MANUAL

AlphaLISA® TNFα and TNFRII (Human) Binding Kit

Product number: AL3121 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess inhibitors of human TNFα and TNFRII binding, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapetics by using competitive binding to human TNFα and TNFRII binding

Sensitivity: $IC_{50:}$ 1.03 nM (average, using anti TNFRII antibody)

Signal to background ratio: 571 using 3 nM TNFα and 3 nM TNFRII

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human TNFα, His tagged human TNFRII and AlphaLISA Immunoassay buffer.

Storage: The kit components must be stored at 4 ˚C in the dark. Reconstituted proteins can be stored at – 20 °C for 3 months.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Tumor necrosis factor alpha (TNFα) is an immunity-modulating cytokine that is required for defense against infectious diseases and carcinogenesis. TNFα activates signals through its two receptors [TNF receptor 1 (TNFRI) and TNF receptor 2 (TNFRII)]. TNFRI is ubiquitously expressed and can be fully activated by both the membrane-bound and soluble trimeric forms of TNFα, whereas TNFRII is found mostly on certain populations of immune cells and respond to the membrane-bound form of the TNFα homotrimer. However, a block of TNFα-mediated host defense often increases the risk of bacterial or viral infection or of the development of lymphoma. Thus, a thorough understanding of the function of the TNFα-TNFR binding is important for the design of optimal therapies against the various TNFα-related autoimmune diseases.

Description of the AlphaLISA Assay

The AlphaLISA detection of human TNFα and TNFRII binding uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged TNFRII and Streptavidin-coated donor beads to capture the biotinylated TNFα. Donor beads and acceptor beads come into proximity through TNFα binding to TNFRII. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1).

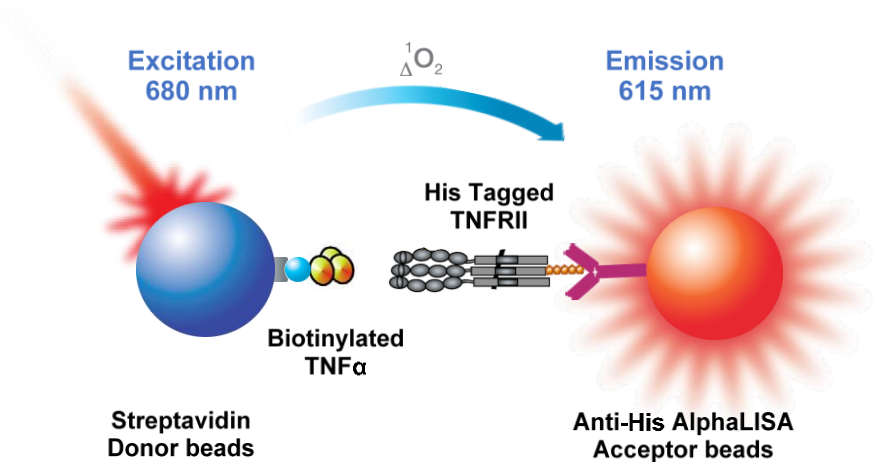


Figure 1. AlphaLISA Assay Principle.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.

Kit Content: Reagents and Materials

Kit components	AL3121C*** (500 assay points)	AL3121F*** (5000 assay points)
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Proclin-300, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	200 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Proclin-300, pH 7.4	20 µL @ 5 mg/mL (1 brown tube, black cap)	200 µL @ 5 mg/mL (1 brown tube, black cap)
Lyophilized TNFα (Biotinylated) *	0.525 µg, lyophilized (1 tube, clear cap)	0.525 µg, lyophilized (10 tubes, clear caps)
Lyophilized TNFRII (His tagged) *	0.801 µg, lyophilized (1 tube, clear cap)	0.801 µg, lyophilized (10 tubes, clear caps)
AlphaLISA Immunoassay Buffer (10X) **	10 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute TNFα and TNFRII in 100 µL Milli-Q® grade H_2O respectively. The reconstituted proteins should be used within 60 minutes. After reconstitution, aliquot and store unused protein at -20 °C for 3 months. Avoid multiple freeze-thaw cycles.

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 20 µL in 384 well plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	Revvity Inc.	6050185
AlphaPlate-384, Shallow Well (ProxiPlate)	Revvity Inc.	6008350 6008359
EnVision®-Alpha Reader	Revvity Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Anti-TNFα Antibody	R&D Systems	MAB610
Human TNF RI/TNFRSF1A Antibody	R&D Systems	MAB225
Recombinant Human TNF-alpha Protein	R&D Systems	210-TA
Mouse IgG1 Isotype Control	R&D Systems	MAB002
Human TNF RII/TNFRSF1B Antibody	R&D Systems	MAB726

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend the beads by vortexing before use. Do not vortex the proteins.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized proteins.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal™-A Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

Competition Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating six inhibition curves with anti-TNFRII antibody in a 20 µL final assay volume (216 wells, triplicate determinations). These calculations do not include excess reagent to account for losses during transfer of solutions or dead volumes. If a different number of samples are tested, the volumes of all reagents must be adjusted accordingly.
- The dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

One Incubation Step Manual described as below:

1) Preparation of 1X AlphaLISA Immunoassay Buffer (for 10 mL):

   Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL H_2O.

2) Preparation of serial dilutions of 4X anti TNFRII antibody in 1X AlphaLISA Immunoassay buffer as follows:

   a. Reconstitute 100 µg of TNFRII with 200 µL PBS to make 0.5 mg/mL (3.33 µM) stock concentration
   b. Prepare serial dilutions of 4X anti-TNFRII antibody in 1X AlphaLISA Immunoassay Buffer as follows, change tips between each dilution:

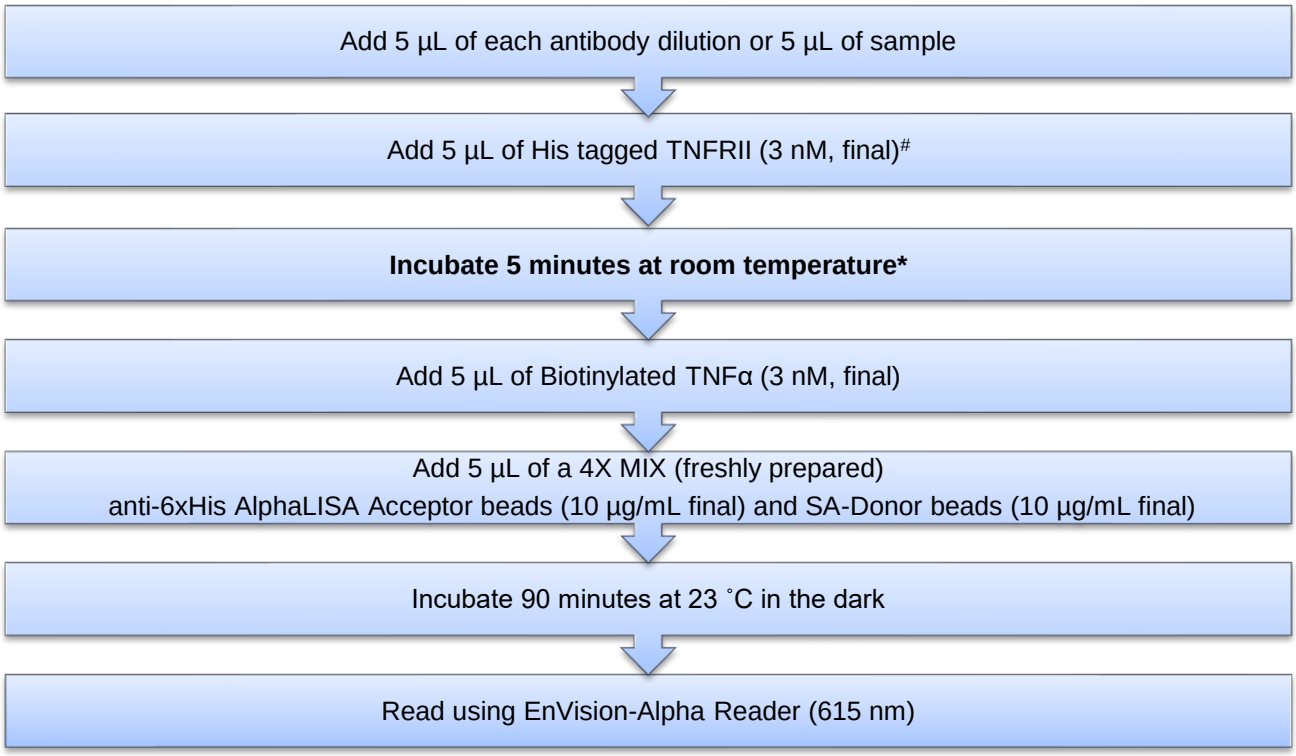
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (nM) (4X)	[Ab] (nM) (1X)
A	12 µL of 3.33 µM stock	88 µL	400	100
B	30 µL of tube A	70 µL	120	30
C	30 µL of tube B	60 µL	40	10
D	30 µL of tube C	70 µL	12	3
E	30 µL of tube D	60 µL	4	1
F	30 µL of tube E	70 µL	1.2	0.3
G	30 µL of tube F	60 µL	0.4	0.1
H	30 µL of tube G	70 µL	0.12	0.03
I	30 µL of tube H	60 µL	0.04	0.01
J	30 µL of tube I	70 µL	0.012	0.003
K	30 µL of tube J	60 µL	0.004	0.001
L	0	70 µL	0	0

3) Preparation of 4X His tagged TNFRII (12 nM):
   a. Reconstitute lyophilized TNFRII (0.801 µg) in 100 µL H_2O to make 300 nM TNFRII.
   b. Add 45 µL of 300 nM TNFRII to 1080 µL 1X AlphaLISA Immunoassay buffer.
   c. Prepare just before use.

4) Preparation of 4X biotinylated TNFα (12 nM):
   a. Reconstitute lyophilized TNFα (0.525 µg) in 100 µL H_2O to make 300 nM TNFα.
   b. Add 45 µL of 300 nM TNFα to 1080 µL 1X AlphaLISA Immunoassay buffer.
   c. Prepare just before use.

5) Preparation of the mix of 4X Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and 4X Streptavidin (SA) Donor beads (40 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 10 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 10 µL of 5 mg/mL SA-Donor beads to 1230 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use.

6) In a ProxiPlate (384 wells):



Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

If screening anti-TNFα antibodies, add TNFα first, then add TNFRII.

* Incubation time was determined optimal for this particular application.

Typical competitive binding Data:

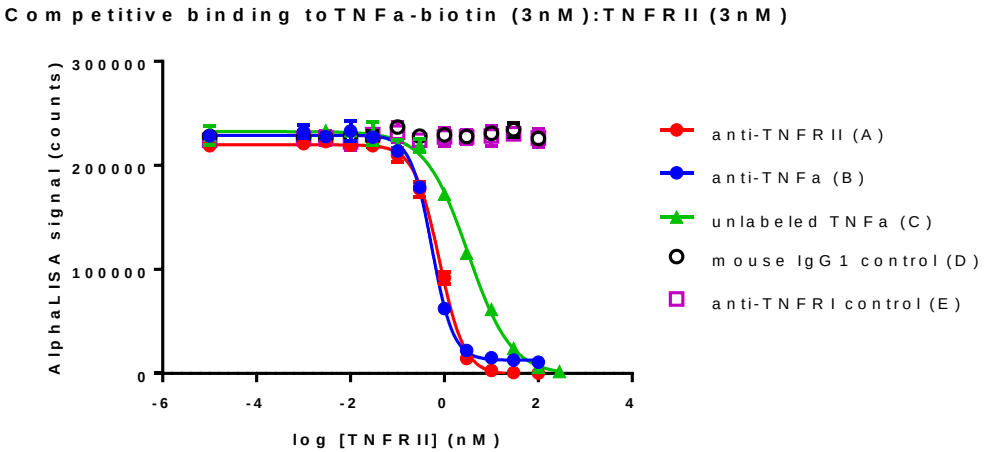


Figure 2. Competitive Binding: Anti-TNFRII antibody (A) and anti-TNFα (B) block TNFa/TNFRII binding with IC_{50} = 0.78 nM and IC_{50}=0.54 nM respectively. Nonbiotinylated TNFα (C) competitively binds to TNFRII with IC_{50} =3.2 nM. Mouse IgG1(D) and anti-TNFRII antibody (E) were measured as negative controls. All IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism 7.

Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

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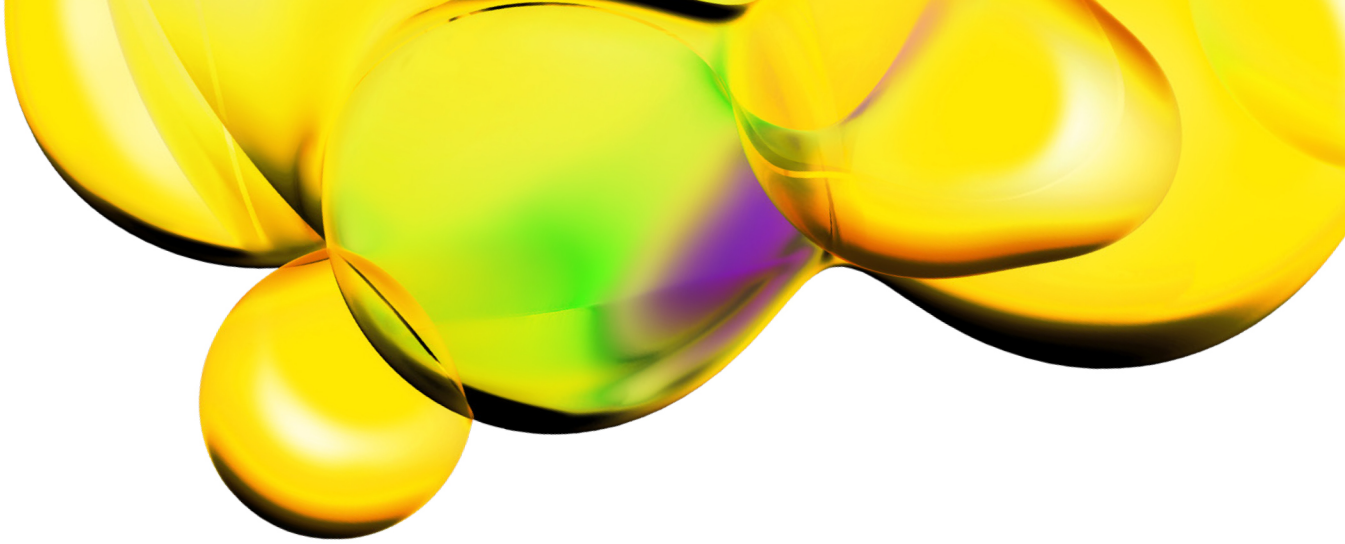

www.revvity.com

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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