

AlphaLISA in urine: Detecting soluble TNFR1 in a complex sample matrix.

Author

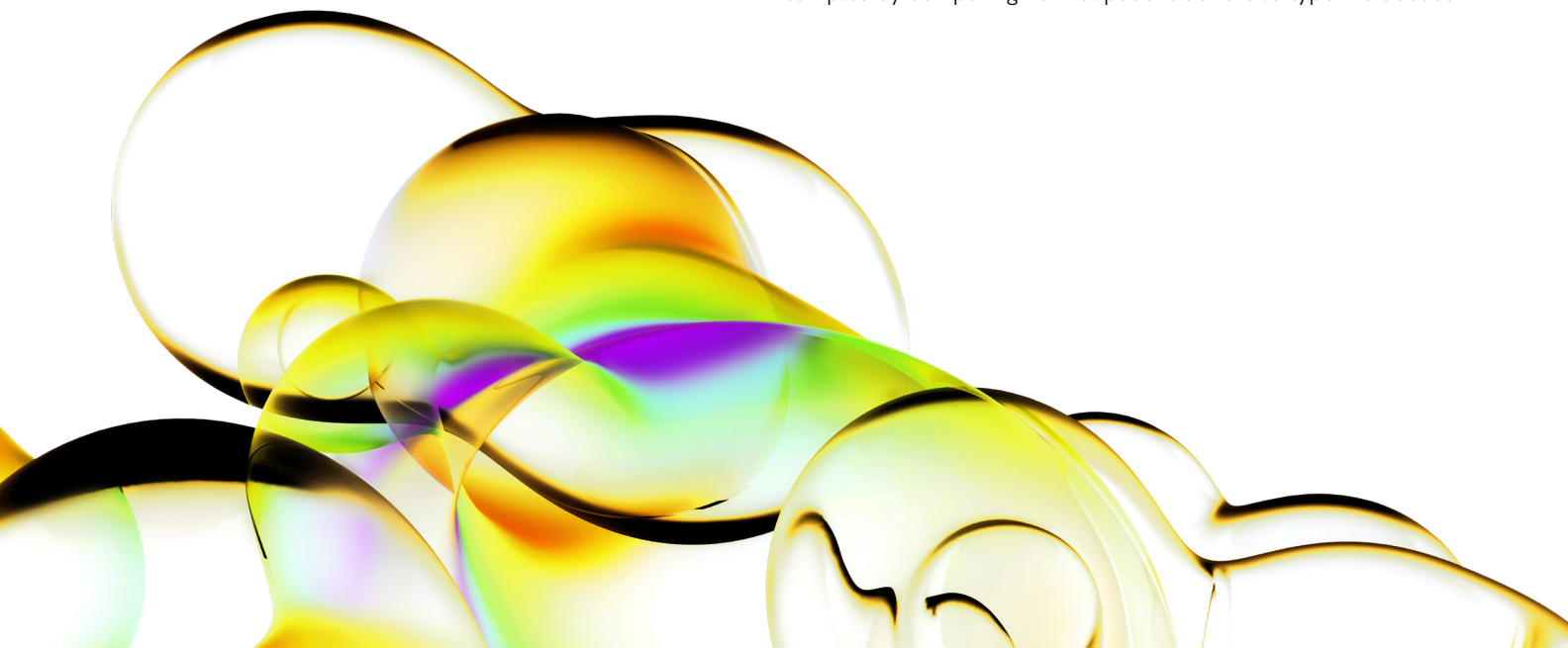
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Introduction

Tumor Necrosis Factors (TNFs) are a superfamily of transmembrane proteins that can be released by extracellular proteolytic cleavage to function as cytokines. They are expressed predominantly by immune cells and coordinate diverse cell functions such as regulation of immune response, inflammation, proliferation, differentiation, and apoptosis. Two receptors, TNFR1 and TNFR2, have been identified which regulate the response to TNFs. Each of these shows high affinity to bind TNF- α or TNF- β , however they remain immunologically distinct.¹ Soluble TNF binding proteins that are capable of neutralizing the biological effects of TNFs have been discovered in serum and urine samples. Researchers have shown these soluble forms to be 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, type 2 diabetes, and can be used as a predictive marker for progression to advanced chronic kidney disease or end-stage renal disease.^{2,3}

Wash-based ELISA assays are typically used to measure increased levels of soluble TNFR1 from complex patient samples. While sensitive, ELISA assays often require large amounts of sample for each assay, and can be very labor intensive. The Human TNFR1 AlphaLISA™ Detection Kit was designed for the quantitative determination of TNFR1 using a homogeneous bead-based assay. This technical note demonstrates how to quantify levels of TNFR1 in human urine samples by comparing normal patient donors to type 2 diabetics.



To ensure accurate detection in urine, linearity of dilution experiments were performed to determine an appropriate diluent for the assay. The selected diluent was further validated through spike-and-recovery testing.

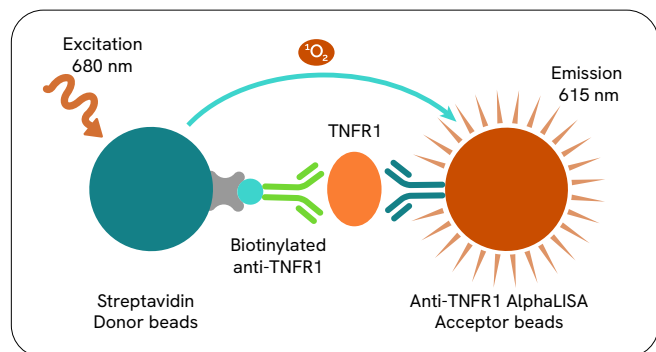


Figure 1: Human TNFR1 Assay Principle. Streptavidin-coated Alpha Donor beads bind the biotinylated anti-TNFR1 antibody; the AlphaLISA Acceptor beads are supplied conjugated to an anti-TNFR1 antibody. Binding of antibodies in the presence of TNFR1 brings the Donor and Acceptor beads into close proximity. 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.

AlphaLISA workflow

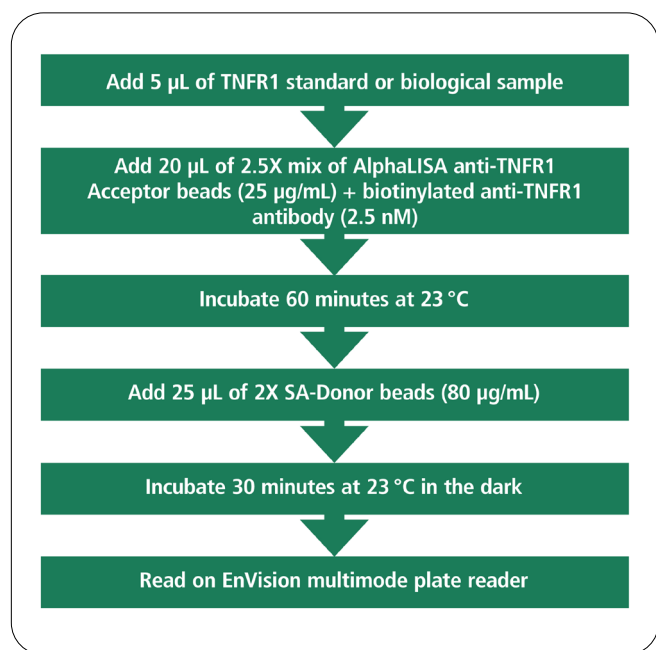


Figure 2: Workflow for Human TNFR1 AlphaLISA Detection Kit. The assay provides sensitive quantification of TNFR1, utilizing only 5 µL of sample and generating results in under three hours.

Reagents

- Human TNFR1 AlphaLISA Detection Kit (Revvity, #AL3088)
- DPBS, no calcium, no magnesium (ThermoFisher, #14190-114)
- 30% Bovine Serum Albumin Solution (Sigma-Aldrich, #A7284)
- Immunoassay Buffer (Revvity, #AL000C)
- 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)
- ViewPlate™-96 F, TC treated (Revvity, #6005182)
- AlphaPlate™-384, light gray (Revvity, #6005350)
- TopSeal™-A Plus Adhesive Sealing Film (Revvity, #6050185)

Data collection and analysis

AlphaLISA assays were measured on a Revvity EnVision™ multimode plate reader using default values for standard Alpha detection. Standard curve determination was performed in parallel to unknown samples in the same diluent. 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. For dilutional linearity experiments R -squared (R^2) values were calculated using linear regression. Acceptable R^2 values (> 0.995) are an indication of goodness-of-fit and help determine the proper diluent. For spike-and-recovery experiments, percent recovery was calculated by dividing the observed result of the urine spike by the observed diluent spike. Acceptable spike-and-recovery values range from 70-130%. Further, 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:

$$\% \text{ Recovery} = (\text{observed sample spike} / \text{observed diluent spike}) \times 100$$

$$\text{LDL} = \text{mean (blanks)} + 3X \text{ standard deviation}$$

$$\text{LLOQ} = \text{mean (blanks)} + 10X \text{ standard deviation}$$

Linearity of dilution for urine samples

Determining the appropriate diluent to match the biological sample is critical for accurate detection in any assay format. Urine is a complex matrix and poses the challenge of not having a standard diluent in use across different technologies. When testing human serum, samples are frequently diluted in fetal bovine serum. In an ELISA the diluent may be the same proprietary buffer used for cell supernatants which often contains added detergent and protein such as BSA. TNFR1 AlphaLISA detection reagents (biotinylated antibody, Acceptor Beads, and Donor beads) comprise 90% of the total assay volume and are prepared in our Immunoassay Buffer which contains 0.1% casein and 0.5% Triton™ X-100 eliminating the need to include detergent in the sample diluent. Diluent testing was performed for the TNFR1 AlphaLISA (Fig. 3) with a standard curve in Immunoassay Buffer compared to DPBS with either 0.1% or 0.5% BSA added to create a “urine-like” diluent.

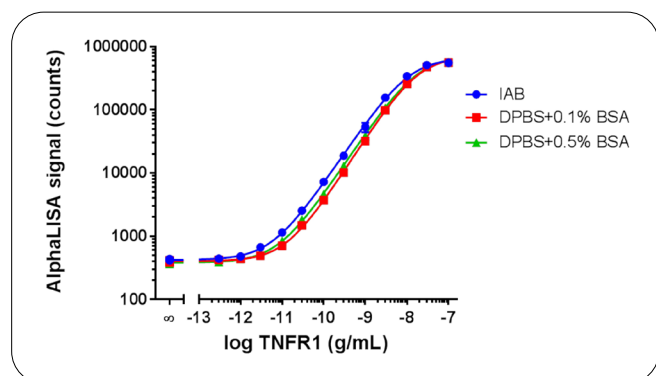


Figure 3: TNFR1 Standard Curve Comparison. Serial dilutions were prepared in parallel for multiple diluents. LDL and LLOQ for each diluent: Immunoassay Buffer (1.02, 3.49 pg/mL), DPBS+0.1% BSA (2.34, 7.88 pg/mL), and DPBS+0.5% BSA (1.28, 4.60 pg/mL).

When preparing standard curves in multiple diluents, minor differences in curve shape may be apparent. These small differences affect the final interpolated values of your sample. This makes it essential to prepare the standard curve in the same diluent as the biological sample for accurate quantification.

TNFR1 in normal human urine has been reported at moderate levels. Attempts to spike TNFR1 from the analyte stock into a pooled normal human urine sample confounded the experimental results. This could be due in part to potential hooking of the antibodies at concentrations beyond the upper limit of detection, or interfering substances in the neat urine samples.

In this case, as might be the case for other targets of interest, taking an approach similar to Parallelism is useful. As a result of moderate to high endogenous levels, the biological sample can simply be serially diluted without performing the spike step. Linearity of dilution results performed with no spike (Fig. 4) suggest DPBS with 0.5% BSA added is an appropriate sample diluent for urine as seen by the linearity achieved after the initial two-fold dilution. Results were similar for DPBS+0.1% BSA, however R^2 values improved with additional BSA.

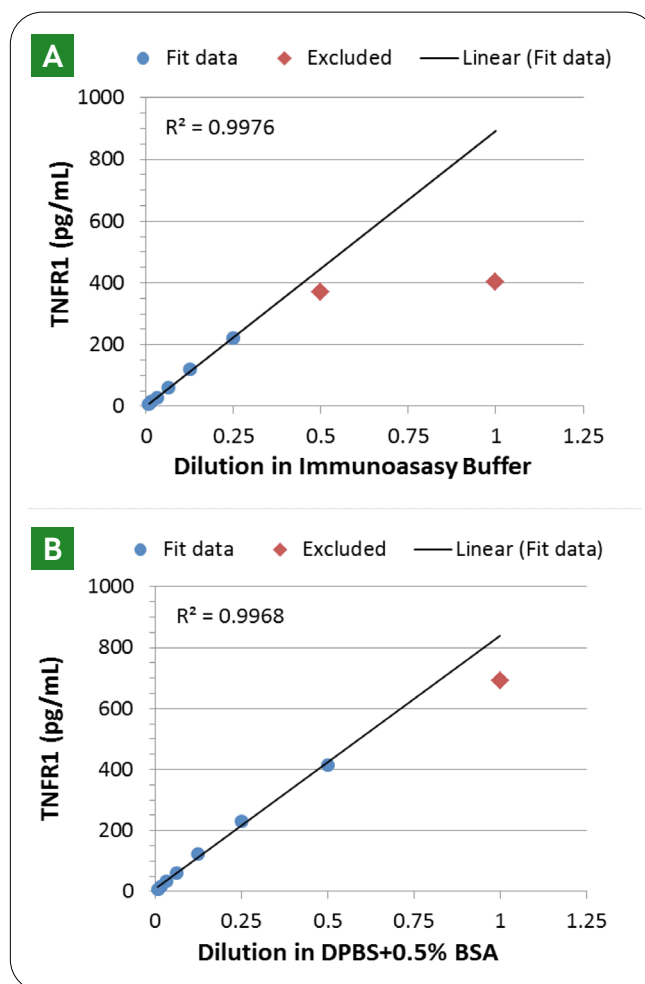


Figure 4: Human TNFR1 AlphaLISA Dilutional Linearity in Urine. Pooled normal human urine was prepared by two-fold serial dilution until linearity was achieved. R^2 values are listed for the apparent linear range after the removal of data points not included in the linear regression. Immunoassay Buffer (A) required a dilution factor of 1:4 to achieve linearity whereas DPBS+0.5% BSA (B) required only a 1:2 dilution factor.

Spike-and-recovery in urine samples

Matrix effects in urine are also reported to have a negative impact on spike-and-recovery outcomes. Interference from substances within the sample such as organic compounds, electrolytes or the pH itself may inhibit antibody binding and overall assay performance. Pre-dilution of the urine sample can be a useful technique to overcome these matrix effects.⁴ Spike-and-recovery was performed using three concentrations of TNFR1 analyte spiked into pooled normal human urine and subsequently diluted for analysis, or spiked directly into urine that was pre-diluted in DPBS+0.5% BSA for comparison. The same concentrations were spiked into DPBS+0.5% BSA diluent for calculations of percent recovery. In general, selected concentrations for spike-and-recovery should cover a broad range across the linear portion of your standard curve including one spike that is close to the LLOQ. The “No Spike” baseline control was subtracted from each spike condition to more readily compare to the

expected spike concentration (Table 1). Reported values for urine samples diluted after spiking (spike then dilute) were corrected for dilution factor to compare to the expected spike concentration.

Spiking TNFR1 directly into the urine sample or diluting after spiking yielded reduced concentrations when compared to the pre-dilution method. Additionally, one of the issues with spiking a low initial concentration is that after dilution it may fall below the LLOQ. Pre-dilution of the pooled normal urine sample improved detection of spiked TNFR1 as anticipated. This can be seen when calculating Percent Recovery (Table 2). Pre-dilution of the pooled normal urine sample \geq four-fold yielded acceptable recovery values whereas neat urine or spike then dilute method did not pass the criteria for recovery (70-130%).

Table 1: Human TNFR1 AlphaLISA Spike-and-Recovery in Urine. Pooled normal human urine and sample diluent spiked with three concentrations of TNFR1 analyte as noted with a No Spike control. Dilutions were performed in DPBS+0.5% BSA either before or after spiking analyte. No Spike values were subtracted from the observed spike concentrations.

Spike pg/mL	Neat		Spike then dilute		Pre-dilute before spike		
	Diluent	Urine	0.25X Urine	0.125X Urine	0.5X Urine	0.25X Urine	0.125X Urine
No Spike	0.0	1361.2	1754.6	2151.2	893.8	496.2	273.7
50	56.7	9.9	< 0	< 0	20.4	45.8	51.4
500	520.0	235.4	210.2	223.9	421.8	421.3	424.0
5000	4944.8	2393.2	3174.5	3184.6	4140.8	3992.5	4630.7

Table 2: Human TNFR1 AlphaLISA Percent Recovery in Urine. Pooled normal human urine and sample diluent spiked with three concentrations of TNFR1 analyte as noted. Dilutions were performed in DPBS+0.5% BSA either before or after spiking analyte. Interpolated spike values in urine were compared to spike values in diluent to calculate percent recovery.

Spike pg/mL	Neat		Spike then dilute		Pre-dilute before spike		
	Diluent	Urine	0.25X Urine	0.125X Urine	0.5X Urine	0.25X Urine	0.125X Urine
50	113.3	17.4	< 0	< 0	36.0	80.9	90.7
500	104.0	45.3	40.4	43.1	81.1	81.0	81.5
5000	98.9	48.4	64.2	64.4	83.7	80.7	93.6

Soluble TNFR1 detection in patient urine samples

Urine was purchased to assay single donor normal patients along with type 2 diabetes patients. Pooled normal human urine was also included for reference. Urine samples were thawed and centrifuged at 10,000 rpm for two minutes to pellet any particulate matter that could further interfere with testing. The donor urine was not filtered or centrifuged by this supplier prior to packaging. Samples were also pH tested to determine if they fell within the normal average range (pH 6.5 - 8). All urine samples were diluted 1:4 in DPBS+0.5% BSA and assayed in triplicate. Both normal donors tested have similar results as the pooled sample while one type 2 diabetes donor (Patient A) showed a marked increase in soluble TNFR1 (Fig. 5).

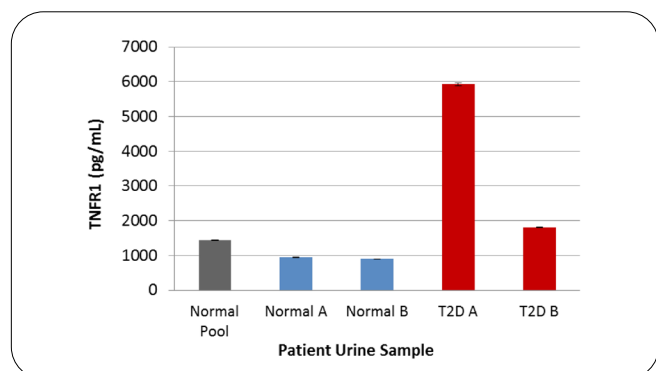


Figure 5: Human TNFR1 Detection in Patient Samples. Two single donor samples of normal and type 2 diabetes patients were tested to show utility of the assay. Donor A for type 2 diabetes shows elevated levels of soluble TNFR1 up to six-fold higher than the normal samples.

Summary

AlphaLISA technology provides a rapid, homogeneous, bead-based assay format for the quantification of an analyte in complex matrices. This technical note demonstrates detection of soluble TNFR1 in human urine. Linearity of dilution experiments performed without the exogenous analyte spike suggested DPBS+0.5% BSA as a suitable

“urine-like” diluent for use in AlphaLISA assays with an R^2 value 0.997 after an initial two-fold dilution. The normal setup for the linearity of dilution experiments was adjusted to accommodate the endogenous levels of soluble TNFR1 present in the pooled normal human urine sample. Similarly, adjustments were made to the standard spike-and-recovery setup as a result of complex matrix components present in urine and the interference on accurate percent recovery measurements. Pre-dilution of urine in the diluent \geq four-fold resulted in acceptable percent recovery for all spike concentrations tested. A moderate level of soluble TNFR1 was seen in the pooled normal human urine sample and in two single donor normal samples. One of the type 2 diabetes patients showed a marked increase in soluble TNFR1 up to six-fold higher than the single donor normal samples. An increased number of patient samples are needed to determine the statistical significance of a disease state relative to normal donor levels; however the data and methods shown here are a proof-of-concept for soluble TNFR1 detection in the complex matrix of urine.

References

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