Sword ELISA Booster

For Human TNFα

Catalog No. SB-HTNFA02-05

For use with the

R&D Systems® DuoSet® for Human TNFα (Cat# DY210 or DY210-05)

This package insert must be read before using this product.
For research use only.
Not for use in diagnostic procedures.

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INTRODUCTION

TNFα is a pleiotropic inflammatory cytokine produced predominantly by macrophages, and serves a variety of functions; possessing both growth stimulating and growth inhibitory processes, as well as having self-regulatory properties.[1] TNFα interacts with receptors TNFR1 and TNFR2, and these are expressed differently on cells and tissues resulting in signaling cascades that lead to a range of responses including: cell death, survival, differentiation, proliferation, and migration.[2] TNFα cannot usually be detected in healthy individuals; however, elevated serum and tissue levels are found in inflammatory and infectious conditions, and serum levels correlate to the severity of infections.[2] Lack of regulation of TNF-α production has been implicated in a variety diseases such as Alzheimer's disease, cancer, and Crohn's disease.[2-4]This ELISA Booster has been optimized for use with the R&D Systems DuoSet ELISA for Human TNFα (DY210 or DY210-05).

MATERIALS

Booster Components

Part	Volume	Supplied
Sword ELISA Booster - Component A (10X)	**9 mL**	**1 - 15mL vial**
Sword ELISA Booster - Component B (10X)	**9 mL**	**1 - 15mL vial**
Sword Diluent - Component C (10X)	**9 mL**	**1 - 15mL vial**
Sword Development Solution - Component D (5X)	**18 mL**	**1 - 25mL vial**
Sword ELISA Blocker for TNFα - Product no. SBL-503-05	**110 mL**	**1 – 125mL bottle**
Sword ELISA Diluent for TNFα - Product no. SDI-806-05	**110 mL**	**1 – 125mL bottle**
Sword ELISA HRP Conjugate - Component E (10,000X)	**30 µL**	**1 – 1 mL vial**

Each Booster provides sufficient reagents for approximately 500 wells using a final reaction volume of 300 µL per assay. **Do not mix reagent components from different Booster lots.**

STORAGE AND HANDLING

Upon receipt, the Booster should be stored at 2-8°C, protected from light. Stored properly, the Booster components should remain stable until the expiration date designated with the booster. Allow reagents to warm to room temperature before opening component containers.

ADDITIONAL MATERIAL REQUIRED

Non-expired R&D Systems DuoSet for Human TNFα (Cat# DY210 or DY210-05)

Reagent Diluent: PBS with 1% BSA freshly prepared

Coating Buffer: PBS

Wash Buffer: PBS with 0.5% Tween-20 freshly prepared

Deionized water

Proper pipettes and pipette tips

Multimode plate reader compatible with Sword ELISA Booster chemistry (see Appendix 1)

EXPERIMENTAL PROTOCOL

The following protocol is designed for use with the R&D Systems DuoSet for Human TNFα (Cat# DY210 or DY210-05). Qualified detectors are listed in Appendix 1.

Preparation of Working Solutions for Sword ELISA Booster

1.1 **Preparation of Sword ELISA Booster substrate solution:**

The following example is to prepare 16 mL Sword ELISA Booster substrate solution, enough for one 96 well plate. If you are not using a full plate, scale accordingly.

- Add the following to 11.2 mL deionized water:
- 1.6 mL Sword Booster - Component A (10X)
- 1.6 mL Sword Booster - Component B (10X)
- 1.6 mL Sword Diluent - Component C (10X)

Sufficient 10X Sword Diluent, Sword Booster Component A, and Sword Booster Component B have been provided to prepare sufficient Sword Booster to run 500 tests. This mixture is stable at 2-8º C for 3 days, but it is best if used within 1 - 3 hours. Protect this solution from air and light. The Sword substrate solution should be yellow in color.

Note: Salts in the Sword Diluent Component C (10X) may precipitate upon prolonged storage at 2-8°C. These salts readily re-dissolve by gentle inversion when the 10X solution is brought to Room Temperature. Check vial before using this reagent.

1.2 **Preparation of 1X Sword Development Solution:**

The following example is to prepare 16 mL Sword Development Solution, enough for one 96 well plate. If you are not using a full plate, scale accordingly.

- Add 3.2 mL 5X Sword Development Solution (Component D) to 12.8 mL deionized water.

Caution: *Both the 5X and 1X Development Solutions are caustic and should not come in contact with the skin*.

Assay Procedure

Plate Preparation

2.1 Reconstitute the Human TNF-α Capture Antibody with 0.5 mL of PBS to prepare a concentrate of mouse anti-human TNF-α capture antibody. Refer to the lot-specific Certificate of Analysis from R&D Systems for the amount supplied. Dilute the concentrated Human TNF-α Capture Antibody to a working concentration of 12 µg/ml in PBS, without carrier protein. Immediately coat a 96-well microplate with 100 µL/well of the diluted Human TNF-α Capture Antibody. Seal the plate and incubate overnight at 2-8°C.

2.2 Aspirate each well and wash a total of three times with 400 µl/well Wash Buffer (PBS + 0.05% Tween®20). Sufficient washing is essential for good assay performance. For each wash, allow Wash Buffer to sit in plate for 15 – 30 s prior to aspiration. Remove liquid completely from each well between each wash. The use of an automated plate washer is highly recommended. After the final wash, remove any excess Wash Buffer by inverting the plate and blotting against clean paper towels.

2.3 Block plates by adding 200 µl Sword ELISA Blocker for TNFα (Product no. SB-503-05). ELISA Blocker to each well. Cover with a plate seal and incubate at room temperature for a minimum of 1 hour.

2.4 Repeat the aspiration/wash as in Step 2.2. The plates are now ready for sample addition.

Assay Procedure

3.1 Reconstitute Human TNFα Standard with 0.5 mL of deionized or distilled water to prepare a concentrate of recombinant human TNFα Standard.

3.2 Prepare dilutions of standard and samples in Reagent Diluent (PBS with 1% BSA). A seven-point standard curve using 4-fold serial dilutions in Reagent Diluent, with a high standard of 1000 pg/ml and a low standard of 0.244 pg/ml, is recommended. For serum and plasma spike recovery, a high standard of 750 pg/ml and a low standard of 0.183 pg/ml is recommended.

3.3 Add 100 µl of Sword Diluent for TNFα (Product no. SD-806-05) to each well. Then, add 100 µl standard or sample per well. Cover with a plate seal and incubate 2 hours at room temperature on a rotator/shaker.

3.4 Repeat the aspiration/wash as in Step 2.2 of Plate Preparation.

3.5 Reconstitute the Human TNFα Detection Antibody with 1.0 mL Reagent Diluent (PBS with 1% BSA) to prepare a concentrate of biotinylated goat anti-human TNFα. Refer to the lot-specific Certificate of Analysis from R&D Systems for the amount supplied. Dilute in Reagent Diluent to the working concentration specified on the lot-specific Certificate of Analysis from R&D Systems.

3.6 Add 100 µl of the diluted Human TNFα Detection Antibody to each well. Cover with a plate seal and incubate 2 hours at room temperature on a rotator/shaker. **Avoid placing the plate in direct light.**

3.7 Repeat the aspiration/wash as in Step 2.2 of Plate Preparation.

3.8 Dilute Sword ELISA HRP Conjugate 1/10,000 in reagent diluent (e.g. 1.5 µL conjugate for 15 mL diluent).

3.9 Add 100 µl of the diluted Sword ELISA HRP-conjugate to each well. Cover with a plate seal and incubate for 20 minutes at room temperature on a rotator/shaker. **Avoid placing the plate in direct light.**

3.10 Repeat the aspiration/wash as in Step 2.2 of Plate Preparation.

3.11 Add 150 µL of the Sword Booster solution (prepared in step 1.1) to each well. Incubate for 15 minutes at room temperature in the dark.

3.12 Add 150 µL of Sword Development solution (prepared in step 1.2). The assay mixture should turn pink upon the addition of Sword Development solution. Incubate for 30 minutes in the dark. Due to the high volume in the wells, it is critical that caution is taken to avoid spilling contents. ***Do not put a lid on the plate.***

3.13 Determine the relative fluorescence units (RFU) of each well, using a microplate reader set to fluorescence with excitation and emission settings depending on the detector as listed in Appendix 1. Use the auto calculate function to determine the optimal gain setting. Signal will be stable for up to 90 minutes after development.

EVALUATION OF RESULTS

A standard curve is generated by plotting the mean signal values from the standard samples against the concentration of the standard samples.

4.1 For the most accurate results, the standard sample data should be fit to a four parameter logistic curve (4PLC) using the appropriate computer software for this iterative fitting process.

The 4PLC equation: $$Y = D + \frac{(A-D)}{\left[1+\left(\frac{X}{C}\right)^{B}\right]}$$

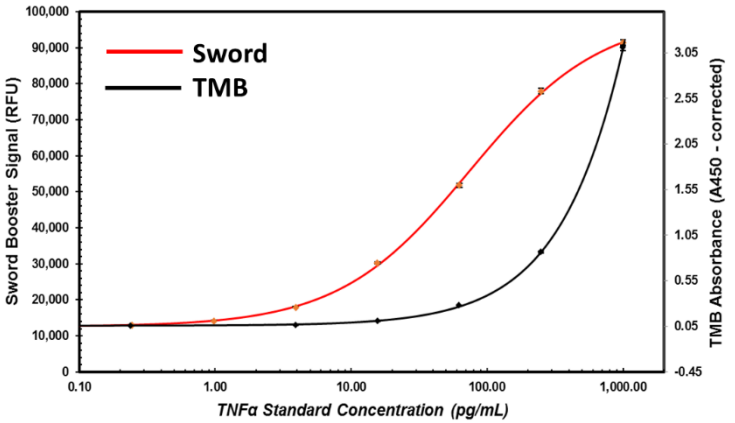
communicated by D. Rodbard[5] has been used by Sword Diagnostics for this fitting process.

PERFORMANCE

Sword Booster Performance Human TNF Alpha		DuoSet® Insert	Sword Boost	Quantikine® Insert	Sword Boost
Assay Range:	**0.98-1000 pg/mL**	15.6-1000 pg/mL	**15.9x**	15.6-1000 pg/mL	**15.9x**
LLOQ:	**0.98 pg/mL**	15.6 pg/mL	**15.9x**	15.6 pg/mL	**15.9x**
Sample Size:	**100 µL**	100 µL		100 µL	
Spike Recovery: (Plasma)	**0.05 pg/mL**				
Spike Recovery: (Serum)	**0.18 pg/mL**				

Sensitivity

Figure 1. Sword ELISA Booster for Human TNFα and TMB Curves



Human TNFα standard curve (red line) generated as described in "Experimental Protocol" section of this document in comparison to TMB (black line). The curve was fit to a four parameter logistic curve (4PLC). Standard error bars are shown with each data point.

The **Limit of Detection** (LOD) of Human TNFα with this procedure is typically **0.73 pg/mL** or less. The limit of detection was established by establishing a background value as the mean value of the Negative Controls plus 2 times the standard deviation of the Negative Control measurement, and determining the corresponding human IL-2 concentration for this background value with 4PLC fitted curve equation. The **Low Limit of Quantitation** (LLOQ) as determined by the lowest concentration where the signal was higher than the background plus 2 SD was also **0.98 pg/mL**. The reported assay range for TMB in the R&D Systems DuoSet for Human TNFα is 15.6 - 1000 pg/mL.[6]

Precision

Table 1: Precision of TNFα Quantification in Human Serum with Sword ELISA Booster

	TNFα Quantified (pg/mL)				
Donor	**Run 1**	**Run 2**	**Run 3**	**Average**	**CV%**
Very Low	0.81	0.91	0.72	0.82	11.6%
Low	1.09	1.29	1.18	1.19	11.9%
Medium	1.42	1.26	1.11	1.26	12.5%
High	3.42	3.25	3.12	3.27	4.6%

Table 1. TNFα levels were quantified in human serum from healthy donors using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα. Donor samples were tested in duplicate in three separate runs.

Table 2: Precision of TNFα Quantification in Human Plasma EDTA with Sword ELISA Booster

	TNFα Quantified (pg/mL)				
Donor	**Run 1**	**Run 2**	**Run 3**	**Average**	**CV%**
Very Low	1.01	0.89	0.77	0.89	19.5%
Low	1.74	1.43	1.58	1.55	9.8%
Medium	3.71	2.68	3.20	3.21	16.0%
High	4.30	4.14	4.00	4.15	3.6%

Table 2. TNFα levels were quantified in human plasma EDTA from healthy donors using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα. Donor samples were tested in duplicate in three separate runs.

Recovery

Table 3: Spike Recovery with Sword ELISA Booster for Human TNFα in Serum

TNFα Spike (pg/ml)	**Quantified IL-1β** (pg/ml)	**Accuracy %**	**CV%**
0.00	1.62	-	10.8%
0.05	1.78	107%	7.7%
0.18	1.92	107%	4.8%
0.73	2.22	95%	2.2%
2.93	4.18	92%	3.9%
11.72	12.07	91%	4.5%
46.9	41.97	87%	2.4%
187.5	160.34	85%	1.4%

Table 3. Human TNFα Reference Standard was spiked into pooled human serum from healthy donors. Human TNFα levels were quantified using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα.

Table 4: Spike Recovery with Sword ELISA Booster for Human TNFα in Plasma EDTA

TNFα Spike (pg/ml)	Quantified IL-1β (pg/ml)	Accuracy %	CV%
0.00	2.654	-	10.9%
0.05	2.151	80%	10.4%
0.18	2.675	94%	9.0%
0.73	3.505	104%	7.5%
2.93	6.074	109%	0.9%
11.72	15.374	107%	1.4%
46.9	51.67	104%	2.3%
187.5	181.084	95%	3.0%

Table 4. Human TNFα Reference Standard was spiked into pooled human plasma EDTA from healthy donors. Human TNFα levels were quantified using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα.

Linearity

Table 5: Linearity with Sword ELISA Booster for Human TNFα in Diluted Serum and Plasma EDTA

	TNFα Quantified (pg/mL)	
Dilution	Serum	Plasma EDTA
1:1	707.299	775.453
1:2	374.228	363.065
1:4	193.886	205.486
1:8	105.363	108.481
1:16	49.145	54.807

Table 5. Human TNFα Reference Standard was spiked into pooled human serum and plasma EDTA from healthy donors. Spiked samples were diluted in calibrator diluent. Human TNFα levels were quantified

using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα.

Quantification

Table 6: Quantification of TNFα in Healthy Human Serum

	TNFα Quantified (pg/mL)	
Donor	**Range**	**Average**
1	1.56 – 2.94	2.25
2	0.32 – 0.59	0.46
3	0.62 – 1.55	1.09
4	0.90 – 1.24	1.07
5	1.08 – 1.25	1.17
6	0.72 – 0.91	0.81
7	1.09 – 1.28	1.19
8	0.22 – 0.36	0.29
9	0.59 – 0.61	0.60
10	3.12 –0 3.42	3.27
11	1.11 – 1.42	1.26
12	0.29 – 0.44	0.36

Table 6. Human TNFα was quantified in human serum from twelve healthy donors using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα.

Table 7: Quantification of TNFα in Healthy Human Plasma

	TNFα Quantified (pg/mL)	
Donor	**Range**	**Average**
1	4.00 – 4.30	4.15
2	1.43 – 1.74	1.58
3	0.38 – 0.68	0.50
4	1.18 – 2.06	1.62
5	1.41 – 1.61	1.51
6	2.68 – 3.71	3.20
7	0.34 – 0.56	0.44
8	0.77 – 1.58	1.18
9	1.13 – 1.37	1.25
10	0.77 – 1.01	0.89
11	0.35 – 0.54	0.44
12	1.22 – 1.56	1.39

Table 7. Human TNFα was quantified in human plasma EDTA from twelve healthy donors using the R&D Systems Human TNFα DuoSet ELISA (DY210) with Sword ELISA Booster for Human TNFα.

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[6]www.rndsystems.com. R&D Systems DuoSet ELISA for Human TNFα, 2016.

TECHNICAL SUPPORT

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APPENDIX 1. COMPATIBLE DETECTORS

Vendor	Model	Sword Compatible	Filter Mode	Mono Chrometer	Red PMT
BioTek	Cytation 5	Yes	Best	Ok	Best
BioTek	Cytation 3	Yes	Good	Ok	Best
BioTek	Synergy H1, H4, 2	Yes	Good	Ok	Best
BioTek	NEO, NEO2	Yes	Best	Good	Best
Tecan	Infinite M1000 Pro	Yes	n/a	Best	n/a
Tecan	Infinite F500 Pro	Yes	Best	n/a	n/a
Tecan	Infinite 200 Pro	Yes	n/a	Good	n/a
Tecan	Spark 10m / 20M	Yes	n/a	Best	n/a
Molecular Devices	SpectroMax Paradigm	Yes	Good	Ok	
Molecular Devices	SpectraMax M Series	No			
Perkin Elmer	Envision	No			
Perkin Elmer	Victor	No			

APPENDIX 2. INSTRUMENT SETTINGS

BioTek Cytation 5 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	730 nm
Emission Bandwidth	40 nm
Optics Position	Top 570 nm
Gain	Extended
Read Height	Calibrate for high well (usually A1)

Note: A red-shifted PMT is recommended for best results, but not critical.

BioTek Synergy H4 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	680 nm*
Emission Bandwidth	30 nm
Optics Position	570
Gain[a]	Extended
Read Height	Calibrate for high well (usually A1)

*Note: *The 680 nm emission filter is recommended for the instruments without a red-shifted PMT. If the H4 being used has a red-shifted PMT, we recommend the 730 nm/40 nm BW emission filter.*

BioTek NEO2 Parameter	Setting
Detection Method	Fluorescence Intensity
Read Type	Endpoint/Kinetic
Optics Type	Filter
Excitation Wavelength	530 nm
Excitation Bandwidth	25 nm
Emission Wave Length	730 nm
Emission Bandwidth	40 nm
Optics Position	Top 570 nm
Gain	Autoscale to high well (usually A1)
Read Height	Calibrate for high well (usually A1)

Note: The NEO2 is equipped with the red-shifted PMT, and it should be used for our application.

Tecan Infinite M1000 Pro Parameter	Setting
Measurements	Fluorescence Intensity
Mode	Top
Excitation Wavelength	530 nm
Excitation Bandwidth	20 nm
Emission Wave Length	700 nm
Emission Bandwidth	20 nm
Flashes	25 (100 Hz)
Integration	20 µsec
Lag Time	0 µsec
Gain[a]	Auto based on high well (usually A1)
Z-Position	Auto based on high well (usually A1)