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THUNDER™ Human TNFα TR-FRET Biomarker Assay Kit

CATALOG NUMBERS KIT-TNFA-100 (100 tests)
KIT-TNFA-500 (500 tests)
KIT-TNFA-2500 (2500 tests)
KIT-TNFA-5000 (5000 tests)
KIT-TNFA-10000 (10000 tests)

Store at -80°C.
For research use only.
Not for use in diagnostic procedures.

PRODUCT DESCRIPTION

This assay kit is designed for the quantitative determination of **tumor necrosis factor alpha (TNFα)** in cell culture supernatants using a simple, rapid and sensitive immunoassay based on the homogeneous (no-wash) THUNDER™ TR-FRET technology. The kit does not include a recombinant **human TNFα** standard, which must be purchased separately from R&D Systems® (catalogue number 210-TA).

SPECIFICITY

This assay kit contains two specific and selective antibodies that recognize both natural and recombinant **human TNFα** in cell culture supernatant samples.

SPECIES REACTIVITY

Human (Swiss-Prot Acc. P01375; Entrez Gene Id 7124).

TR-FRET ASSAY PRINCIPLE

The THUNDER™ **human TNFα** assay kit is a homogeneous time-resolved Förster resonance energy transfer (TR-FRET) sandwich immunoassay (Figure 1). The assay workflow consists of a one-step reagent addition (Figure 2). **Human TNFα** in the cell supernatant sample is detected with a pair of fluorophore-labeled antibodies in a simple "add-incubate-measure" format (no wash steps). One antibody is labeled with a donor fluorophore (Europium chelate; Eu-Ab1) and the second with a far-red acceptor fluorophore (FR-Ab2). The binding of the two labeled antibodies to distinct epitopes on the target cytokine takes place in solution and brings the two dyes into close proximity. Excitation of the donor Europium chelate molecules with a flash lamp (320 or 340 nm) or a laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which in turn emit a TR-FRET signal at 665 nm. Residual energy from the Eu chelate generates light at 615 nm. The signal at 665 nm is proportional to the concentration of **human TNFα** in the cell supernatant. Data can be expressed as either the signal at 665 nm or the 665 nm/615 nm ratio. A distinct advantage of the THUNDER™ TR FRET technology is that it does not require addition of potassium fluoride to ensure signal stability. The non-toxic nature of THUNDER™ makes it an ideal candidate for multiplexing with other assay technologies using a sequential assay protocol.

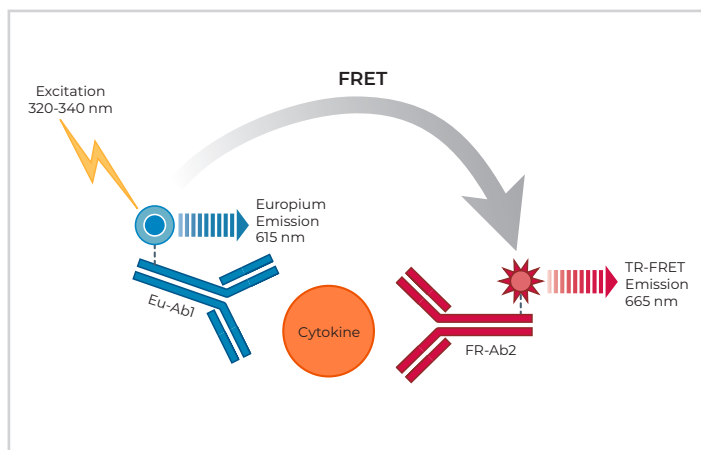


Figure 1 Schematic representation of the TR-FRET assay principle.



Figure 2 THUNDER™ human TNFα assay workflow

Upon receipt, store kit in the dark at -80°C.

THUNDER™ ASSAY PROTOCOL

This protocol must be read in its entirety prior to beginning the assay.

- We cannot guarantee the performance of the product outside the conditions detailed in this Assay Protocol.
- Bring all reagents to room temperature prior to running the assay.
- Centrifuge all tubes before use to improve recovery of content (2000x g, 10-15 sec).
- Use ultrapure water (Milli-Q® grade water; 18 M Ω cm) to dilute Assay Buffer.
- It is recommended to test all standards in triplicate and samples at least in duplicate.

KIT COMPONENTS

	100 points*	500 points*
Eu-labeled anti-TNF α antibody (Eu-Ab1)	5 μ L (1 clear tube, red cap)	25 μ L (1 clear tube, red cap)
Acceptor-labeled anti-TNF α antibody (FR-Ab2)	20 μ L (1 brown tube, blue cap)	100 μ L (1 brown tube, blue cap)
Assay Buffer 2 (5X)**	1 mL (1 tube, yellow cap)	3 mL (3 tubes, yellow cap)

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

**Extra Assay Buffer can be ordered separately (cat # TRFRET-AB2).

ADDITIONAL MATERIALS REQUIRED

	Recommended source	Catalog No.
Recombinant human TNF α	R&D Systems®	210-TA
Ultrapure laboratory grade water	Many options available	NA
Low-volume 384-well microplate, white	PerkinElmer Greiner Corning	6007290 784075 4513
Multi- and single-channel pipettes	Many options available	NA
Adhesive sealing film for plates	Many options available	NA
A plate reader equipped with the TR-FRET option	Many options available	NA

REAGENT PREPARATION

- The instructions described below are for testing the entire number of assay points in each kit. Adjust volumes accordingly for testing of fewer assay points.
- Prepare only as much reagent as is needed on the day of the experiment.

STEP 1 PREPARATION OF 1X ASSAY BUFFER 2

- Mix end-over-end the 5X Assay Buffer 2 before use.
- The thawed 5X Assay Buffer 2 can be stored at 4°C for 1-2 weeks. For longer periods of time, buffer should be stored at -80°C.
- Unused 1X Assay Buffer 2 can be stored at 4°C for 2 days.

For the 100-point kit

Add 1 mL of 5X Assay Buffer 2 to 4 mL of water.

For the 500-point kit

Add 3 mL of 5X Assay Buffer 2 to 12 mL of water.

STEP 2 PREPARATION OF TNF α STANDARD SERIAL DILUTIONS

IMPORTANT NOTE

The analyte TNF α is not included in the kit. The kit has been validated using human TNF α recombinant protein from R&D Systems® (Catalog number 210-TA). Other commercial recombinant human TNF α may also work but have not been tested with this kit.

- Prepare working standards just before use and use within one hour. Do not store working standards.
- Each well requires 15 μ L of working standard.
- Change tip between each standard dilution.
- Mix each tube thoroughly before the next transfer.
- The following section describes the preparation of a standard curve for triplicate measurements (recommended).

NOTE

The Working Stock Solution should be used within one hour or aliquoted into screw-capped polypropylene vials and stored at -80°C for long term storage.

REAGENT PREPARATION (CONTINUED)

- A.** Follow the manufacturer's instructions to reconstitute the analyte (typically at 100-200 $\mu\text{g}/\text{mL}$) to create a Stock Solution.
- B.** Create a 1 $\mu\text{g}/\text{mL}$ Working Stock Solution by diluting the Stock Solution in either 1X Assay Buffer 2 if the standard curve is done in Assay buffer or in culture medium with 10% fetal bovine serum (FBS) if the standard curve is done in culture medium. Use this Working Standard Solution to prepare a standard dilution series.
- C.** Label 12 tubes, one for each working standard. Alternatively, serial dilutions can be conducted using a polypropylene 96-well plate.
- D.** Prepare serial dilutions for the standard curve as indicated in the table below using either 1X Assay Buffer 2 or culture medium with 10% FBS (for testing of culture supernatant samples):

Tube	Volume of TNF	Vol. of 1X Assay Buffer or culture medium +10% FBS (μL)	[TNF α] in standard curve (g/mL in 15 μL)
1	10 μL diluted TNF α (1E-6 g/mL)	90	1.0E-7
2	30 μL of tube 1	70	3.0E-8
3	30 μL of tube 2	60	1.0E-8
4	30 μL of tube 3	70	3.0E-9
5	30 μL of tube 4	60	1.0E-9
6	30 μL of tube 5	70	3.0E-10
7	30 μL of tube 6	60	1.0E-10
8	30 μL of tube 7	70	3.0E-11
9	30 μL of tube 8	60	1.0E-11
10	30 μL of tube 9	70	3.0E-12
11	30 μL of tube 10	60	1.0E-12
12*	0	70	0

* In order to calculate the Limit of Detection (LOD), three additional zero standard tubes (13-15) are assayed in triplicate (12 wells in the 384-well plate).

STEP 3 PREPARATION OF 4X ANTIBODY DETECTION MIX

- Prepare and mix just before use.
- Each well requires 5 μL of 4X Antibody Detection Mix
- The unused 4X Antibody Detection Mix may be stored at 4°C for 2 days or aliquoted and stored at -80°C. Avoid repeated freeze/thaw cycles.

For the 100-point kit

NOTE

Due to the low reagent volumes in the 100-point kit, the antibodies are diluted with 1X Assay Buffer 2 directly in the vial.

- A.** Add 255 μL of 1X Assay Buffer 2 into the vial containing 5 μL of Eu Ab1 stock solution.
- B.** Add 240 μL of 1X Assay Buffer 2 into the vial containing 20 μL of FR Ab2 stock solution.
- C.** Mix gently 260 μL of pre-diluted Eu Ab1 with 260 μL of pre-diluted FR-Ab2.

For the 500-point kit

- A.** Mix gently 1,275 μL of 1X Assay Buffer 2 with the 25 μL of Eu Ab1 stock solution.
- B.** Mix gently 1,200 μL of 1X Assay Buffer 2 with the 100 μL of FR-Ab2.
- C.** Mix gently 1,300 μL of pre-diluted Eu Ab1 with 1,300 μL of pre-diluted FR Ab2.

SAMPLE PREPARATION

- Each well requires 15 μ L of sample (cell supernatant).
- Just after their collection, put the samples at +4°C and assay immediately or aliquot and store -80°C. Avoid repeated freeze/thaw cycles.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- If the analyte concentration in the sample possibly exceeds the highest point of the standard curve, prepare one or more 10-fold dilutions of the sample. Prepare serial dilutions using the same medium used to culture the cells.

ASSAY PROCEDURE

- Samples and standards must be assayed at least in duplicate each time the assay is performed.
- When loading reagents in the low-volume 384-well microplate, change tips between each standard or sample addition and after each set of reagents.
- If using a multichannel pipettor, always use a new disposable reagent reservoir.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
 - Add 15 μ L of each working standard or 15 μ L of cell supernatant sample.
 - Add 5 μ L of 4X Antibody Detection Mix (Eu-Ab1 + FR-Ab2) to each of the assay wells.
 - Cover the plate with a plate sealer and incubate 2 hours at room temperature.
 - Gently remove the adhesive plate sealer. Read plate on a TR-FRET compatible microplate reader.

NOTE *The same plate can be read several times without any negative effect on the assay performance.*

TR-FRET PLATE READER SETTINGS

For optimal assay performance, we recommend that you read the TR FRET assays at two wavelengths, detecting both the emission from the Europium chelate donor fluorophore at 615 nm, and the acceptor fluorophore at 665 nm. The following instrument settings are provided as guidelines.

Parameter	TR-FRET Compatible Plate Reader*	
	Flash lamp excitation	Laser excitation
Excitation filter	320 nm (or 340 nm)	Not applicable
Emission filter	615 nm (or 620 nm)	615 nm (or 620 nm)
Delay time	90 μ s	50 μ s
Flash energy level	100% or High	100%
Number of flashes	100-200	20
Window (integration time)	300 μ s	100 μ s

* These settings are provided as guidelines only. Settings should be optimized for each reader. For optimal settings, visit bioauxilium.com/resources.

DATA ANALYSIS

- TR-FRET data are typically calculated and presented ratiometrically using the following formula: $[(665 \text{ nm}/615 \text{ nm}) \times 1,000]$
- Alternatively, the signals at 665 nm can be used directly to process your data.
- Calculate the TR-FRET ratio for each well.
- Since TR-FRET signal is read in a time-resolved mode, background subtraction is usually not necessary.
- Create a standard curve by analyzing data according to a nonlinear regression using the 4 parameter logistic equation (sigmoidal dose-response curve with variable slope) and a $1/Y^2$ data weighting. For correct analysis, the highest standard point should be removed from the curve if it is present after the hook point (i.e., if it shows lower counts compared to the next standard).
- Do not use a value of zero for the zero standard. Use instead the actual background values obtained with the zero standard.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE

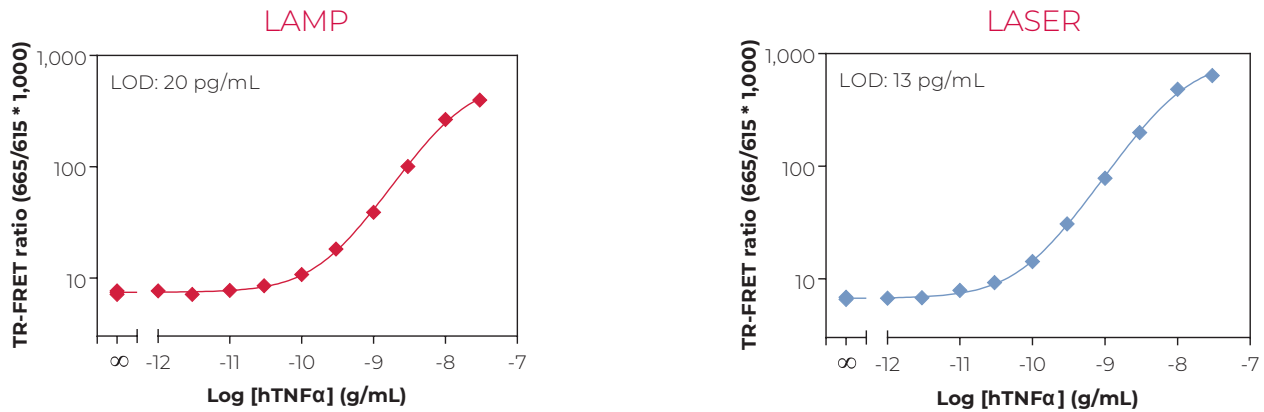


Figure 3 Typical nonlinear human TNF α standard curves in Assay Buffer 2. Left: lamp excitation, Right: laser excitation. Data represent the mean \pm standard deviation of triplicate measurements (3 wells) for each standard. The highest standard (1.0E-7) was omitted from the standard curve. The data was generated using a low-volume 384-well white plate read on the EnVision[®] equipped with TR-FRET option.

VALIDATION DATA

The THUNDER[™] human TNF α assay kit has been validated using human TNF α protein from R&D Systems[®] (Catalog number 210-TA; NOT included in the kit).

HUMAN TNF α THUNDER [™] ASSAY SUMMARY		
Assay Type	Homogeneous sandwich immunoassay	
Format	384-well plate	
Hands-on time	15 minutes	
Incubation time	2 hours	
Sample type (volume)	Cell culture supernatant (15 μ L)	
Specificity	Natural and recombinant human TNF α	
	Laser excitation	Lamp excitation
Limit of Detection (LOD)	13 pg/mL	20 pg/mL
Lower Limit of Quantification (LLOQ)	62 pg/mL	114 pg/mL
EC ₅₀	8.6 ng/mL	12 ng/mL
Dynamic Range	13 – 30,000 pg/mL	20 – 30,000 pg/mL

SENSITIVITY

The Limit of Detection (LOD) was calculated by adding two standard deviations to the mean background counts of 12 zero standard replicates and calculating the corresponding concentration on the standard curve.

The Lowest Level of Quantitation (LLOQ) was calculated by adding 10 standard deviations to the mean background counts of 12 zero standard replicates and calculating the corresponding concentration on the standard curve.

	ASSAY BUFFER 2		DMEM +10% FBS		RPMI +10% FBS	
	Laser	Lamp	Laser	Lamp	Laser	Lamp
# of standard curves	9	9	6	6	6	6
LOD (pg/mL)	13	20	31	42	18	35
LLOQ (pg/mL)	62	114	144	215	75	171

PRECISION

INTRA-ASSAY PRECISION

Three samples of known concentration (low, medium, high) were tested twenty times each on the same plate to assess intra-assay precision. The assay was run in a 384-well plate with standards and samples prepared in Assay Buffer 2. The resulting low %CV (<10%) for each sample indicates good reproducibility within an assay.

Sample concentration	Low	Med	High
# of samples	21	21	21
Mean (pg/mL)	753	2117	11359
SD (pg/mL)	54	65	629
CV (%) Intra-Assay	7.2	3.1	5.5

INTER-ASSAY PRECISION

Three samples of known concentration were tested in separate assays to assess inter-assay precision. A total of seven independent experiments were performed by two operators using two different kit lots: The assay was run in 384-well plates with standards and samples prepared in Assay Buffer 2 and tested in triplicate. The resulting low %CV (<10%) for each sample indicates good reproducibility between assays.

Sample concentration	Low	Med	High
# of samples	30	30	30
Mean (pg/mL)	842	2408	9991
SD (pg/mL)	61	204	890
CV (%) Intra-Assay	8.9	8.5	7.3

RECOVERY

To assess the accuracy of the assay, spike-to-recovery experiments were conducted. Each assay consisted of one standard curve and three spike concentrations (low, medium and high), assayed in triplicate in Assay Buffer 2, DMEM and RPMI (supplemented with 10% FBS). The average recovery was calculated from the average of 9 wells and compared to a standard curve prepared in the corresponding diluent. Data show recovery values within the range of 80–120% demonstrating assay accuracy.

	Spike level	Expected (pg/mL)	Observed (pg/mL)	Average % Recovery
Assay Buffer 2	High	10000	11560	116
	Medium	3000	3513	117
	Low	1000	944	94
DMEM + 10% FBS	High	10000	8268	83
	Medium	3000	2641	88
	Low	1000	858	86
RPMI + 10% FBS	High	10000	10184	102
	Medium	3000	3361	112
	Low	1000	879	88

LINEARITY

To assess the linearity of the assay, samples were spiked with a high concentration of analyte in culture medium supplemented with 10% FBS and diluted with the same culture medium with 10% FBS. The assay was run in a 384-well plate with standards prepared in culture media with 10% FBS. Each sample was measured in triplicate. Data show recovery values within 80-120% confirming assay linearity.

	Expected (pg/mL)	Observed (pg/mL)	Average % Recovery
DMEM + 10% FBS	25 000	26,175	105
	12 500	12,614	101
	6 250	6,214	99
	3 125	3,581	115
	1 563	1,726	110
RPMI + 10% FBS	25 000	29,184	117
	12 500	15,006	120
	6 250	7,142	114
	3 125	3,652	117
	1 563	1,817	116

