TECHNICAL NOTE

AlphaPlex Technology

Cellular Normalization: Using α-tubulin Duplexing to Normalize an IL-1β Assay

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Introduction

One benefit of duplexing with AlphaPlex™ technology is the ability to normalize cellular assays based on cell count. α-tubulin is an intracellular protein that is part of the microtubule cytoskeleton of eukaryotic cells. α-tubulin is ubiquitous in human cells and highly preserved across eukaryotic organisms. α-tubulin is not typically modulated by stimulation or inhibition. As such, α-tubulin can be used as a tool for the normalization of cellular protein per well. In a cell-based assay, it is important to normalize for cell number to ensure that any assay results are due to treatment or modulation, rather than cell density fluctuations or variations.

AlphaPlex technology allows for an all-in-one-well measurement of both normalization protein and the target of interest in a highly sensitive, quantitative, reproducible and user-friendly mode. In an AlphaPlex assay, biotinylated anti-target and anti-α-tubulin antibodies bind to the streptavidin-coated Alpha Donor beads, while another anti-target antibody is conjugated to AlphaLISA® Acceptor beads. A second anti-α-tubulin antibody is bound to the AlphaPlex 545 Acceptor beads. In the presence of the analytes, the beads come into close proximity. The excitation of the Donor beads provokes the release of singlet oxygen molecules that triggers a cascade of energy transfer in the Acceptor beads, resulting in a peak of light emission at 615 nm and 545 nm (Fig. 1).

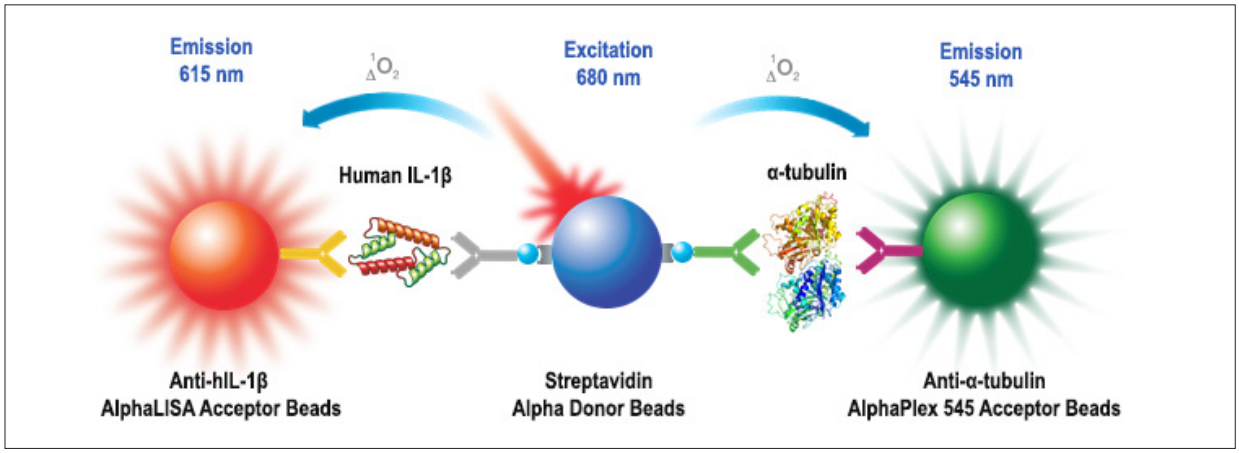

Figure 1. AlphaPlex assay for multiplexing with α-tubulin.

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In this technical note, we multiplexed an AlphaLISA human IL-1β (hIL-1β) assay with the AlphaPlex 545 α-tubulin assay. The AlphaPlex α-tubulin assay enables normalization of AlphaLISA cell-based biomarker detection assays to α-tubulin protein concentrations to control for cell number.

Materials and Methods

Reagents

- AlphaLISA human IL-1β detection kit (PerkinElmer #AL220)
- AlphaPlex 545 α-tubulin detection kit (PerkinElmer #AP383TB)
- 384-well Optiplate™ assay plates (PerkinElmer #6007290)
- AlphaScreen® mirror block (PerkinElmer #2102-5900)
- Alpha filter for Europium (PerkinElmer #2100-5090)
- Alpha filter for Terbium (PerkinElmer #2100-5930)
- TopSeal®-A (PerkinElmer #6050185)
- EnVision® multimode plate reader with Alpha standard module and filter options*
- 96-well CulturPlates™ (PerkinElmer #6005680)

** EnVision systems that are not already equipped with Alpha or AlphaPlex detection technology can be upgraded. Please contact your local sales or service representative for details.*

Cell Culture

U937 cells (ATCC® #CRL1593.2) were differentiated for 48 hours with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) (Sigma #P1585-1MG), then changed into phenol red-free RPMI (Sigma #R7509) media with no FBS and challenged for 21 hours with lipopolysaccharides (LPS; 10 µg/mL) (Sigma #L4516-1MG) in 96-well CulturPlates.

Cell Treatment

The U937 cells were differentiated using PMA and removed from the plate using cell dissociation buffer (Sigma #C5914-100ML). Then various concentrations of cells were plated in a 96-well CulturPlate and subjected to treatment with LPS to induce cytokine secretion. The supernatants were removed, cells washed with PBS, and lysed with 100 µL of AlphaLISA Lysis Buffer (#AL003C). For inhibition studies, U937 cells were differentiated with PMA for 48 hours and then treated simultaneously with LPS (10 µg/mL) and increasing concentrations of dexamethasone (Sigma #D2915-100MG) for 21 hours.

Protocol

The basic protocol used for duplexing is illustrated in Figure 2.

Instrumentation

There are two options available to read AlphaPlex depending on the EnVision model (Fig. 3): sequential read or simultaneous read. In simultaneous mode, a special dichroic mirror is needed (PerkinElmer #2102-5900), whereas the standard AlphaScreen mirror (PerkinElmer #2101-4010 or 2102-5910) can be used in the sequential mode. Suitable emission filters for Europium and Terbium are needed independent of the measuring mode.

Instructions for installing the mirrors and filters, and setting up the detection protocols, can be found in the EnVision AlphaPlex Instrument Set-Up Guide.

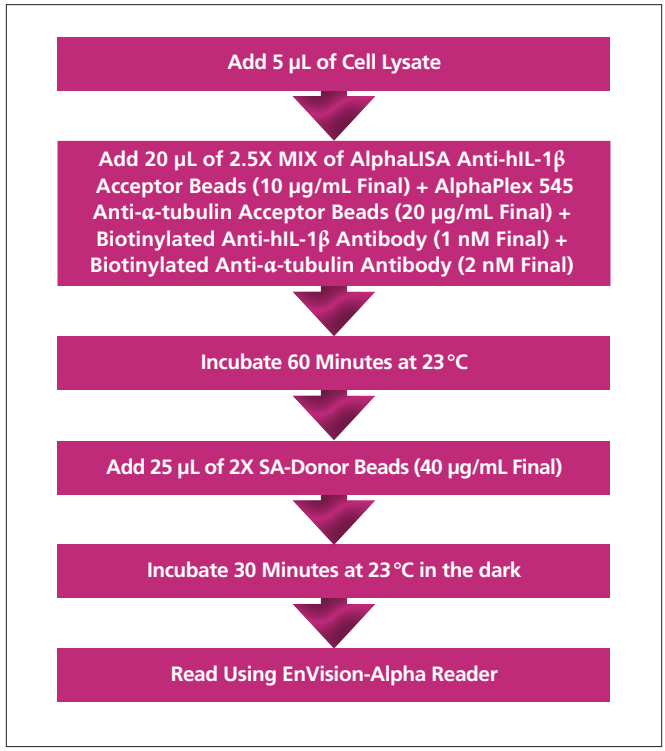

Figure 2. Workflow for an IL-1β assay duplexing with the α-tubulin AlphaPlex kit.

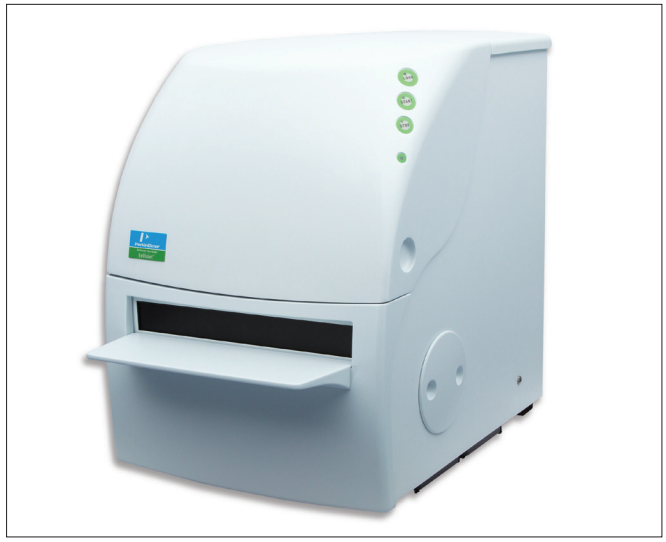

Figure 3. EnVision multimode plate reader.

Data Analysis

A spreadsheet was used to perform a crosstalk correction of the data. This worksheet can define the optical crosstalk between the Europium anti-IL-1β beads and the Terbium anti-α-tubulin beads. The worksheet can also be used once the crosstalk has been defined to correct the data from any experiment both for standard curve and samples.

AlphaPlex assays utilizing both AlphaLISA and AlphaPlex 545 beads in the same well require an optical crosstalk correction to the data. For a detailed explanation on how to perform these optical crosstalk corrections, see the AlphaPlex Quick Start Guide.

For the AlphaPlex assays in this technical note, control reactions with each Acceptor bead alone were used to perform optical cross-talk correction. These controls measure any Europium signal in the Terbium channel and vice versa and can be used to correct the data. The AlphaPlex Development Guide provides in-depth information for advanced users to further develop their own assays.

Once the crosstalk was measured and the data corrected, the following procedure was used:

- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting in GraphPad Prism (version 7.0).
- Analyze inhibition data using a nonlinear regression inhibition dose-response curve (variable slope - four parameters) in GraphPad Prism (version 7.0).

IL-1β data were normalized to α-tubulin protein concentration where indicated by dividing the crosstalk-corrected AlphaLISA 615 nm IL-1β signal by the crosstalk-corrected AlphaPlex 545 (Terbium) α-tubulin signal.

Results

Standard Curves and Inhibition With Dexamethasone

Standard curves were run for both the AlphaLISA human IL-1β kit and the AlphaPlex 545 (Terbium) α-tubulin assay. Data are plotted as AlphaLISA signal vs. concentration of analyte (Fig. 4A). Next, cells were treated with increasing concentrations of dexamethasone, a chemical known to have an inhibitory effect on IL-1β. As shown in Figure 4B, a concentration-dependent inhibitory effect is seen on IL-1β production. As expected, the signal for α-tubulin remained unchanged.

Normalization of IL-1β Data to α-tubulin Protein Levels

Next, an analysis was performed to compare raw data for the AlphaLISA IL-1β assay to data normalizing to α-tubulin levels. Figure 5A shows raw data for treatment of different concentrations of cells with dexamethasone. Figure 5B shows the data normalized to α-tubulin levels. As expected, the AlphaLISA raw signal increases with increasing numbers of cells.

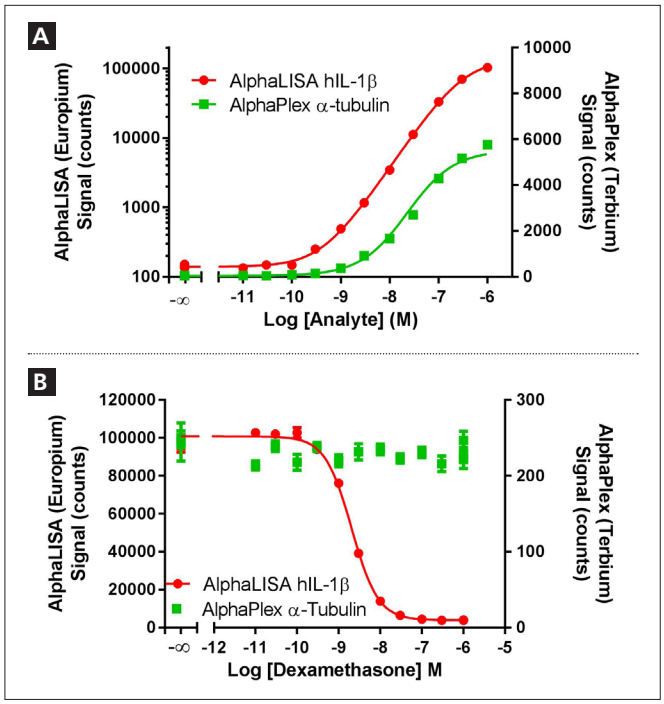

Figure 4. A) Standard curves of AlphaLISA hIL-1β and AlphaPlex 545 α-tubulin in AlphaLISA Immunoassay Buffer. B) hIL-1β inhibition with dexamethasone, 50K cells/well.

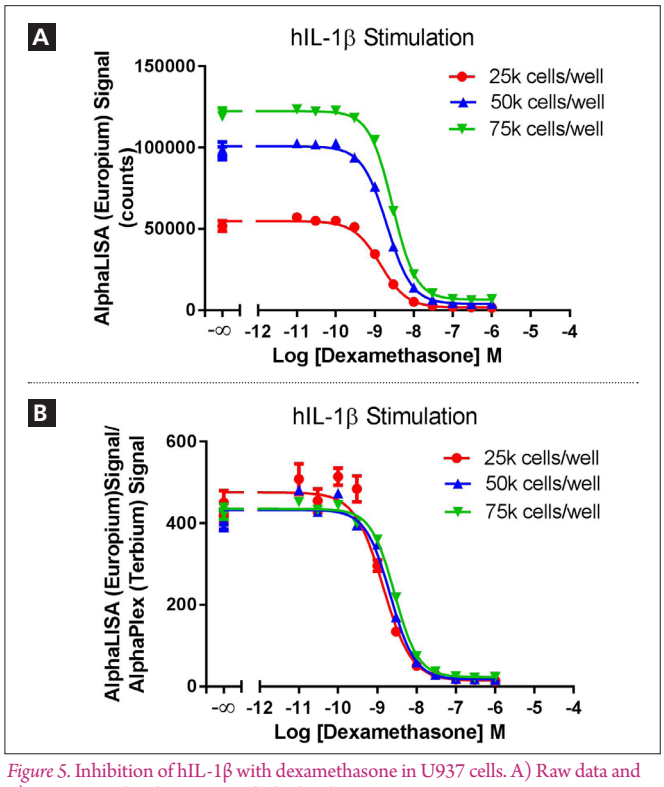

Figure 5. Inhibition of hIL-1β with dexamethasone in U937 cells. A) Raw data and B) Data normalized against α-tubulin level.

However, when data are corrected by normalizing to α-tubulin protein levels, the normalized data overlap to remove the effect of cell number on the signal. The IC_{50} values remain unaffected by normalization (25K cells/well: IC_{50} = 1.34 nM; 50K cells/well: IC_{50} = 2.04 nM; 75K cells/well: IC_{50} = 2.83 nM).

Simulated Pipetting Variability

As a final analysis, we simulated an experiment where the user has high variability in cell numbers from well to well. We randomly chose various cell numbers (25,000 – 75,000 cells/well) and plotted the dexamethasone inhibition data. We then compared the raw data from inhibition of U937 cells with dexamethasone to normalized data. As seen in Figure 6, variability of the data was improved by normalizing to α-tubulin levels. Although this is an example of an extreme case of mispipetting, the data clearly demonstrates how normalization can significantly improve the data. This allowed a better fit of an inhibition curve to generate a more accurate IC_{50} value for dexamethasone.

Conclusions

α-tubulin can act as an internal normalization tool for cellular assays. AlphaPlex 545 (Terbium) assays, together with the EnVision Multimode Plate Reader, provide a robust platform to measure cytokine levels per cell in a single well, homogeneously, in less than three hours. Assay quality is improved by normalization against an internal control and allows for the correction of errors resulting from cell pipetting or compound toxicity.

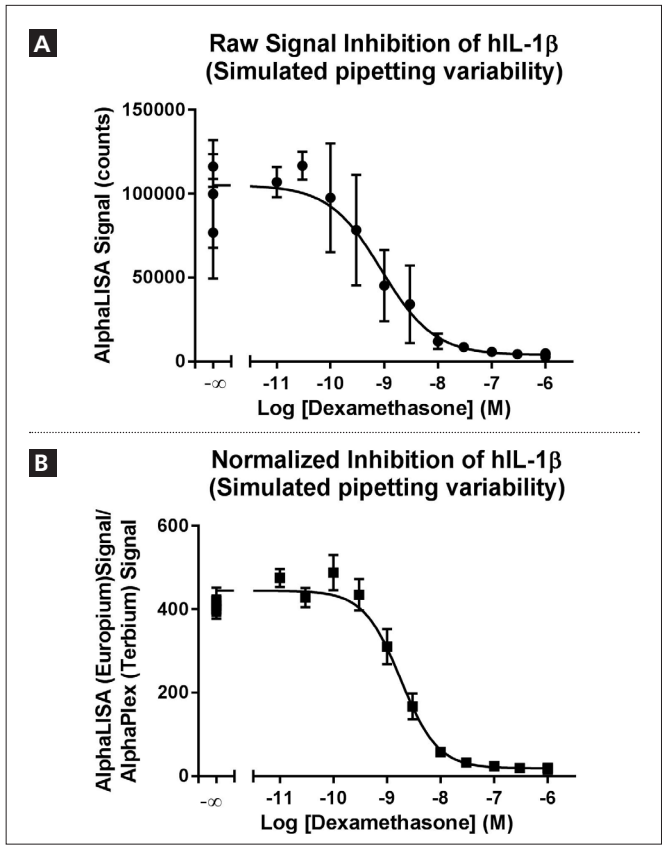

Figure 6. Inhibition of hIL-1β with dexamethasone in U937 cells. A) Raw data and B) Data normalized against α-tubulin levels. Normalized data more accurately represents an IC_{50} of dexamethasone in 50K cells/well (Raw IC_{50} = 0.89 nM; Normalized IC_{50} =1.80 nM).

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