



## TNF- $\alpha$ (human) TR-FRET Biomarker Assay Kit

*Powered by Bioauxilium's THUNDER™ TR-FRET Technology*

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Item No. 500234

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## GENERAL INFORMATION

### Materials Supplied

Item Number	Item Name	96 wells Quantity/Size	480 wells Quantity/Size
400284	Europium-Labeled TNF- $\alpha$ Antibody	1 vial/5 $\mu$ l	1 vial/25 $\mu$ l
400285	Acceptor-Labeled TNF- $\alpha$ Antibody	1 vial/20 $\mu$ l	1 vial/100 $\mu$ l
400296	Assay Buffer 2 (5X)	1 vial/1 ml	3 vials/1 ml

To request this kit in bulk quantities, please contact our Sales department. If any of the items listed above are damaged or missing, please contact our Customer Service department. Sales and Customer Service departments can be reached at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.



**WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.**

### Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

## Precautions

**Please read these instructions carefully before beginning this assay.**

Do not mix or substitute reagents or materials from other kit lots or kits. Kits are quality control tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

We cannot guarantee the performance of the product outside the conditions detailed in this kit booklet.

## Before You Start

**Please note the following:**

ONLY white plates should be used for TR-FRET.

DO NOT modify the assay protocol or volumes.

## If You Have Problems

### **Technical Service Contact Information**

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

## Storage and Stability

This kit will perform as specified if stored as directed at -80°C and used before the expiration date indicated on the outside of the box.

## THUNDER™ General Information

THUNDER™ TR-FRET Biomarker Assay Kits are designed for the quantitative measurement of human cytokines in cell culture supernatants using homogeneous (no wash) TR-FRET technology.

THUNDER™ TR-FRET Biomarker Assay Kits are based on Bioauxilium's enhanced proprietary time-resolved Förster resonance energy transfer (TR-FRET) technology. THUNDER™ assays can be read on most commercially available TR-FRET-compatible plate readers (a list of suitable TR-FRET readers can be found at [www.Bioauxilium.com](http://www.Bioauxilium.com)). TR-FRET-based assays are homogeneous because they do not require any washing or separation steps. In addition, the THUNDER™ assays use a standardized, simple, and rapid "add-incubate-measure" protocol with a single step reagent addition. This streamlined assay protocol dramatically decreases hands-on time and provides a powerful alternative to cumbersome, error-prone and time-consuming techniques such as Western blot and ELISA.

THUNDER™ TR-FRET Biomarker Assay Kits do not require the addition of potassium fluoride to ensure signal stability. The non-toxic nature of THUNDER™ kits make them ideal candidates for multiplexing with other assay technologies using a sequential assay protocol.

THUNDER™ TR-FRET Biomarker Assay Kits contain the essential reagents necessary to carry out the measurement of human cytokines in cell culture supernatants, with the exception of the standard and the plate(s).

## Materials Needed But Not Supplied

1. A plate reader equipped with a TR-FRET option
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. A source of ultrapure water, with a resistivity of 18.2 MΩ·cm and total organic carbon (TOC) levels of <10 ppb, is recommended. Pure water - glass-distilled or deionized - may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000).*
4. Low-volume 384-well, or half-area 96-well, white plates
5. Recombinant human TNF-α standard, available from R&D Systems® (Catalog No. 210-TA).
6. Adhesive sealing film for plates

## INTRODUCTION

### Background

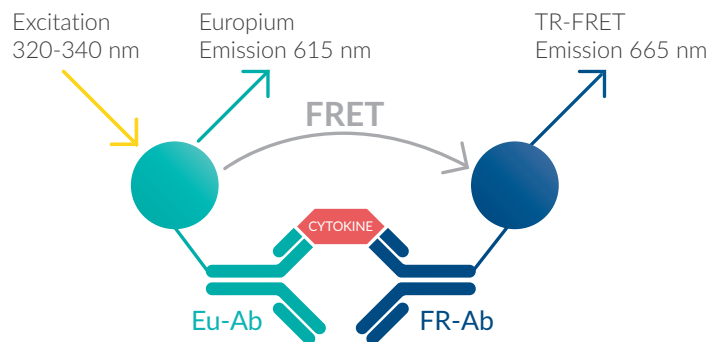
TNF-α is a cytokine and member of the TNF/TNF receptor (TNFR) cytokine superfamily.<sup>1</sup> TNF-α is produced as a 233-amino acid transmembrane precursor protein from which mature, 157-amino acid soluble TNF-α is formed.<sup>2</sup> It is primarily produced by activated macrophages but can also be produced by a variety of other cells, such as T cells, natural killer cells, and osteoblasts.<sup>3,4</sup> TNF-α binds to and activates its receptors, TNFR1 and TNFR2, which are associated with intracellular protein complexes that activate caspases to induce cell death, induce p38 MAPK signaling, and initiate NF-κB or AP-1-mediated transcription of immune and inflammatory mediators.<sup>5</sup> TNF-α promotes inflammation partly by inducing endothelial cells to express adhesion molecules, COX enzymes, and pro-coagulant factors.<sup>4</sup> TNF-α is produced in the inflamed tissues of patients with inflammatory diseases such as rheumatoid arthritis, and neutralizing antibodies to TNF-α reduce the levels of TNF-α *in vitro* and in mouse models of the disease.<sup>4</sup> Additionally, anti-TNF-α monoclonal antibodies are used to inhibit the actions of TNF-α in patients with chronic inflammatory disorders.<sup>6</sup> *Tnf*<sup>-/-</sup> mice exhibit increased susceptibility to certain microbial infections and lack resistance to leishmaniasis, indicating a role in infectious disease resistance.<sup>5</sup> *Tnf* knockout mice are also resistant to certain types of cancer, including chemically induced skin carcinogenesis.<sup>1</sup> Exogenous TNF-α induces death of cancer cells *in vitro*, as well as disrupts tumor vascularization and induces necrosis *in vivo*, but it has tumor promoting properties when produced in the cancer microenvironment.<sup>1,7</sup>

### About This Assay

This TNF-α (human) TR-FRET Biomarker Assay Kit uses a homogenous TR-FRET sandwich immunoassay method amenable to rapid measurement of TNF-α levels in cell culture supernatants. The standard curve spans a dynamic range of 13-10,000 pg/ml and a lower limit of quantification (LLOQ) of 62 pg/ml using the assay buffer included in the kit with laser excitation. The amount of reagents provided is sufficient for testing either 96 or 480 wells, depending on the size of the kit.

## Principle Of This Assay

This assay is a homogenous TR-FRET-based sandwich immunoassay (Figure 1, below). Native or recombinant human TNF- $\alpha$  in cell culture supernatant samples is detected with a pair of fluorophore-labeled antibodies reactive to human TNF- $\alpha$ .



**Figure 1. Schematic of the TR-FRET biomarker assay principle**

The first antibody is labeled with a long-lifetime donor fluorophore (a europium chelate; Eu-Ab) and the second with a far-red acceptor fluorophore (FR-Ab). 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 Eu chelate molecules with a flash lamp (320 or 340 nm) or laser (337 nm) triggers a FRET from the donor to the acceptor molecules, which, in turn, emit a TR-FRET signal at 665 nm. The signal at 665 nm is proportional to the concentration of human TNF- $\alpha$  in the cell culture supernatant. Residual energy from the Eu chelate generates light at 615 nm.

TR-FRET assays exhibit very low background fluorescence levels and high signal-to-background (S/B) ratios. The data can be expressed and analyzed as either the signal at 665 nm or the 665 nm/615 nm ratio. The ratiometric measurement further increases assay reproducibility and robustness.

## PRE-ASSAY PREPARATION

### Reagent Preparation

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

Bring all reagents to room temperature prior to use.

Centrifuge all tubes before use to improve recovery of content (2,000 xg, 10-15 seconds).

Use ultrapure water (18 M $\Omega$ -cm) to dilute the Assay Buffer 2 (5X).

**NOTE:** It is recommended to test all standards in triplicate and samples at least in duplicate.

#### 1. Assay Buffer 2 (1X)

Mix the Assay Buffer 2 (5X) (Item No. 400296) end-over-end before use. The thawed Assay Buffer 2 (5X) will be stable for 1-2 weeks when stored at 4°C or for longer periods of time when stored at -80°C.

Add 1 ml (96 wells) or 3 ml (480 wells) of Assay Buffer 2 (5X) with 4 ml (96 wells) or 12 ml (480 wells) of ultrapure water. Store unused Assay Buffer 2 (1X) at 4°C; it will be stable for approximately two days.

## 2. TNF- $\alpha$ Standard Serial Dilutions

Recombinant human TNF- $\alpha$  is not included in the kit. The kit has been validated using recombinant human TNF- $\alpha$  from R&D Systems® (Catalog No. 210-TA) in a comparable manner. Other commercial recombinant human TNF- $\alpha$  proteins may also work but have not been tested with the kit.

Prepare standard solutions just before use and use within one hour. Do NOT store the standard solutions.

Each well requires 15  $\mu$ l of serially diluted standards.

Reconstitute the analyte following the manufacturer's instructions (typically 100-200  $\mu$ g/ml) to create a stock solution. Dilute the stock solution in either Assay Buffer 2 (1X) or culture medium containing 10% FBS, depending on which will be used for the standard curve dilutions, to create a 1  $\mu$ g/ml TNF- $\alpha$  working stock solution. The working stock solution will be used to prepare a standard dilution series.

*NOTE: If all of the working stock solution will not be used within one hour, aliquot into screw-capped polypropylene vials and store at -80°C for long-term storage. The undiluted stock solution may also be stored at -80°C.*

To prepare the standard solutions for use: obtain 12 clean test tubes and label them #1-12. Alternatively, serial dilutions can be conducted using a 96-well polypropylene plate.

Prepare serial dilutions for the standard curve as indicated in Table 1, on page 11, using either Assay Buffer 2 (1X) or culture medium containing 10% FBS. Change the pipette tip between each standard dilution. Mix each tube thoroughly between each transfer.

Tube	Volume of TNF- $\alpha$ Standard	Volume of Assay Buffer 2 (1X) OR Culture Medium + 10% FBS ( $\mu$ l)	TNF- $\alpha$ in standard curve (g/ml in 15 $\mu$ l)
1	10 $\mu$ l diluted TNF- $\alpha$ (1 $\mu$ g/ml)	90	1.0E-7
2	30 $\mu$ l of tube 1	70	3.0E-8
3	30 $\mu$ l of tube 2	60	1.0E-8
4	30 $\mu$ l of tube 3	70	3.0E-9
5	30 $\mu$ l of tube 4	60	1.0E-9
6	30 $\mu$ l of tube 5	70	3.0E-10
7	30 $\mu$ l of tube 6	60	1.0E-10
8	30 $\mu$ l of tube 7	70	3.0E-11
9	30 $\mu$ l of tube 8	60	1.0E-11
10	30 $\mu$ l of tube 9	70	3.0E-12
11	30 $\mu$ l of tube 10	60	1.0E-12
12*	0	70	0

**Table 1. Standard curve serial dilutions**

\*In order to calculate the limit of detection (LOD), three additional zero-standard tubes per plate should be prepared and assayed in triplicate.

### 3. Antibody Detection Mix (4X)

*NOTE: Due to the low reagent volumes, the antibodies are diluted with Assay Buffer 2 (1X) directly in the vial when assaying only 96 wells.*

*NOTE: Each well requires 5 µl of Antibody Detection Mix (4X).*

Prepare and mix just before use.

**Antibody Detection Mix (4X) (96 wells):** Add 255 µl of Assay Buffer 2 (1X) into the vial containing 5 µl of Europium-Labeled TNF-α Antibody (Eu-Ab) (Item No. 400284). Add 240 µl of Assay Buffer 2 (1X) into the vial containing 20 µl of Acceptor-Labeled TNF-α Antibody (FR-Ab) (Item No. 400285). Gently mix the diluted Eu-Ab and FR-Ab solutions together.

OR

**Antibody Detection Mix (4X) (480 wells):** Gently mix 25 µl of Eu-Ab (Item No. 400284) with 1,275 µl of Assay Buffer 2 (1X). Gently mix 100 µl of FR-Ab (Item No. 400285) with 1,200 µl of Assay Buffer 2 (1X). Gently mix the diluted Eu-Ab and FR-Ab solutions together.

Store unused Antibody Detection Mix (4X) at 4°C; it will be stable for approximately two days. If all unused Antibody Detection Mix (4X) will not be used within two days, aliquot and store at -80°C. Avoid repeated freeze/thaw cycles.

### Sample Preparation

Each well requires 15 µl of sample (cell culture supernatant).

After collection, place the samples at 4°C and assay immediately or aliquot and store at -80°C.

Avoid repeated freeze/thaw cycles. Thaw samples at room temperature. Do NOT use a water bath to thaw samples.

Prepare serial dilutions using the same media used to culture the cells. 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.

## ASSAY PROTOCOL

### Workflow

The THUNDER™ TR-FRET Cell Signaling Assay workflow consists of a one-step assay reagent addition followed by an incubation period.



Figure 2. Assay workflow

### Performing the Assay

Samples and standards **MUST** be assayed at least in duplicate (triplicate recommended) each time the assay is performed.

When loading reagents in the low-volume 384-well microplate, change pipette 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, ensure the liquids are at the bottom of the well.

1. Add 15 µl of each working standard or 15 µl of the supernatant sample to the designated wells.
2. Add 5 µl of Antibody Detection Mix (4X) to each of the assay wells.
3. Cover the plate with adhesive plate-sealing film and incubate for 2 hours at room temperature.
4. Gently remove the adhesive plate-sealing film. Read the plate on a TR-FRET-compatible microplate reader (see Table 2, page 15).

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

### TR-FRET Plate Reader Settings

We recommend reading the TR-FRET assays at two wavelengths, detecting both the emission from the Eu chelate donor fluorophore at 615 nm and the acceptor fluorophore at 665 nm. Table 2, below, provides instrument settings to be used as guidelines.

Parameter	TR-FRET-compatible Plate Reader	
	Flash lamp excitation	Laser excitation
Excitation filter	320 nm (or 340 nm)	N/A
Emission filter	615 nm (or 620 nm)	615 nm (or 620 nm)
Delay time	90 µs	50 µs
Flash energy level	100% or High	100%
Number of flashes	100-200	20
Window (integration time)	300 µs	100 µs

Table 2. Recommended TR-FRET plate reader settings



## Calculations

Because the TR-FRET signal is read in a time-resolved manner, background subtraction is usually not necessary.

Do not use a value of zero for the zero standard. Use 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.

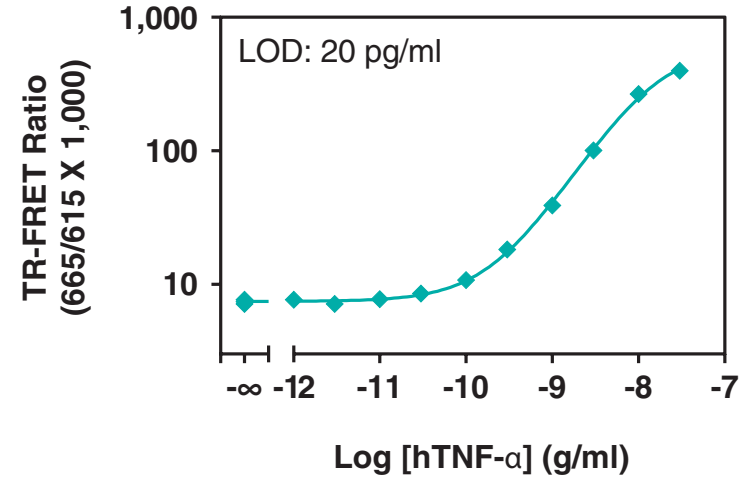
1. TR-FRET data are typically calculated and presented ratiometrically using the following formula:

$$[(665 \text{ nm}/615 \text{ nm}) \times 1,000]$$

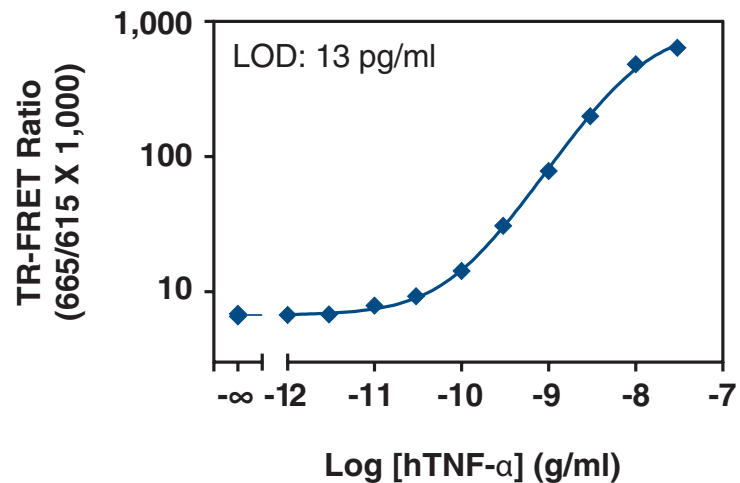
2. Calculate the TR-FRET ratio for each well. Alternatively, the signals at 665 nm can be used directly to analyze the data.
3. Create a standard curve by analyzing the data according to a nonlinear regression using the four-parameter logistic equation (sigmoidal dose-response curve with variable slope) 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).

## Performance Characteristics

### Representative Data



**Figure 3. Typical standard curve using lamp excitation** Data represent the mean  $\pm$  standard deviation of triplicate measurements (three wells) for each standard dilution. 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 with an EnVision® Multilabel Plate Reader equipped with a TR-FRET option.



**Figure 4. Typical standard curve using laser excitation** Data represent the mean  $\pm$  standard deviation of triplicate measurements (three wells) for each standard dilution. 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 with an EnVision® Multilabel Plate Reader equipped with a TR-FRET option.

### 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 lower limit of quantification (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 (1X)		DMEM + 10% FBS		RPMI + 10% FBS	
	Laser	Lamp	Laser	Lamp	Laser	Lamp
# of Standard Curves	9	9	6	6	6	6
Standard Curves LOD (pg/ml)	13	20	31	42	18	35
LLOQ (pg/ml)	62	114	144	215	75	171

**Table 3. Sensitivity of the TNF-α (human) TR-FRET Assay Kit**

## 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 the standards and samples prepared in Assay Buffer 2 (1X). The resulting low %CV (<10%) for each sample indicates good reproducibility within an assay.

	Sample Concentration		
	Low	Medium	High
# of Samples	21	21	21
Mean (pg/ml)	753	2,117	11,359
SD (pg/ml)	54	65	629
CV (%) Intra-Assay	7.2	3.1	5.5

Table 4. Intra-assay precision

### 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 the standards and samples prepared in Assay Buffer 2 (1X) and tested in triplicate. The resulting low %CV (<10%) for each sample indicates good reproducibility between assays.

	Sample Concentration		
	Low	Medium	High
# of Samples	30	30	30
Mean (pg/ml)	842	2,408	9,991
SD (pg/ml)	61	204	890
CV (%) Intra-Assay	8.9	8.5	7.3

Table 5. Inter-assay precision

## Recovery

To assess the accuracy of the assay, spike-to-recovery experiments were conducted using Assay Buffer 2 (1X) and culture media. Each assay consisted of one standard curve and three spike concentrations (low, medium, high) assayed in triplicate in Assay Buffer 2 (1X), DMEM (supplemented with 10% FBS), or RPMI (supplemented with 10% FBS). The percent recovery is reported as the mean concentration of nine wells determined using a standard curve prepared in the corresponding diluent. The data shows recovery values within the range of 80-120% demonstrating assay accuracy.

	Spike Level	Expected (pg/ml)	Observed (pg/ml)	% Recovery
Assay Buffer 2 (1X)	High	10,000	11,560	116
	Medium	3,000	3,513	117
	Low	1,000	944	94
DMEM + 10% FBS	High	10,000	8,268	83
	Medium	3,000	2,641	88
	Low	1,000	858	86
RPMI + 10% FBS	High	10,000	10,184	102
	Medium	3,000	3,361	112
	Low	1,000	879	88

Table 6. Recovery in assay buffer or culture media

## Linearity

To assess the linearity of the assay, a high concentration of TNF- $\alpha$  was spiked into 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 the same type of supplemented culture medium. Each sample was measured in triplicate. Data shows recovery values within 80-120% confirming assay linearity.

	Expected (pg/ml)	Observed (pg/ml)	% Recovery
DMEM + 10% FBS	25,000	26,175	105
	12,500	12,614	101
	6,250	6,214	99
	3,125	3,581	115
RPMI + 10% FBS	1,563	1,726	110
	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

Table 7. Dilutional linearity in culture medium

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 µl)	
Specificity	Native 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 <sub>50</sub> Value	8.6 ng/ml	12 ng/ml
Dynamic Range	13-30,000 pg/ml	20-30,000 pg/ml

Table 8. Assay validation summary for the TNF-α (human) TR-FRET Biomarker Assay Kit

## RESOURCES

### References

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## NOTES

### Warranty and Limitation of Remedy

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