TECHNICAL NOTE

Comparison of AlphaLISA and ELISA methods for TNFR1 detection in a panel of complex sample matrices.

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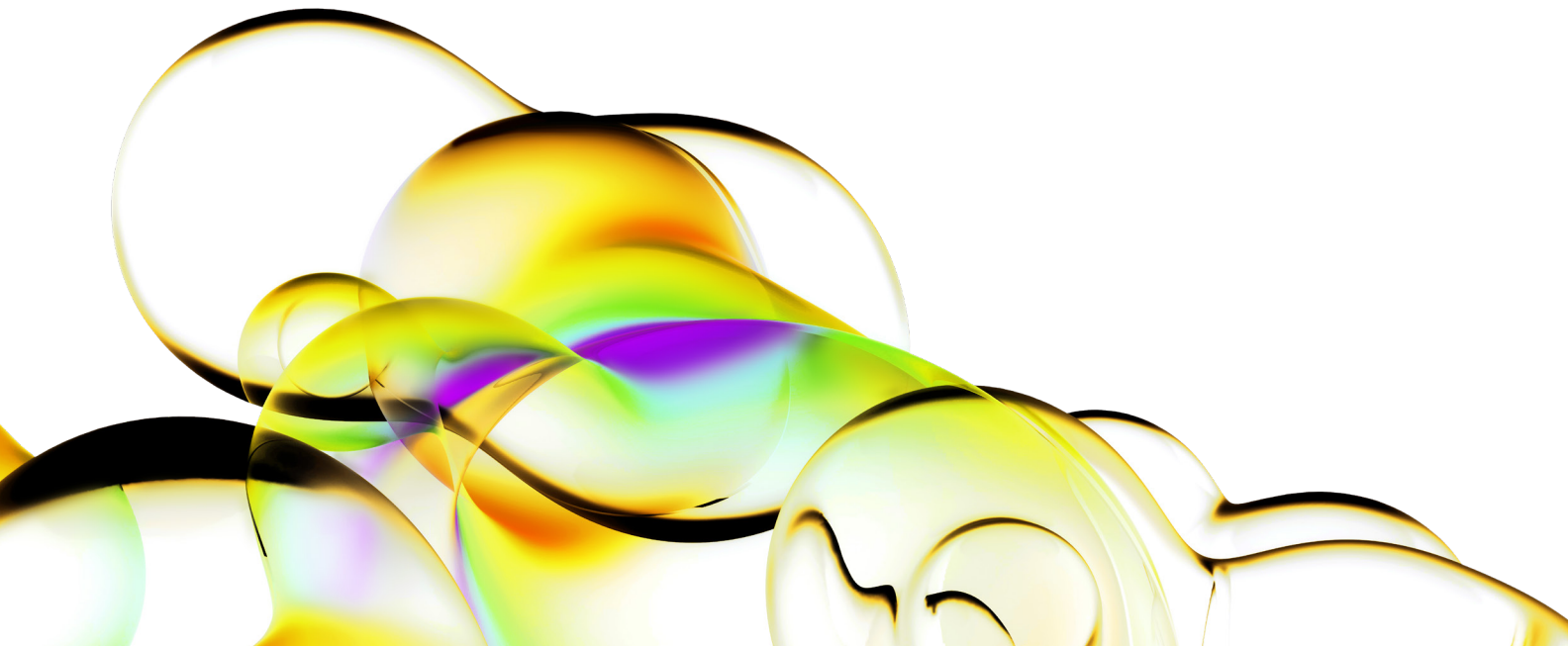
Introduction

A traditional enzyme-linked immunosorbent assay (ELISA) is often used to detect and quantify the levels of an analyte from a range of complex sample matrices (e.g. human serum, urine, or blood) where an abundance of other proteins and molecules are present. While sensitive, an ELISA often requires large amounts of sample for each assay and can be very labor intensive. Typical working time in the lab for a traditional ELISA ranges from four to six hours, depending on the number of wash steps and access to liquid handling instrumentation. ELISA methods are susceptible to the constraint of a narrow linear range for the optical density (OD), which is common to absorbance-based measurements, according to the Lambert-Beer Law which defines the linear relationship between concentration and absorbance. The smaller dynamic range (~2 logs) of the assay decreases the likelihood that your unknown sample concentration will fall within the standard curve and introduces the challenge of testing multiple dilutions from the same potentially limited sample. Repeat dilution testing of the same sample also requires additional assay wells and often leads to purchasing another kit or coating additional assay plates to accurately quantify all your samples.



Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA™) is a bead-based assay technology used to study biomolecular interactions in a microplate format. Binding of molecules captured on the beads leads to an energy transfer from one bead to the other, ultimately producing a luminescent signal (Figure 1). This format is robust and highly reproducible with a short total assay time, producing results in less than three hours. AlphaLISA requires only 5 µL of sample per well and provides sensitive quantification of the target analyte. The larger dynamic range (up to ~5 logs) also provides an easier target to hit for samples of unknown concentration after selecting the appropriate diluent for the sample matrix. This technical note compares ELISA kits from two suppliers to the Human TNFR1 AlphaLISA™ Detection Kit to demonstrate the ease of use and benefits of Alpha technology. For each kit, dynamic range, sensitivity and quantification from three biological sources (cell supernatants, human urine and serum samples) were examined.

Soluble tumor necrosis factor receptor 1 (TNFR1), along with TNFR2, regulates the response to Tumor Necrosis Factors (TNFs) which are a superfamily of transmembrane proteins that can be released by extracellular proteolytic cleavage to function as cytokines. TNFs are expressed predominantly by immune cells and coordinate diverse cell functions. TNFR1 and TNFR2 show high affinity to bind either TNF-α or TNF-β however they remain immunologically distinct. TNFR1 is expressed ubiquitously on almost all cell types, whereas TNFR2 expression is limited to hematopoietic and endothelial cells.[1] Soluble TNF binding proteins capable of neutralizing the biological effects of TNFs were discovered in serum and urine samples. Researchers have shown these soluble forms are truncated versions of the receptor produced by shedding of the extracellular domains. The soluble portions of the receptor retain the ability to bind TNFs and are found in healthy and diseased patients alike. Increased soluble TNFR1 is an indicator for disease states such as infection, rheumatoid arthritis, type 2 diabetes, and can be used as a predictive marker for progression to advanced chronic kidney disease or end-stage renal disease.[2-4]

Reagents

- Human TNFR1 AlphaLISA Detection Kit (Revvity, #AL3088)
- Quantikine® ELISA Human TNF R1 Immunoassay Kit (R&D Systems, #DRT100)
- Human sTNF-R1 EASIA Kit (ThermoFisher, #KAC1761)
- DPBS, no calcium, no magnesium (ThermoFisher, #14190-114)
- 30% Bovine Serum Albumin Solution (Sigma-Aldrich, #A7284)
- Immunoassay Buffer (Revvity, #AL000C)
- Pooled Normal Human Serum (Innovative Research, #IPLA-SER)
- Normal Single Donor Human Serum (BioIVT)
- Normal Single Donor Chronic Kidney Disease (BioIVT)
- Normal Single Donor Rheumatoid Arthritis (BioIVT)
- Pooled Normal Human Urine (Innovative Research, #IR100007P)
- Normal Single Donor Human Urine (Innovative Research, #IR100007)
- Single Donor Human Type II Diabetes Urine (Innovative Research, #IR14005)
- THP-1 cells (ATCC, #TIB-202)
- Primary Peripheral Blood Mononuclear Cells; PBMC (ATCC, #PCS-800-011)
- RPMI-1640 (ATCC, #30-2001)
- Fetal Bovine Serum; FBS (ThermoFisher, #26140-079)
- DPBS, no calcium, no magnesium (ThermoFisher, 14190-114)
- Phorbol 12-myristate 13-acetate; PMA (Tocris, #1201)
- Lipopolysaccharide; LPS (Sigma, #L4516)
- ViewPlate™-96 F, TC treated (Revvity, #6005182)
- AlphaPlate™-384, light gray (Revvity, #6005350)
- TopSeal™-A Plus Adhesive Sealing Film (Revvity #6050185)

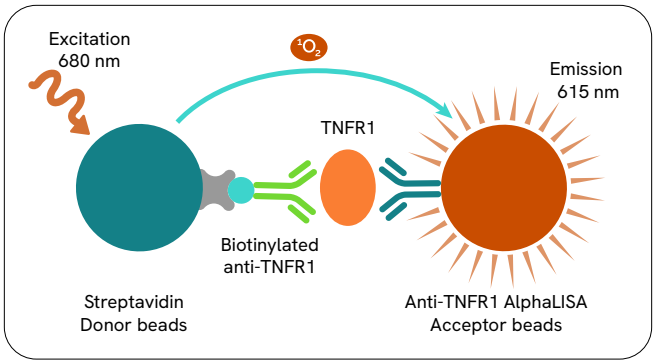


Figure 1: **Human TNFR1 AlphaLISA schematic**. Streptavidin-coated Alpha Donor beads bind the biotinylated anti-TNFR1 antibody; the AlphaLISA Acceptor beads are supplied conjugated to an anti-TNFR1 antibody. Binding of antibodies in the presence of TNFR1 brings the Donor and Acceptor beads into proximity. Excitation of the Donor beads at 680 nM promotes the release of singlet oxygen molecules that trigger a cascade of energy transfer in the Acceptor beads, resulting in a sharp peak of light emission at 615 nM.

Data collection and analysis

AlphaLISA

Revvity: Human TNFR1 AlphaLISA assay was performed following the standard protocol (Figure 2). AlphaLISA signal was measured on a Revvity EnVision™ multimode plate reader using default values for Alpha detection. Serum samples were prepared in fetal bovine serum (FBS) at a 1:4 dilution factor. Urine samples were prepared in DPBS with 0.5% BSA added (a urine-like diluent as previously determined) at a 1:4 dilution factor.[5] Cell supernatants were tested neat in the culture media (RPMI+10% FBS). Standard curve determination was performed in parallel to unknown samples in the same diluent (FBS, DPBS+0.5% BSA, or cell culture media). Curves were plotted in GraphPad Prism® with nonlinear regression fitting using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. Reported values of biological samples were adjusted for dilution factor.

Assay workflow comparison

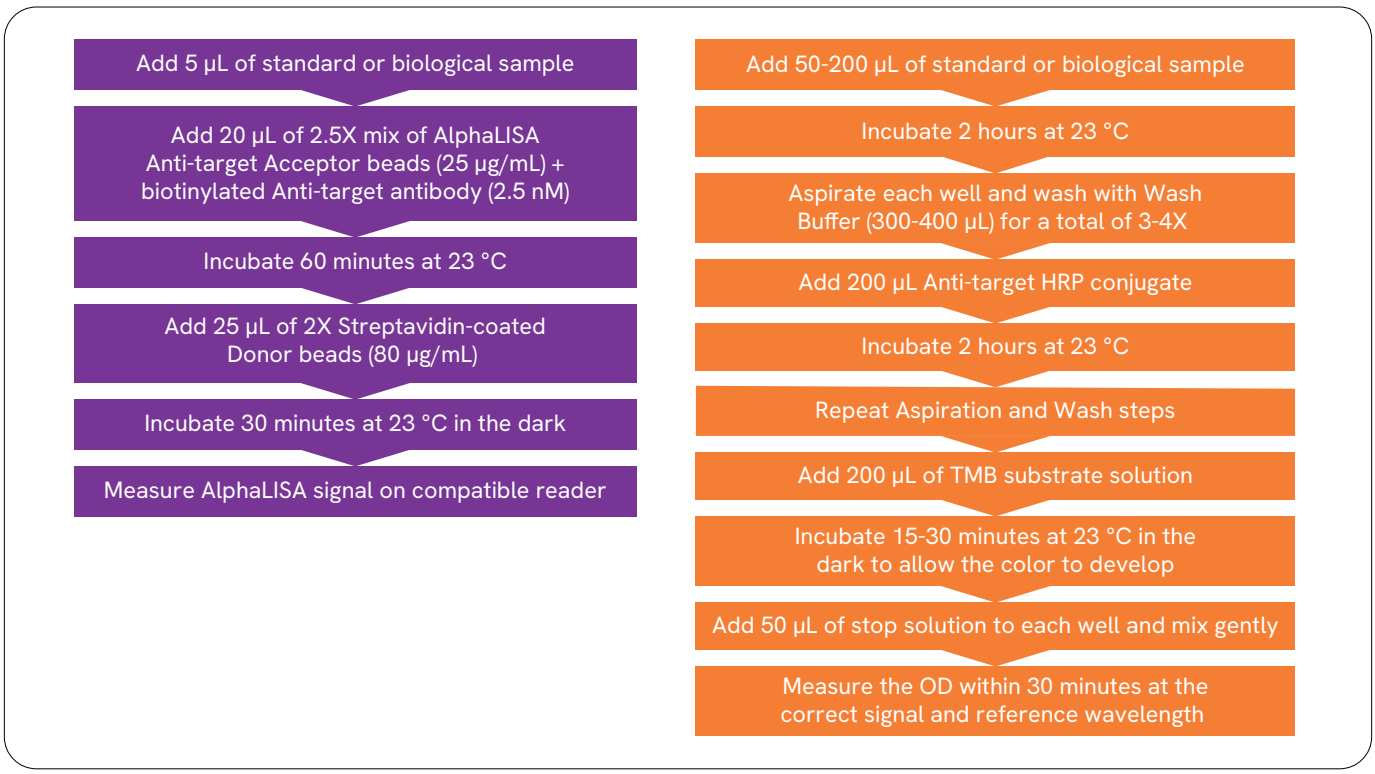


Figure 2: **Workflow for typical AlphaLISA detection kit and traditional colorimetric ELISA format.** ELISA strip-well plates pre-coated with capture antibody were provided in both kits. No preparation was required prior to addition of biological samples. For a standard ELISA, additional time is needed to coat and block the plates, generally overnight incubation adding one day to the timeline. The R&D Systems TNFR1 Kit follows the traditional ELISA workflow shown here. The ThermoFisher TNFR1 kit has a slightly shorter workflow (only one initial sample/reagent incubation step, and one set of washes) cutting the total assay time in half.

ELISA methods

R&D Systems: Quantikine® ELISA was performed following the recommended protocol using the pre-coated strip well plate in the traditional antibody sandwich format. Serum samples were diluted a minimum of a 1:10 dilution factor in the RD60 calibrator diluent. Cell supernatants were tested neat with the exception of one analyte sample requiring a 1:10 dilution factor in RD5-5 calibrator diluent. Human urine samples were diluted a minimum of 1:10 dilution factor in RD5-5 calibrator diluent. Two separate analyte stocks were prepared in RD60 and RD5-5 to match the biological samples with standard curves generated from each. Optical density (OD) was measured at 450 nM with a reference wavelength of 540 nM for correction. The buffer only (0 pg/mL) average result was background subtracted from all sample OD values. Data was plotted in GraphPad Prism® with log-log line fitting and interpolation of unknown sample concentration. Reported values of biological samples were adjusted for dilution factor.

ThermoFisher: EASIA (enzyme amplified sensitivity immunoassay) was performed following the recommended protocol using the pre-coated strip well assay plate in a modified antibody sandwich format. This modified ELISA method utilizes a blend of pre-coated TNFR1 antibodies, includes shaking during the capture step and reduces the number of total wash steps to shorten the workflow. Individual vials of analyte standards at pre-determined concentration were prepared according to the manual. The kit includes a buffer control and two quality control (QC) samples of low and medium concentration. The QC samples safely met the 70-130% accepted criteria of the expected concentration and as such the assay runs passed. Biological samples were tested neat with the exception of the human rheumatoid arthritis serum which was run at a 1:4 dilution factor in the buffer control as recommended. Samples were tested at OD 450 nM with a reference correction at 630 nM. The buffer only (0 pg/mL) average result was background subtracted from all sample OD values. The polychromatic OD method listed in the kit was not necessary for OD range extension as no samples were beyond the upper limit of the supplied standards. Data was plotted in GraphPad Prism® with log-log line fitting and interpolation of unknown sample concentration. Reported values of biological samples were adjusted for dilution factor.

Sensitivity and dynamic range

For all assay formats, the lower detection limit (LDL) and lower limit of quantification (LLOQ) were calculated using the standard curve blanks (buffer only). The calculated LDL or LLOQ numbers were interpolated onto the standard curve to provide a result in pg/mL of analyte. Below are the formulas:

LDL = mean (blanks) + 3X standard deviation

LLOQ = mean (blanks) + 10X standard deviation

Dynamic range described here spans from the LDL to the highest analyte standard tested as the ELISA curve fitting does not allow for a 95% confidence interval value at the upper limit to establish the top value of the range.

Assay metrics: Dynamic range, LDL, and LLOQ

Standard curves were prepared from the analyte provided in each kit and are shown in Figure 3. The concentration range of the TNFR1 analyte varied widely across the three assays. Table 1 contains the assay metrics calculated. The R&D Systems ELISA is sensitive in terms of the lower range of detection; however, the top end of the assay is limited to 500 pg/mL. The opposite is true of the ThermoFisher kit that has a standard curve with the lowest dose starting at 900 pg/mL and extending almost to the top end of the AlphaLISA standards. AlphaLISA provides the largest dynamic range of the three assays tested with more than one additional log of coverage on average. The LDL results for each method are similar, with the exception of diluent-specific increase for the AlphaLISA and the decreased sensitivity of the ThermoFisher kit (55.8 pg/mL determined experimentally).

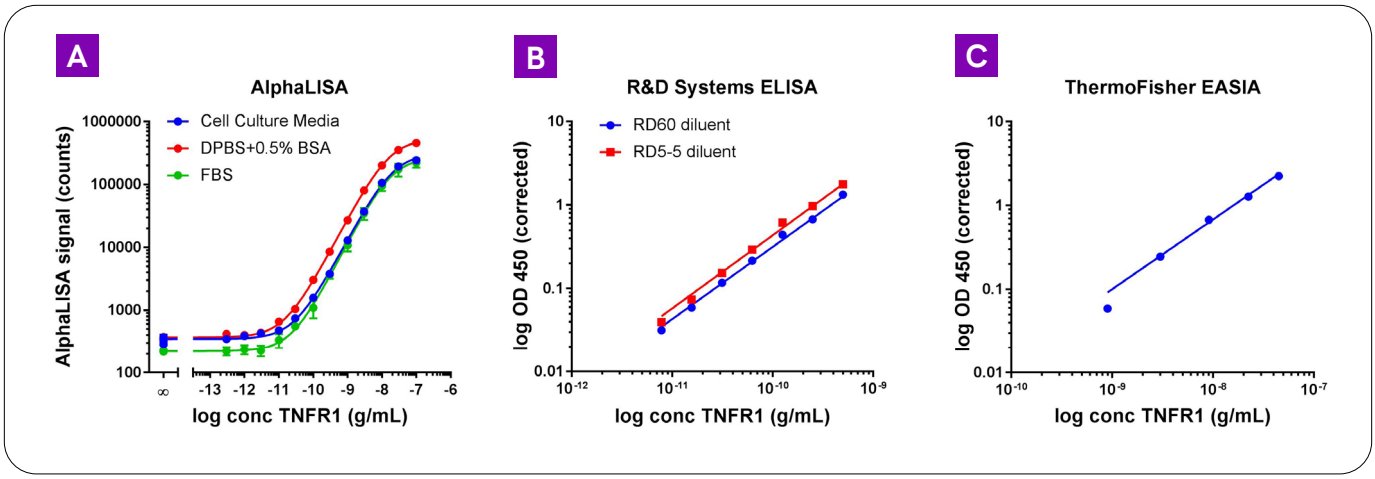


Figure 3: **TNFR1 standard curve comparison**. Serial dilutions were prepared in parallel for multiple diluents in each assay following manufacturer's protocol. (A) AlphaLISA, (B) R&D Systems ELISA, and (C) ThermoFisher EASIA.

Table 1: **Assay information and metrics.** LDL and LLOQ were calculated for each kit in the appropriate diluent listed. Dynamic range described here spans from the LDL to the top of the analyte standard as the ELISA fitting does not allow for a 95% confidence interval value at the upper limit.

	Analyte standard range pg/mL	Samples and diluent	LDL pg/mL	LLOQ pg/mL	Dynamic range
AlphaLISA	0.3 – 100,000	Serum (FBS)	7.4	23.6	~ 4 logs
		Supernatant (Media)	9.6	34.4	~ 4 logs
		Urine (DPBS+BSA)	2.8	10	~ 5 logs
R&D Systems ELISA	7.8 - 500	Serum (RD60)	2.9	4.0	~ 2.5 logs
		Supernatant and Urine (RD5-5)	2.0	2.6	~ 2.5 logs
ThermoFisher EASIA	900 – 45,000	Serum, Supernatant and Urine (Assay Buffer)	55.8	141	~ 3.5 logs

Quantification of TNFR1 in biologically relevant samples

Serum and urine samples were used from the same lot after careful storage of aliquots prepared from the original supplier stock. THP-1 and PBMC cell supernatants were prepared in bulk with the stimulation conditions listed for each. After removal of supernatants and centrifugation to avoid carryover of cells, aliquots were made and kept frozen until the time of testing as previously described.[6] Data was compiled in Table 2 which shows the soluble TNFR1 in each sample as measured by AlphaLISA and the two ELISA formats. Additionally, the correlation plot shown in Figure 4 comparing AlphaLISA data to both ELISA methods shows an excellent linearity across sample types. The correlation coefficient calculated in Excel is 0.990 for R&D Systems and 0.979 for ThermoFisher. A correlation coefficient of +1.0 indicates a perfect positive correlation, which means that as variable X increases, variable Y increases and while variable X decreases, variable Y decreases. The absolute concentrations do not match across technologies with AlphaLISA generally yielding a higher concentration of soluble TNFR1; however the cell-based stimulation trends and rank order of serum and urine samples are consistent.

Table 2: **Quantification of TNFR1 present in biological samples.** Results are shown as pg/mL of soluble TNFR1 as quantified by each kit and corrected for any dilution factor. The majority of cell supernatant (sup) samples fall well below the lowest standard for the ThermoFisher kit; however the increase of TNFR1 is still evident in both cell types with PMA treatment.

Sample	AlphaLISA	R&D Systems ELISA	ThermoFisher EASIA
Pooled Normal Serum	3393.3	1457.6	1865.3
Single Normal Serum	2784.4	1294.7	1845.4
Single Chronic Kidney Disease Serum	8820.4	5782.8	8119.2
Single Rheumatoid Arthritis Serum	23389.6	22061.6	39907.9
Pooled Normal Urine	1430.8	681.2	825.0
Single Normal Urine	949.5	314.9	325.9
Single Type II Diabetes Urine	5930.2	3560.4	4912.8
THP-1 Sup (Untreated Cells)	304.1	100.3	130.9
THP-1 Sup LPS 100 ng/mL	678.3	183.6	188.0
THP-1 Sup PMA 100 nM	2937.3	1838.7	2382.1
PBMC Sup (Untreated Cells)	61.0	23.4	62.5
PBMC Sup LPS 100 ng/mL	155.3	64.8	85.6
PBMC Sup PMA 100 nM	352.2	140.5	148.1

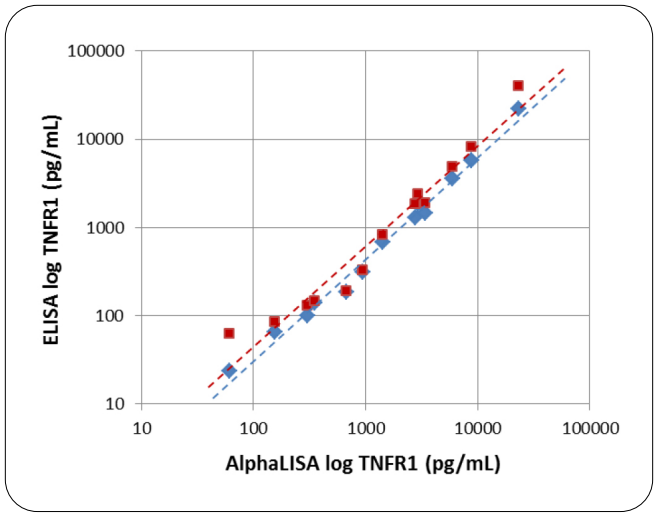


Figure 4: **Correlation plot of soluble TNFR1 present in biological samples**. Average results are plotted by pg/mL of soluble TNFR1 as quantified by each kit and corrected for any dilution factor. AlphaLISA results for each biological sample were plotted on the x-axis with the corresponding ELISA result on the y-axis (R&D Systems = blue diamonds, and ThermoFisher = red squares) with each showing a dotted line linear fit for visual reference. The ThermoFisher data begins to deviate from the linear fit at concentrations below the lowest standard curve dose (900 pg/mL). These samples represent the low soluble TNFR1-containing cell supernatants.

Summary

This technical note demonstrates detection of soluble TNFR1 in human biological samples from three main sources: cell culture supernatant, patient derived urine and serum samples. All three assays were effective at detecting soluble TNFR1 in complex matrices when following manufacturer's recommended protocols and sample handling, however there were some observed differences in the overall assay workflow and performance.

Time to results

The AlphaLISA protocol is considerably faster than the R&D Systems traditional ELISA, while the ThermoFisher EASIA protocol time falls in the middle of the two. The absence of wash steps in the AlphaLISA reduces the time to obtain results and decreases the volume of waste disposal in the lab.

Sample consumption

Another advantage of the AlphaLISA is the amount of sample required to perform the assay. The data shown here suggest that 5 µL of sample in the AlphaLISA is sufficient compared to 200 µL and 50 µL of total sample volume from the R&D Systems and ThermoFisher kits respectively.

Assay metrics: Dynamic range and sensitivity

The key differences occur in the measured assay metrics. The dynamic range of AlphaLISA covers the largest concentration range of the three offerings, up to ~5 logs compared to half that with the ELISA methods. The LDL for Alpha technologies is known to be dependent on the assay diluent chosen. As seen in Table 1, the addition of FBS in cell culture media, or used as a surrogate for human serum, decreases the lower sensitivity of the assay. The R&D Systems RD60 diluent used for serum samples is a proprietary formula with no disclosure of the composition of the buffer. Likewise, there was no diluent required for use with the ThermoFisher kit unless samples exceeded the upper limits of the analyte standard. This suggests the AlphaLISA is more stringent in the setup ensuring the analyte standard is matched with biological samples in a suitable composition of diluent. Manufacturer-reported LDL values of the TNFR1 kits for R&D Systems and ThermoFisher are 0.77 pg/mL and 50 pg/mL respectively with the AlphaLISA reported at 1.1 pg/mL in AlphaLISA Immunoassay Buffer. LDL results determined experimentally here show AlphaLISA to be on par with R&D Systems and lower than the ThermoFisher kit. LDL calculations from the ELISA manufacturers are calculated using 20 control points of buffer alone. For practical purposes (i.e., cost of each kit), fewer samples were run here as blanks for this calculation but experimental LDL results matched the manufacturer-reported values. Overall, the AlphaLISA microplate format allows greater flexibility for determining assay sensitivity, saves on precious sample volume and total time in lab while delivering reliable and reproducible quantification of analyte concentration.

References

1. Parameswaran, N, et al. Tumor necrosis factor-α signaling in macrophages. Critical Reviews in Eukaryotic Gene Expression (2010); 20:87-103.
2. Giai, C et al. Shedding of tumor necrosis factor receptor 1 induced by protein A decreases tumor necrosis factor alpha availability and inflammation during systemic staphylococcus aureus infection. Infection and Immunity (2013); 81(11): 4200-4207
3. Carlsson, A, et al. Association of soluble tumor necrosis factor receptors 1 and 2 with nephropathy, cardiovascular events, and total mortality in type 2 diabetes. Cardiovascular Diabetology (2016); 15(40).
4. Saulnier, PJ, et al. Association of circulating biomarkers (Adrenomedullin, TNFR1, and NT-proBNP) with renal function decline in patients with type 2 diabetes: a French prospective cohort. Diabetes Care (2017); 40:367–374.
5. Carlson, A. Revvity Technical Note (2018): AlphaLISA in Urine: Detecting Soluble TNFR1 in a Complex Sample Matrix.
6. Carlson, A. Revvity Technical Note (2018): Quantifying TNFR1 in Both Soluble and Membrane Bound Form Using AlphaLISA Technology.

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