TECHNICAL NOTE

Biotin-free AlphaLISA assays for the detection of cytokines in PBMC supernatants.

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Introduction

Peripheral blood monocytes (PBMCs) are a mixed population of mononuclear immune cells consisting of lymphocytes (T cells, B cells, NK cells) monocytes and dendritic cells. To understand the effects of interaction with antigen presenting cells *in vitro* or to preferentially expand T cells (as would occur in an immune response), PBMCs can be stimulated with mitogenic lectins such as phytohemagglutinin and concanavalin A to induce an immune response and proliferation of T cells. The costimulatory molecules CD3 and CD28, found on antigen presenting cells, also control proliferative and functional properties of T cells and PBMCs. We used Dynabeads® (beads coated with anti-CD3 and anti-CD28 antibodies) to stimulate PBMCs and evaluated cytokine secretion using AlphaLISA® no-wash chemiluminescent technology. The interaction of CD3/CD28 Dynabeads® with PBMCs has been widely used to induce T cell activation and expansion and to measure and profile the different cytokines secreted and collected as cell culture supernatant.[1, 2] Supernatant samples must be measured in the culture media in which the cells are grown. Certain media, including RPMI which is commonly used to culture PBMCs, contain high levels of biotin that can interfere with assays dependent on streptavidin/biotin interactions for detection. Revvity offers biotin-free AlphaLISA kits utilizing a digoxigenin (DIG)/anti-DIG interaction instead of a streptavidin/biotin interaction for use with samples containing high levels of endogenous biotin.[3]

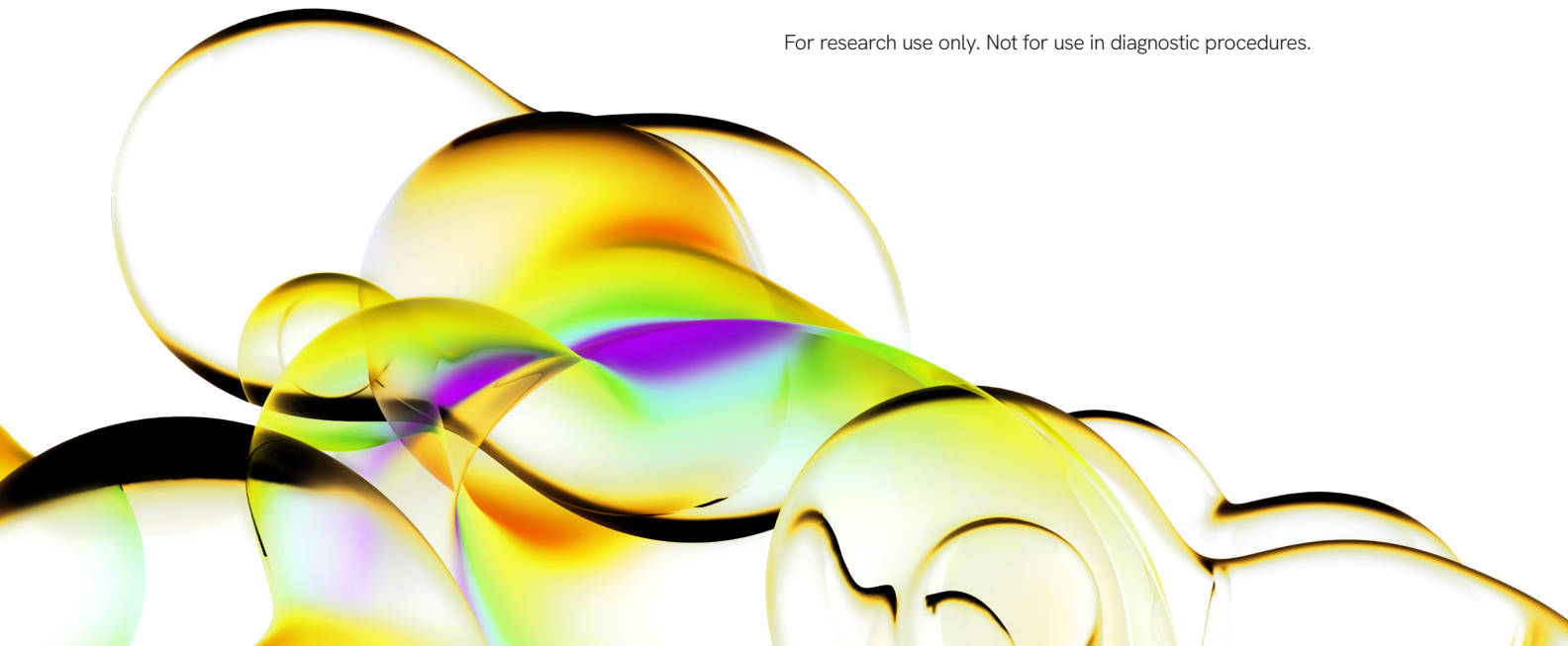

Figure 1 illustrates the AlphaLISA biotin-free assay. AlphaLISA technology allows the detection of molecules of interest in a no-wash, highly sensitive, quantitative assay. In an AlphaLISA biotin-free assay, a DIG-labeled anti-analyte antibody binds to the anti-DIG-coated Donor beads while another anti-analyte antibody is directly conjugated to AlphaLISA Acceptor beads. In the presence of the analyte, the beads come into close proximity. Donor bead excitation with light at 680 nm causes the release of singlet oxygen molecules that triggers a cascade of energy transfer in nearby Acceptor beads, resulting in a sharp peak of light emission at 615 nm.

In this technical note, AlphaLISA biotin-free kits were used to detect and quantify the cytokines interleukin 2 (IL-2), tumor necrosis factor alpha (TNFα), interferon gamma (IFN-γ), and interleukin 6 (IL-6) in supernatants secreted by human PBMCs cultured for two days with or without Dynabead® stimulation. The assay procedure followed a simple workflow that is illustrated in Figure 2.

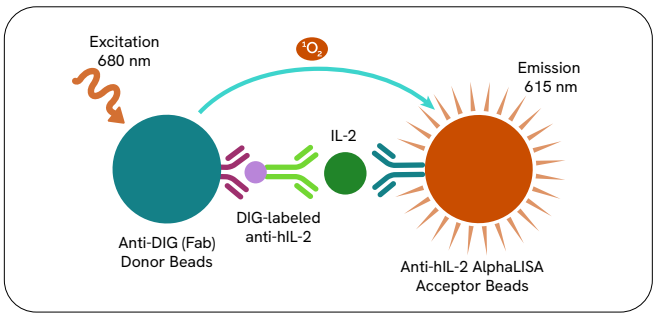

Figure 1: Example of an AlphaLISA biotin-free assay for detection of immune checkpoint markers.

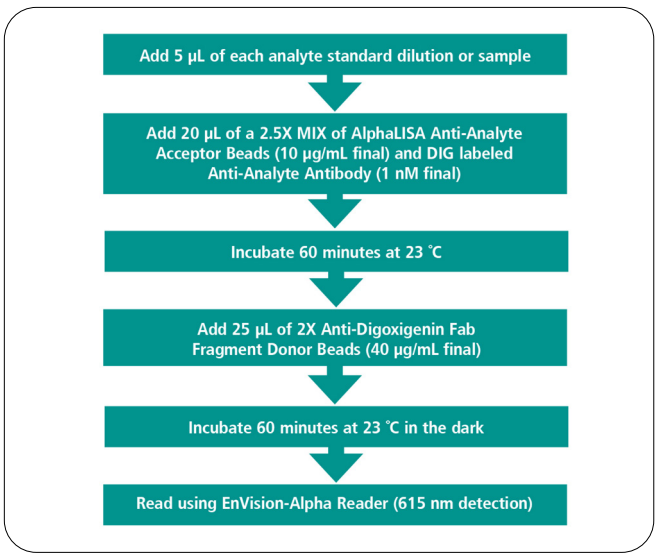

Figure 2: Representative workflow for AlphaLISA biotin-free kits, which provide sensitive detection of cytokines and only require 5 µL of sample.

Materials and methods

Activation of PBMCs by Dynabeads®

Frozen normal human primary PBMCs (ATCC®, #PCS-800-011™) were rapidly thawed, transferred to ice-cold Hanks Buffered Salt Solution (HBSS, ThermoFisher, #14025-134), and counted with a hemacytometer. Cells were rinsed in HBSS, spun down, and resuspended in RPMI-1620 media (ATCC, #30-2001) supplemented with 10% FBS (ThermoFisher, #26140-079). PBMCs were seeded at 100,000 cells per well (40 µL/well) into the wells of a ½ Area ViewPlate™-96 microplate (Revvity, #6005760). After seeding cells, half of the wells were activated by adding 40 µL of Human T-activator CD3/CD28 Gibco Dynabeads® (ThermoFisher, #11131D) diluted in culture media at a standard ratio of one bead to one cell. The other half were control wells which received just culture media. Cells were incubated for two days at 37 °C. After two days, the plates were centrifuged briefly (300 x *g* for five minutes) and placed on a magnet in order to bring the Dynabeads® to the bottom. 55 µL of lysate samples were then transferred to a polypropylene StorPlate-96V (Revvity, #6008290) and kept up to two weeks at -20 °C before AlphaLISA assays were run.

AlphaLISA assays

All AlphaLISA assays were run according to their respective kit manuals, generally following the workflow outlined in Figure 2. Supernatants from the PBMCs were divided to separate assay plates (AlphaPlate™-384 microplates, Revvity, #6005350) and four AlphaLISA biotin-free kits [IL-2 (Revvity, #AL333C), TNFα (Revvity, #AL325C), IFN-γ (Revvity, #AL327C), IL-6 (Revvity, #AL3025C)] were used to measure cytokine concentrations. Recombinant proteins provided in each kit were titrated in culture media to produce standard curves that were used for interpolation of sample data to quantify concentrations of cytokines in culture supernatants. Figure 3 shows the standard curves for recombinant human TNFα titrated in assay buffer and in culture media (RPMI + 10% FBS). All AlphaLISA assays were measured on the EnVision® 2105 multimode plate reader using standard Alpha settings.

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. Cytokine concentrations were determined by interpolating the counts measured from the standard curve in culture media. All data points are an average of measurements of samples from three independent culture wells. The lower detection limit (LDL) of an AlphaLISA assay was calculated by interpolating the average background counts (of 12 wells with no analyte) plus three times the standard deviations value from the standard curve.

Results and discussion

Standard curves for each cytokine were run in buffer and culture media (+ 10% FBS). As demonstrated in Figure 3, the excellent correlation (overlap) between standard curves in buffer and RPMI and lack of effect on sensitivity (LDL) illustrates the effectiveness of biotin-free AlphaLISA kits for use when a significant amount of biotin is present in a sample matrix.

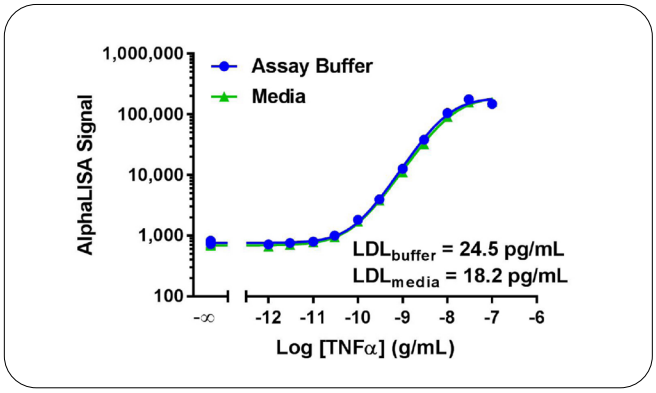

Figure 3: Standard curve for the AlphaLISA biotin-free TNFα assay run in assay buffer and culture media (RPMI-1640 with 10% FBS).

PBMCs were cultured and stimulated for two days with CD3/CD28 Dynabeads®. Four cytokines released by PBMCs into culture media were measured and the concentrations were quantified by interpolating the AlphaLISA signal generated for each cytokine to a corresponding AlphaLISA standard curve. As shown in Figure 4, biotin-free AlphaLISA assays were able to simply and rapidly measure cytokines secreted into media by heterogeneous cell populations like PBMCs with and without Dynabead® stimulation.

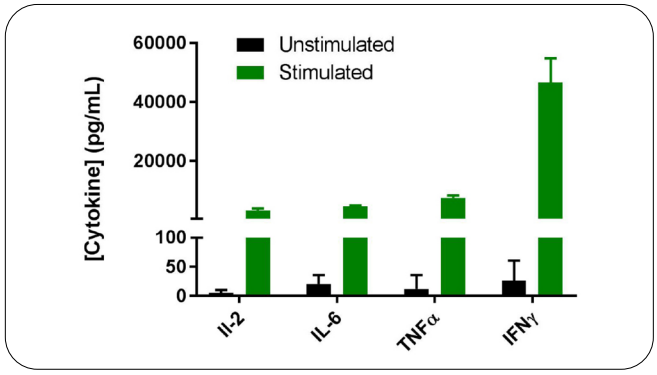

Figure 4: Quantification of cytokines secreted from PBMCs with or without Dynabead® stimulation.

Conclusion

We demonstrate here the ease of quantitating secreted cytokines from biotin-enriched supernatants generated by PBMC stimulation using AlphaLISA biotin-free assay kits. AlphaLISA technology allows for the sensitive detection of biomolecules in a quick no-wash format utilizing only 5 µL of sample – making it an ideal platform for testing primary cells.

References

1. Herr, W. et.al. *In Vitro* Stimulation and Expansion of Human Tumor-Reactive CD8+ Cytotoxic T Lymphocytes by Anti-CD3/CD28/CD137 Magnetic Beads. Basic Immunology (2011); 74, 155-164.

2. Whiteside, TL. et.al. *In Vitro* Cytokine Production by Normal Human Peripheral Mononuclear Cells as a Measure of Immunocompetence or the State of Activation. Clin Diagn Lab Immunol (1994); 1(3), 261-268.

3. Avoiding Biotin Interference in AlphaLISA Assays. Revvity Application Note (2017).

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