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TECHNICAL NOTE

Detection of immune checkpoint markers in peripheral blood mononuclear cell lysates with AlphaLISA.

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Introduction

Peripheral blood mononuclear cells (PBMCs) are a heterogeneous population of blood cells having round nuclei. They consist of monocytes, macrophages, dendritic cells and lymphocytes (T cells, B cells, and NK cells). In order to induce and upregulate the expression of specific immune checkpoint markers in the PBMC populations, we used Dynabeads®, polystyrene-coated microspheres with antibodies against human CD3 and CD28 proteins covalently coupled to the surface. The binding of the antibodies to CD3 and CD28 proteins on PBMCs mimic the *in vivo* effects of antigen presenting cells (APCs), stimulating cultured T lymphocytes to proliferate and further differentiate.[1] Once activated, T cells can upregulate a variety of immune checkpoint molecules such as LAG3 (Lymphocyte activation gene-3). Immune checkpoint molecules that are found typically on APCs can also be found within the PBMC population on macrophages and dendritic cells. VISTA, HVEM, CD86 (B7-2) and CD276 (B7-H3) are typically expressed on the APCs and are expected to be expressed within the PBMC population of cells.[2]

When using traditional wash-based ELISAs, one of the challenges in assessing expression levels of multiple immune checkpoint molecules is the high sample consumption (typically 100 – 200 µL per detection molecule of interest) which can be cumbersome when culturing and treating primary cells such as PBMCs. One way to quickly and easily quantitate immune checkpoint markers using a small amount of sample (5 µL per detection molecule of interest) is using AlphaLISA™ no-wash chemiluminescent technology. Figure 1 shows an example of an AlphaLISA assay set up used to detect immune checkpoint markers.

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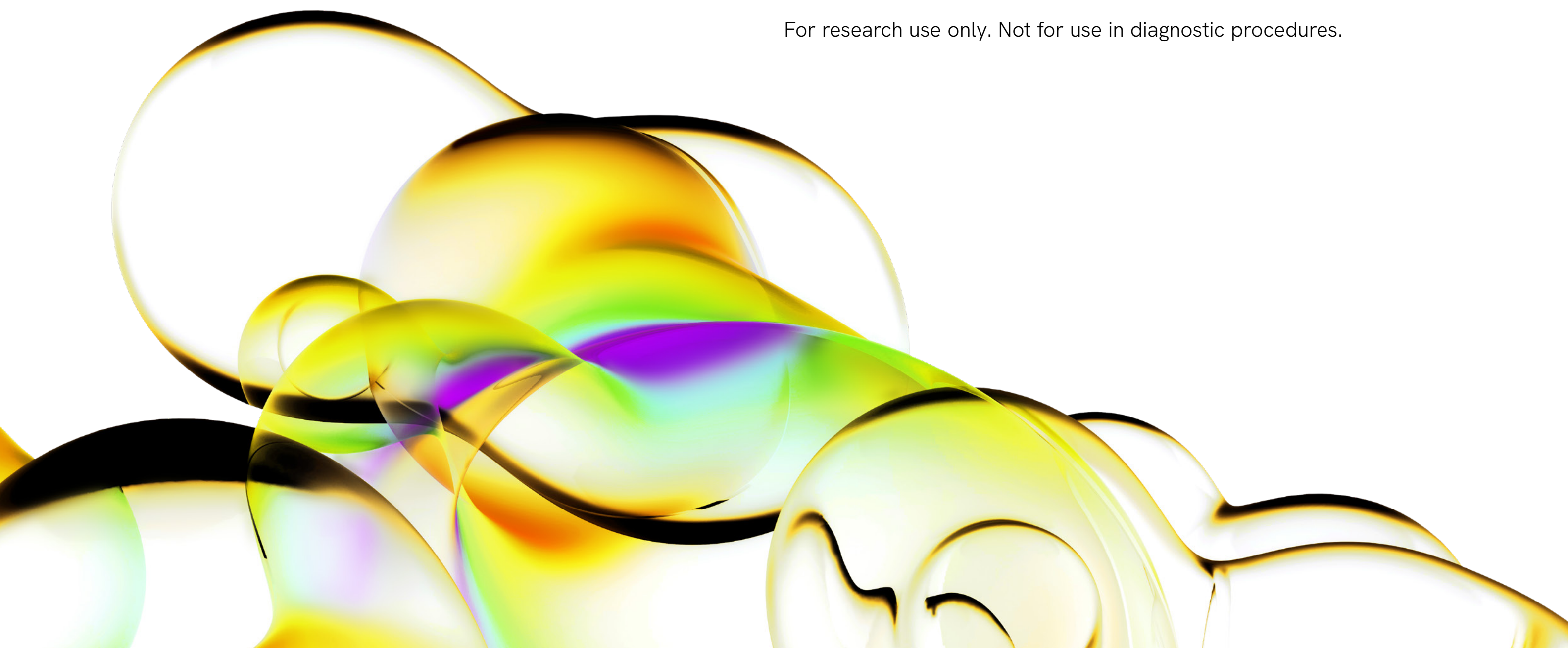

Streptavidin Donor beads bind a biotinylated anti-analyte antibody and an anti-analyte antibody is directly conjugated to the AlphaLISA Acceptor beads. When the analyte is present in the sample, the antibodies bind the analyte and bring the Donor and Acceptor close in space. Upon excitation at 680 nm, the Donor beads generate singlet oxygen which can activate the AlphaLISA Acceptor beads, resulting in light production at 615 nm. In the absence of analyte, no signal is generated. Previously, we showed that CTLA-4 and CD28 expression is increased in PBMCs after activation with Dynabeads®.[3] In this technical note, we show how AlphaLISA can be used to detect and quantify additional immune checkpoint molecules within the PBMC population with and without activation using Dynabeads®.

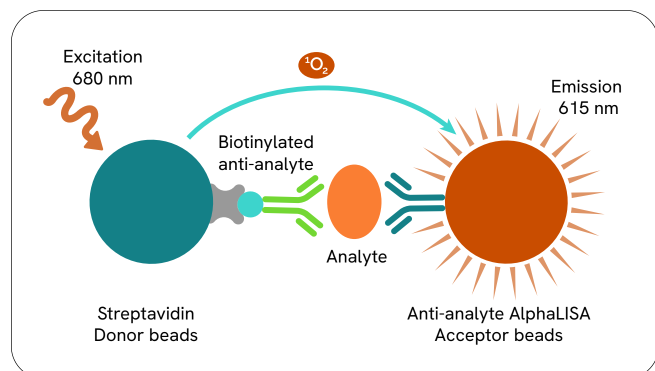

Figure 1: Example of an AlphaLISA Assay for detection of immune checkpoint markers.

Materials and methods

Activation of PBMCs

Normal human primary PBMCs (ATCC® , #PCS-800-011™) stored in liquid nitrogen were rapidly thawed, transferred to ice-cold Hanks Buffered Salt Solution (HBSS, ThermoFisher, #14025-134), and counted with a hemacytometer. Cells were spun down, supernatant removed, and resuspended in RPMI-1620 media (ATCC, #30-2001) supplemented with 10% FBS (ThermoFisher, #26140-079) to achieve the concentrations needed to add 100,000, 50,000 and 25,000 cells in 40 µL to wells of a ½ Area ViewPlate™-96 microplate (Revvity, #6005760). After transferring cells to a microplate, half of the wells were activated with Human T-activator CD3/CD28 Gibco Dynabeads® (ThermoFisher, #11131D) at a standard ratio of one bead to one cell by adding 40 µL of beads suspended in culture media and the other half were control wells which received just culture media. Cells were incubated for two days at 37 °C.

Additional wells with the same conditions were plated to assess relative proliferation rates with ATPlite™ 1step Luminescence Assay System (Revvity, #6016731). After the two day culture period, 55 µL of media was removed and 25 µL of 2X AlphaLISA Immunoassay buffer (#AL000C) was added to each well (to get 1X final concentration) and the microplate was shaken (at 600-700 RPM on a DELFIA™ PlateShaker) for 20 minutes at room temperature. The microplate was then placed on a magnet in order to bring the Dynabeads® to the bottom and 55 µL of lysate samples were transferred to a polypropylene StorPlate-96V (Revvity, #6008290) and kept up to two weeks at -20 °C before AlphaLISA assays were run. The work-flow is shown in Figure 2.

AlphaLISA assay, ATPlite 1step assay and imaging

All AlphaLISA assays were run according to their respective assay manuals. Lysates from the PBMCs were divided into separate assay plates (AlphaPlate™-384 microplates, Revvity, #6005350) and five AlphaLISA kits were run on 5 µL lysate samples from the same wells to measure expression levels of various checkpoint markers (HVEM (#AL3054C), VISTA (#AL3059C), LAG3 (#AL3058C), CD276 (#AL3060C), and CD86 (#AL3045C)). Recombinant proteins provided in each kit were titrated in a mixture of RPMI media (with 10% FBS) and 2X Immunoassay buffer (1:1) to produce standard curves that were used for interpolation of sample data to quantify protein concentrations in the PBMC samples. Figure 3 shows an example standard curve that was used for quantifying CD276 levels. All AlphaLISA assays were measured on the EnVision™ 2105 multimode plate reader using standard Alpha settings.

Cells were imaged using the EnSight™ multimode plate reader prior to running the ATPlite 1step assays to assess cellular proliferation and viability. Assays were run directly in the culture plate by removing 40 µL of media and adding 40 µL of ATPlite 1step substrate solution and shaking (at 700 RPM) for five minutes. White BackSeals™ (Revvity, #6005199) were added and luminescence was measured on the EnSight.

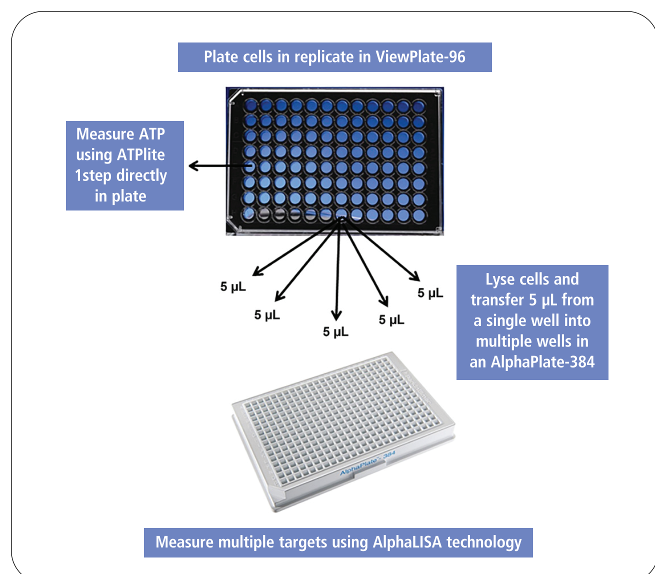

Figure 2: Work-flow for quantifying multiple immune checkpoint markers from a single well.

Data analysis

Standard curves were plotted in GraphPad Prism® using nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting (Figure 3). Protein concentrations were determined by interpolating the counts measured onto the standard curve. Relative levels of markers were normalized to approximate cell number by dividing the interpolated value by the average ATPlite 1step luminescence signal of four replicate wells for each condition and multiplying by a factor (10^6).

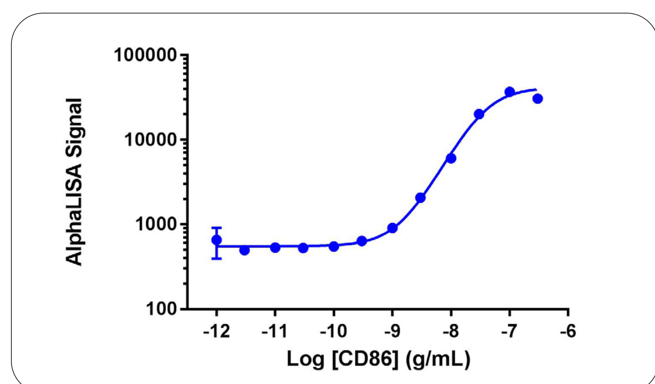

Figure 3: Example of a standard curve used to quantify levels of markers in lysates. Recombinant CD276 was detected in a 1:1 mixture of RPMI/10% FBS and Immunoassay buffer to mimic lysis conditions.

Results

Three concentrations of PBMCs were plated and stimulated with anti-CD3/anti-CD28 Dynabeads® resulting in increased proliferation of the cells at all concentrations plated. Images of PBMC cultures in Figure 4 illustrate the proliferation and clustering of T cells in response to stimulation with Dynabeads®. Relative levels of proliferation and cellular viability were assessed using ATPlite 1step. As expected, the addition of Dynabeads® caused an increase in the proliferation of the PBMCs as shown by an increase in ATP-dependent luminescence in Figure 5.

Next, PBMC lysates were assessed for concentrations of five different immune checkpoint markers using AlphaLISA biomarker detection kits. Four of these immune checkpoint markers, HVEM, VISTA, CD86, and CD276, were increased with both cell number plated and with stimulation, as shown in Figure 6. LAG3 is a cell surface protein expressed on many different subpopulations, including NK, dendritic, B cells and activated T cells, so it is expected to increase with PBMC activation. As shown in Figure 7, LAG3 expression levels increased after stimulation by Dynabeads®.

Next, in order to assess if the increase in immune checkpoint expression within the PBMC population was due to the increase in cell proliferation observed in Figure 5, the ATPlite 1step luminescence data was used to normalize the relative levels to relative cell number. As shown in Figure 8, no significant changes in expression levels that were normalized to the ATPlite 1step signal were observed for the four proteins shown in Figure 6, suggesting that the increased expression levels of these immune checkpoint markers were due to changes in cell proliferation rates and not due to further activation or differentiation of T cells. However, the increased expression of LAG3 observed here can be linked directly to the activation of specific ellular subtypes and not just due to increases in cell number.

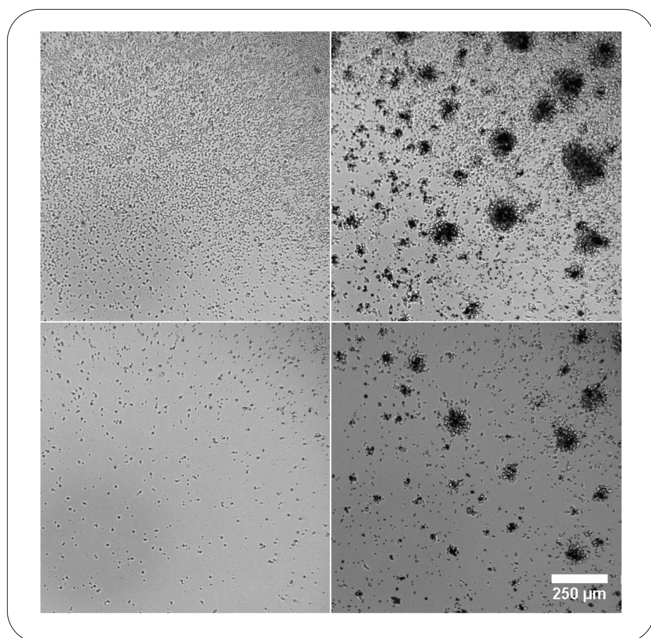

Figure 4: Representative images of microplate wells that were seeded with 100K PBMCs (top) and 25K cells (bottom) and the effect of Dynabeads® (right-side images) on these cultures.

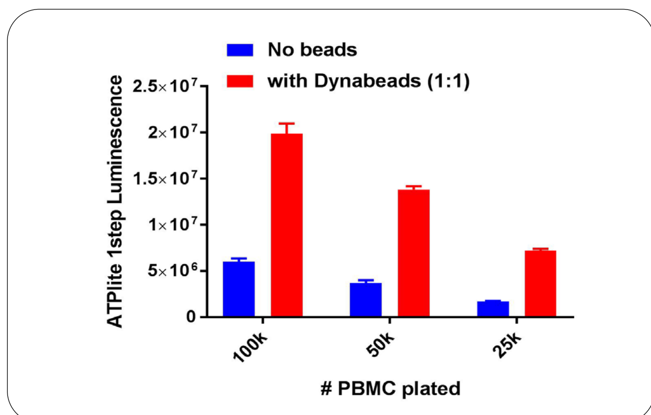

Figure 5: ATPlite 1step assay results indicate increased cell proliferation of PBMCs with Dynabead® stimulation.

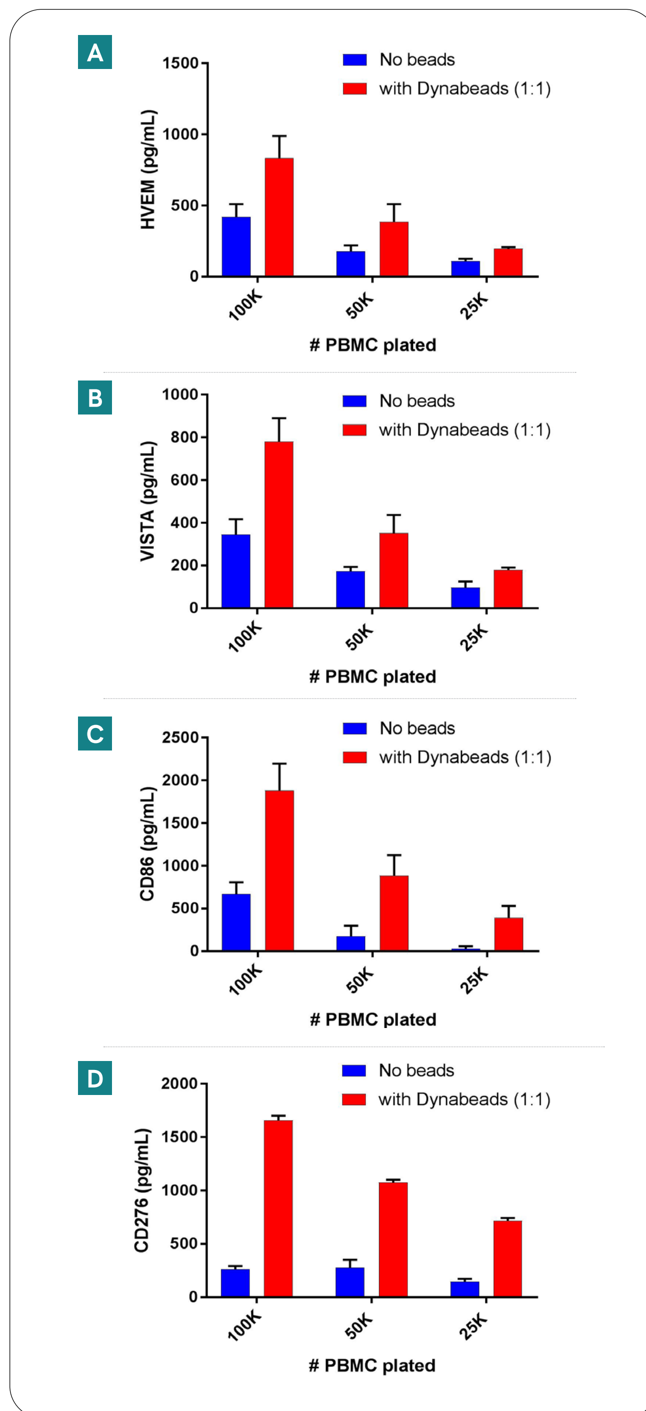

Figure 6: Quantification of four immune checkpoint markers with and without stimulation using AlphaLISA detection assays illustrate how cell number and stimulation enhance the concentration of these proteins in PBMC cultures. Concentrations of HVEM (A), VISTA (B), CD86 (C), and CD276 (D) increased both as cell number increased and upon stimulation with Dynabeads®.

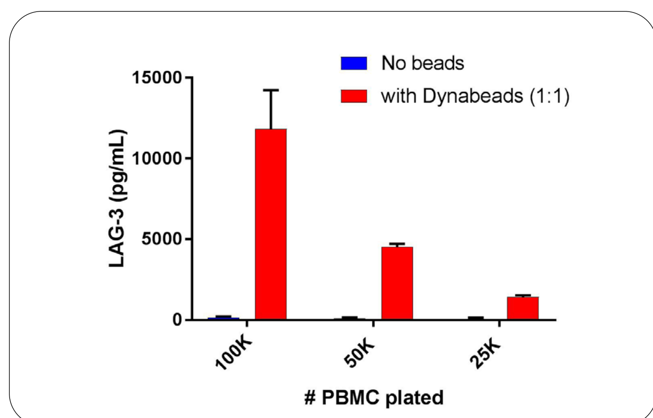

Figure 7: LAG3 is highly upregulated in PBMCs with Dynabead® stimulation. Quantification of LAG3 with and without stimulation is shown.

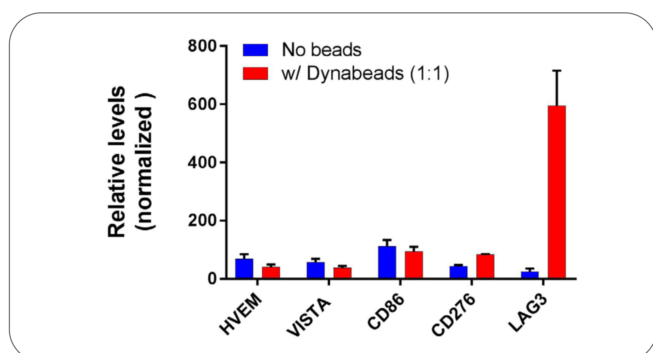

Figure 8: Relative levels of Immune checkpoint markers after normalization to approximate cell number using the data from wells plated with 100K PBMCs. Data were normalized to ATPlite 1step luminescence signal and multiplied by an arbitrary factor of 106 to adjust the y-axis values above zero.

Conclusion

Here, we were able to measure and quantitate five different immune checkpoint proteins in lysates from Dynabead®-stimulated human PBMCs with minimal sample consumption (5 µL of sample per target of interest) and with no wash steps using AlphaLISA technology. We were also able to quickly and easily assess cellular proliferation using the ATPlite 1step kit. Finally, by normalizing the AlphaLISA signal to the ATPlite signal we were able to distinguish upregulation of LAG3 driven by T cell activation vs. an increase in the HVEM, VISTA, CD276, and CD86 protein levels driven solely by increased cellular proliferation.

References

1. Martkamchan, S. et al. The Effects of Anti-CD3/CD28 Coated Beads and IL-2 on Expanded T Cell for Immunotherapy. Adv Clin Exp Med (2016); 25, 5, 821-828.
2. Assal A. et al. Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1. Immunotherapy (2015); 7(11), 1169-1186.
3. Quantifying Changes in CD28 and CTLA-4 Levels in Peripheral Blood Mononuclear Cells with AlphaLISA Technology. Revvity Application Note (2017).

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