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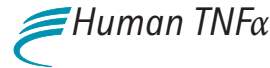
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## HTRF® package insert

Document reference : 62TNFPEB rev07 (July 2008)

For in vitro research use only  
 Storage temperature : 2-8°C

**Packaging details :**

62TNFPEB	384-well low volume plate (20 µL)
	1,000 tests

### 1. Assay description and intended use

This kit is intended for the quantitative determination of human Tumor Necrosis Factor α.

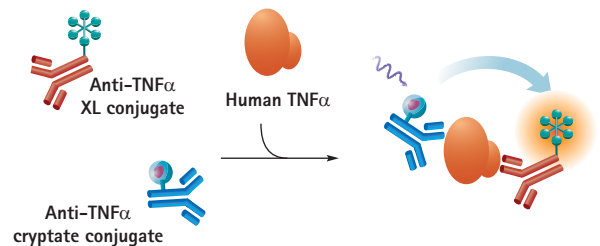
Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown below, the TNFα is detected by an anti-TNFα MAb labeled with XL665, the second MAb is labeled with Cryptate. These mouse MABs recognize distinct epitopes of human TNFα. They do not crossreact with TNFα from other species.

Specific signal (i.e. energy transfer) is proportional to the concentration of TNFα in the sample or standard.

The human TNFα assay can be run under two different protocols:

1. The "supernatant" protocol (see §3), a standard TNFα assessment in cell supernatant : i) cells are stimulated in a regular cell culture plate, and ii) cell supernatant is then transferred to the assay plate.
2. The "cell-based" protocol (see §4) is carried out in a single plate and allows the quantification of TNFα directly on stimulated cells, without any transfer steps.

**CAUTION ! Reagent reconstitution differs between the two protocols. Make sure to reconstitute the conjugates according to the chosen protocol's specifications.**



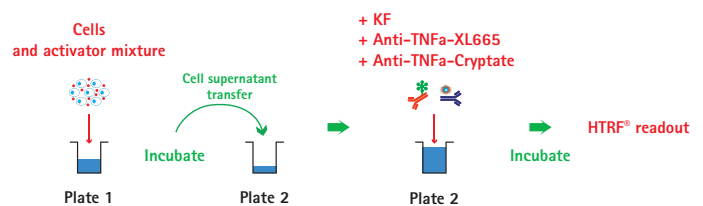
### 2. Background

Tumor Necrosis Factor alpha is a 17 kDa cytokine secreted by activated macrophages and monocytes with a large spectrum of antiviral immunoregulation, metabolic and inflammatory properties. This factor is cytotoxic for some tumor cell lines in vitro and causes the necrosis of certain tumors in vivo. TNFα acts via binding to specific cell surface receptors.

### 3. Supernatant assay protocol

The supernatant assay protocol must be run in two distinct microplates :

- i) a culture plate for cell stimulation and ii) an assay plate in which cytokine detection is carried out.



#### 3.1. Supplied reagents and reconstitution

Supplied reagents	Reagent reconstitution (stock solutions)	Working solutions
Anti-TNFα-Cryptate**	1 vial Lyophilized*	Add 5 mL of reconstitution buffer to each vial. Mix gently
Anti-TNFα-XL665	1 vial Lyophilized*	

Supplied reagents (continued)	Reagent reconstitution (stock solutions)	Working solutions
TNF $\alpha$ calibrator. Concentrated recombinant TNF $\alpha$ .	2 vials Lyophilized*	See label indications for reconstitution volume and concentration. Mix gently.
TNF $\alpha$ control. Concentrated recombinant TNF $\alpha$ .	1 vial Lyophilized*	

Reconstitution buffer 50 mM Phosphate buffer, pH 7.0, 0.8M KF	1 vial See volume on the label
Diluent 50 mM Phosphate buffer, pH 7.0, 0.2 % BSA, 0.02 % NaN <sub>3</sub> , preservatives	1 vial of 20 mL

\* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing BSA protease free and stabilizers.

\*\* The Cryptate conjugate concentration was optimized for a maximum assay sensitivity and to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference PHERAstar Plus reader (BMG LABTECH).

Allow the reagents to warm up at room temperature for at least 30 mins and reconstitute all vials as indicated above.

**Precaution :** HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the XL665 and Cryptate-conjugates will impair the assay's quality.

## 3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels. Once reconstituted, unused Cryptate and XL665 conjugates are stable up to one week at 4°C. They can be refrozen (at -80°C) and thawed once only. TNF $\alpha$  is not stable once reconstituted. Always use fresh-made TNF $\alpha$  solutions and discard unused reagent. Two vials of maximum calibrator are supplied with the kit. Extra vials can be ordered separately (ref 62TNFCDA).

## 3.3. Calibration curve preparation

Reconstitute the maximum calibrator according to the indications printed on the label and follow the dilution sequence shown in the table below to constitute the calibration curve. Dilution must be carried out with the diluent (or with freshly made PO<sub>4</sub> 50 mM, BSA 0.2% pH7).

Calibrator	Working concentration in pg/mL	Preparation
Cal 7 (max calibrator)	2000	200 $\mu$ L of stock solution + 800 $\mu$ L of diluent
Cal 6	1000	500 $\mu$ L Cal 7 + 500 $\mu$ L diluent
Cal 5	500	500 $\mu$ L Cal 6 + 500 $\mu$ L diluent
Cal 4	200	400 $\mu$ L Cal 5 + 600 $\mu$ L diluent
Cal 3	100	500 $\mu$ L Cal 4 + 500 $\mu$ L diluent
Cal 2	50	500 $\mu$ L Cal 3 + 500 $\mu$ L diluent
Cal 1	20	400 $\mu$ L Cal 2 + 600 $\mu$ L diluent

## 3.4. Sample preparation

Dilute all samples to be assayed with the diluent (or with freshly made PO<sub>4</sub> 50 mM, BSA 0.2% pH7). Consecutive dilutions should be made within the 0 to 2000 pg/mL range (working solution).

## 3.5. Assay protocol for 384-well low volume plate (20 $\mu$ L)

⇒ Dispense the reagents in the following order :

- 10  $\mu$ L standard or sample \*
- 5  $\mu$ L Anti-TNF $\alpha$ -Cryptate
- 5  $\mu$ L Anti-TNF $\alpha$ -XL665

\* For negative control, replace the first reagent by 10  $\mu$ L of diluent.

⇒ Cover the plate with a plate sealer and let the incubation take place at room temperature for 3 to 24 hours.

⇒ Read on compatible HTRF® reader (more information about compatible reader at [htrf-assays.com/readers](http://htrf-assays.com/readers)).

## 3.6. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using a 384- well low volume plate in 20  $\mu$ L final assay volume (HTRF® packaged basis).

To move to other plate formats (96 half-well or 1536-well) and final volumes (100  $\mu$ L to less than 10  $\mu$ L), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20  $\mu$ L final assay volume. For instance, in the case of the 1536-well format in 10  $\mu$ L final volume, half as much material per well is used, thereby allowing 2,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Assay components	Volume proportion	Assay format		
		1536-well (10 $\mu$ L)	384-well low volume (20 $\mu$ L)	96 half-well (100 $\mu$ L)
Sample	2 volumes	5 $\mu$ L	10 $\mu$ L	50 $\mu$ L
XL665 conjugate	1 volume	2.5 $\mu$ L	5 $\mu$ L	25 $\mu$ L
Cryptate conjugate	1 volume	2.5 $\mu$ L	5 $\mu$ L	25 $\mu$ L
		2,000 tests	1,000 tests	200 tests

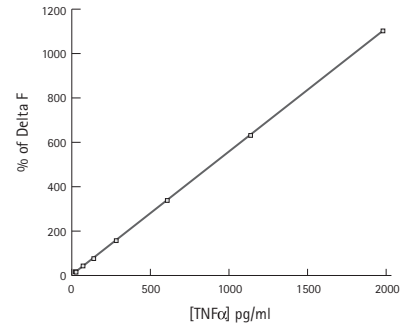
Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

### 3.7. Data reduction and example of calibration curve

This data should not be substituted for that obtained in the laboratory.

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
Negative control	1459 1392	35178 34331	415 405	410	1.6	
<b>[calibrator]</b> pg/mL						
20	1808 1582	38139 33638	474 470	472	0.6	15
50	1977 1924	33411 33011	592 583	587	1.1	43
100	2386 2452	33555 33511	711 732	721	2.0	76
200	3395 3312	32101 31510	1058 1051	1054	0.4	157
500	6092 6129	34426 33688	1770 1819	1794	2.0	338
1000	10416 10615	34612 35547	3009 2986	2998	0.5	631
2000	16650 17239	34411 34339	4839 5020	4929	2.6	1102
TNFa control*	8192 7794	35631 34843	2299 2237	2268	1.9	453

- Ratio =  $\frac{A_{665nm}}{B_{620nm}} \times 10^4$
- Mean Ratio =  $\frac{\sum \text{Ratios}}{2}$
- CV =  $\frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$
- Delta F =  $\frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$   
(Ratio<sub>neg</sub> = negative control)



### 3.8. Evolution of the estimated concentration of a positive sample\*

\*Sample : Supernatant from LPS-induced THP-I cells, cultured in RPMI with Glutamax

	1h	3h	4h	5h	6h	24h
Sample (pg/mL)	310	289	282	273	273	280

### 3.9. Analytical characteristics

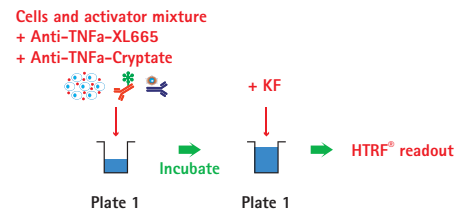
Detection limit (dose of mean zero + 2 SD)	Hook effect	Linear range
5 pg/mL	31,000 pg/mL	up to 2,000 pg/mL

The HTRF® TNFα assay was calibrated against the NIBSC international standard 87/650. The following equivalence was found :

1 pg TNFα HTRF® ⇔ 1.9 pg TNFα NIBSC 87/650 (i.e. : 0.076 IU)

## 4. Cell-based assay protocol

In this protocol, reagent reconstitution and distribution have been modified in order to allow the direct measurement of TNFα on stimulated cells. This protocol is particularly well adapted to secondary screening.



### 4.1. Reagent reconstitution

Anti-TNFα - Cryptate	⇒	Reconstitute each vial with 2 mL of <b>culture medium</b> . Mix gently.
Anti-TNFα - XL665	⇒	
TNFα calibrator	⇒	<ul style="list-style-type: none"> <li>• Calculate the reconstitution volume for each vial by dividing the reconstitution volume indicated on the labels of the vials by 1.67 (i.e. if 500 μL is printed on the calibrator label, the reconstitution volume will be 299.4 μL).</li> <li>• Use <b>culture medium</b> NOT WATER to reconstitute both calibrator and control.</li> </ul>
TNFα control	⇒	
Reconstitution buffer 50 mM Phosphate buffer, pH 7.0, 0.8M KF	⇒	Ready to use : in the cell-based assay, the reconstitution buffer is not used for conjugate reconstitution. It is added as KF buffer at the end of the incubation to allow HTRF® readout.

## 4.2. Reagent storage and stability

Refer to § 3.2.

## 4.3. Calibration curve preparation

Reconstitute the maximum calibrator with the culture medium volume indicated on the label, divided by 1.67. Follow the same dilution sequence as shown in the table in § 3.3., replacing the diluent by culture medium.

## 4.4. Sample preparation

Cell density optimization is a key step in the TNF cell-based assay. Typically, the level of TNF produced by cells must fall within the 100-2000 pg/mL range. Optimization consists of testing a broad range of cell concentrations (e.g. between 100 and 20,000 cells per well) in the presence or the absence of a direct activator of TNF $\alpha$  production, e.g. LPS at 10  $\mu$ g/mL. In practice, resuspend the cells in the culture medium supplemented with the activator(s) or inhibitor(s) so that the desired number of cells will be dispensed under 6  $\mu$ L.

## 4.5. Cell-based assay protocol for 384-well low volume (20 $\mu$ L)

Dispense the reagents in the following order:

- 6  $\mu$ L standard or cell suspension
- 2  $\mu$ L anti-TNF $\alpha$  Cryptate
- 2  $\mu$ L anti-TNF $\alpha$  XL665

\* For negative control, replace the first reagent by 6  $\mu$ L of culture medium.

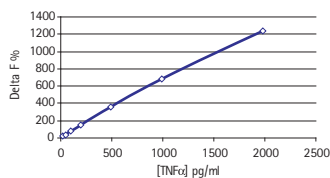
- Incubate the plate for 18 to 24 hours at 37°C in a CO<sub>2</sub> incubator with a fully humidified atmosphere.
- Add 10  $\mu$ L of reconstitution buffer.
- Read on a compatible HTRF® reader (more information about compatible reader at [htrf-assays.com/readers](http://htrf-assays.com/readers)).

## 4.6. Case study : TNF $\alpha$ quantification in peripheral blood mononucleated cells (PBMC)

- PBMC were separated on a ficoll density gradient (d=1.077) (Sigma). Cells were washed twice and viability was evaluated by trypan blue dye exclusion. Cells were then resuspended in RPMI1640 culture medium complemented with 10% heat inactivated calf serum, and stimulated with 10  $\mu$ g/mL of LPS.
- A graduated number of cells per well were distributed in 6  $\mu$ L of culture medium including the LPS activator, according to the cell-based assay protocol described above.

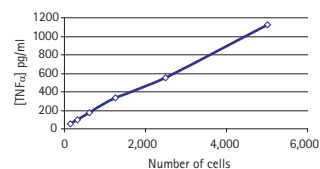
TNF $\alpha$  calibration curve in culture medium

[TNF $\alpha$ ] pg/mL	Delta F % (Overnight incubation, readout after KF addition)
19.8	19
49.5	40
99	86
198	148
495	358
990	681
1980	1240



TNF $\alpha$  quantification in PBMC

Number of cells / well	[TNF $\alpha$ ] pg/mL
5,000	1128.3
2,500	556.4
1,250	330.5
625	173.7
300	95.7
150	53



The data shows that the co-incubation of the stimulated cells with the labeled antibody pair does not alter HTRF® readout or assay performances. TNF $\alpha$  quantification is proportional to the number of cells per well. In this example, the cell density selected for a secondary screen to determine inhibitors of TNF $\alpha$  production would be 5,000 cells/well. In all instances, cell density should be carefully optimized, as the secretion of TNF $\alpha$  may vary between cell types.