Exploring best practices to measure cytokines in human whole blood samples using AlphaLISA technology.

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Introduction

Whole blood is a complex matrix consisting of all normal blood components, including red blood cells (erythrocytes), white blood cells (leukocytes), platelets (thrombocytes) and plasma. Traditionally, isolated blood components, such as plasma, serum, and peripheral blood mononuclear cells (PBMCs) have been validated for use with many immunoassay technologies; however, measurement of biomarkers in whole blood samples has been limited. Use of whole blood may be advantageous in some applications as it provides a more physiologically relevant environment to assess biomarker (particularly cytokine) profiles, and offers improved cell viability compared to PBMC cultures. Moreover, working with whole blood may be more efficient, with fewer processing steps (such as time-sensitive separation or purification) required before analysis.[1,2] Thus, despite potential challenges—for example, "matrix effects" (i.e. compounds in the sample matrix which may interfere with immunoassay measurement), or assay signal quenching due to the sample color or opacity—there is growing interest in analyzing biomarkers in whole blood samples by immunoassay.[1,3]

Revvity's AlphaLISA™ technology is a homogeneous, no-wash, bead-based luminescent amplification assay. This highly sensitive platform uses anti-analyte Donor and Acceptor Beads that bind to the target analyte and upon excitation at a specific wavelength, the Donor Bead transfers singlet oxygen to the Acceptor Bead if they are within proximity (≤200 nm), resulting in light production by the Acceptor Beads which can be measured for quantitation of the analyte. AlphaLISA High Performance (HP) Cytokine Detection Kits are the newest generation of AlphaLISA kits for cytokine detection, developed by utilizing high performing and highly specific monoclonal antibody pairs for each assay (Figure 1).

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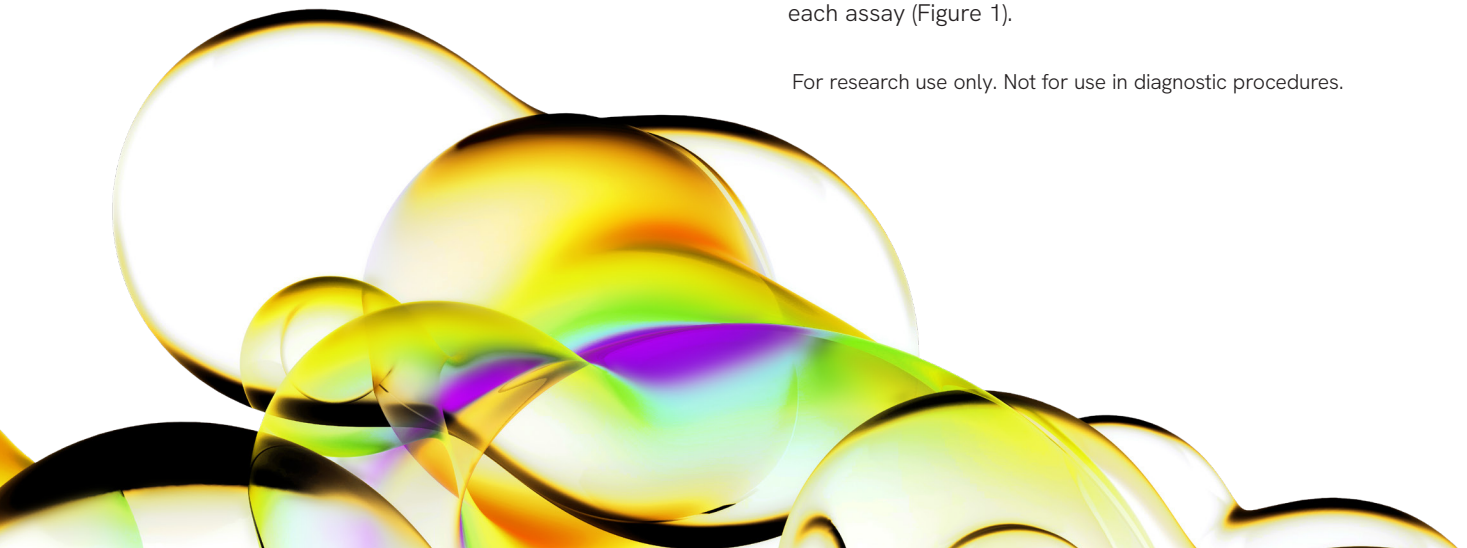


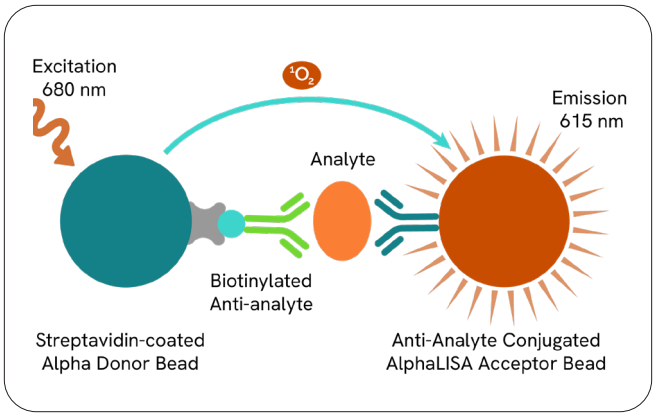


Figure 1: Example of an AlphaLISA High Performance cytokine assay

AlphaLISA products are routinely validated for use with buffered solutions, cell lysates, and serum/plasma. Although AlphaLISA technology is considered to have broad sample compatibility, there is potential for components in complex biological matrices, such as blood, to impact measurement of the target analyte.[4] Therefore, it is important to conduct methodological and biological validations of the assay technology when working with novel or untested sample types. Furthermore, the presence of hemoglobin in blood, which absorbs light across much of the same wavelengths as Alpha beads emit light, may complicate detection due to quenching of the signal.[4,5]

Despite potential challenges, the robust, sensitive, and versatile nature of AlphaLISA technology makes it well-suited for complicated sample types. Here, we conducted experiments to explore best practices for analyzing human whole blood samples using Revvity's AlphaLISA High Performance cytokine kits, and then demonstrated successful measurement of cytokines in human whole blood. To this end, we: 1) compared different sample processing techniques (e.g. blood collection tube types, dilution factors, and mechanical spin-down steps) which may enhance sample measurement; 2) conducted analytical assay validations (linearity and spike-recovery experiments) to demonstrate suitable diluents, dilution ranges, and recovery of expected analyte concentrations with AlphaLISA; and finally, 3) performed LPS-stimulation of blood samples to establish the AlphaLISA HP cytokine kits could detect biologically meaningful differences in cytokine concentrations across samples.

Methods

Samples

Freshly collected human whole blood samples were sourced from established biological sample vendors: BioIVT (Hicksville, NY) or Research Blood Components, LLC (Watertown, MA). Following collection, samples were transported and stored at refrigerated temperatures (~4 °C) to prolong sample integrity. For analytical validation experiments (e.g. linearity, spike-recovery), a pooled sample consisting of at least two individuals was used to account for the fact that blood composition may vary somewhat between individuals, therefore a pooled sample may be more representative. For subsequent experiments comparing cytokine levels in stimulated blood, samples were collected from two separate donors to account for known variation in cytokine responses between individuals.[6]

Blood collection tube type is known to affect the outcome of some laboratory analyses;[7] therefore, as part of our validations, we conducted a brief experiment to test whether AlphaLISA signal varied between two different blood collection tube types. Matched samples were collected in tubes containing either 3.2% Sodium Citrate (Na Citrate) or Lithium Heparin (Li Heparin) anticoagulants. Based on results (below), 3.2% Na Citrate was found to be superior, and used in all experiments unless noted otherwise. However, an in-depth comparison of additional blood collection tube types should be considered in future work.

Assay protocol and analysis

To demonstrate Revvity AlphaLISA HP cytokine assays can be used to measure cytokines in whole blood samples, two kits were chosen for this study: TNFα (#AL3157) and IL-1β (#AL3160). Both assays are sandwich format, with the analyte of interest captured between two antibodies. TNFα is a multifunctional proinflammatory cytokine synthesized mainly by nucleated blood cells which plays a role in lipid metabolism, coagulation, and endothelial function.[8] IL-1β is a central player of the immune response and is involved in inflammation at local and systemic levels by triggering kinase signaling factors that lead to activation of transcription factors.[8]

Assays were run in 384-well OptiPlate™ (Revvity, #6007290) using 50 µL total volume, with minor adjustments to the kit TDS protocol. The TNFα kit TDS describes an optional "high concentration" protocol, which uses a lower volume of concentrated (10X) Acceptor beads compared to the standard protocol (2.5X). We followed the "high concentration" protocol for TNFα, and then adapted the IL-1β kit to use the same protocol, as it appeared to improve recovery when working with blood samples, compared to the standard protocol (data not shown). In addition, when working with complex sample types, the diluent used to prepare the standard curve should closely match the sample matrix, in order to obtain a more accurate measurement.[9-11] Linearity experiments (results below) demonstrated fetal bovine serum (FBS; ThermoFisher Scientific, #A3840001) to be the optimal diluent for samples and standards, and thus was used in all assays, unless otherwise noted. In all assays, Acceptor and Donor Bead mixtures were prepared in the included kit Immunoassay Buffer, as per the manual. Samples and standards were run in triplicate.

Assays were read on an EnVision® 2105 multimode plate reader using the default values for Alpha detection. Data were analyzed using GraphPad Prism®, with standard curves fit using the four-parameter logistic non-linear regression equation and $1/Y^2$ weighting. Analyte concentrations were interpolated based on the standard curve, with results presented in pg/mL and adjusted for dilution factor as appropriate.

Using AlphaScreen Omnibeads to explore Alpha signal quenching

Hemoglobin, present in red blood cells, is known to quench Alpha signal.[4] Therefore, as an initial experiment, we investigated how Alpha signal was impacted by hemoglobin and sample color/opacity using AlphaScreen Omnibeads (Revvity, #6760626). Omnibeads are helpful for troubleshooting applications, as they contain all the chemicals necessary for a strong Alpha signal without the need for binding of an Acceptor Bead; thus, any detected variation in signal can be traced to either the dispensing of the beads into the plate or the direct reading of the signal from the wells. For our purposes, this allowed for determination of signal quenching that occurred due to sample characteristics (e.g. hemoglobin content, color) versus potential assay binding interference due to the sample matrix.

To mirror the conditions of our assay protocol, 5 µL of whole blood was added to the wells of an OptiPlate, along with 45 µL of diluted Omnibeads (20 µg/mL final concentration) for a total assay volume of 50 µL. Blood samples were tested across a range of dilutions spanning from undiluted (neat) to 1:256 (using 2-fold serial dilutions) in two diluents: 100% FBS or PBS + 0.1% bovine serum albumin (BSA) (ThermoFisher, PBS: #10010023; BSA: #AM2616). Separate sets of Omnibeads were prepared in each diluent type for addition to the diluted blood samples. In addition, Omnibeads prepared in each diluent type were run neat (no blood, diluent only) to determine absolute signal.

As a secondary test, we explored whether addition of a mechanical "spin down" step to separate out the red blood cells just prior to assay could reduce the Alpha signal quenching observed with blood samples, without impacting subsequent measurement of the target analyte. Thus, a separate set of whole blood samples were prepared as described above in a V-bottom StorPlate (Revvity, # 6008299). The plate was then gently centrifuged (1000 rpm, 10 min, room temperature (RT)) which resulted in the red blood cells settling to the bottom and the remainder of the sample (i.e. "supernatant") remaining at the top of the well with a clear/translucent appearance (Figure 2). The supernatant portion (containing no visible red coloration) was then transferred to the assay plate. Resultant Alpha signal was compared between samples that were spun down versus those that remained mixed (no spin).

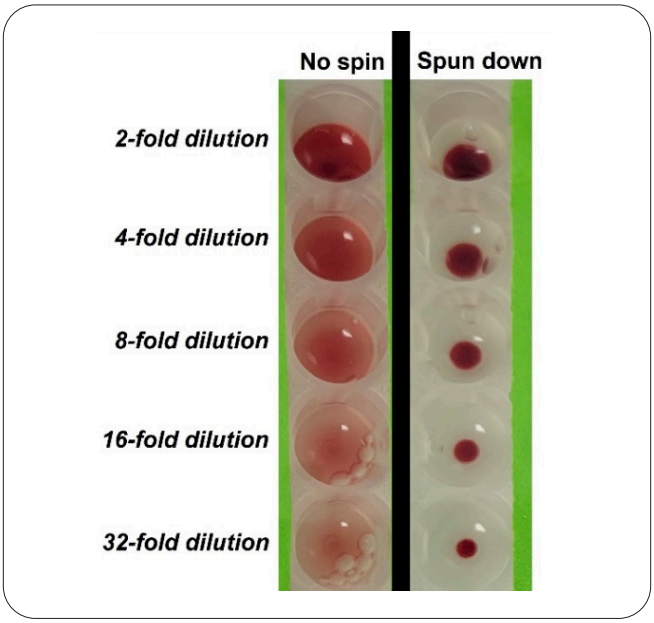


Figure 2: Comparison of whole blood sample coloration based on dilution factor ("no spin"; left) and separation of red blood cells in diluted samples that were centrifuged ("spun down"; right).

Linearity test of diluted whole blood samples

An important initial step when working with a new sample type is to identify an appropriate diluent for the samples and standard curve. Linearity experiments were performed to determine an appropriate diluent for whole blood and to confirm that the resultant sample calculations were similar across a range of dilutions. Diluents tested included AlphaLISA Immunoassay Buffer (the standard buffer included in the kits), FBS, and PBS + 0.1% BSA. Whole blood samples were diluted 2-fold (1-part whole blood + 1-part diluent) in each of the diluent types. This sample was spiked with a high concentration of the standard analyte (3 ng/mL for TNFα; 1 ng/mL for IL-1β). The spiked 2-fold diluted sample was then serially diluted using 2-fold steps up to a 64-fold dilution. The dilution curve was spun down prior to transfer to the assay plate, and only the supernatant portion was taken to the assay (as described above). The spike-in analyte (prepared in diluent only) was also run to determine the concentration of analyte as measured in each diluent type. To assess linearity, the interpolated concentrations of the diluted spiked blood samples versus the dilution factor were plotted. A linear regression was performed, and correlation coefficients compared (ideally r^2 is >0.995, indicating the target analyte can be measured across a range of dilutions). [Note: performing this experiment using undiluted (neat) whole blood significantly hindered measurement by AlphaLISA (data not shown). Thus, as is common for complex sample types, an initial ≥2-fold dilution of the sample was required to achieve linearity]. For more information, Revvity offers several guides for conducting linearity tests.[9-11]

Spike-recovery tests using whole blood samples

Spike-recovery experiments are important to confirm the selected diluent and dilutions allow for accurate measurement of analyte, and that there are no substances in the sample matrix that interfere with measurement/recovery of the target analyte. Results are given as percent recovery (% recovery) of the exogenous spiked-in analyte, with an acceptable range considered to be 70-130% recovery. Examples of spike-recovery assay setups can be found on Revvity's website.[9-11]

For this study, whole blood was diluted 2-fold or 32-fold in FBS. Diluted blood samples were transferred to a V-bottom plate and then spiked with a low, medium, or high concentration of standard analyte. Spike-in concentrations of 30, 300, and 3,000 pg/mL were used for TNFα, and 12, 120, and 1,200 pg/mL for IL-1β. The same addition volume was used for all spike-in concentrations to ensure the original blood dilution factor was not altered by the spike-in. For the 2-fold diluted samples, following addition of the spike-in, the plate was centrifuged (1000 rpm, 10 min, RT) to gently separate the red blood cells. Only the supernatant portion was taken to the assay. For the 32-fold diluted blood, the mixed, spiked samples were transferred without spin separation to the assay. Non-spiked diluted blood samples (both 2-fold and 32-fold dilution) were also assayed to determine basal concentrations of analyte measured in the samples (this background concentration was subtracted in the final % recovery calculations). Additionally, each concentration of spike-in analyte was prepared in FBS diluent only. These diluent-only spike-ins were used to determine the concentration of the spike-in measured by the assay, allowing for calculation of the percent recovered in the diluted blood sample. An additional set of samples, spike-ins, and standards were prepared in in PBS + 0.1% BSA for comparison to FBS diluent.

Measurement of cytokines using best practices

Based on data from previous experiments, we established two possible workflows for preparing whole blood samples for AlphaLISA; outlined in Figure 3. A final experiment was conducted to demonstrate these two workflows could be used to measure differences in cytokine concentrations across blood samples. Whole blood samples from two separate donors were diluted 2-fold and 32-fold in FBS before addition to 96-well tissue culture plates (Corning #3610). Samples were then treated with 100 ng/mL lipopolysaccharide solution (LPS; Invitrogen, #00497693) or FBS diluent (as a negative control) before incubating at 37 °C. Plates were prepared in duplicate, allowing for samples to be assayed at two different time points (6-hour or 24-hour) post-stimulation to account for transient cytokine production. At each time point, treatment and control samples were collected and assayed immediately. For the 32-fold diluted samples, the mixed samples were taken to assay without centrifugation. For 2-fold diluted samples, blood was transferred to a V-bottom plate (Greiner bio-one, #651-180) and centrifuged (10 min, 1000 rpm, RT) to separate the red blood cells, before transferring only the supernatant portion to the assay. Measured cytokine concentrations (adjusted for dilution factor) were compared between LPS-treated and non-treated samples and between donors. For samples that were undetectable by the assay (i.e. no interpolated value), the concentration of the lowest standard was used as a proxy.

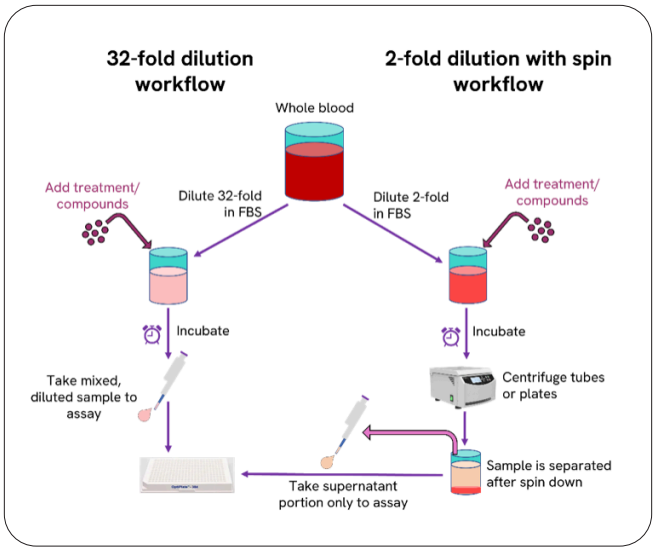


Figure 3: Schematic of two recommended workflows for preparing whole blood samples for cytokine detection using AlphaLISA. Human whole blood samples can be diluted 32-fold in FBS before taking the sample directly to assay (left workflow); alternatively, when needed, whole blood can be diluted 2-fold in FBS, then a spin-down step can be used to separate out the interfering red blood cells, and the supernatant portion only is taken to the assay (right workflow).

Results and discussion

Using AlphaScreen Omnibeads to explore Alpha signal quenching

Omnibead generated Alpha signal was variable in blood samples based on dilution factor, diluent type, and dependent on whether a spin down step was performed to separate the red blood samples prior to assay (Figure 4). Overall, Alpha signal was lower in FBS-diluted samples compared to samples diluted in PBS + 0.1% BSA, with the absolute signal of FBS-diluted beads approximately 33% lower than that of PBS + BSA-diluted beads. This difference may be due to the inherent color of serum (versus clear PBS) which could contribute to a lower signal.

Despite the reduced signal level, the signal patterns were similar in FBS- and PBS + BSA-diluted samples. Following a standard dilution curve, signal quenching was observed with whole blood samples until ~32-fold, or greater, dilution. However, when a spin down separation of the sample was performed prior to transfer to assay, the sample signal was close to the absolute bead signal at only a 2-fold dilution. Importantly, this highlights that incorporating a spin down step into the sample preparation protocol may reduce the need for high dilution factors often required with whole blood on immunoassays. Undiluted (neat) whole blood showed signal quenching regardless of spin down step. Although Omnibead signal cannot be considered a direct substitute for Alpha signal generated in an actual immunoassay, this experiment provided valuable preliminary insight into sample processing and dilution limits of whole blood samples on AlphaLISA technology.

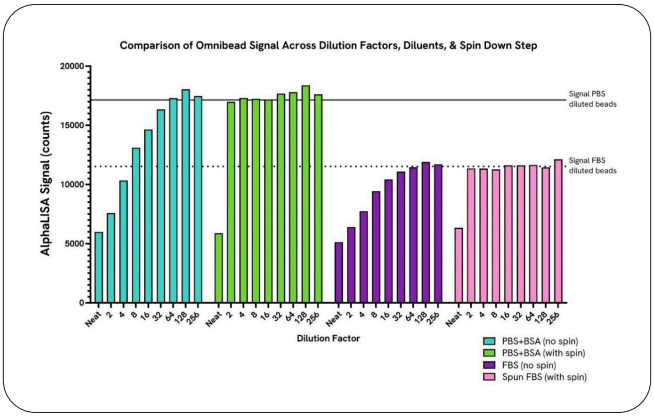


Figure 4: Comparison of Omnibead generated Alpha signal in whole blood samples across various dilution factors, diluents, and mechanical processing steps (spin down). Blood samples were 2-fold diluted starting with neat (undiluted) blood up to a 1:256 dilution in either: PBS + 0.1% BSA (blue and green bars) or fetal bovine serum (FBS; purple and pink bars). For each diluent type, one set of samples was left completely mixed ("no spin"; blue and purple bars); the other set of diluted samples was gently centrifuged to separate the red blood cells before taking the supernatant portion to the assay ("with spin"; green and pink bars). Absolute bead signal—Omnibead signal measured in diluent only—is given by the horizontal lines (solid line: PBS + BSA-diluted beads; dotted line: FBS-diluted beads).

Linearity test of diluted whole blood samples

For both cytokine kits, the standard curves showed slight variation depending on the type of diluent used for preparation (Figure 5). In particular, the FBS-diluted curves showed a signal shift compared to the Immunoassay Buffer and PBS + BSA curves which generally overlapped. This is noteworthy, as it may account for variability in linearity and percent recovery results based on diluent type. Although the FBS diluted curves were shifted, this may ultimately improve measurement of diluted whole blood samples, as the matrix of standard used for interpolation was more similar to that of the samples.

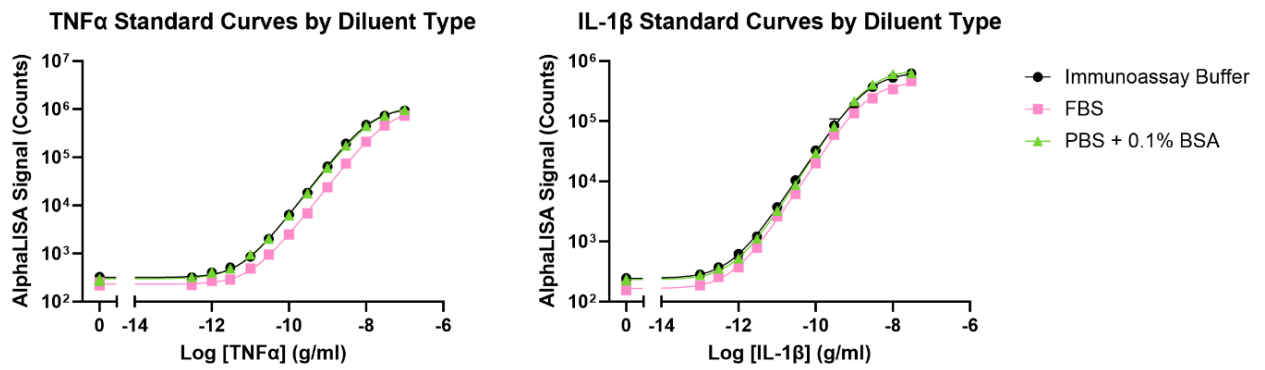
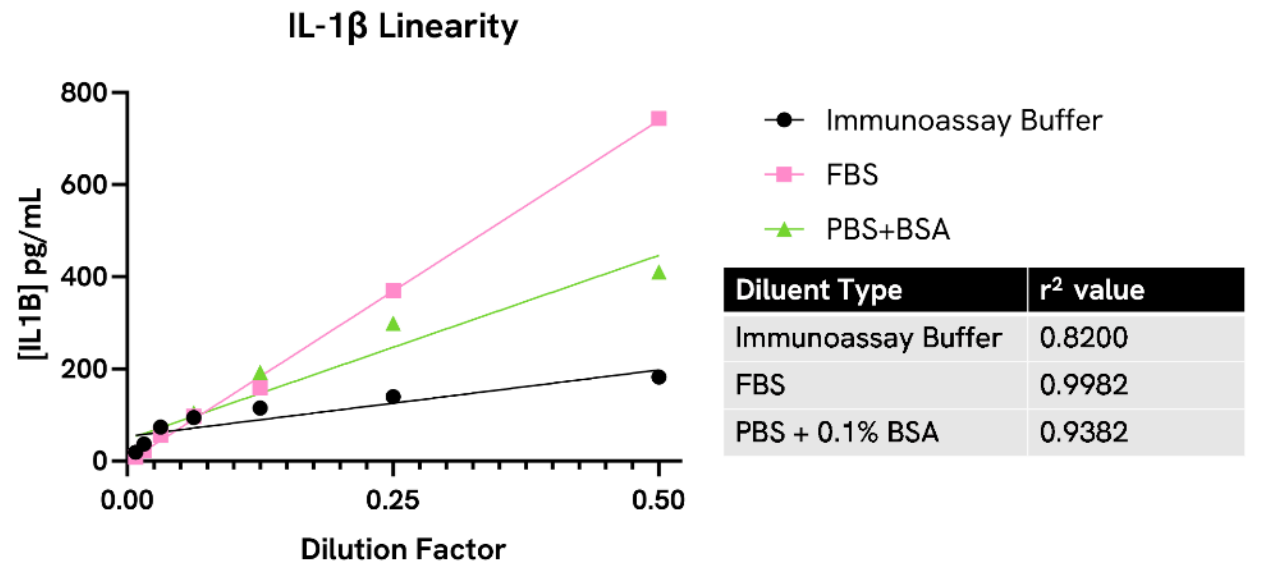


Figure 5: Comparison of standard curves prepared in three different diluent types: the included kit immunoassay buffer (black circles), fetal bovine serum (pink squares), or PBS + 0.1% BSA (green triangles) run on both cytokine assays.

Linearity tests showed FBS to be the best diluent option for both cytokine kits. Linear regression produced r^2 values >0.995 on both assays for FBS-diluted samples (Figures 6 and 7), whereas Immunoassay Buffer and PBS + BSA were below 0.995. A comparison of the linearity curves for three buffer types can be seen on Figure 5 below for IL-1β, with similar results observed for the TNFα kit (not shown).

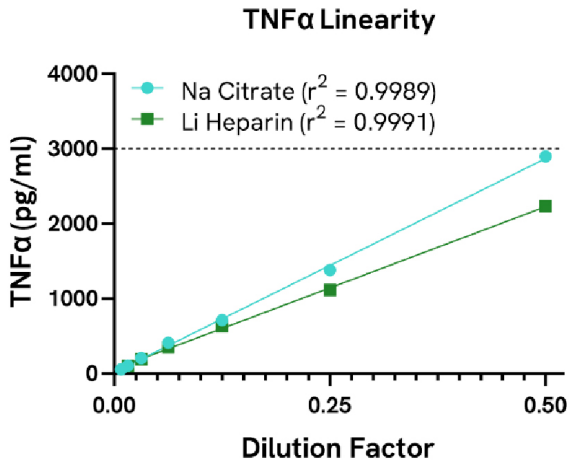
In particular, the Immunoassay Buffer proved to be a poor diluent choice for whole blood samples, as it caused lysis of the red blood cells (likely due to detergents in the buffer), and as a result, even when a spin down step was incorporated prior to assay, the samples did not separate, and signal quenching and/or interference was apparent.



Diluent Type	r^2 value
Immunoassay Buffer	0.8200
FBS	0.9982
PBS + 0.1% BSA	0.9382

Figure 6: Linearity results for 2-fold diluted whole blood samples (including a spin down step prior to assay) on the IL-1β HP cytokine AlphaLISA. Linear regression (r^2 values) showed that FBS-diluted samples (pink squares) produced acceptable linearity results compared to samples diluted in Immunoassay Buffer (black circles) or PBS + 0.1% BSA (green triangles).

When comparing linearity results based on blood collection tube type, r^2 values for 2-fold FBS-diluted samples were similar when using Na Citrate or Li Heparin blood collection tubes (0.9989 and 0.9991, respectively; Figure 7). However, when considering the percent recovery of TNFα analyte based on dilution factor, blood from Na Citrate tubes was more closely aligned with expected values compared to blood from Li Heparin tubes (Figure 7). It is pertinent to note the comparison of blood collection tubes was limited in this study (only two collection tube types tested and only TNFα kit tested). Further in-depth exploration of blood collection tube types and the effects of other pre-analytical/sample handling variables on AlphaLISA measurement would be valuable to conduct. However, based on these results, Na Citrate tubes were used for all further experiments.



Dilution Factor [expected concentration]	% recovery of expected TNFα concentration: Na Citrate Collection Tube	Li Heparin Collection Tube
2-fold [3,000 pg/mL]	97%	74%
4-fold [1,500 pg/mL]	92%	74%
8-fold [750 pg/mL]	95%	84%
16-fold [375 pg/mL]	110%	94%
32-fold [187.5 pg/mL]	109%	101%
64-fold [93.75 pg/mL]	113%	109%

**Calculated as: (Interpolated concentration / Expected concentration of spike-in) * 100*

Figure 7: Comparison of TNFα linearity results for FBS-diluted whole blood samples collected in either Sodium Citrate (Na Citrate, blue circles) or Lithium Heparin (Li Heparin, green squares) blood collection tubes. When assessing the percent of recovered TNFα analyte compared to the expected spiked-in concentration across a range of dilutions, Na Citrate collection tubes showed improved percent recovery compared to Li Heparin tubes (table on right).

Spike-recovery tests using whole blood samples

For TNFα, overall, percent recovery was lower in 2-fold PBS + 0.1% BSA-diluted samples (both no spin and with spin) compared to FBS-diluted samples (Table 1). Percent recovery was also below the defined acceptable range (70-130%) for PBS + 0.1% BSA-diluted samples. Given these findings, combined with the linearity results, PBS + BSA was excluded from further use as a diluent.

For 2-fold FBS-diluted blood, a spin down step was necessary to achieve acceptable recovery, as only 36-42% recovery was observed without spin down (Table 1). Importantly, the use of a spin down step (conducted after addition of the spike-in) did not appear to negatively impact recovery and the percent recoveries for all spike-in concentrations were within the acceptable range (104-124%). Based on results of the Omnibead test, it was expected that assay signal was likely to be quenched below approximately 32-fold dilution if no spin down was used. This was confirmed when testing an 8-fold FBS-diluted blood sample (no spin), which showed percent recovery below the acceptable range (57-75%); however, at 32-fold dilution, percent recoveries close to 100% were observed (Table 1). This suggests a 32-fold dilution of whole blood may be sufficient to remove matrix interference and signal quenching for AlphaLISA High Performance cytokine kits.

Table 1: Comparison of percent recoveries for spiked-in TNFα analyte in FBS and PBS + 0.1% BSA diluted whole blood samples (with and without spin down steps to separate red blood cells). Acceptable percent recovery is considered 70-130%.

	Percent recovery TNFα in FBS diluted blood				Percent recovery TNFα in PBS+0.1% BSA diluted blood	
TNFα spike-in concentration (pg/mL)	2-fold dilution, with spin	2-fold dilution, no spin	8-fold dilution, no spin	32-fold dilution, no spin	2-fold dilution, with spin	2-fold dilution, no spin
30	104%	36%	57%	91%	55%	20%
300	106%	42%	72%	103%	65%	25%
3,000	124%	42%	75%	102%	63%	22%
Conclusion:	Acceptable recovery	Poor recovery	Sub-optimal recovery	Acceptable recovery	Sub-optimal recovery	Poor recovery

Percent recovery of spiked-in IL-1β in FBS-diluted blood was acceptable at both a 2-fold dilution (with spin down) and a 32-fold dilution (no spin down). Similar to the TNFα results, this suggests either dilution factor could be applied for this assay, and that a spin-down step did not negatively impact recovery of IL-1β.

Table 2: Percent recoveries of spiked-in IL-1β in 2-fold diluted (with spin down step) and 32-fold diluted (no spin down step) whole blood samples. Sample dilutions were prepared in FBS.

	Percent recovery IL-1β in FBS-diluted blood	
IL-1β spike-in concentration (pg/mL)	2-fold dilution, with spin	32-fold dilution, no spin
12	87%	92%
120	89%	93%
1,200	94%	97%
Conclusion:	Acceptable recovery	Acceptable recovery

Measurement of stimulated cytokine production using best methods

LPS-stimulated blood samples showed elevated concentrations of both TNFα and IL-1β compared to controls, suggesting that AlphaLISA technology can measure biologically meaningful differences in cytokine concentrations in human whole blood samples (Figure 8). LPS is known to induce cytokine production, thus elevated cytokine levels would be expected in treated samples (LPS+) compared the control (LPS-, no LPS treatment) samples. This pattern was consistent in both 32-fold diluted samples and 2-fold diluted samples when a spin down step was incorporated into the sample preparation.

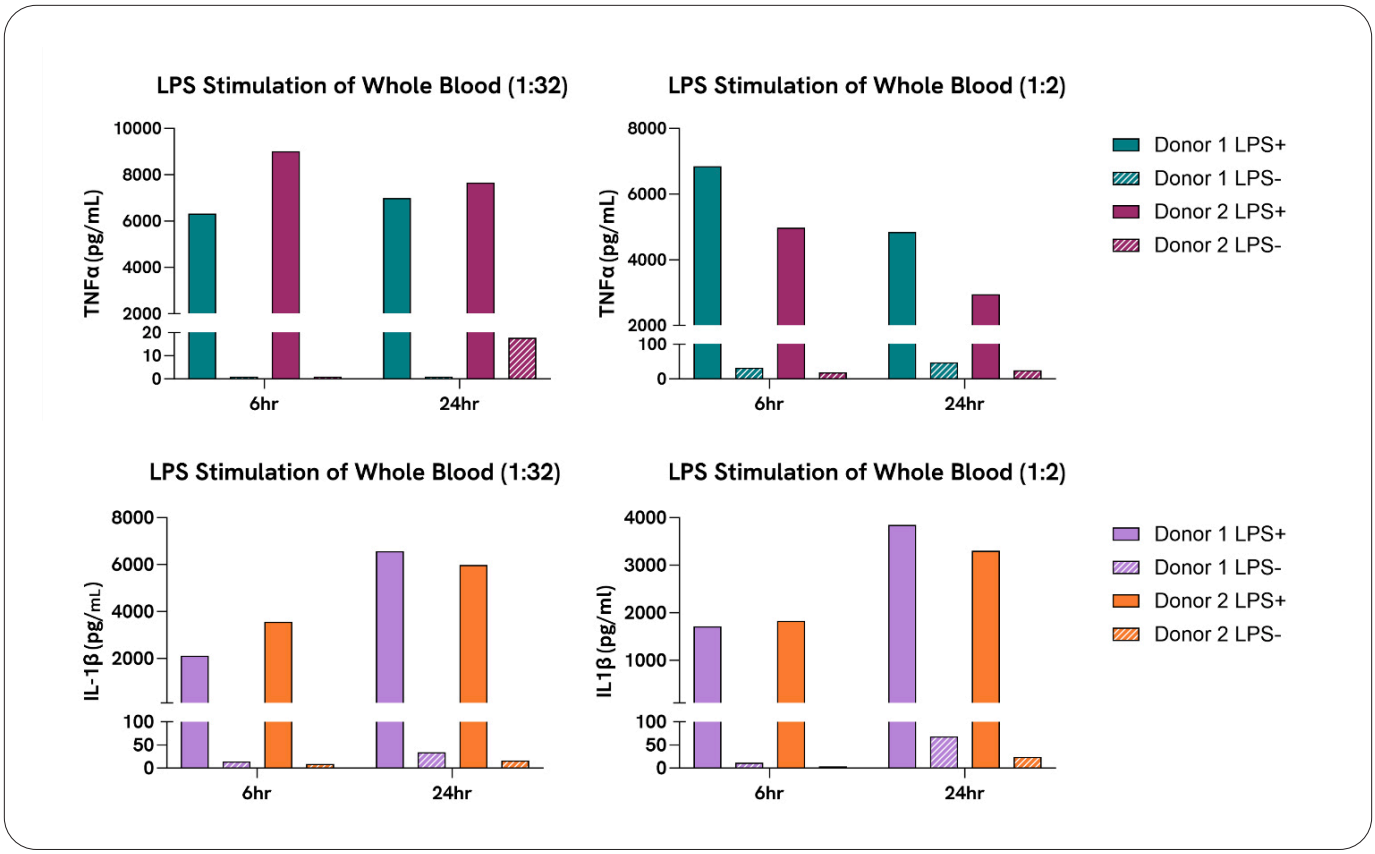


Figure 8: Measured TNFα (top row) and IL-1β (bottom) concentrations in LPS-stimulated (LPS+, solid color bars) versus control/untreated (LPS-, striped bars) blood samples from two separate donors. Cytokines were measured at 6-hour and 24-hour post-stimulation to account for transient cytokine production. Results are shown for matched samples run using different sample processing workflows: 32-fold dilution (left panels), or 2-fold dilution, with spin down (right side panels).

In comparing data generated across the two dilution factors, cytokine levels in stimulated samples (LPS+) tended to be higher in 32-fold (no spin) diluted samples versus 2-fold (with spin) diluted samples. During this experiment, we observed some natural clotting of the diluted blood during the 6 and 24-hour incubation periods, with more extensive clotting noted in the 2-fold diluted samples. It is possible this clotting may have slightly reduced recovery of the analyte. Alternatively, since the interpolated concentrations are adjusted for dilution factor (i.e. multiplied by the dilution factor), it is possible the higher (32-fold) dilution factor may somewhat artificially elevate the final reported number, and could contribute to the difference observed in final values. However, importantly, the relative patterns remained generally similar across the two dilution factors. Also notable, when measuring control (LPS-) samples, cytokine concentrations were quite low, and in some cases, indistinguishable from background on the assay—this was particularly the case for 32-fold diluted samples, especially in the TNFα assay. Therefore, using a 2-fold dilution (with spin) protocol may allow for improved measurement of analytes with low basal concentrations. Overall, either workflow appears to be useable with whole blood samples, although selecting a single dilution factor and workflow for use in experiments is likely preferable to ensure comparability of results.

Conclusion

Human whole blood is a complex matrix which can be challenging to use with many immunoassay technologies. Here we demonstrate that cytokines can be measured in human whole blood samples using AlphaLISA High Performance cytokine kits when samples are properly prepared for assay. This study compared various sample processing and assay practices, and offers two potential workflows for measuring whole blood samples on AlphaLISA HP cytokine kits. Based on our findings the following recommendations can be made from this study:

- Undiluted (neat) human whole blood demonstrates significant signal quenching and/or interference when used on AlphaLISA technology. However, cytokines were measurable in whole blood samples diluted ≥32-fold, particular for analytes at higher concentrations (either stimulated or basal levels). Different analytes or assay kits may show differing dilution thresholds and users should conduct their own dilution tests.
- In situations where detection of low concentrations of analyte are critical, a workflow combining a 2-fold dilution paired with a spin down step and transfer of the supernatant portion to assay, may be a preferable alternative to using high dilution factors often needed with mixed whole blood samples. Our study suggests a simple spin down step post-sample dilution (as opposed to separation of plasma/serum after initial blood collection) can be used without impacting recovered analyte concentrations.
- Preparation of samples and standards in appropriate diluent is a critical consideration when working with whole blood samples on AlphaLISA. Fetal bovine serum was the best tested diluent in this study, but other similar serum or blood component-based diluents may also be suitable.
- The findings presented here are general guidelines and may be applicable (potentially with slight modifications) for other cytokine targets and AlphaLISA kits. However, it is always good practice to for users to conduct their own analytical assay validations (e.g. linearity and spike-recovery) for new targets or kits, particularly when working with non-traditional sample types, to ensure confidence in results. Additional modifications may further improve whole blood measurements by AlphaLISA.

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