APPLICATION NOTE

Utilization of AlphaLISA technology to accurately detect asthma biomarkers in human serum.

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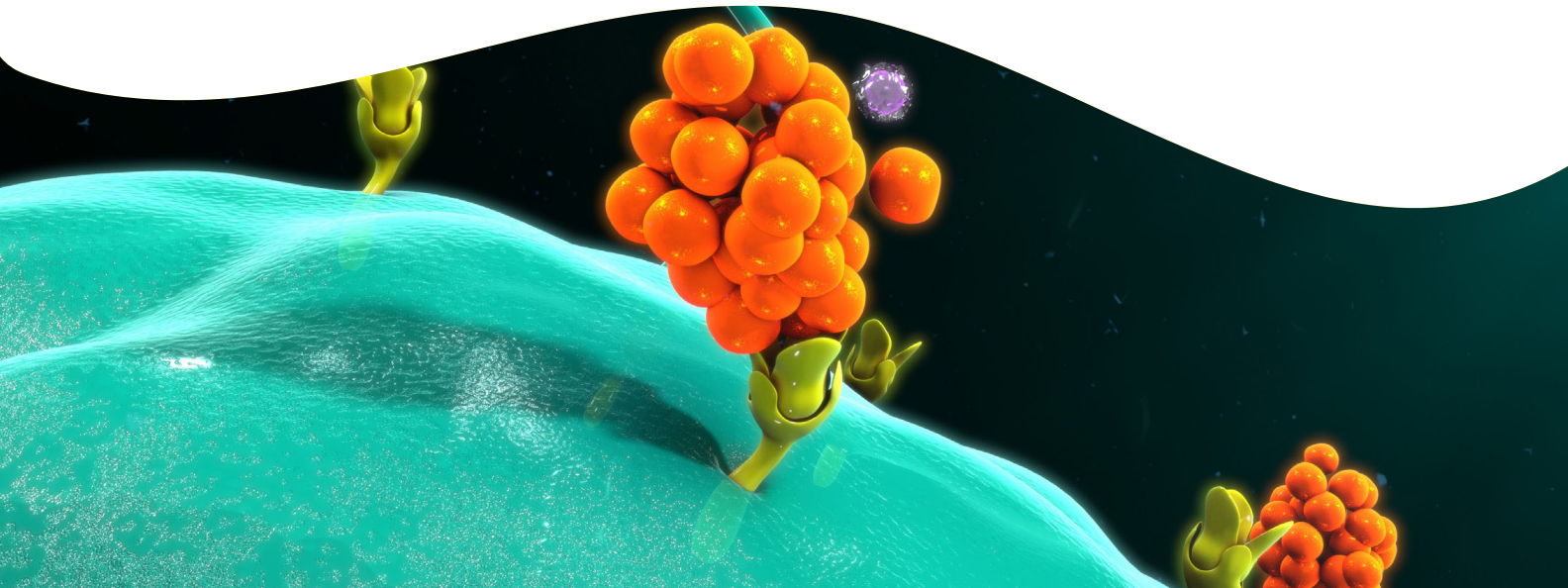
Introduction

Asthma is a chronic inflammatory disease of the lung airways. Hundreds of millions of people around the world are affected by the disease, while hundreds of thousands fall victim to the disease each year. Asthmatic attacks can be triggered easily and usually without notice, triggered by allergens and other irritants. As such there is increasing demand for methods to study asthma and to discover novel medications to better control and prevent asthma. One approach of particular interest is the detection of specific asthma biomarkers in serum that will help diagnose asthma early in life. Such biomarkers can identify sensitizations to specific allergens and also monitor disease and treatment progression.

Amplified luminescence proximity homogenous assay (Alpha) technology allows for the detection of molecules of interest using a homogeneous, no-wash format. Figure 1 shows an example of AlphaLISA™ technology, whereby a streptavidin-conjugated Donor bead is bound to a biotinylated anti-analyte antibody and Acceptor beads are directly conjugated to another anti-analyte antibody. When the specific analyte is present the antibodies bring the Donor bead and Acceptor bead within close proximity of each other. Upon excitation with a 680 nm laser, the Donor beads generate singlet oxygen molecules that transiently diffuse in solution to activate nearby Acceptor beads, which through a series of reactions emits light at 615 nm. The light emission (AlphaLISA signal) is then detected on an Alpha-enabled instrument.



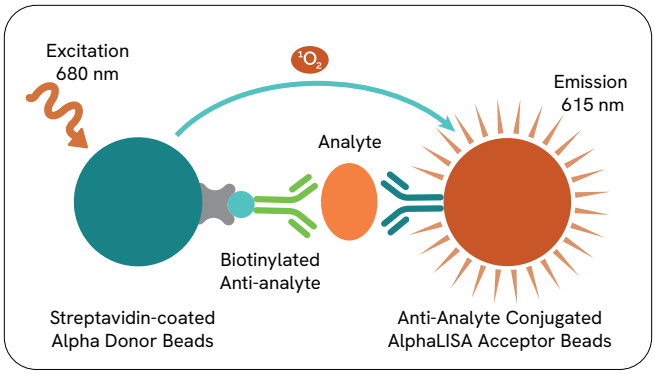


Figure 1: AlphaLISA assay schematic. In the presence of analyte, antibodies sandwich the analyte and bring the Donor beads and Acceptor beads within close proximity. Upon excitation singlet oxygen from the Donor beads diffuses and activates nearby Acceptor beads to generate light that is proportional to the amount of analyte.

Herein, we demonstrate how AlphaLISA technology can be used to quantify biomarker level in serum samples. Specifically, we used AlphaLISA detections kits for Human IL-21, IL-33, and TNFα. To ensure proper and accurate detection of biomarkers in serum, dilution linearity and spike-and-recovery experiments were performed to determine the proper diluents and dilution factors required for accurate recovery. Assays were detected using the EnVision™ multilabel plate reader. Finally, demonstration of measuring cytokine levels in asthmatic patient samples was shown by testing five normal human and four asthma patient serum samples for presence of IL-21, L-33 and TNFα cytokines.

Materials and methods

Human serum samples

Human serum samples were purchased from BioreclamationIVT. We obtained five normal patient serum samples, as well as two mild and two moderate asthma patient serum samples. Patient samples were carefully chosen to limit any possible effects of patient medications on biomarker levels. All normal patient samples had no prescribed medications. All asthma patients were limited to one asthma related medication, an albuterol (bronchodilator) inhaler, to limit the effect of multiple drugs on inflammatory biomarker levels. Normal human serum used for dilution linearity and spike-and-recovery experiments was purchased from Innovative Research (Cat. # IPLA-SER).

Detection of IL-21, IL-33, and TNFα using Alpha technology

AlphaLISA immunoassays for IL-21 (Revvity AL3043), IL-33 (Revvity AL3042), and TNFα (Revvity AL208) were performed according to the recommended protocols provided with each kit. Each kit supplies the necessary Acceptor beads, biotinylated antibody, Donor beads, analyte, and the recommended assay buffer to perform the assay. Standard analytes and samples were prepared in the appropriate diluent according to the protocols. For IL-21 assays, 5 µL of prepared standard analyte or samples were added to a 384-well white OptiPlate (Revvity 6007290) followed by the addition of 10 µL of an Acceptor bead mix. Then the plate was sealed with TopSeal A-PLUS (Revvity 6050185) and incubated for 30 minutes at room temperature. TopSeal-A PLUS is recommended for preventing evaporation during incubations. Next, 10 µL of biotinylated antibody was added to each well of the plate.

The plate was sealed and incubated for 60 minutes at room temperature. Afterwards, 25 µL of streptavidin Donor beads were added under subdued light to prevent photobleaching; in the dark or under green filters (Rosco 389). The plate was sealed, covered with a black lid (Revvity 6000027), and incubated at room temperature for 30 minutes prior to measuring AlphaLISA signal. For IL-33 and TNFα assays, 5 µL of prepared standard analyte or samples were added to a 384-well white OptiPlate followed by the addition of 20 µL of an Acceptor bead and biotinylated antibody mix. Then the plate was sealed with TopSeal A-PLUS and incubated for 60 minutes at room temperature. Next, 25 µL of streptavidin Donor beads were added under subdued light. The plate was sealed, covered with a black lid, and stored at room temperature for 30 minutes prior to measuring AlphaLISA signal. Further, a plate can be read with or without the TopSeal A-PLUS with no measureable difference. Finally, if solutions do not appear to be settled at the bottom of the well (i.e. droplets on well wall) gently tapping the plate flatly on a surface may be sufficient to settle solutions, if not it's recommended to briefly centrifuge the plate at 500 x *g* for about 10 seconds.

Dilution linearity experiments

Linearity of dilution experiments were performed for IL-21, IL-33, and TNFα AlphaLISA kits. The following solutions were tested as the optimal serum diluent: AlphaLISA Immmunoassay buffer (Revvity AL000), Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.1% BSA (Revvity CR84-100), and fetal bovine serum (FBS).

In the case of IL-33, 1:1 fetal bovine serum and normal human serum (FBS/NHS) was also tested as a diluent. Standard curves were prepared in each diluent tested following the same dilution scheme proposed in each kits technical data sheet. A known amount of standard analyte was spiked into a 100% serum sample then diluted in twofold increments in the chosen diluents. In the case of 1:1 FBS/NHS, a known amount of analyte was spiked into 100% normal human serum then diluted twofold in 100% FBS then sequentially diluted in twofold increments in 1:1 FBS/NHS, thus maintaining a final percentage of serum at 50%. The remainder of the assay was performed following the appropriate AlphaLISA protocol highlighted above. It is critically important to note that all Acceptor bead, Donor bead, and biotinylated antibody dilutions must be performed in the diluent mentioned in each kits technical data sheet, which in all cases here was AlphaLISA Immunoassay buffer.

Spike-and-recovery experiments

Spike-and-recovery experiments were performed for IL-21, IL-33, and TNFα AlphaLISA kits. Standard curves were prepared in the optimal diluent determined from the dilution linearity experiment. A known amount of standard analyte was spiked into a 100% serum sample then diluted to the optimal dilution factor in the appropriate diluent determined by the dilution linearity experiment. In the case of 1:1 FBS/NHS, for IL-33, the analyte standard curve was prepared in 1:1 FBS/NHS and analyte was spiked into 100% normal human serum then diluted twofold in 100% FBS and any additional dilutions would be performed in 1:1 FBS/NHS. The remainder of the assay was performed following the appropriate Alpha protocols highlighted above. It is critically important to note that all Acceptor bead, Donor bead, and biotinylated antibody dilutions must be performed in the diluent mentioned in each kits technical data sheet, which in all cases here was AlphaLISA Immunoassay buffer.

Instrumentation

All Alpha assays were measured on the Alpha-enabled EnVision multilabel plate reader (Figure 2), using the 640as mirror module (#444) and the M570w emission filter (#244). Standard Alpha measurement settings were used: total measurement time 550 ms and excitation time 180 ms. Importantly, white 384-well OptiPlates were used for all assays. The specific plate type should be selected in the Alpha protocol settings in the EnVision software, which is located under Select Protocol → Protocol-General settings → Plate type → 384 OptiPlate.

Figure 2: EnVision multilabel plate reader.

Data analysis

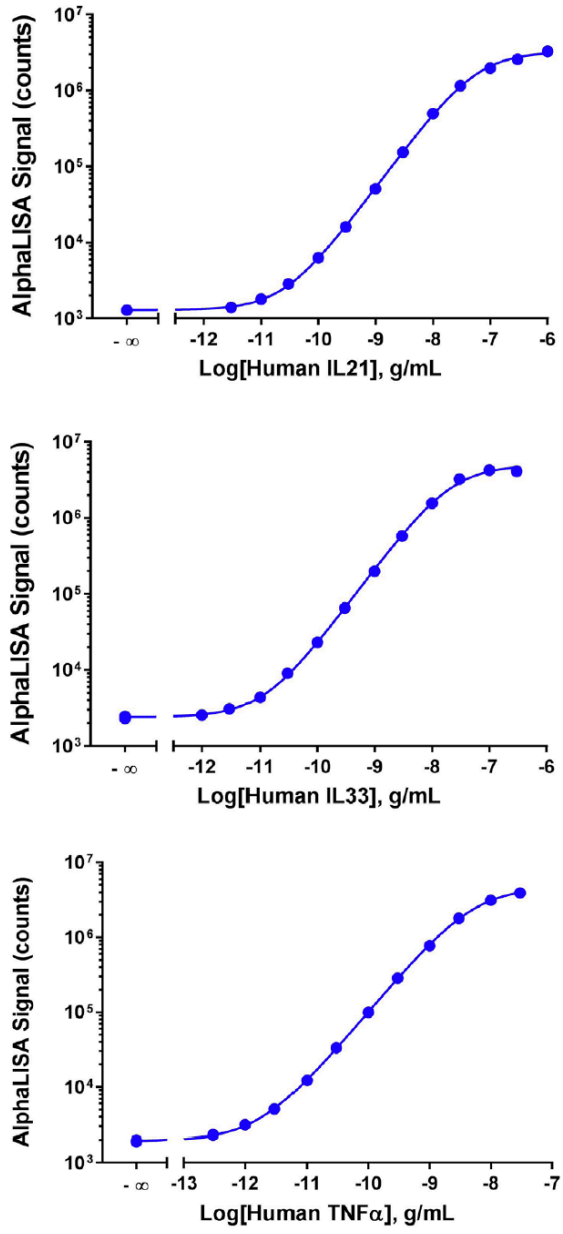
Diluted samples were compared to standard curves prepared in the same diluent. Standard curves were prepared using the recombinant standards provided in each AlphaLISA kit. Standard curves were plotted in GraphPad Prism version 7.0 using nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) with $1/Y^2$ weighting method. For dilution linearity experiments R-squared values were calculated to represent linearity and approximate percent recovery was calculated by dividing the interpolated value (from the standard curve) by the expected value then multiplied by 100 to convert to percent. For spike-and-recovery experiments percent recovery was calculated by dividing interpolated serum spikes by the associated interpolated diluent spikes then multiplied by 100 to covert to percent. Spike values were determined by comparing to a standard curve prepared in the diluent used. Additionally, all real sample concentrations were interpolated onto a standard curve prepared in the same diluent as that used to dilute the sample. Further, lower limit of detection (LDL) was calculated by averaging the blank wells (wells without analyte) and adding three times the standard deviation then interpolating the concentration from the standard curve. Below are the formulas.

% Recovery = (spiked sample value / expected sample value) X 100

LDL = mean (blanks) + 3 X SD

Results

AlphaLISA assays are simple, quick and offer exceptional assay performance



Kit	LDL, pg/mL	Dynamic range	S/B
IL-21	5.3	5.3 pg/mL to 1 µg/mL	2558
IL-33	1.5	1.5 pg/mL to 100 ng/mL	1780
TNFα	0.3	0.3 pg/mL to 30 ng/mL	2022

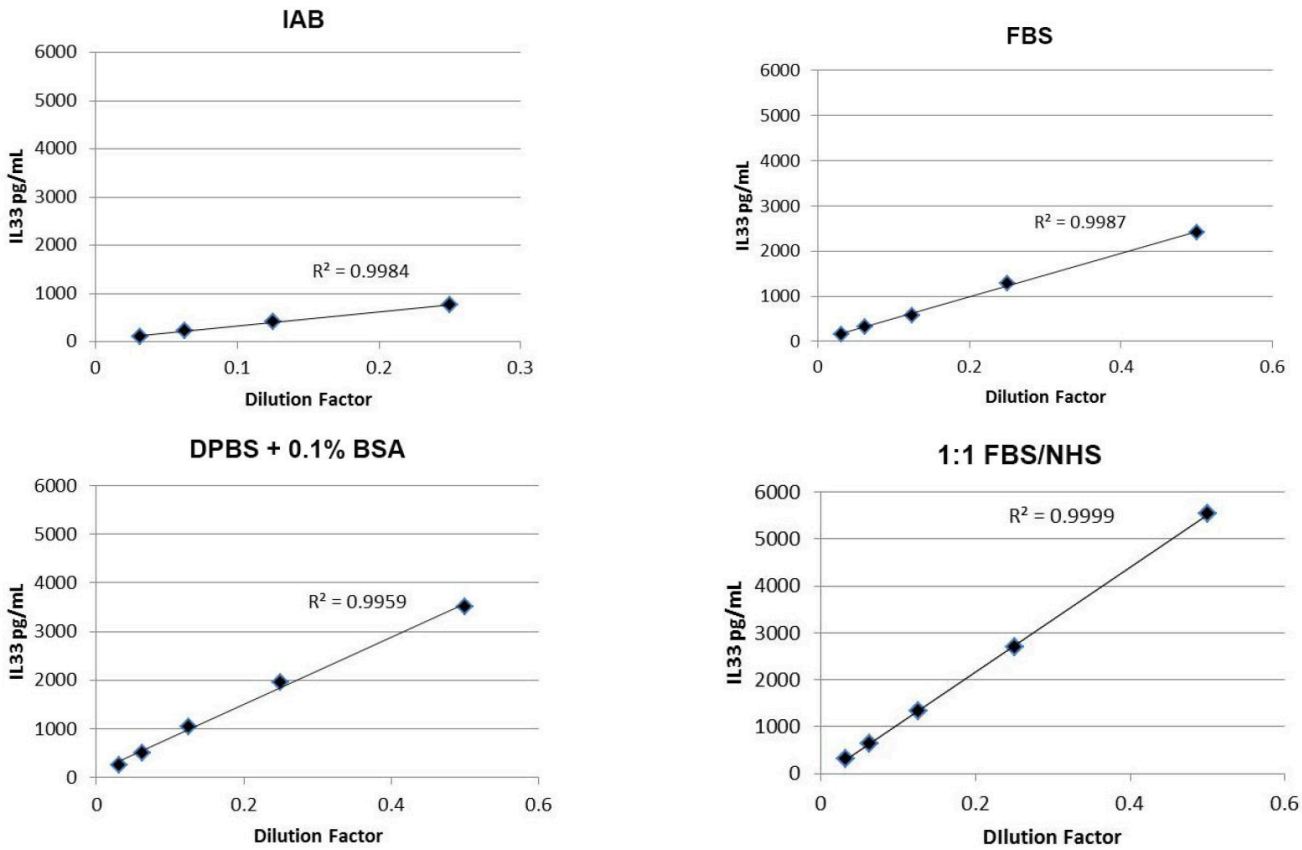
Figure 3: Performance of IL-21, IL-33, and TNFα AlphaLISA Immunoassays. Standard curves are shown for each assay performed in AlphaLISA Immunoassay buffer. The table at the bottom right displays the performance characteristics for each assay.

AlphaLISA technology is a sensitive, straightforward and robust bead-based immunoassay technology that utilizes a homogenous format that does not require washing or additional signal development steps. Furthermore, AlphaLISA detection kits are available for a wide range of relevant biomarkers. To demonstrate some of the benefits of Alpha technology standard curves were prepared for IL-21, IL-33, and TNFα AlphaLISA kits using the recommended diluents and assay protocols published in each kits respective technical data sheet. Figure 3 shows standard curves for IL-21, IL-33, and TNFα using AlphaLISA Immunoassay Buffer as the standard diluent in white 384-well OptiPlates. Each assay demonstrated exceptional performance characteristics as shown in the table in Figure 3 where low pg/mL sensitivities, wide dynamic ranges spanning more than 4-logs, and high signal-to-background ratios > 1500 were observed.

Linearity of dilution experiments help determine the optimal assay diluent

Another benefit of Alpha technology is its compatibility with complex biological matrices like human serum. However, due to Alpha's homogenous design matrices such as human serum may interfere with assay recovery. To ensure accurate recovery, assays should be optimized by performing a linearity of dilution experiment. The purpose of this experiment is to determine the optimal assay diluent for preparing your standard curve. Further, the experiment will identify the extent of sample dilution required to ensure accurate quantification of the analyte. The optimal diluent is one that achieves assay linearity (R-squared value ≥ 0.995), provides acceptable percent recovery of the analyte (between 70 – 130%) and maintains exceptional sensitivity. To demonstrate the human IL-33 AlphaLISA kit was used to perform the dilution linearity experiment shown in Figure 4. Standard curves were prepared in AlphaLISA Immunoassay buffer (IAB), fetal bovine serum (FBS), Dulbecco's phosphate buffer saline (DPBS) + 0.1% BSA, and a 1:1 mixture of FBS and normal human serum (FBS/NHS). Each of the diluents tested achieved linearity after only a twofold dilution of a mock normal human serum sample spiked with a known amount of analyte, except for IAB which required a fourfold dilution. Non-spiked normal human serum showed no detectable levels of IL-33. Assay recovery was observed to vary significantly by diluent type highlighting the importance of choosing diluents that are similar to the sample type tested. In the table below the graphs,

the recoveries for each dilution series were calculated by determining the spiked value recovered from the associated standard curve and comparing those to the expected recoveries. The FBS/NHS diluent demonstrated the most optimal recoveries with a twofold dilution obtaining about 111% recovery. Further, sensitivities of 1.5, 3.2, 7.5, and 23 pg/mL were achieved for IAB, FBS, DPBS +0.1% BSA, and FBS/NHS, respectively. In this case, FBS/NHS was chosen as the most optimal assay diluent because it required the lowest dilution of sample, provided the best analyte recovery, and retained exceptional assay sensitivity. Refer to the appendix for step-by-step guidance on performing the linearity of dilution experiment.



	% Recovery compared to standard curve			
DF	IAB	FBS	DPBS + 0.1% BSA	1:1 FBS/NHS
1.00	21.7%	54.9%	56.5%	91.9%
0.50	25.3%	48.1%	70.1%	110.6%
0.25	30.5%	50.9%	78.2%	108.1%
0.13	33.2%	46.5%	82.6%	106.4%
0.06	36.0%	50.9%	79.9%	101.7%
0.03	36.7%	51.0%	80.7%	101.7%

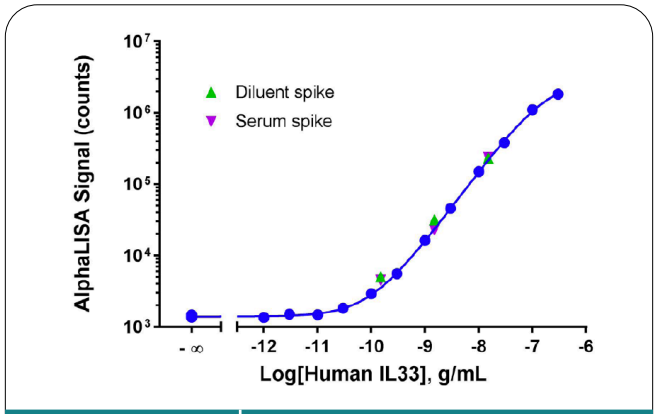
Figure 4: Dilution linearity experiments performed using the IL-33 AlphaLISA kit. The four graphs represent the dilution series performed in IAB (top left), FBS (top right), DPBS +0.1% BSA (bottom left), and 1:1 FBS/NHS (bottom right). The y-axis is the interpolated concentrations of IL-33 at each dilution and the x-axis is the dilution factor where one represents no dilution and 0.5, 0.25, 0.125, 0.625, and 0.03125 represents a 2, 4, 8, 16, and 32-fold dilution. High points were excluded until the R-squared value was greater than 0.995. At the bottom is a table of calculated percent recoveries at each point in the dilution series where the interpolated values were divided by expected spike value X 100.

Excellent analyte recovery was observed when using the optimal assay diluent

To ensure the chosen diluent is optimal for recovering and quantifying analyte in serum samples a spike-and-recovery experiment was performed. The experiment was performed by preparing diluent spikes and mock sample spikes by spiking a high, medium, and low concentration of the human IL-33 into FBS/NHS and normal human serum. The samples were diluted twofold in FBS/NHS for the diluent spikes and diluted twofold in FBS for the mock samples and compared to a standard curve prepared in NHS/FBS (Figure 5). As shown in the figure the triangles represent where the sample concentrations fell along the curve. In the table to the right, percent recovery was calculated by first interpolating the spiked concentrations from the standard curve then dividing the serum spike concentration by the respective diluent spike concentration and multiplying by 100. Good recovery was observed at all concentrations tested. This further supported that FBS/NHS was the optimal assay diluent to achieve accurate recovery of spiked samples. However, it is important to note that using this particular diluent may constrain assay functionality for some assay kits. This is because the assay diluent is 50% normal serum meaning our background only wells will contain 50% of the basal analyte levels, thus constraining the dynamic range for some kits. In cases where the analyte levels are expected to decrease in disease samples it is not recommended to use this type of assay diluent. In cases where analyte levels are expected to increase in samples this type of diluent is fine. Fortunately, the levels of IL-33 present in normal human serum are very low and disease samples are expected to have greater than basal levels, thus using the FBS/NHS assay diluent did not affect assay functionality. Refer to the appendix for step-by-step guidance on performing the spike-and-recovery experiment.

Increased serum IL-33 levels were detected in samples from moderate asthmatic patients

Human serum samples were acquired from five normal patients and four asthmatic patients (two mild and two moderate). Human IL-21, IL-33, and TN-Fα AlphaLISA detection kits were used to detect and quantify the presence of these cytokines in the samples. Patient samples were diluted to the optimal level using the best diluents for each kit. Samples were diluted eightfold in FBS for IL-21 and a standard was prepared in FBS. Samples were diluted twofold in FBS for both IL-33 and TNFα, but standard curves were prepared in NHS/FBS and FBS for IL-33 and TNFα, respectively. TNFα was detected in all patient samples tested, but little or no difference was observed between samples. For IL-21, all of the samples displayed no elevation in expression. Most interestingly, IL-33 was significantly elevated in samples of patients with moderate asthma, while levels did not increase in normal or mild asthma patient samples.



Kit	% Recovery
Spikes	Compared to Spiked Diluent Samples
High (15 ng/mL)	102%
Medium (1.5 ng/mL)	76%
Low (0.15 ng/mL)	88%

Figure 5: Spike-and-recovery experiment performed using IL-33 AlphaLISA kit. On the top is a figure displaying the standard curve for IL-33 performed in the 1:1 FBS/NHS diluent. The green upward facing and purple downward triangles represent spikes at high, medium, and low concentrations in diluent and serum, respectively. On the bottom is a table showing the percent recovery of the serum spike samples compared to the diluent spike samples.

Conclusion

AlphaLISA is a bead-based assay technology that requires no washing and provides excellent assay performance with regard to signal-to-background, dynamic range, and sensitivity. In this application note, these benefits were demonstrated using AlphaLISA to quantitate important asthma biomarkers IL-21, IL-33, and TNFα in complex matrices. Particularly, these assays were shown to be optimized to achieve accurate recoveries in serum samples with dilution linearity and spike-and-recovery experiments to find the optimal assay diluent. It is very critical to highlight that using the proper diluent for diluting samples and preparing standard curves is crucial for accurate recovery from samples. In cases where the optimal diluent was used, accurate recoveries were observed with minimal dilution of samples and good sensitivity was retained.

Table 1: Human IL-21, IL-33, and TNFα AlphaLISA assays were performed to quantify levels of cytokines in patient samples. A standard curve for IL-21, IL-33, and TNFα was performed in FBS, 1:1 FBS/NHS, and FBS, respectively. The LDL values are reported for each standard curve. The average detected cytokine levels for five normal patient samples were reported in pg/mL. The average detected cytokine levels for 2 mild asthmatic and 2 moderate asthmatic patient samples were reported in pg/mL.

	Assay LDL, pg/mL	Normal patient serum, pg/mL	Mild asthmatic patient serum, pg/mL	Moderate asthmatic patient serum, pg/mL
TNFα	0.9	5.5	6.7	3.8
IL-33	12.0	Below detectable limits	Below detectable limits	109
IL-21	1.6	Below detectable limits	Below detectable limits	Below detectable limits

To assess these asthma biomarkers in more biologically-relevant models, nine patient serum samples comprised of normal, mild asthmatic, and moderate asthmatic conditions were tested. TNFα was detected and quantified in all samples tested with no significant difference between them. For asthma patients, TNFα is often elevated[1]. One possible explanation for the discrepancy is that the albuterol use by asthmatic patients may have dampened the expected physiological increases for these biomarkers. The sample size for these experiments was also small. Additionally, no increased levels were observed in any samples for IL-21.

Most interesting was IL-33, which was observed at lower than detectable limits for normal and mild asthmatic samples and significantly increased levels in the serum samples from patients with moderate asthma. This observation correlates well with previous studies that showed increasing levels of IL-33 mRNA was associated with asthma severity, where the levels significantly increased when comparing endobronchial biopsy samples between normal and asthma subjects[2]. IL-33 is an IL-1 family cytokine thought to be a potent driver of T helper-2 cytokine production. As such diseases like asthma are of particular interest because IL-33 levels could be one factor leading to an overactive immune system. However, it was previously shown that existing ELISA kits for measuring IL-33 levels in serum was difficult partly due to interference from the serum samples[3]. We believe the incorporation of serum into our assay diluent makes our approach unique as it allows for these inherent serum interferences to be taken into account. Thus, Alpha technology can be highly useful for measuring difficult to study biomarkers in complex matrices.

These data demonstrate how Alpha technology is an exceptional immunoassay technology due to its no-wash format, excellent sensitivity, reproducibility, and its ability to be optimized to function with complex matrices such as serum. The importance of choosing the proper diluent must be highlighted to ensure accurate recovery and quantification of analyte in complex samples. Finally, Alpha technology can be simply optimized to function well with complex samples by performing dilution linearity and spike-and-recovery experiments.

References

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Appendix

Linearity of dilution

Purpose: To determine the optimal diluent that allows for dilution linearity of spiked mock samples (R-squared ≥ 0.995), good recovery compared to curve (> 70% and < 130%), sufficient sensitivity, and a dilution factor for accurate sample recovery.

For this example the human IL-33 was used. Each kits technical data sheet should be referred to for concentrations and volumes of each addition.

- Prepare standard curve in the diluent(s) to be tested
  - A diluent that is more similar to the sample typically works best
- Prepare a single spiked sample and a non-spiked 100% serum sample then dilute in twofold increments in the chosen diluent. See Table 2 for an example of the dilution scheme.
  - The non-spiked serum sample is necessary to determine basal levels in order to adjust for percent recovery
- Add 5 µL of standard and dilution linearity samples to wells of a white 384-well OptiPlate
  - Or the recommended volume for sample in the kits technical data sheet
- Prepare a 2.5X mix of Anti-IL-33 AlphaLISA Acceptor beads (25 µg/mL) and biotinylated Anti-IL-33 antibody (2.5 nM)
  - Example of preparing 1 mL of the 2.5X mix. Add 5 µL of Anti-IL-33 AlphaLISA Acceptor beads (5 mg/mL stock) and 5 µL of biotinylated anti-IL-33 antibody (500 nM stock) to 990 µL of AlphaLISA Immunoassay Buffer.
- Add 20 µL of the 2.5X mix of Acceptor beads and biotinylated antibody to each well of the plate
- Cover with TopSeal A-PLUS and incubate for one hour at room temperature
- Prepare a 2X solution of streptavidin-coated Alpha Donor beads in subdued light
  - Example of preparing 1 mL of 2X solution of Alpha Donor beads. Add 16 µL of streptavidin-coated Donor beads (5 mg/mL stock) to 984 µL of AlphaLISA Immunoassay buffer.
  - The Alpha Donor beads are light sensitive and should be handled in subdued light only to avoid photobleaching
- Add 25 µL of the 2X solution of Donor beads to each well of the plate
- Cover with TopSeal A-PLUS, cover with a black lid and incubate for 30 minutes at room temperature
  - The plate can be stored in the dark if no black lid is available.
- Read the plate on an Alpha enabled reader
  - See materials and methods section for instrument settings and data analysis

Criteria for choosing the optimal assay diluent

- Achieved assay linearity (R-squared > 0.995)
- Accurate recovery compared to expected values (between 70 – 130%)
- Sufficient assay sensitivity and allows for dilution factor

Table 2: Dilution scheme for linearity experiments.

Tube	Dilution factor	Volume of analyte	Volume of diluent	Concentration in tube (ng/mL)
1	1	10 µL of 10x diluted IL-33 Stock (0.3 µg/mL)	90 µL of sample	30
2	0.5	50 µL of Tube 1	50 µL	15
3	0.25	50 µL of Tube 2	50 µL	7.5
4	0.13	50 µL of Tube 3	50 µL	3.8
5	0.06	50 µL of Tube 4	50 µL	1.9
6	0.03	50 µL of Tube 5	50 µL	0.9

Spike-and-Recovery

Purpose: To assess whether the optimal diluent(s) and extent of sample dilution determined in the dilution linearity experiments are sufficient for accurate recovery (i.e between 70% and 130% recovery) from spiked mock samples.

For this example the human IL-33 was used. Each kits technical data sheet should be referred to for concentrations and volumes of each addition.

- Prepare standard curve in the diluent(s) to be tested
  - A diluent that is more similar to the sample typically works best
- Prepare a three spiked samples and a high, medium, and low concentration spike in diluent and 100% serum then dilute to the appropriate level determined from the dilution linearity experiment. See Table 3 for an example of the dilution scheme.
  - The non-spiked serum sample is necessary to determine basal levels in order to adjust for percent recovery
  - The spike amounts may need to be adjusted based on the specific kit
- Add 5 µL of standard and dilution linearity samples to wells of a white 384-well OptiPlate
  - Or the recommended volume for sample in the kits technical data sheet
- Prepare a 2.5X mix of Anti-IL-33 AlphaLISA Acceptor beads (25 µg/mL) and biotinylated Anti-IL-33 antibody (2.5 nM)
  - Example of preparing 1 mL of the 2.5X mix. Add 5 µL of Anti-IL-33 AlphaLISA Acceptor beads (5 mg/mL stock) and 5 µL of biotinylated anti-IL-33 antibody (500 nM stock) to 990 µL of AlphaLISA Immunoassay Buffer.
- Add 20 µL of the 2.5X mix of Acceptor beads and biotinylated antibody to each well of the plate
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  - The Alpha Donor beads are light sensitive and should be handled in subdued light only to avoid photobleaching
- Add 25 µL of the 2X solution of Donor beads to each well of the plate
- Cover with TopSeal A-PLUS, cover with a black lid and incubate for 30 minutes at room temperature
  - The plate can be stored in the dark if no black lid is available.
- Read the plate on an Alpha enabled reader
  - See materials and methods section for instrument settings and data analysis

% Recovery = ((interpolated serum spike value – basal levels) / interpolated diluent spike value) X 100

% Recovery is considered acceptable when between 70 – 130%

Table 3: Dilution scheme for spike-and-recovery experiments.

Tube	Volume of analyte	Volume of diluent	Dilution factor	Expected recovery (ng/mL)
1	10 µL of 10x Diluted IL-33 Stock (0.3 µg/mL)	90 µL of Sample or Diluent	2	15
2	10 µL of Tube 1	90 µL of Sample or Diluent	2	1.5
3	10 µL of Tube 2	90 µL of Sample or Diluent	2	0.15
4	0	100 µL of Sample or Diluent	2	0

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