Human Tumor Necrosis Factor Alpha (TNF-α) Detection Kit (TR-FRET)

Catalog Number：FRT-C02

Assay Tests: 100 tests & 500 tests

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

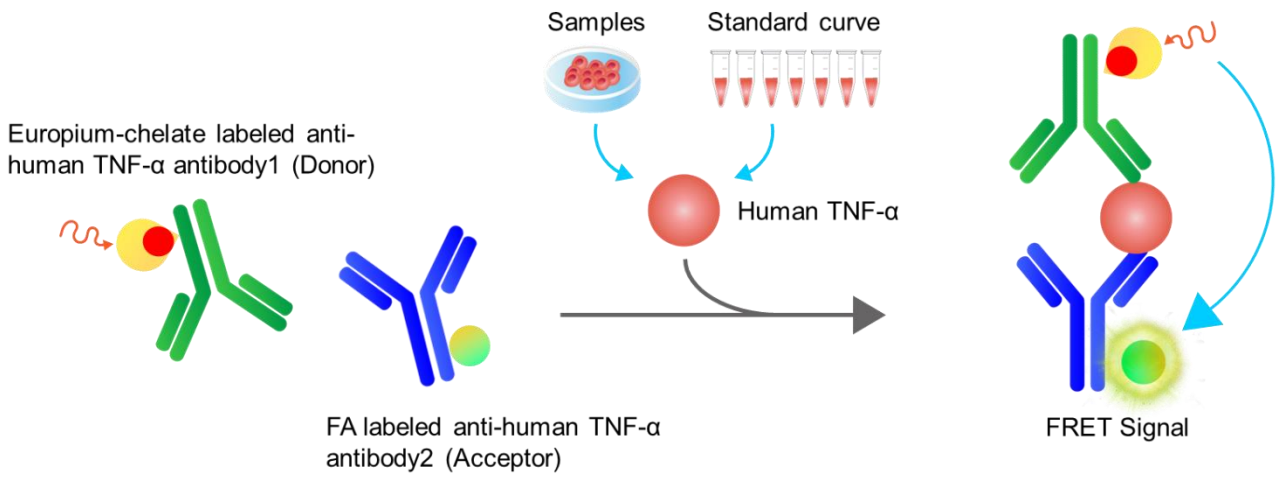
IMPORTANT: Please carefully read this manual before performing your experiment.

PRODUCT OVERVIEW

The Human Tumor Necrosis Factor Alpha (TNF-α) Detection kit (TR-FRET) can detect the Human Tumor Necrosis Factor Alpha (TNF-α) in homogeneous system within 2 hours, it is highly sensitive, short detection time and easy to use. This kit is specifically designed for the accurate quantitation of human TNF-alpha from cell culture supernatant, serum and plasma.

ASSAY PRINCIPLE

This human Tumor Necrosis Factor Alpha (TNF-α) detection kit is based on a TR-FRET sandwich assay using two different specific antibodies. One antibody is labeled with a europium chelate (as the donor), while the second is labeled with FA (as the acceptor). In the presence of human TNF-α, the donor and acceptor are in close proximity because both labeled antibodies bind to the human TNF-α. When excited by a specific light source (337 nm), the donor emits a 620 nm signal, which is absorbed by the acceptor and results in a 665 nm emission.



NOTE:

1. For Research Use Only. Not For Use in Diagnostic or Therapeutic Procedures.
2. The kit should not be used beyond the expiration date on the kit label.
3. Do not mix or substitute reagents with those from other lots or sources.

CONTENTS AND STORAGE

Store the unopened kit at 2-8 °C. Do not use the kit after the expiration date indicated on the kit label.

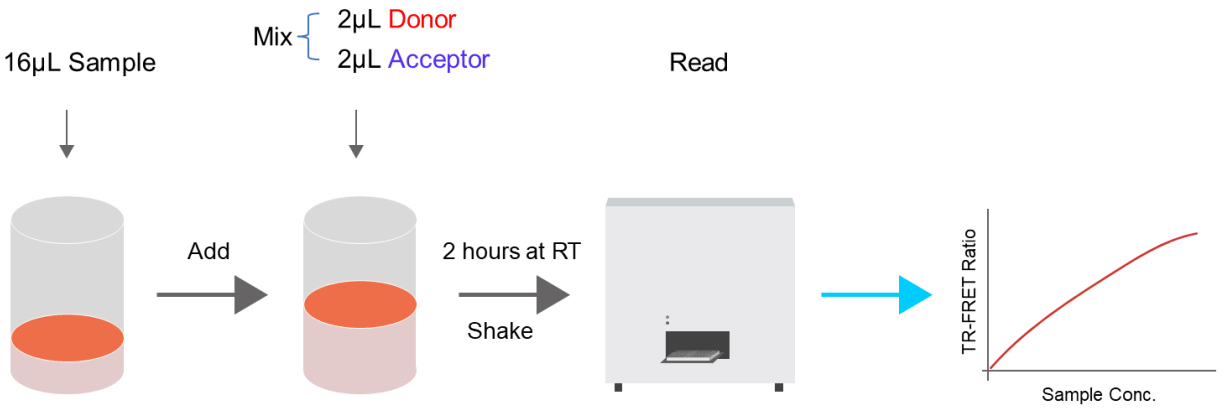
Catalog	Contents	Size (100 tests)	Size (500 tests)	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
FRTC02-C01	Anti-TNF-α Antibody Europium-chelate	100 tests	500 tests	-70℃, avoid light
FRTC02-C02	FA Labeled Anti-TNF-α Antibody	100 tests	500 tests	-70℃, avoid light
FRTC02-C03	Human TNF-α Standard	15 μg	15 μg	-70℃
DB-04	TR-FRET Sample Dilution Buffer, pH7.4	50 mL	50 mL	2-8℃
DB-05	TR-FRET Detection Buffer, pH7.4	50 mL	50 mL	2-8℃

Note: Anti-TNF-α Antibody Europium-chelate and FA Labeled Anti-TNF-α Antibody should be protected from light.

REQUIRED MATERIALS NOT SUPPLIED

	Items	Specifications
Instrument	Microplate reader	Plate reader capable of measuring signals at 665nm/620nm in TR-FRET mode. Example: BMG Labtech CLARIOstar Plus.
	Microporous plate shaker	-
Reagents	Deionized, ultrapure or distilled water	-
Consumables	Single channel and multichannel channel pipettes	There must be calibrated pipettes, with 10 μL, 200 μL and 1000 μL precision
	Pipette tips	Low adsorption pipette tips, all tips need to fit the pipettes
	96 or 384-well white plate	Non-transparent 96 or 384-well low volume white plates typically give the lowest background signal. e.g., 384-well white plate (iSTAR, Cat. No. GT247.008)
	EP Tubes	For to prepare standard dilutions and working solutions.

QUICK GUIDE



REAGENT PREPARATION

1. Bring all reagents to room temperature (20℃-25℃) before use. If crystals have formed in the buffer solution, warm to room temperature and mix gently until the crystals have completely dissolved.
2. Reconstitute the provided lyophilized materials to stock solutions with **Deionized, ultrapure or distilled water** as recommended in the following table 1 and solubilize for 15 to 30 minutes at room temperature with occasional gentle mixing.
3. Avoid vigorous shaking or vertexing. The reconstituted stock solutions should be stored at -70℃. It is recommended not to freeze-thaw more than 2 times.

Note: Anti-TNF-α Antibody Europium-chelate and FA Labeled Anti-TNF-α Antibody stock solution should be protected from light.

Table 1. Reconstitution methods for 100 tests and 500tests

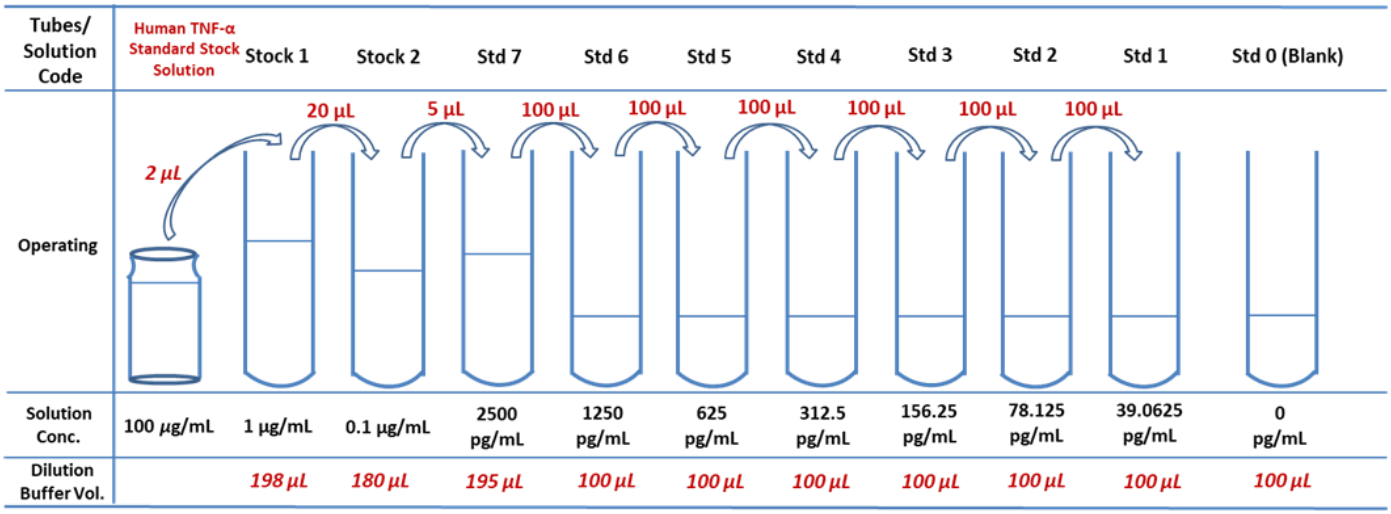
Catalog	Components	The volume of buffer required for reconstitution		Stock Solution Conc.
		Size (100 tests)	Size (500 tests)	
FRTC02-C01	Anti-TNF-α Antibody Europium-chelate	300 μL water	300 μL water	About 4 μg/mL
FRTC02-C02	FA Labeled Anti-TNF-α Antibody	60 μL water	300 μL water	About 64 μg/mL
FRTC02-C03	Human TNF-α Standard	150 μL water	150 μL water	100 μg/mL

PROCEDURE OF ASSAY

- Each well requires 16 µL of standard or sample， and 4 µL of the pre-mix donor & acceptor solution.
- Serially dilute the standard stock solution with **DB-04 (TR-FRET Sample Dilution Buffer, pH7.4)** or **a solution with the same matrix as the test samples**.
- It is recommended to prepare the standard curve using a solution with the same matrix as the test samples, so as to reduce interference from matrix effects and obtain more accurate back-calculated sample concentrations.

1. Prepare standard serial dilutions.
   - Label a tube "**Stock 1**". Add 2 µL of the reconstituted human TNF-α Standard (100 µg/mL) and 198 µL of **Sample Dilution Buffer (DB-04 or a solution with the same matrix as the samples)** to tube **Stock 1**, gently mix well.
   - Label a tube "**Stock 2**". Add 20 µL of Stock 1 and 180 µL of **Sample Dilution Buffer** to tube **Stock 2**, gently mix well.
   - Label 7 tubes, one for each standard point: Std.-1, Std.-2, Std.-3, Std.-4, Std.-5, Std.-6, Std.-7.
   - Add 5 µL of the liquid from **Stock 2** and 195 µL of **Sample Dilution Buffer** to tube Std.-7, thoroughly mix (Std.-7 =2500 pg/mL).
   - Prepare 1:1 serial dilutions for the standard curve as follows: Add 100 µL of **Sample Dilution Buffer** to each tube (Std.-1, Std.-2, Std.-3, Std.-4, Std.-5, Std.-6).
   - Transfer 100 µL of liquid from Std.-7 to the tube Std.-6, and thoroughly mix (Std.-6 = 1250 pg/mL).
   - Continue to transfer 100 µL of liquid from previous dilution tube to the next dilution tube until add liquid to tube Std.-1.
   - Sample Dilution Buffer serves as zero standard (blank).

Figure 1. Preparation of serial dilutions of the human TNF-alpha standard



2. Prepare Biological Samples. Each well requires **16 µL** of sample. All samples with a concentration above the highest standard (Std 7) must be diluted in **Sample Dilution Buffer**.
3. Prepare Donor Working Solution. Dilute the Anti-TNF-α Antibody Europium-chelate stock solution 1:4 with DB-05.
4. Prepare Acceptor Woking Solution. Dilute the FA Labeled Anti-TNF-α Antibody stock solution 1:4 with DB-05.
5. Prepare the pre-mix solution. Pre-mix the Donor and Acceptor working solution 1:1 (v/v).
6. Add 16 µL of standards or samples, 4 µL of the pre-mix donor & acceptor solution to each well. Refer to **followed figure 2 and Table 2** for the design of microplate layout according to the experimental requirements and add the corresponding reaction solution into the corresponding plate holes
7. Seal the plate with microplate sealing film and incubate at room temperature (20℃-25℃) for **2 hours** on orbital shaker at 400-600 rpm.
8. Data Recording. Use the TR-FRET module of a microplate reader to read the fluorescence signal at 665nm and 620nm (the excitation wavelength is 337 nm).

Note:

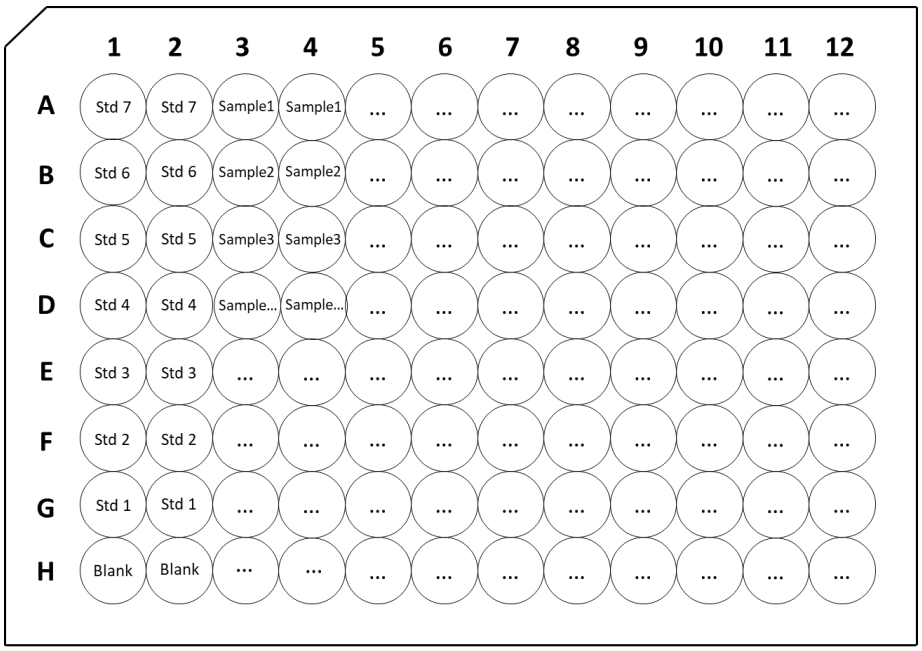


Figure 2. Plate layout

Table 2. Samples adding to microplate

	1	2	3	4
A	16 μL Std7 4 μL pre-mixed TNFα antibodies	16 μL Std7 4 μL pre-mixed TNFα antibodies	16 μL Sample1 4 μL pre-mixed TNFα antibodies	16 μL Sample1 4 μL pre-mixed TNFα antibodies
B	16 μL Std6 4 μL pre-mixed TNFα antibodies	16 μL Std6 4 μL pre-mixed TNFα antibodies	16 μL Sample2 4 μL pre-mixed TNFα antibodies	16 μL Sample2 4 μL pre-mixed TNFα antibodies
C	16 μL Std5 4 μL pre-mixed TNFα antibodies	16 μL Std5 4 μL pre-mixed TNFα antibodies	16 μL Sample3 4 μL pre-mixed TNFα antibodies	16 μL Sample3 4 μL pre-mixed TNFα antibodies
D	16 μL Std4 4 μL pre-mixed TNFα antibodies	16 μL Std4 4 μL pre-mixed TNFα antibodies	16 μL Sample... 4 μL pre-mixed TNFα antibodies	16 μL Sample... 4 μL pre-mixed TNFα antibodies
E	16 μL Std3 4 μL pre-mixed TNFα antibodies	16 μL Std3 4 μL pre-mixed TNFα antibodies	...	...
F	16 μL Std2 4 μL pre-mixed TNFα antibodies	16 μL Std2 4 μL pre-mixed TNFα antibodies	...	...
G	16 μL Std1 4 μL pre-mixed TNFα antibodies	16 μL Std1 4 μL pre-mixed TNFα antibodies	...	...
H	16 μL Sample Dilution Buffer 4 μL pre-mixed TNFα antibodies	16 μL Sample Dilution Buffer 4 μL pre-mixed TNFα antibodies	...	...

CALCULATION OF RESULTS

1. Calculate the TR-FRET Ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4.$$

2. Calculate the Mean Ratio for each sample, standard or control and subtract average zero standard Ratio to obtain the ΔRatio value.

3. The standard curve is plotted with the standard concentration as x-axis and the ΔRatio as y-axis. Establish a standard curve by processing the data using computer software capable of executing a **four-parameter logistic (4-PL)** curve fitting. Normal range of Standard curve: R2⩾0.9900.

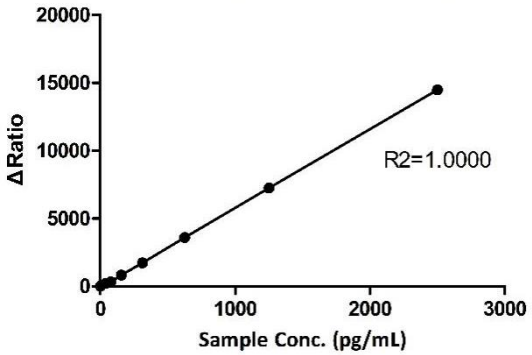
4. Detection range: 39.0625 pg/mL-2500 pg/mL. If the Ratio value of the sample to be tested is higher than 2500 pg/mL, the sample shall be diluted with dilution buffer and assay repeated. If the ΔRatio value of the sample to be tested is lower than 39.0625 pg/mL, the sample should be reported < 39.0625 pg/mL.

TYPICAL DATA

Note: *For each experiment, a standard curve needs to be set for each microplate, and the specific Ratio value may vary depending on different laboratories, testers, or equipment. The following example data is for reference only. The sample concentration was calculated based on the results of the standard curve.*

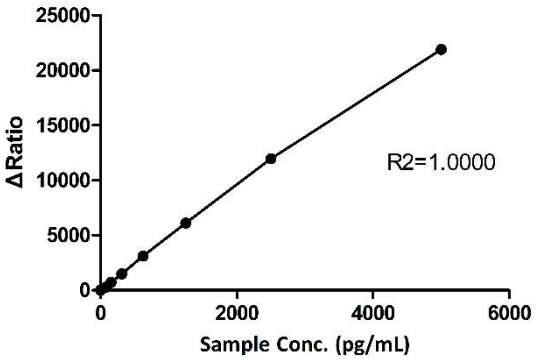
Standard curve generated in TR-FRET Sample Dilution Buffer, pH7.4 (DB-04):

Human TNF-alpha Standard (pg/mL)	TR-FRET Ratio	Δ Ratio	CV(%)
2500	15677	14479	1.8
1250	8446	7248	7.5
625	4785	3587	3.4
312.5	2928	1730	3.1
156.25	2019	821	4.3
78.125	1521	322	3.5
39.0625	1409	211	6.7
Blank	1198	0	2.2



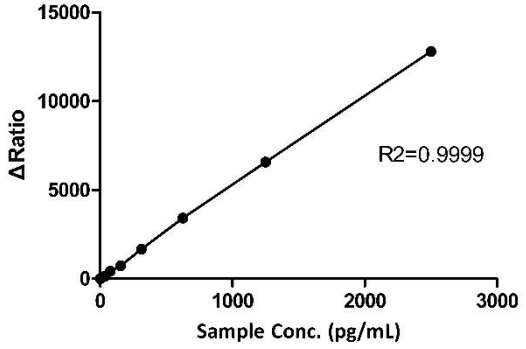
Standard curve generated in DMEM with 10% FBS:

Human TNF-α Standard (pg/mL)	TR-FRET Ratio	Δ Ratio	CV (%)
5000	23706	21912	0.5
2500	13756	11963	1.2
1250	7901	6107	2.2
625	4916	3123	3.0
312.5	3283	1489	1.4
156.25	2521	727	0.6
78.125	2077	283	2.7
Blank	1794	0	3.8



Standard curve generated in RPMI 1640 with 10% FBS:

Human TNF-α Standard (pg/mL)	TR-FRET Ratio	Δ Ratio	CV (%)
2500	14251	12797	2.3
1250	8029	6574	4.6
625	4870	3415	1.8
312.5	3124	1669	2.3
156.25	2184	730	0.9
78.125	1882	427	4.7
39.0625	1615	160	2.9
Blank	1454	0	1.1



PERFORMANCE CHARACTERISTICS

Sensitivity

The minimum detectable concentration was determined by adding twice standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

Matrix	TR-FRET Sample Dilution Buffer, pH7.4 (DB-04)	DMEM with 10% FBS	RPMI 1640 with 10% FBS
Assay range	39.0625 pg/mL-2500 pg/mL	78.125 pg/mL-5000 pg/mL	39.0625 pg/mL-2500 pg/mL
Limit of detection (LOD)	18.59 pg/mL	28.91 pg/mL	25.19 pg/mL
Limit of Quantitation (LOQ)	39.0625 pg/mL	78.125 pg/mL	39.0625 pg/mL

Linearity

To assess the linearity of the assay, samples spiked with high concentrations of human TNF-α were serially diluted with TR-FRET Sample Dilution Buffer, pH7.4 (DB-04) to produce samples with values within the dynamic range of the assay.

Dilution factor		Serum	Plasma			Cell culture medium	
			Sodium citrate	Heparin	EDTA	DMEM	RPMI1640
1:4	Average Recovery (%)	92.37	81.79	85.52	83.84	99.28	94.15
	Range (%)	88.53-97.26	78.27-86.10	82.10-93.72	80.85-91.69	96.03-103.11	88.16-98.80
1:8	Average Recovery (%)	98.48	91.40	92.01	95.73	100.25	97.70
	Range (%)	94.07-103.36	86.24-97.06	89.51-95.92	90.02-102.62	97.41-102.37	92.62-103.41
1:16	Average Recovery (%)	94.39	94.54	94.84	98.85	103.28	97.51
	Range (%)	92.00-101.40	93.32-97.92	86.15-101.07	86.59-110.35	91.89-110.13	87.70-108.85

Inter-Assay/Intra-Assay Precision

Five Samples of known concentrations were tested repeatedly 16 times in batches to assess within-batch precision and accuracy. Five Samples of known concentrations were tested repeatedly across three independent analytical batches to evaluate between-batch precision and accuracy.

	Intra-assay Precision					Inter-assay Precision				
Sample Conc. (pg/mL)	**2500**	**1875**	**500**	**78.13**	**39.06**	**2500**	**1875**	**500**	**78.13**	**39.06**
Number of Replicate	16	16	16	16	16	3	3	3	3	3
Mean (pg/mL)	2408.34	1791.18	458.68	81.87	50.31	2438.62	1838.90	483.87	79.05	39.33
Standard Deviation	40.52	35.25	15.67	8.10	6.85	142.26	75.01	21.19	12.51	6.42
CV (%)	1.68	1.97	3.42	9.89	13.62	5.83	4.08	4.38	15.83	16.32
Recovery (%)	96.33	95.53	91.74	104.79	128.79	97.54	98.07	96.77	101.18	100.68

Calibration

This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human TNF-α produced at ACROBiosystems. To convert sample values obtained with the Human TNF-α Detection kit (TR-FRET) to approximate NIBSC (17/232) nominally assigned mass values, use the equation below.

NIBSC/WHO (17/232) approximate value (U/mL) = 0.1 × ACRO Human TNF-α value (pg/mL).

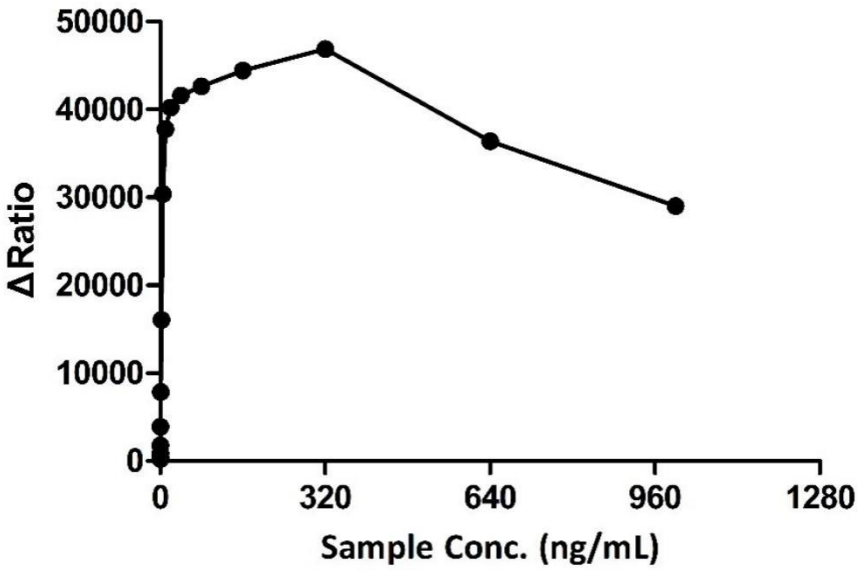
Specificity

No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines at up to 1 μg/mL.

Human		
IL-2	IL-11	G-CSF
IL-3	IL-12B	M-CSF
IL-4	IL-15	SCF
IL-5	IL17A	BMP-2
IL-6	IL-18	FGF basic
IL-7	IL-21	Flt-3 Ligand
IL-8	IFN-alpha 1	VEGF165
IL-9	IFN-gamma	Thrombopoietin
IL-10	GM-CSF	TGF-Beta 1

Hook Effect

Not be affected by the concentration of human TNF-α up to 320 ng/mL.



TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Large CV	* Inaccurate pipetting * Air bubbles in wells	* Check pipettes * Remove bubbles in wells
High background	* Reagent contamination * Interfering components	* Avoid contaminating the reagents. * Ensure the purity of the samples or dilute it to reduce interference
Hook	* Inappropriate sample detection concentration * The usage concentration of the donor/acceptor is not applicable to certain special samples	* Sample dilution optimization * Optimize the usage concentration of donors/acceptor
Very low readings across the plate	* Incorrect wavelengths or gain value set * Insufficient reaction time	* Check filters/gain/reader * Increase reaction time
Samples are reading too high, but standard curve looks fine	* Samples contain target levels above assay range	* Dilute samples and run again
Matrix interference	* Sample matrix does not match the standard curve	* Dilute the sample with a larger dilution factor to reduce matrix interference and perform spike recovery experiments. * Prepare the standard curve with the same matrix as the test sample in order to reduce the interference of matrix effects and obtain more accurate sample back-calculated concentrations.
Low instrument detection sensitivity	* Instrument parameters are not appropriate	* Set the instrument's delay time to 50-100μs, the Integration time to 100-400μs, and select the instrument gain within an appropriate range to avoid signal oversaturation or too low.