TECHNICAL NOTE

AlphaLISA Technology

Quantifying TNFR1 in Both Soluble and Membrane Bound Form Using AlphaLISA Technology

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Introduction

Tumor Necrosis Factors (TNFs) are cytokines that are the primary modifiers of inflammatory and immune response. TNFs play a beneficial role mediating host resistance to infection and tumor formation. 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. TNFR1 is expressed ubiquitously on almost all cell types, whereas TNFR2 expression is limited to hematopoietic and endothelial cells.[1] Researchers have shown that a soluble form of TNFR1 (sTNFR1) is a truncated version of the receptor produced by the disintegration and extracellular release of membranous protein on the cell surface (ectodomain shedding). sTNFR1 ectodomain shedding is often regulated by members of a family of metalloproteinases such as ADAM17 (also called TACE, TNF-α converting enzyme), and can be further stimulated with treatments of lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA). The soluble portion of the receptor retains the ability to bind and inhibit TNFs resulting in a neutralizing biological effect. sTNFR1 is found in healthy and diseased patients alike, however increased sTNFR1 levels are an indicator for disease states such as inflammation, infection, and asthma.[2,3]

A common way to detect soluble TNFR1 ectodomain shedding into cell culture media is by using labor intensive wash-based ELISA assays. Here we present a way to measure TNFR1 using a homogeneous bead-based AlphaLISA assay. The human TNFR1 AlphaLISA® detection kit was designed for the quantitative determination of soluble TNFR1 in serum and cell culture media. This technical note further demonstrates the functionality of the kit by detecting sTNFR1 in cell supernatant as well as TNFR1 on the cell membrane.

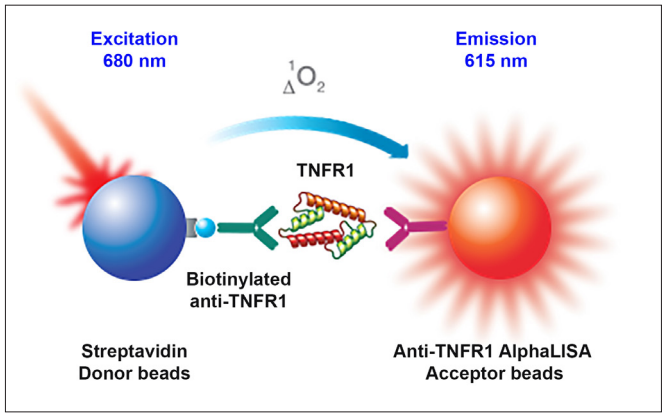

Figure 1. Human TNFR1 assay principle. Streptavidin-coated Alpha Donor beads bind the biotinylated anti-TNFR1 antibody, the AlphaLISA Acceptor beads are supplied conjugated to an anti-TNFR1 antibody. Binding of antibodies in the presence of TNFR1 brings the Donor and Acceptor beads into close proximity.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

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AlphaLISA Workflow

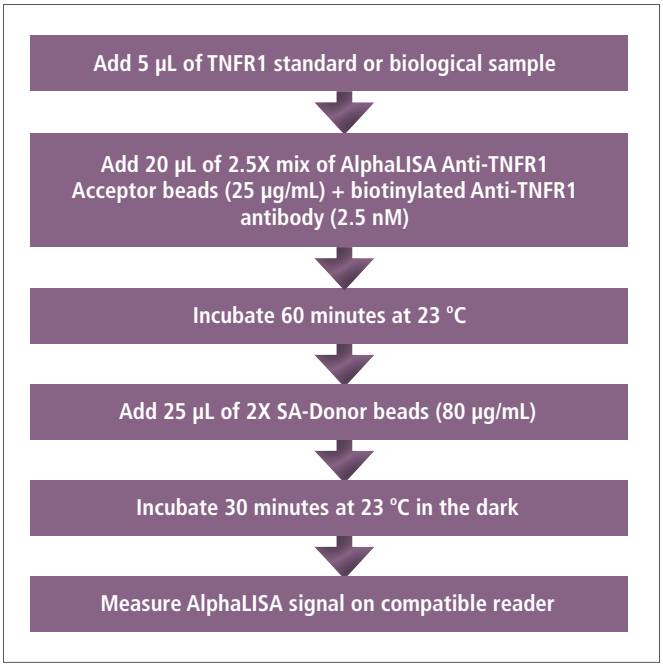


Figure 2. Workflow for TNFR1 AlphaLISA detection assay. The assay provides sensitive quantification of TNFR1, utilizing only 5 µL of sample and generating results in under three hours.

Reagents

- TNFR1 (human) AlphaLISA Detection Kit (PerkinElmer, #AL3088)
- 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)
- 5X AlphaLISA Lysis Buffer (PerkinElmer, #AL003C)
- cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Sigma, #4693159001)
- Phorbol 12-myristate 13-acetate; PMA (Tocris, #1201)
- Lipopolysaccharide, LPS (Sigma, #L4516)
- Phytohemagglutinin PHA-M (Sigma, #L8902)
- ViewPlate™-96 F, TC treated (PerkinElmer, #6005182)
- AlphaPlate™-384, light gray (PerkinElmer, #6005350)
- TopSeal™-A Plus Adhesive Sealing Film (PerkinElmer, #6050185)

Data Collection and Analysis

AlphaLISA assays were measured on a PerkinElmer EnVision® multimode plate reader using default values for standard Alpha detection. Standard curve determination was performed in parallel to unknown samples. Curves were plotted in GraphPad Prism® according to nonlinear regression fitting using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) and $1/Y^2$ data weighting. The lower limit of detection (LDL) and lower limit of quantification (LLOQ) were calculated using the standard curve blanks (buffer only). The calculated LDL or LLOQ number was then interpolated onto the standard curve to provide a result in pg/mL of analyte.

LDL = mean (blanks) + 3X standard deviation

LLOQ = mean (blanks) + 10X standard deviation

Quantification of Soluble TNFR1 from Cell Supernatants

THP-1 cells expressing endogenous TNFR1 were dispensed manually into a ViewPlate and grown in culture up to 48 hours. Supernatants were collected at the time point noted, centrifuged at 1200 rpm for five minutes to pellet any remaining cells, aliquoted further and kept frozen at -20 °C until testing. For each assay replicate, 5 µL was transferred to an AlphaPlate-384 and analyzed for sTNFR1 released in the absence of additional stimulation. The standard curve was prepared in RPMI supplemented with 10% FBS to correspond with the cell culture media.

Additionally, THP-1 cells and PBMCs were incubated with reagents known to stimulate ectodomain shedding of TNFR1. Samples were prepared in bulk in a 12-well dish, 1 mL final volume per well. THP-1 cells were dispensed at 8 x 10^5 cells/mL and treated for 48 hours. PBMCs were used at 2 x 10^6 cells/mL and treated for 72 hours. Treatment conditions included media alone, 100 ng/mL LPS, 100 nM PMA, or 10 µg/mL PHA-M. At each harvest time point, supernatants were removed from the 12-well dish, pooled in a 15 mL tube and centrifuged at 1200 rpm for five minutes to pellet remaining cells. Supernatants were immediately transferred to new 15 mL tubes, centrifuged again and then aliquoted further for storage in a -80 °C freezer until testing.

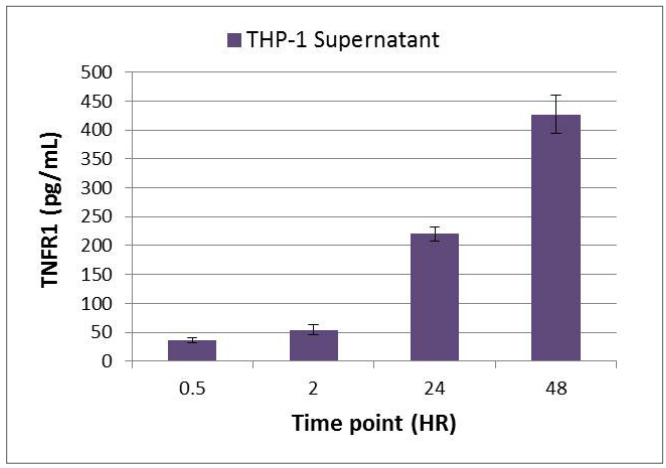
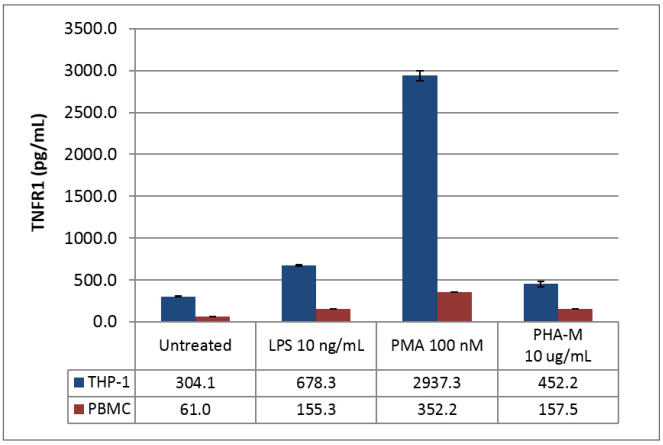


Figure 3. Baseline level of TNFR1 shed from THP-1 cells into culture media over time in the absence of additional stimulation. THP-1 cells were grown in RPMI + 10% FBS. 100,000 cells/well were plated in 96-well ViewPlates. Supernatant samples were collected at 0.5, 2, 24, and 48 hours and assayed by AlphaLISA. Calculated LDL and LLOQ in RPMI + 10% FBS were 3.7 and 11.4 pg/mL respectively.



	Untreated	LPS 10 ng/mL	PMA 100 nM	PHA-M 10 ug/mL
THP-1	304.1	678.3	2937.3	452.2
PBMC	61.0	155.3	352.2	157.5

Figure 4. Stimulation of THP-1 cells or PBMCs increases TNFR1 concentration in the supernatant above baseline levels. In both cell types, treatment with 100 nM PMA induced strong shedding of TNFR1.

Quantification of Membrane Bound TNFR1 in Cell Samples

THP-1 cells were harvested directly from the culture flask and washed two times in DPBS to remove growth media. Three cell densities were plated in 80 µL of fresh media (RPMI + 10% FBS) and immediately lysed with either 20 µL of 5X Immunoassay Buffer (diluted from 10X stock provided in the assay kit) or 20 µL of 5X AlphaLISA Lysis Buffer (prepared with protease inhibitor cocktail). The assay plate was placed on an orbital shaker set to 700 rpm for 25 minutes following suggested harvest protocol for membrane-bound proteins.[4] Lysates were transferred to a storage plate and kept frozen at -20 °C until performing the AlphaLISA. One standard curve was prepared in each mixture of media with 1X lysis buffer (Immunoassay or AlphaLISA lysis) to match the cell lysate preparation.

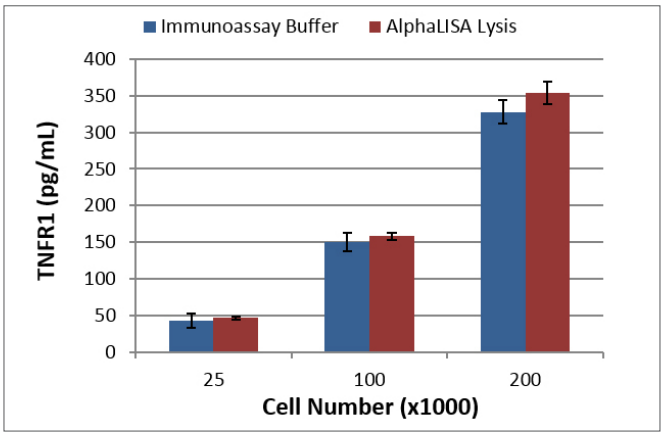


Figure 5. Detection of membrane-bound TNFR1 in THP-1 cells. Comparable results were achieved when Immunoassay Buffer or AlphaLISA Lysis Buffer was used to harvest cell lysates. Titration of cell density yields a corresponding decrease in the quantity of TNFR1 detected. Calculated LDL and LLOQ values were similar in both lysis buffer mixtures. LDL of 5.82 and 5.18 pg/mL, LLOQ of 16.6 and 15.6 pg/mL in Immunoassay Buffer and AlphaLISA Lysis Buffer mixture respectively.

Summary

AlphaLISA technology provides a rapid, no wash assay format for the quantification of a target analyte from cell culture supernatants and lysed cell samples, as well as other complex matrices. This technical note shows examples of TNFR1 measurement in the supernatant collected from two different cell types exhibiting baseline levels of ectodomain shedding as well as further stimulation of sTNFR1 release by LPS, PMA, or PHA-M treatment. Additionally, the data shown here suggest this kit can be used to determine membrane bound TNFR1. This population includes intact TNFR1 that has not undergone shedding. Investigation of intact TNFR1 could be useful for understanding soluble TNF-α binding in the context of host defense and inflammation.

References

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2. Sakimoto T, et al. Release of soluble tumor necrosis factor receptor 1 from corneal epithelium by TNF-α–converting enzyme-dependent ectodomain shedding. Investigative Ophthalmology and Visual Science (2009); 50(10).
3. Yoshida S, et al. Elevation of serum soluble tumour necrosis factor (TNF) receptor and IL-1 receptor antagonist levels in bronchial asthma. Clinical Experimental Immunology (1996); 106:73-78
4. Roby P, Hinterneder J. Protocol optimization for detection and quantification of membrane-bound proteins using AlphaLISA technology. PerkinElmer Application Note (2017).

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