MANUAL

LANCE *Ultra* TNFα (Human) Detection Kit

Product number: TRF1208 C/M

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed for the quantitative determination of hTNFα in media using a homogeneous LANCE *Ultra* assay (no wash steps).

Sensitivity: Lower Detection Limit (LDL): 9.3 pg/mL

Lower Limit of Quantification (LLOQ): 47.4 pg/mL

EC_{50}: 6.3 ng/mL

Dynamic range: Kit designed to detect hTNFα between: 9.3– 30,000 pg/mL (Figure 1).

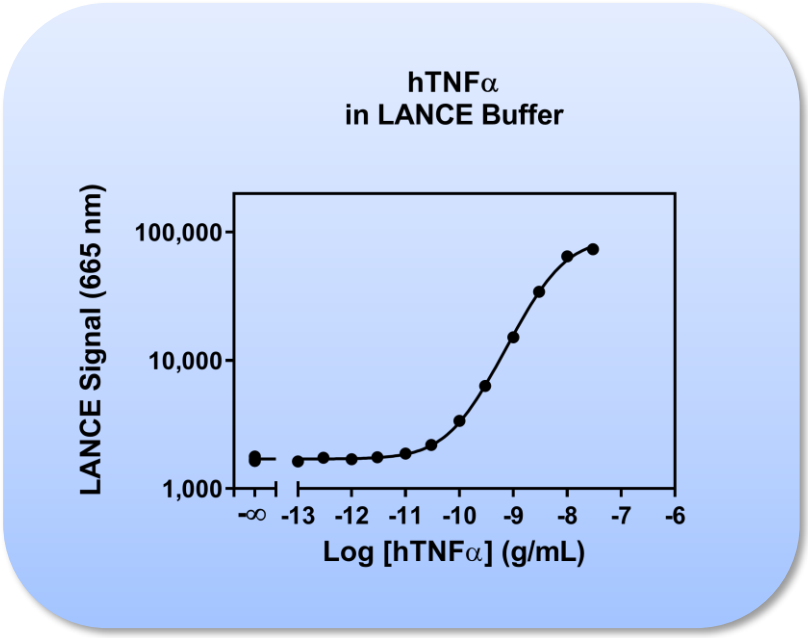

Figure 1. Typical sensitivity curves in LANCE Assay Buffer. The data was generated using a white Optiplate™-384 microplate and the VICTOR *X*, ViewLux, EnVision or EnSpire Multilabel Plate Reader equipped with TR-FRET option

Storage: Store kit in the dark at +4˚C. Store reconstituted analyte at -20°C.

Stability: This kit is stable for at least 6 months from the manufacturing date when stored in its original packaging and the recommended storage conditions. Note: Once reconstituted, the hTNFα analyte is stable for at least 45 days when stored at -20°C.

Analyte of Interest

Tumor necrosis factor alpha (TNFα) is a multifunctional proinflammatory cytokine synthesized mainly by nucleated blood cells as a 233 aa type II transmembrane protein which is cleaved by ADAM17 between aa 76-77 to form a soluble homotrimeric complex. TNFα plays a role in lipid metabolism, coagulation, and endothelial function and has been associated with cancer, infection and inflammation (including inflammatory bowel disease), ischemia/reperfusion injury and heart failure, and insulin resistance.

Description of the LANCE *Ultra* Assay

LANCE® and LANCE® (Lanthanide chelate excite) *Ultra* are our TR-FRET (time-resolved fluorescence resonance energy transfer), homogeneous (no wash) technologies. One antibody of interest is labeled with a donor fluorophore (a LANCE Europium chelate) and the second molecule is labeled with an acceptor fluorophore [U*Light*™ dye]. Upon excitation at 320 or 340 nm, energy can be transferred from the donor Europium chelate to the acceptor fluorophore if sufficiently close for FRET (~10 nm). This results in the emission of light at 665 nm.

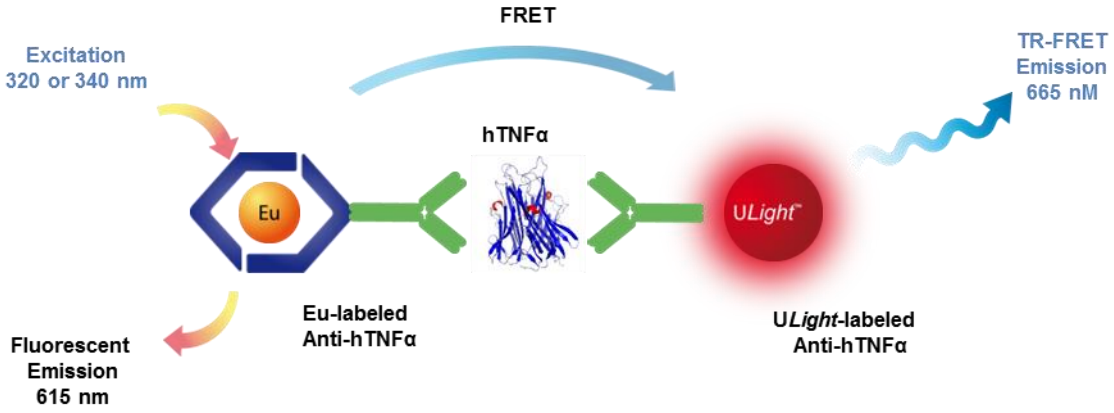

Figure 2. LANCE assay principle.

Precautions

- All blood components and biological materials should be handled as potentially hazardous.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.

Kit Content: Reagents and Materials

Kit components	TRF1208C (500 assay points***)	TRF1208M (10 000 assay points***)
Lance Ultra Eu-labeled Anti-hTNFα Antibody stored in TSA buffer, 0.1% BSA	10 µL @ 500 nM (1 clear tube, yellow cap)	120 µL @ 500 nM (1 clear tube, orange cap)
Lance Ultra U*Light*-labeled Anti-hTNFα Antibody stored in TSA buffer, 0.1% BSA	60 µL @ 500 nM (1 brown tube, blue cap)	1200 µL @ 500 nM (1 brown tube, green cap)
hTNFα Analyte* lyophilized	0.1 µg (1 tube, clear cap)	0.1 µg (1 tube, clear cap)
LANCE Assay Buffer (10X)**	2 mL, 1 small bottle	100 mL, 1 large bottle

* Reconstitute hTNFα in 100 µL Milli-Q® grade H_2O. The reconstituted analyte should be used within 60 minutes or aliquoted into screw-capped polypropylene vials and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles. It has been demonstrated that reconstituted hTNFα is stable for at least 45 days at -20°C. One vial contains an amount of hTNFα sufficient for performing 10 standard curves. Additional vials can be ordered separately (cat # TRF1208S).

** Extra buffer can be ordered separately (cat # TRFLAB002: 2 mL, cat # TRFLAB100: 100 mL).

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

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Plus Adhesive Sealing Film	Revvity Inc.	6050185
VICTOR X, ViewLux, EnVision or EnSpire Multilabel Plate Reader equipped with TR-FRET option	Revvity Inc.	-

Recommendations

General recommendations:

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec).
- Re-suspend all reagents by vortexing before use.
- Use Milli-Q® grade H_2O (18 MΩ•cm) to dilute Buffer.
- When diluting the standard or samples, change tips between each standard or sample dilution. When loading reagents in the assay microplate, change tips between each standard or sample addition and after each set of reagents.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal-A Adhesive Sealing Films to reduce evaporation during incubation. LANCE *Ultra* TR-FRET assays cannot be read with the TopSeal-A Film attached. Please remove before reading.
- LANCE signal is detected using a VICTOR X, ViewLux, EnVision or EnSpire Multilabel Reader equipped with the TR-FRET. Use an excitation wavelength of 320 or 340 nm to excite the LANCE Europium chelate. We recommend you read this assay in dual emission mode, detecting both the emission from the Europium donor fluorophore at 615 nm, and the acceptor fluorophore (at 665 nm for U*Light* dye). The raw FRET signal at 665 nm can be used to process your data.
- Signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.
- The standard curves shown in this technical data sheet are provided for information only. A standard curve must be generated for each experiment. The standard curve should be performed in LANCE Assay Buffer

Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating one standard curve in a 20 µL final assay volume (48 wells, triplicate determinations) and 452 samples. The manuals also include testing samples in 384 well plates. If different amounts of samples are tested, the volumes of all reagents must be adjusted accordingly, as shown in the table below. ****These calculations do not include excess reagents to account for losses during transfer of solutions or dead volumes.*
- The standard dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.
- Use of four background points in triplicate (12 wells) is recommended when LDL/LLOQ is calculated. One background point in triplicate (3 wells) can be used when LDL/LLOQ is not calculated.

		Volume			
Format	**# of data points**	**Final**	**Sample**	**Eu-Antibody/U*Light*-Antibody MIX**	**Plate recommendation**
TRF1208C	250	40 µL	30 µL	10 µL	White OptiPlate-96 (cat # 6005290) White ½ AreaPlate-96 (cat # 6005560)
	500	20 µL	15 µL	5 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290)
	1 250	8 µL	6 µL	2 µL	ProxiPlate™-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	2 500	4 µL	3 µL	1 µL	White OptiPlate-1536 (cat # 6004290)
TRF1208M	5 000	20 µL	15 µL	5 µL	White ½ AreaPlate-96 (cat # 6005560) White OptiPlate-384 (cat # 6007290)
	12 500	8 µL	6 µL	2 µL	ProxiPlate-384 Plus (cat # 6008280) White OptiPlate-384 (cat # 6007290)
	25 000	4 µL	3 µL	1 µL	White OptiPlate-1536 (cat # 6004290)

General Manual (1-step manual): Dilute standards, samples, and assay components in 1X LANCE Assay Buffer.

Each manual described below is designed for <u>500 assay points</u> including one standard curve (48 wells) and samples (452 wells).

Standard Preparation:

1) <u>Preparation of 1X LANCE Assay Buffer</u>:
    a. Add 1 mL of 10X Assay LANCE Assay Buffer to 9 mL H_2O.

2) <u>Preparation of hTNFα analyte standard dilutions</u>:
    a. hTNFα analyte is provided at 0.1 µg in lyophilized form. Reconstitute with 100 µL H_2O to create a 1 µg/mL solution. Prepare standard dilutions as follows (change tip between each standard dilution):

Tube	Vol. of hTNFα (µL)	Vol. of diluent (µL) *	[hTNFα] in standard curve	
			(g/mL in 15 µL)	(pg/mL in 15 µL)
A	10 µL of reconstituted hTNFα	90	1.00E-07	100 000
B	30 µL of tube A	70	3.00E-08	30 000
C	30 µL of tube B	60	1.00E-08	10 000
D	30 µL of tube C	70	3.00E-09	3 000
E	30 µL of tube D	60	1.00E-09	1 000
F	30 µL of tube E	70	3.00E-10	300
G	30 µL of tube F	60	1.00E-10	100
H	30 µL of tube G	70	3.00E-11	30
I	30 µL of tube H	60	1.00E-11	10
J	30 µL of tube I	70	3.00E-12	3
K	30 µL of tube J	60	1.00E-12	1
L	30 µL of tube K	70	3.00E-12	0.3
M ** (background)	0	100	0	0
N ** (background)	0	100	0	0
O ** (background)	0	100	0	0
P ** (background)	0	100	0	0

* At low concentrations of analyte, a significant amount of analyte can bind to the vial. Therefore, load the analyte standard dilutions in the assay microplate within 60 minutes of preparation.

** Four background points in triplicate (12 wells) are used when LDL is calculated. If LDL does not need to be calculated, one background point in triplicate can be used (3 wells).

3) Preparation of 4X MIX Eu-labeled anti-hTNFα Antibody (1.2 nM) + U*Light* labeled anti-hTNFα Antibody (12 nM):
   a. Add 6 µL of 500 nM Eu-labeled anti-hTNFα Antibody and 60 µL of 500 nM U*Light*-labeled anti-hTNFα Antibody to 2434 µL of 1X LANCE Assay Buffer.
   b. Prepare just before use.

4) In a white Optiplate (384 wells):

Add 15 µL of each analyte standard dilution or 15 µL of sample

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Add 5 µL of a 4X MIX Eu-labeled anti-hTNFα Antibody (0.3 nM final) + U*Light* labeled anti-hTNFα Antibody (3 nM final)

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Incubate 60 minutes at 23˚C

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Read using LANCE TRF Laser (in TR-Fret mode)

Important: LANCE signal is detected using an EnVision Multilabel Reader equipped with the TR-FRET. Use an excitation wavelength of 320 or 340 nm to excite the LANCE Europium chelate. We recommend you read this assay in dual emission mode, detecting both the emission from the Europium donor fluorophore at 615 nm, and the acceptor fluorophore (at 665 nm for U*Light* dye).

Data Analysis

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the LANCE counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze 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 (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- The LDL is calculated by interpolating the average background counts (12 wells without analyte) + 2 x standard deviation value (average background counts + (2xSD)) on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value (average background counts + (10xSD)) on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Read from the standard curve the concentration of analyte contained in the samples.
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Assay Performance Characteristics

LANCE Ultra assay performance described below was determined using the 1 step manual.

Assay Sensitivity

The LDL and LLOQ were calculated as described above. The values correspond to the lowest concentration of analyte that can be detected in a volume of 15 µL using the recommended assay conditions.

LDL (pg/mL)	LLOQ (pg/mL)	Buffer	# of experiments
9	47	LANCE Assay	9
10	37	DMEM	6
20	69	RPMI	6

Assay Precision:

The following assay precision data were calculated from the three independent assays using two different kit lots. In each lot, the analytes were prepared in LANCE Assay Buffer. Each assay consisted of one standard curve comprising 12 data points in triplicate and 12 background wells containing no analyte. The assays were performed in a 384-well format using LANCE Assay Buffer.

Intra-assay precision:

The intra-assay precision was determined using 3 independent experiments for a total of 16 independent determinations in triplicate. CV% were calculated for each individual experiment then averaged. Shown is the average intra-experimental CV%.

hTNFα (CV%)	Buffer
3	LANCE Assay
2	DMEM
3	RPMI

Inter-assay precision:

The inter-assay precision was determined using the data across 3 independent experiments with 16 measurements in triplicate. CV% was calculated by comparing the same measurement in each experiment. The CV% for all 16 measurements were then averaged. Shown is the inter-experimental CV%.

hTNFα (CV%)	Buffer
17	LANCE Assay
12	DMEM
12	RPMI

Spike Recovery:

In four experiments, three known concentrations of hTNFα were spiked into 3 separate media and performed triplicate. The spiked samples were referenced to the hTNFα analyte curve produced in the corresponding media.

Spiked hTNFα (ng/mL)	% Recovery		
	LANCE Assay Buffer	DMEM + 10% FBS	RPMI + 10% FBS
3	84	98	92
1	83	98	84
0.3	85	97	96

Troubleshooting Guide

You will find detailed recommendations for common situations you might encounter with your LANCE *Ultra* Assay kit at:

www.revvity.com

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