EP161-EN.02

TNF-alpha : TNFR2[Biotinylated] Inhibitor Screening ELISA Kit

Pack Size: 96 tests

Catalog Number: EP-161

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use In Diagnostic Or Therapeutic Procedures

INTENDED USE

This kit is designed for screening of inhibitors of binding between human TNF-alpha and human TNFR2.

It is intended for research use only (RUO).

PRINCIPLE OF THE ASSAY

This inhibitor screening ELISA kit is designed to facilitate the identification and characterization of new TNF-alpha pathway inhibitors. The assay takes advantage of our in house-developed binding of biotinylated human TNFR2 to immobilized human TNF-alpha in a functional ELISA assay and employs a simple colorimetric ELISA platform. Briefly, we provide you with a human Biotinylated TNFR2 protein, a human TNF-alpha protein, an anti-TNF-alpha neutralizing antibody (as method verified Std.), and Streptavidin-HRP reagent. Your experiment will include 4 simple steps:

1) Coat the plate with human TNF-alpha.

2) Add your molecule of interest to the tests.

3) Add human TNFR2-Biotin to bind the coated human TNF-alpha.

4) Add Streptavidin-HRP followed by TMB or other colorimetric HRP substrate.

Finally, the half maximal inhibitory concentration (IC50) of your compound to TNF-alpha: TNFR2 binding will be determined by comparing OD readings among different experimental groups.

MATERIALS PROVIDED

TABLE 1. MATERIALS PROVIDED (pls modify according to COA)

Catalog	Components	Size (96 tests)	Format	Storage	
EP161-C01	High-bind Plate	1 plate	Solid	2-8°C	
EP161-C02	Human TNF-alpha	20 µg	Powder	2-8°C	-70°C after reconstitution,
EP161-C03	Biotinylated Human TNFR2	10 µg	Powder	2-8°C	

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EP161-C04	Anti-TNF-alpha Neutralizing Antibody	20 μg	Powder	2-8℃	avoid freeze-thaw cycles
EP161-C05	Streptavidin-HRP	5 μg	Powder	2-8℃, avoid light	
EP161-C06	Coating Buffer	12 mL	Liquid	2-8℃	
EP161-C07	20×Washing Buffer	50 mL	Liquid	2-8℃	
EP161-C08	Blocking Buffer	50 mL	Liquid	2-8℃	
EP161-C09	Substrate Solution	12 mL	Liquid	2-8℃, avoid light	
EP161-C10	Stop Solution	7 mL	Liquid	2-8℃	

REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

Single or dual wavelength microplate reader with 450 nm/630nm filter;

Centrifuge;

37 ºC Incubator;

Single channel or multichannel pipettes with 10 μL, 200 μL and 1000 μL precision;

10 μL, 200 μL and 1000 μL pipette tips;

Test Tubes;

Graduated cylinder;

Deionized or distilled water for dilution;

STORAGE AND VALIDITY INSTRUCTIONS

Unopened kit should be stored at 2℃-8℃ upon receiving. Find the expiration date on the outside packaging and do not use reagents past their expiration date.

The kit should be stored as TABLE 1 after the reconstitution of lyophilized materials. The shelf life is 30 days from the date of opening.

Note:

a. Do not use reagents past their expiration date.

b. Find the expiration date on the outside packaging.

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REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (20℃-25℃) before use.

2. Reconstitute the provided lyophilized materials to stock solutions with sterile deionized water as recommended in Tab.2, Solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing. Avoid vigorous shaking or vortex. The reconstituted stock solutions should be stored at -70℃. **Avoid freeze-thaw cycles.**

Note: *Streptavidin-HRP stock solution should be protected from light.*

TABLE 2. RECONSTITUTION METHODS FOR 96 TESTS

Catalog	Components	Amount	Stock Solution	Reconstitution Buffer and
EP161-C02	Human TNF-alpha	20 μg	200 μg/mL	100 μL, water
EP161-C03	Biotinylated Human TNFR2	10 μg	100 μg/mL	100 μL, water
EP161-C04	Anti-TNF-alpha Neutralizing Antibody	20 μg	200 μg/mL	100 μL, water
EP161-C05	Streptavidin-HRP	5μg	50 μg/mL	100 μL, water

RECOMMENDED PROTOCOL

1. Working solution preparation

1.1 Preparation of 1×Washing Buffer:

Dilute 25 mL 20×Washing Buffer with ultrapure water/deionized water to 500 mL.

1.2 Preparation of Dilution Buffer:

10 mL Blocking Buffer (EP161-C08) add 30 mL 1×Washing Buffer.

2. Coating

1) Dilute Human TNF-alpha stock solution (200 μg/mL) to 0.5 μg/mL with Coating Buffer to make Human TNF-alpha working solution.

2) Add 100 μL of Human TNF-alpha working solution (0.5 μg/mL) to each well and leave a couple of wells uncoated for No-Coating Control, seal the plate with microplate sealing film and incubate overnight (or 16 hours) at 4℃.

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3. Washing

Remove the remaining solution by aspiration, add 300μL of 1×Washing Buffer to each well, gently tap the plate for 1 minute, remove any remaining 1×Washing Buffer by aspirating or decanting, invert the plate and blot it against paper towels. Repeat the washing step above for three times.

Note: *For best results, the complete removal of the Human TNF-alpha solution is essential. The use of a manifold dispenser or an auto-washer may be necessary.*

4. Blocking

Add 300 μL Blocking Buffer to each well, seal the plate with microplate sealing film and incubate at 37℃ for 1.5 hours.

5. Washing

Repeat step 3. At the same time, you can start to prepare your samples.

6. Add Samples

1) Make serial dilution of the samples as appropriate.

2) If you intend to use the provided Anti-TNF-alpha Neutralizing Antibody as a reference (Std.), you may dilute the antibody as recommended in Figure 1.

3) Add 50μL of sample solution to each well according to our recommendation (Figure 2) or your own plate setup.

4) For No-Coating Control wells, please add 50 μL Dilution Buffer.

7. Binding

1) Dilute Biotinylated Human TNFR2 stock solution (100 μg/mL) to 0.25 μg/mL with Dilution Buffer to make Biotinylated Human TNFR2 working solution.

2) For No-binding control wells, please add 50μL Dilution Buffer.

3) For all other wells, please add 50 μL Biotinylated Human TNFR2 working solution to the wells and mix the samples by gently tapping the plate. Seal the plate with microplate sealing film and incubate at 37℃ for 1 hour.

Note: The working solution should be prepared immediately before use and should not be stored.

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FIG.1 PREPARATION OF 1:1 SERIAL DILUTIONS OF THE Anti-TNF-alpha Neutralizing Antibody

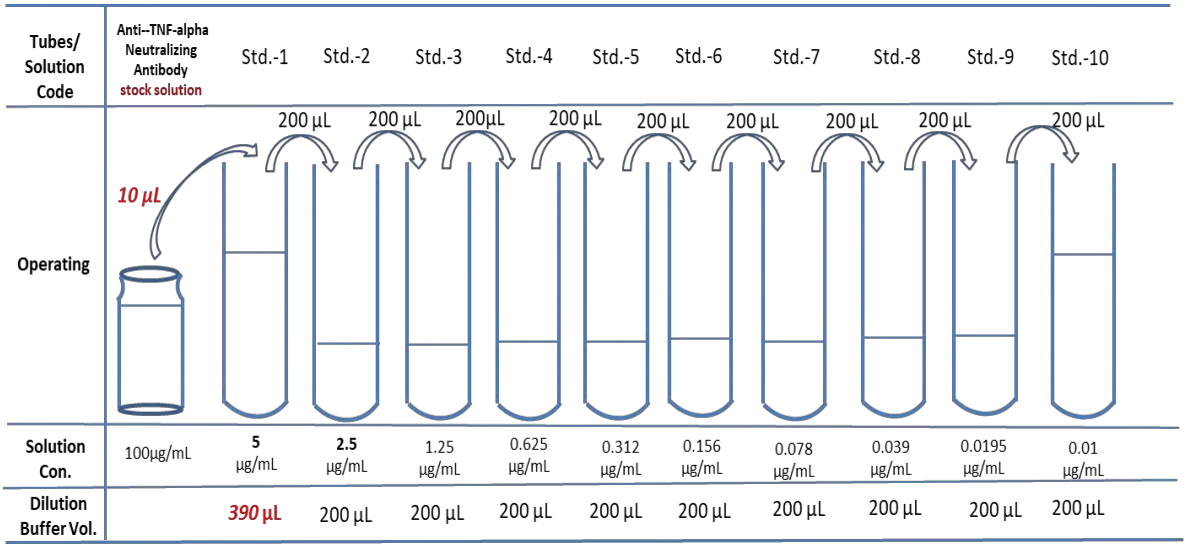
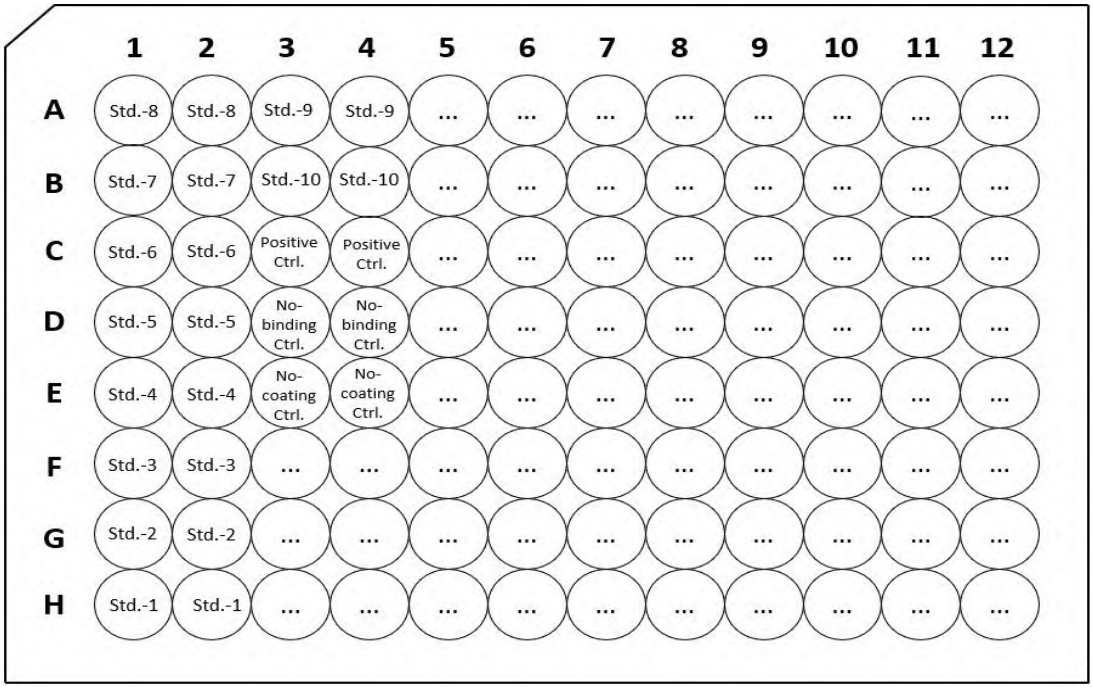


FIG.2 PLATE LAYOUT



8. Washing

Repeat step 3.

9. Add Streptavidin-HRP

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1) Dilute Streptavidin-HRP stock solution (50 μg/mL) to 0.1 μg/mL with Dilution Buffer to make Streptavidin-HRP working solution.

2) For all wells, add 100 μL Streptavidin-HRP working solution, seal the plate with microplate sealing film and incubate at 37℃ for 1 hour, avoid light.

10. Washing

Repeat step 3.

11. Substrate Reaction

Add 100 μL Substrate Solution to each well. Seal the plate with microplate sealing film and incubate at 37℃ for 20 minutes. Avoid light.

12. Termination

Add 50 μL Stop Solution to each well, and gently shake the plate to allow thorough mixing.
Note: *the color in the wells should change from blue to yellow.*

13. Data Recording

Read the absorbance at 450 nm using UV/Vis microplate spectrophotometer.
Note: *Subtracting the value read at* $OD_{450\,nm}$ *with* $OD_{630\,nm}$ *can be used to reduce the background noise.*

SIMPLIFIED PROTOCOL

TABLE. 3 ASSAY PROTOCOL

Steps Code	Steps	Reagents & Instruments	Reaction Conditions	Samples	No-binding Ctrl.	No-coating Ctrl.	Positive Ctrl.
1	Working fluid preparation	N/A	N/A	N/A	N/A	N/A	N/A
2	Coating	Human TNF-alpha Working Solution	4℃ for overnight	100 μL	100 μL	—	100 μL
3	Washing	1×Wash Buffer	Wash for 3 times	300 μL	300 μL	300 μL	300 μL
4	Blocking	Blocking Buffer	37℃ for 1.5 hours	300 μL	300 μL	300 μL	300 μL
5	Washing	1×Wash Buffer	Wash for 3 times	300 μL	300 μL	300 μL	300 μL
6	Add Samples	Samples	—	50 μL	—	—	—

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		Dilution Buffer		—	50 µL	50 µL	50 µL
7	Binding	Biotinylated Human TNFR2 Working Solution	Mix by gentle tapping, incubate at 37℃ for 1 hours	50 µL	—	50 µL	50 µL
		Dilution Buffer		—	50 µL	—	—
8	Washing	1×Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
9	Streptavidin-HRP	Streptavidin-HRP Working Solution	37℃ for 1 hours	100 µL	100 µL	100 µL	100 µL
10	Washing	1×Wash Buffer	Wash for 3 times	300 µL	300 µL	300 µL	300 µL
11	Substrate Reaction	Substrate Solution	37℃ for 20 minutes	100µL	100µL	100µL	100µL
12	Termination	Stop Solution	Mix by gentle tapping	50 µL	50 µL	50 µL	50 µL
13	Data Recording	UV/Vis spectrophotometer	Measure absorbance at 450 nm, with the correction wavelength set at 630 nm				

Note for TAB. 3:

1） Samples: Your samples of interest.

2） No-binding Ctrl.: Reaction without Biotinylated Human TNFR2 added. The absorbance should be around 0.05(< 0.1) at 450 nm.

3） No-coating Ctrl.: Reaction without Human TNF-alpha coated on the wells. The absorbance should be around 0.05(< 0.1) at 450 nm.

4） Positive Ctrl.: Determined the max value in 450nm absorbance, when out of inhibitors.

5） It is recommended that all samples, controls and standards should be done in duplicates.

PRECAUSIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.
2. This kit should be used according to the provided instructions.
3. Do not mix reagents from different lots.
4. Bring all reagents and samples to room temperature (20℃-25℃) before use.
5. This kit should be stored at 2℃-8℃.
6. Please prepare the working solution of each component according to the needs of the experiment. All prepared working solution is for one-time use and cannot be stored.

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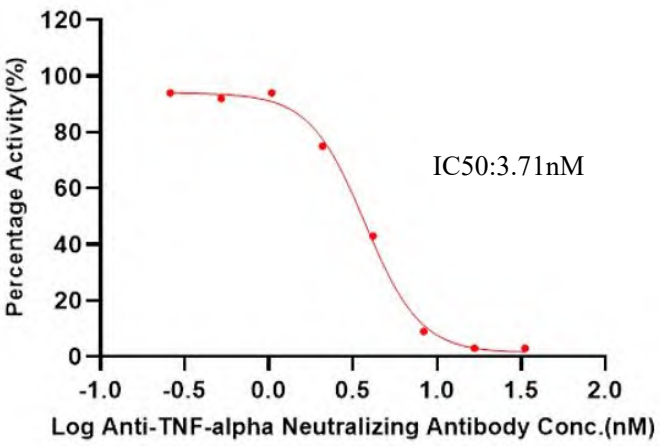
METHOD VERIFICATION

INHIBITION OF TNF-alpha: TNFR2 [Biotinylated] BINDING BY ANTI-TNF-alpha NEUTRALIZING ANTIBODY



Anti-TNF-alpha Neutralizing Antibody Conc.(μg/ml)	Anti-TNF-alpha Neutralizing Antibody Conc.(nM)	Mean Abs.(OD450)	Percentage Activity(%)
0	0.000	2.507	100%
0.039	0.260	2.359	94%
0.078	0.521	2.315	92%
0.156	1.042	2.354	94%
0.313	2.083	1.869	75%
0.625	4.167	1.086	43%
1.25	8.333	0.23	9%
2.5	16.667	0.087	3%
5	33.333	0.07	3%
No Coating		0.061	
No Binding		0.060	

Serial dilutions of Anti-TNF-alpha Neutralizing antibody (Catalog # EP161-C04) (1:1 serial dilution, from 5μg/mL to 0.039μg/mL) was added into TNF-alpha: TNFR2 [Biotinylated] binding reactions. The assay was performed according to the protocol described below. Background was subtracted from data points prior to log transformation and curve fitting.

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