Application Note

Screening for Inhibitors to TNFα/sTNFR1 Binding Using AlphaScreen™ Technology

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Introduction

Tumor necrosis factor alpha (TNFα) is a cytokine involved in a wide variety of inflammatory and immunological processes. Downstream biological effects of TNFα are produced following its binding to specific cell surface receptors. Two distinct TNF receptors have been identified: TNF receptor I (p55 or TNFRI)[1] and TNF receptor II (p75 or TNFRII).[2] While TNFRI is highly expressed in a variety of cells, that of TNFRII is largely restricted to lymphoid cells. TNFRI has a molecular weight of 55 kDa and binds competitively to both TNFα and TNFβ analogs. The soluble form of TNFRI (sTNFRI) is produced by proteolytic cleavage and two molecular weight forms have been detected in serum and urine samples (32 kDa and 48 kDa).[3,4] The sTNFRI acts as a physiological inhibitor of TNF analogs when it scavenges these molecules and prevents their interactions with membrane-bound receptors.

Numerous studies have demonstrated that both TNFα and sTNFRI levels are significant in disease. Elevated levels are particularly apparent in patients suffering from both rheumatoid arthritis[5] and systemic lupus erythematosus.[6] TNFα and sTNFRI are also present at increased levels in patients infected with Human Immunodeficiency Virus.[7]

This AlphaScreen™ TNFα Binding Assay kit has been designed to perform homogeneous, non-radioactive high throughput screens for inhibitors to TNFα - sTNFRI binding.

Principles of AlphaScreen

AlphaScreen is a bead-based non-radioactive Amplified Luminescent Proximity Homogeneous Assay. When a biological interaction brings the beads together, a cascade of chemical interactions act to produce a greatly amplified signal. On laser excitation, a photosensitizer in the Donor bead converts ambient oxygen to a more excited singlet state. The singlet state oxygen molecules diffuse across to react with a thioxene derivative in the Acceptor bead, generating chemiluminescence at 370 nm that further activates fluorophores contained in the same bead. The fluorophores subsequently emit light at 520-620 nm.

In the absence of a specific biological interaction, the singlet state oxygen molecules produced by the Donor bead go undetected without the close proximity of the Acceptor bead. As a result only a very low background signal is produced.

AlphaScreen provides a highly versatile, sensitive, time-resolved, homogeneous and miniaturizable means to efficiently perform assay development and high throughput screening (HTS) resulting in higher throughput at lower costs.

To maximize AlphaScreen signal detection, the AlphaQuest®-HTS and Fusion™α Multilabel Readers were developed with the capability to measure assays in multi-well plates. These instruments use a highly efficient laser diode emitting at 680 nm, fiber optics and specially optimized photomultiplier tubes. Use of the OptiPlate-384 NEW microplates is also recommended for best performance. Fusionα, AlphaScreen, AlphaQuest-HTS and OptiPlate-384 NEW microplates are available from PerkinElmer Life Sciences company.

For further details on the AlphaScreen technology, refer to Application Note ASC-001: Principles of AlphaScreen.

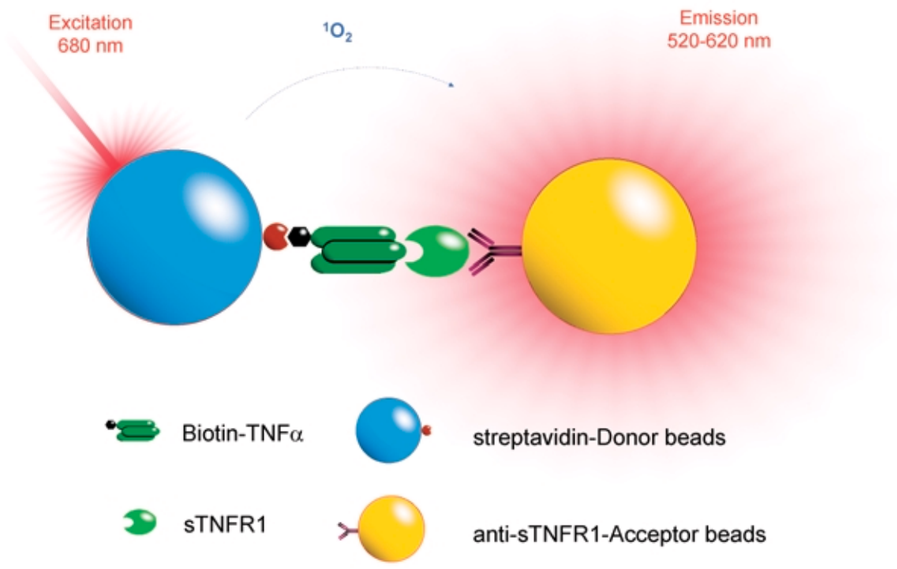

Figure 1: *Schematic Representation of the AlphaScreen TNFα Binding Assay*

Principles of the TNFα Binding Assay

The AlphaScreen TNFα/sTNFR1 assay kit is intended for the screening of molecules that can displace the ligand-receptor interaction, either potential agonists or antagonists. As shown in Figure 1, sTNFR1 captured by anti-sTNFR1 Acceptor beads binds to a biotinylated derivative of TNFα captured by the streptavidin Donor beads to generate the AlphaScreen signal. Molecules binding to either TNFα or the sTNFR1, at either site of interaction, will compete for binding and therefore result in a signal decrease.

Materials and Methods

The AlphaScreen TNFα Assay Kit (Cat # 6760622C (500 Assay Points), 6760622M (10,000 Assay Points), 6760622R (50,000 Assay Points)) is composed of biotinylated-TNFα, Donor-streptavidin beads and Acceptor anti-sTNFR1 beads. The soluble TNFR1 receptor is not provided in the kit (see below for commercial source).

Materials and Preparation

- Assay buffer: 25 mM Hepes, pH 7.4, 100 mM NaCl, 0.1% Tween 20 and 0.1% BSA
- Anti-sTNFR1 Acceptor beads and biotin-TNFα mix. From the 5 mg/mL stock of anti-sTNFR1 Acceptor beads and 250 nM stock of biotin-TNFα, dilute in assay buffer the anti-sTNFR1 Acceptor beads to 50 µg/mL (20 µg/mL final) and biotin-TNFα to 0.75 or 2.5 nM (0.3 or 1.0 nM depending on source of sTNFR1 used; see Figure 2 and Table 1 for more details). **(Solution A)**.
- TNFα (PeproTech cat. 300-01A); dilute to 0.5 µM – 50 pM (0.1 µM – 10 pM final) in assay buffer **(Solution range B)**.
- TNFβ (PeproTech cat. 300-01B); dilute to 0.5 µM – 50 pM (0.1 µM – 10 pM final) in assay buffer **(Solution range C)**.
- EGF (PeproTech cat. 100-15); dilute to 0.5 µM – 50 pM (0.1 µM – 10 pM final) in assay buffer **(Solution range D)**.
- sTNFR1 (comprised of residues 22-211 of the extracellular domain of TNFRI; R&D Systems cat. 636-R1; PeproTech cat. 310-07); dilute to 1.5 nM (0.3 nM final) in assay buffer **(Solution E)**.
- Streptavidin Donor beads (5 mg/mL); dilute to 100 µg/ml in assay buffer (20 µg/ml final) **(Solution F)**.

Methods

A. Competition Assay

The AlphaScreen TNFα competition assay involves the following addition steps to a 384-well white opaque plate (Corning cat# 3705):

1) Add 10 µL of anti-sTNFRI Acceptor bead / biotin-TNFα mix **(Solution A)**.
2) Add 5 µL of competitor at various concentrations **(Solution ranges B-D)**.
3) Add 5 µL of diluted sTNFR1 **(Solution E)**. Incubate for 45 minutes at 23ºC in the dark.
4) Add 5 µL of Strepavidin Donor beads **(Solution F)**. Incubate for 60 minutes at 23ºC in the dark and read on the AlphaQuest or Fusionα readers.

B. DMSO Tolerance

To mimic screening conditions where organic solvents such as DMSO are used as the carrier for compounds, the competition assay as described in (A) above was performed with the following modification:

1) TNFα (PeproTech cat. 300-01A) was diluted (0.5 µM – 50 pM (0.1 µM – 10 pM final)) in assay buffer supplemented with DMSO to give a final DMSO concentration of 0, 0.5, 1, 2 and 5%.

C. Assay Performance

Intra-assay precision for the TNFα assay was measured statistically by evaluation of the Z' values. Studies were performed using the following modifications to the competition assay as described in (A) above:

1) For a control condition where maximal signal is obtained, 5 µL of assay buffer was added to a total of 96 wells in step (A2) above (0 nM TNFα).
2) To mimic a maximally displaced signal 5µL of a saturating concentration of TNFα of was added to a total of 96 wells in step (A2) above (100 nM TNFα).
3) In both steps (1) and (2) in this section, a final DMSO concentration of 1% was used to simulate screening conditions.

Results and Discussion

Soluble TNFR1 is commercially available; two sources were tested for compatibility with our screening system in this study. It was observed that variations occurred between different lots of receptor even from the same supplier. These variations were in the form of activity or binding capacity, where the maximum AlphaScreen signal varied. In order to provide a more consistent assay system, the maximal signal variation between lots was overcome by titrating the concentration of the biotinylated-TNFα. Figure 2 shows the ability of TNFα and TNFβ to compete for the interaction occurring between biotinylated-TNFα and sTNFR1 (Method A). As can be seen, varying the biotinylated-TNFα between 0.3 and 1.0 nM allows similar performance and pharmacology to be attained between lots and suppliers (Figure 2 and Table 1). For example, a signal to background ratio of 20 was obtained at 0.3 nM biotinylated-TNFα for the higher activity R&D Systems lot CWU010081, where for the lower activity R&D Systems lot CWU081071, a ratio of 29 was obtained at 1.0nM biotinylated-TNFα (Figure 2; Table 1). R&D Systems lot CWU010081 was used for the remainder of this study. For this lot, the IC_{50} values shown in Table 1 were converted to K_i values using a biotinylated-TNFα concentration of 0.3 nM and the K_d for [^{125}I]-TNFα binding obtained from Marsters *et al.*[8] TNFα (K_i = 0.26 nM) was more potent at competing for the binding of biotinylated-TNFα to sTNFR1 compared to its homologue TNFβ (K_i = 2.3 nM). This shift in potency for TNFβ relative to TNFα is in agreement with data obtained by other groups using different detection technologies.[8] In order to ensure that this assay was compatible with the high-throughput screening environment, the tolerance of the assay to DMSO, an organic solvent commonly used to store compound libraries, was tested (Method B). Thus, relevant levels of DMSO were tested where typically a final concentration of 1% DMSO may be found in a screening assay. In this study, the tolerance of the sTNFR1 binding assay to DMSO was up to 1% where no significant impact on the assay was observed (Figure 3, Table 2).

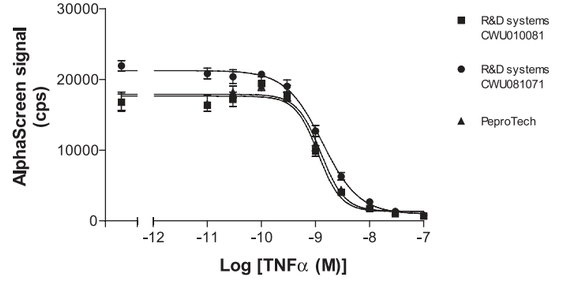

Figure 2: *Comparison of pharmacological performance between different suppliers and lots of sTNFR1. Competition curves for R&D Systems lots CWU010081 and CWU081071 were performed using 0.3 and 1.0 nM biotin-TNFα and Peprotech lot 108156L148 used 0.3 nM biotin-TNFα*

Supplier	Lot	IC_{50} (nM)		Maximal AlphaScreen Signal (cps) with assay buffer alone	Minimal AlphaScreen Signal (cps) at 0.1 μM TNFα	[biotin-TNFα] (nM)
		TNFα	TNFβ			
R&D Systems	CWU010081	1.5±0.4	28.1±0.6	16340±1649	815±100	0.3
R&D Systems	CWU081071	2.0±0.4	30.0±1.1	21746±3255	746±35	1.0
PeproTech	108156L148	1.1±0.2	7.9±0.7	18768±736	766±131	0.3

Table 1: *Comparison of pharmacological performance between different suppliers and lots of sTNFR1.*

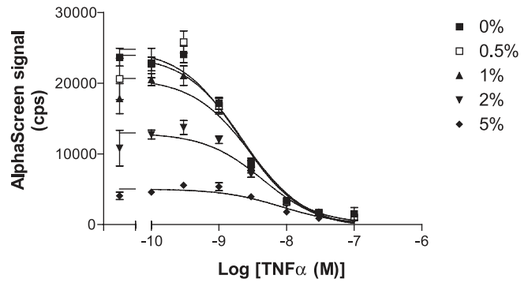

Figure 3: *Effect of DMSO on TNFα competition curves*

[DMSO] (%)	IC_{50} (nM)	SB
0	2	23.3
0.5	2.3	20.5
1	3	19.4
2	4.5	14.4
5	8.3	5.3

Table 2: *DMSO Tolerance*

A method has been established for the assessment of assay performance that can be used for any detection technology. This method uses a Z' calculation[9] to assess the suitability of a given assay for screening compounds in an HTS format. The method incorporates the assay window, comprised of the difference between maximal signal and that from NSB and the variation, expressed as a standard deviation, in both signals:

$$z' = 1 - \left(\frac{3 \cdot \sigma_{Bound} + 3 \cdot \sigma_{NSB}}{Signal_{Bound} - Signal_{NSB}} \right)$$

A Z' value of 0.5 has been verified as an indicator that an assay under development for screening purposes will successfully transition into a HTS. From Table 3, a Z' value > 0.5 was found which indicates that the AlphaScreen TNFα Binding assay can be used for HTS applications and inhibitory compounds identified with high confidence (Method C). These data are shown graphically in Figure 4.

TNFα (nM)	Mean Signal (cps)	Standard Deviation (cps)	CV (%)	Z'
0 (Total)	18504	1846	9	0.67
100 (Non-specific)	1062	97	15	

Table 3: *TNFα Binding Assay Characteristics*

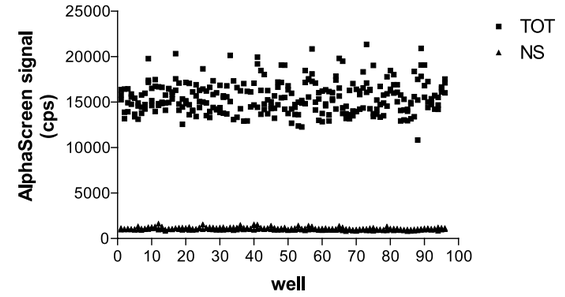

Figure 4: *Assay Performance Characteristics*

Conclusions

Using AlphaScreen, we have developed a homogeneous non-radioactive assay to screen compounds acting specifically on the TNFα-sTNFR1 interaction. The assay can be performed rapidly with only four successive reagent additions, which greatly simplifies automation of the assay. The concentration of biotinylated-TNFα used in the assay can be optimized so that different sources and lots of sTNFR1 can be used without a compromise in assay performance. Using the recommended assay protocol, excellent Z' values in the presence of 1% DMSO can be achieved indicative of a performance that is superior to the minimum requirements for HTS applications. Furthermore, the determined pharmacology of the sTNFRI is in agreement with that found elsewhere using more familiar detection techniques. Lastly, the AlphaScreen platform offers significant throughput advantages relative to competing detection technologies for screening of compounds against TNFα binding. These attributes suggest that the AlphaScreen TNFα Binding kit is the leading technology commercially available for the HTS application of TNFα binding and further, the data from this study open up the opportunity of developing additional cytokine based AlphaScreen assays.

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