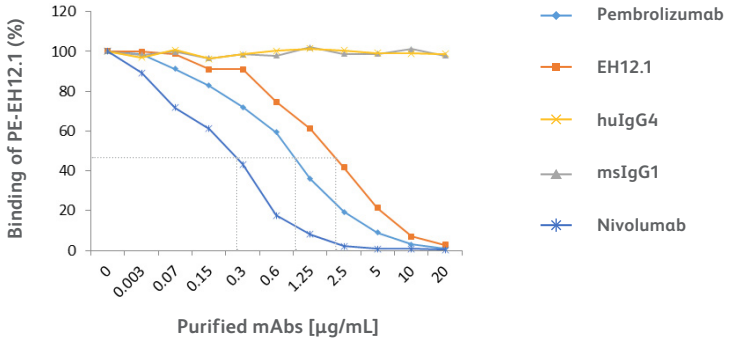

Figure 4. Comparison of pembrolizumab and nivolumab binding to PD-1 on activated T cells

PBMCs from one donor were stimulated and stained as described in Figures 2 and 3. The graph represents PE-EH12.1 binding on $CD8^+$ T cells, gated as described in the text. **Results:** Binding curves for EH12.1/pembrolizumab (light blue) and EH12.1/nivolumab (dark blue) were generated and compared to the EH12.1 reference binding curve (orange). For this donor's sample, lower doses of nivolumab than pembrolizumab or EH12.1 were required to disrupt PE-EH12.1 binding to $CD8^+$ T cells, suggesting that nivolumab binds with higher strength to PD-1 than pembrolizumab or EH12.1.

For contrast, we also performed an antibody-binding competition assay using BD Pharmingen™ PE Mouse Anti-Human CD279, clone MIH4 (PE-MIH4) in combination with the purified antibodies. In this case (Figure 5), both the pembrolizumab and nivolumab binding curves greatly diverged from the reference binding curve, suggesting that MIH4 may bind to a different epitope.

Figure 5

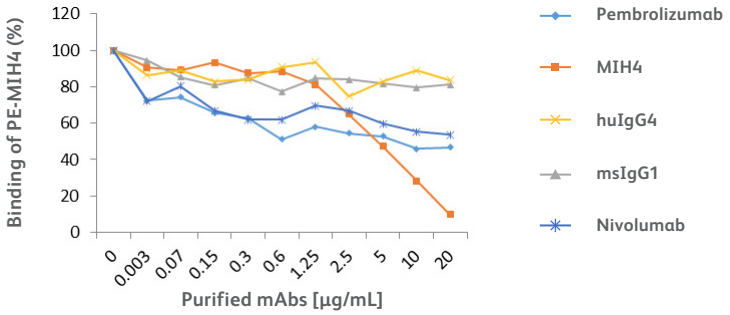

Figure 5. Antibody competition assay using PE-MIH4 as detection reagent

PBMCs from one donor were stimulated and stained as described in Figures 2 and 3, except that PE-MIH4 was used as the detection reagent instead of PE-EH12.1. The cells were stained with PE-MIH4 at a fixed saturating concentration of 0.5 µg/mL and with serial two-fold dilutions (ranging from 0.003 µg/mL to 20 µg/mL) of the purified antibodies shown. **Results:** The pembrolizumab (light blue) and nivolumab (dark blue) binding curves greatly diverged from the reference MIH4 curve (orange), suggesting that these antibodies may bind to different epitopes in the PD-1 receptor.

Finally, we performed an assay to assess PD-1 receptor occupancy on $CD3^+$ T cells in vitro after anti-CD3-stimulated PBMCs were treated for 3 days with 200 µg/mL of pembrolizumab, nivolumab or human IgG4 isotype control. PE-EH12.1 detected PD-1 in untreated cells as well as those cultured with control human IgG4. In contrast, PE-EH12.1 signal was minimal in the cells treated with pembrolizumab or nivolumab, suggesting that the PD-1 receptors were occupied by the blocking antibodies (Figure 6).

Figure 6

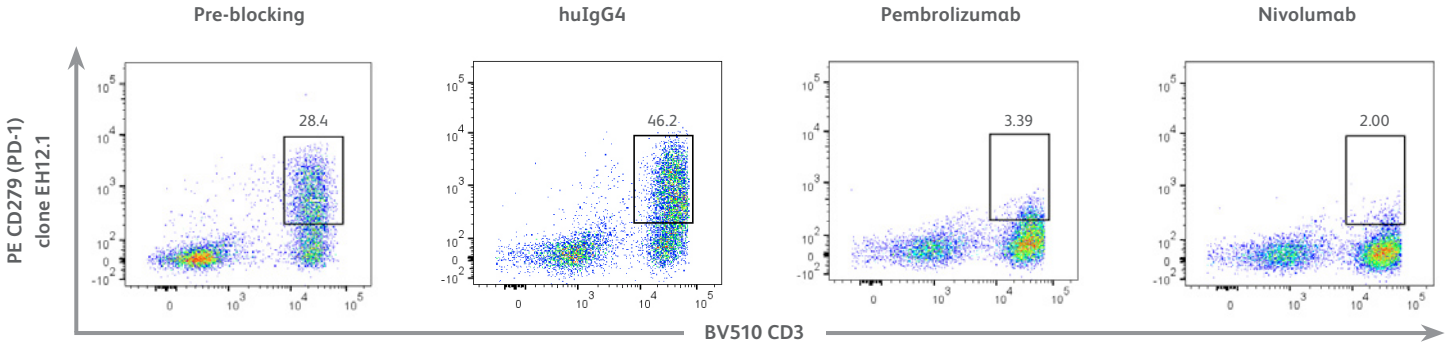

Figure 6. Assessment of PD-1 receptor occupancy in vitro after pembrolizumab or nivolumab treatment

PBMCs from one donor were stimulated overnight with 10 µg/mL of immobilized BD Pharmingen NA/LE Anti-Human CD3. PD-1 expression was assessed on the BD FACSCelesta and then nivolumab, pembrolizumab or human IgG4 isotype control was added to the cultures. The cells were cultured with the blocking anti-PD-1 antibodies as well as immobilized anti-CD3 and 10 ng/mL of BD Pharmingen™ Recombinant Human IL-2 for 3 days. The cells were washed and stained with BD Horizon™ BV510 Mouse Anti-Human CD3 and PE-EH12.1. **Results:** Analysis of the correlated expression of PD-1 and CD3 on gated lymphocytes showed that PE-EH12.1 detected PD-1 in the cultures with huIgG4 control. However, only minimal PE-EH12.1 signal was detected in the cells that were cultured with pembrolizumab or nivolumab, suggesting that these antibodies occupied PD-1 receptors and blocked the interaction between the PE-EH12.1 clone and PD-1.

In summary, PE-EH12.1 (but not PE-MIH4) can effectively be used to detect PD-1 free receptors after cell treatment with pembrolizumab because both antibodies may recognize the same PD-1 epitope with similar affinity. Nivolumab also competed with the EH12.1 clone. However, because nivolumab seems to bind to PD-1 with greater strength, EH12.1 may not be an ideal detection reagent for nivolumab receptor occupancy.

The application reported in this data sheet provided a comprehensive analysis of the binding of pembrolizumab and nivolumab monoclonal antibodies to primary T cells. It successfully revealed the relative strength with which these reagents bind to their target, and provided a robust analysis of the expression of PD-1 on T-cell subsets. Similar binding curves can be used as reference for future drug discovery.

Ordering information

Systems and software	
Description	Cat. No.
BD FACSCelesta™ Flow Cytometer, BVUV Configuration	660346
BD FACSCelesta™ Flow Cytometer, BVR Configuration	660344
BD FACSCelesta™ Flow Cytometer, BVYG Configuration	660345
BD FACSCelesta™ Flow Cytometer, BV Configuration	660343
BD FACSCelesta™ High Throughput Sampler (HTS) Option	658946

Reagents		
Description	Clone	Cat. No.
BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD3	UCHT1	555329
BD Horizon™ BV421 Mouse Anti-Human CD3	UCHT1	562426
BD Horizon™ BV510 Mouse Anti-Human CD3	UCHT1	563109
BD Horizon™ BUV395 Mouse Anti-Human CD3	UCHT1	563546
BD Horizon™ BUV395 Mouse Anti-Human CD4	SK3	563550
BD Horizon™ BV510 Mouse Anti-Human CD4	SK3	562970
BD Pharmingen™ PE Mouse Anti-Human CD279 (PD-1)	EH12.1	560795
BD Pharmingen™ PE Mouse Anti-Human CD279 (PD-1)	MIH4	557946
BD Pharmingen™ Purified NA/LE Mouse Anti-Human CD279 (PD-1)	EH12.1	562138
BD Pharmingen™ Purified Mouse Anti-Human CD279 (PD-1)	MIH4	557823
BD Pharmingen™ Purified NA/LE Mouse IgG1 κ Isotype Control	107.3	554721
BD Pharmingen™ Recombinant Human IL-2		554603
BD Pharmingen™ Human BD Fc Block™		564220
BD Horizon™ Brilliant Stain Buffer Plus		566385

References

1. Gandhi L, Rodriguez-Abreu D, Gadgeel S, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. *N Engl J Med*. 2018;doi: 10.1056/NEJMoa1801005.
2. Hellman MD, Ciuleanu TE, Pluzanski A, et al. Nivolumab plus ipilimumab in lung cancer with a high tumor mutational burden. *N Engl J Med*. 2018;doi: 10.1056/NEJMoa1801946.

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BD Life Sciences, San Jose, CA, 95131, USA

bdbiosciences.com