# MANUAL

**Technology:** HTRF™ Cytokine

# HTRF High Performance Human TNF alpha Kit

| | |
|---|---|
| **Part number:** | 62HTNFAV2PET |
| **Test size** | 96 tests |

**Storage:** ≤ -16°C

**Version:** 01 **Date:** January 2025

## ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of human TNFα in supernatant and offers a fast alternative to ELISA.

The detection principle of this kit is based on HTRF™ technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, TNFα is detected in a sandwich assay by using anti- TNFα antibody labeled with Europium cryptate (donor), and anti- TNFα antibody labeled with d2 (acceptor).

When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the TNFα concentration.

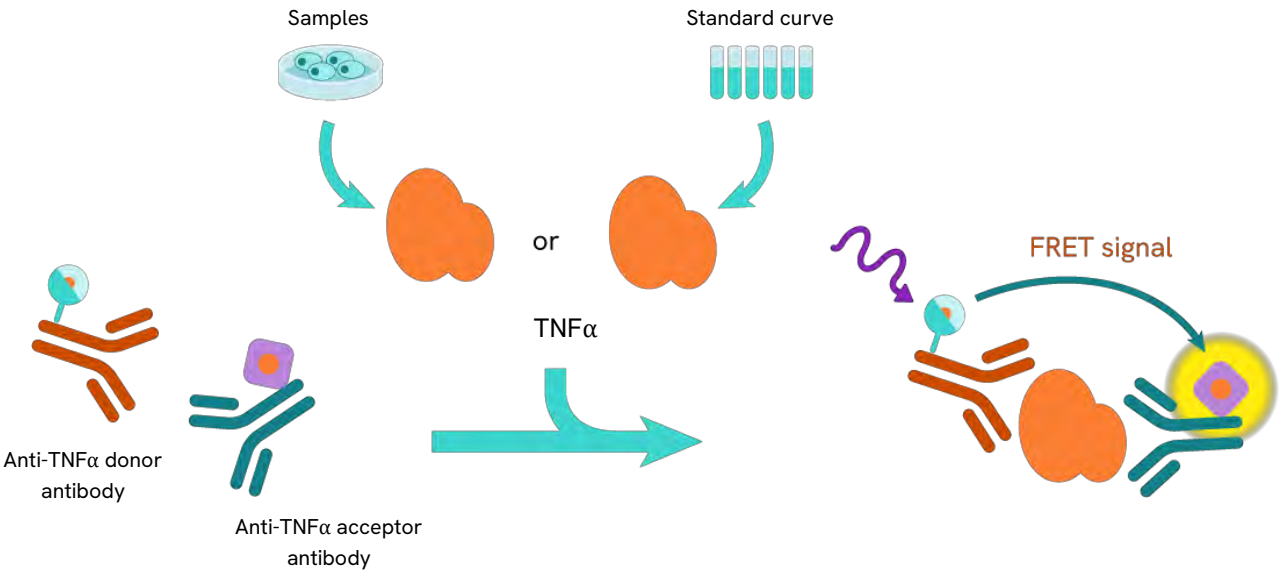
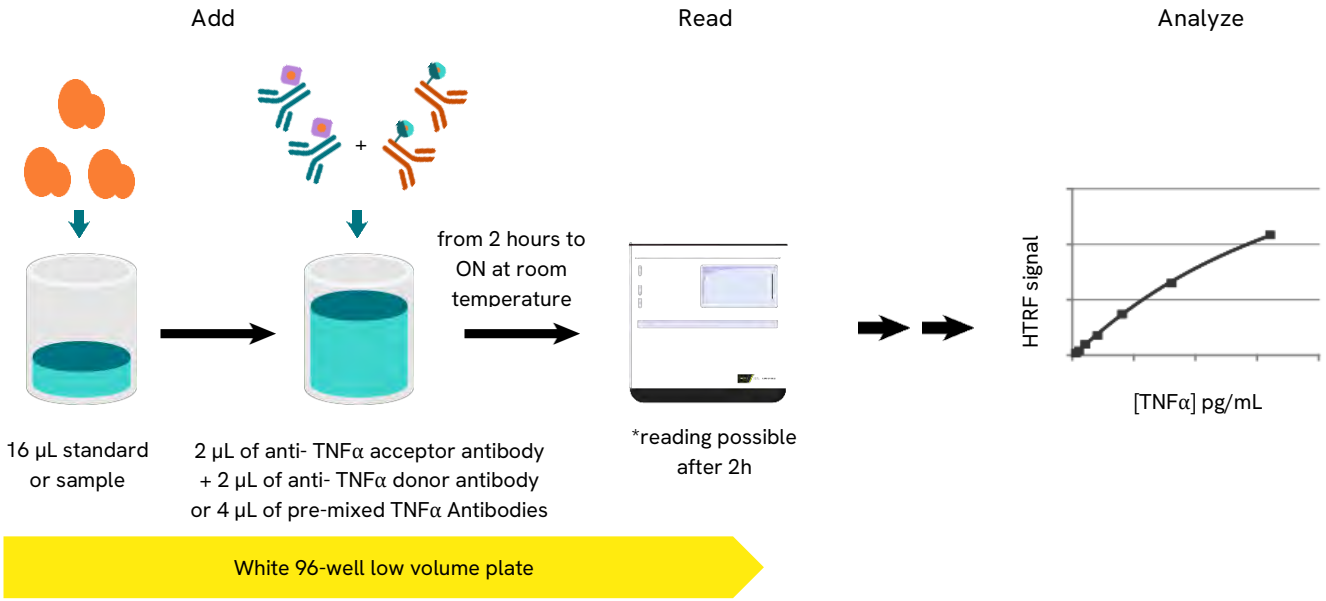



Figure 1: Principle of HTRF TNFα sandwich assay

## PROTOCOL AT A GLANCE






Make sure to use the set-up for Eu Cryptate.

## MATERIAL PROVIDED

| KIT COMPONENTS | 96 TESTS | | |
|---|---|---|---|
| TNFα Standard Lyophilized |  | green cap | 1 vial |
| TNFα Eu Cryptate Antibody Frozen 20X |  | orange cap | 1 vial<br>10 µL |
| TNFα d2 Antibody Frozen 20X |  | blue cap | 1 vial<br>10 µL |
| Diluent* #1 |  | Transparent cap | 1 vial<br>20 mL |
| **Detection Buffer** #3**<br>**Ready-to-use** |  | Transparent cap | 1 vial<br>0.5 mL |
| Plate | | | 1 plate<br>HTRF 96-well low volume plate |

* To prepare working standard solutions, culture medium can be an alternative the diluent.
** The Detection Buffer is used to prepare working solutions of acceptor and donor reagents

### Purchase separately

- HTRF™-Certified Reader. For a list of HTRF-compatible readers and set-up recommendations, please visit our website.
- 96-well or 384-well small volume (SV) detection microplates. For more information about microplate recommendations, please visit our website.

## STORAGE AND STABILITY

- Store the kit at -16°C or below.
- Under proper storage conditions, reagents are stable until the expiry date indicated on the label.

Once reconstituted, standard stock solution may be frozen, and can be thawed only once.
Once thawed, antibody solutions can be frozen once.
To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at ≤-16°C.
Volume of standard and antibody aliquots should not be under 10 µL.
Thawed diluent and detection buffer can be stored at 2-8°C on your premises.

## REAGENT PREPARATION

### Before you begin

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature.
- Before use, allow all kit's reagents to warm up at room temperature then
    - homogenize buffer and diluent with a vortex
    - centrifuge (NEVER vortex) the antibodies to gather all liquid at the bottom of the vial
- It is recommended to filter buffers before use.
- Antibody solutions must be prepared in individual vials and can be mixed prior to dispensing.

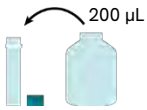
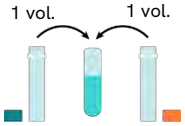
**Take care to prepare stock and working solutions according to the directions for the kit size you have purchased.**

## To prepare reagent stock solutions

| 96 TESTS | |
|---|---|
| **Anti-TNFα Eu Cryptate antibody** | |
| Thaw the TNFα Eu Cryptate antibody. Centrifuge.<br>This stock solution can be frozen and stored at ≤-16°C |  |
| **Anti-TNFα d2 antibody** | |
| Thaw the TNFα d2 antibody. Centrifuge.<br>This stock solution can be frozen and stored at ≤-16°C. |  |
| **TNFα Standard** | |
| Reconstitute the TNFα standard with distilled water.<br>Volume of reconstitution is indicated on the vial label.<br>The reconstituted standard solution can be frozen and stored at -60°C or below. |  |
| **Diluent** | |
| The Diluent is ready-to-use. | |

## To prepare antibody working solutions

Each well requires 4 µL of pre-mixed TNFα antibodies. Prepare the two antibody solutions in separate vials.

| 96 TESTS | |
|---|---|
| **TNFα Eu Cryptate antibody** | |
| Dilute the stock solution (thawed reagent) of TNFα Eu Cryptate-antibody with detection buffer #3:<br>Add 200µL of detection buffer directly in the thawed Eu Cryptate-antibody stock solution. |  |
| **TNFα d2 antibody** | |
| Dilute the stock solution (thawed reagent) of TNFα d2 antibody with detection buffer #3:<br>Add 200µL of detection buffer directly in thawed d2 antibody stock solution. |  |
| **Antibody Mix** | |
| Pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents: e.g. 210 µL of d2 antibody + 210 µL of Eu Cryptate antibody |  |

To prepare working standards solutions

- Each well requires 16 µL of standard.
- Serially dilute the standard stock solution with diluent #1 or with the cell culture medium used to prepare your samples supplemented with BSA or 10% FCS.
- Due to the stability of the TNFα, it is mandatory to prepare the standard curve just before the assay.
- In order to check for a potential interference effect from your own assay buffer when using the assay for the first time, we highly recommend the parallel preparation of a standard curve in your own supplemented cell culture medium and in diluent.
- In order to counteract any standard sticking we recommend changing tips between each dilution.

## A recommended standard dilution procedure is listed and illustrated below:

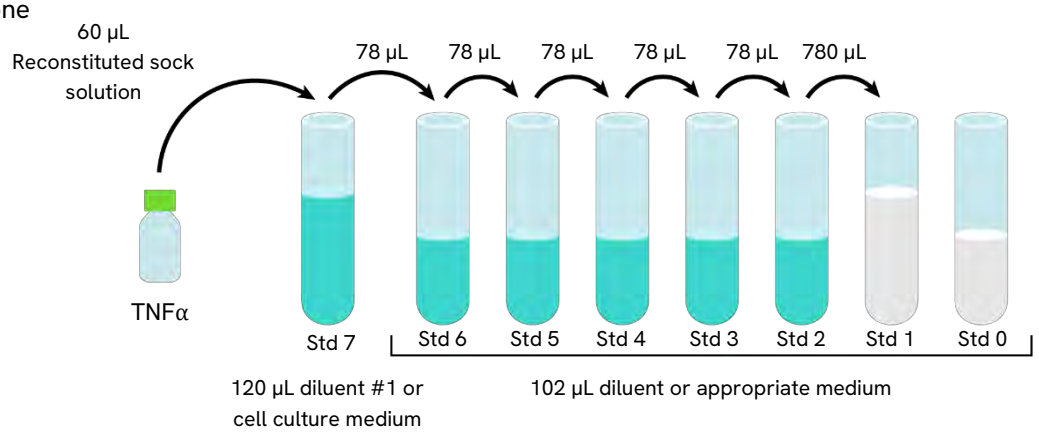
1. Reconstitute the standard vial with the volume indicated on the vial label using distilled water.
2. Prepare the following dilutions:

- Dilute the reconstituted standard stock solution 3-fold with diluent or with cell culture medium.

In practice: take 60 µL of stock solution and add it to 120 µL of diluent or cell culture medium. Mix gently. This yields the high standard (Std 7: 5 500 pg/mL) for the top of the curve.

- Use the high standard (Std 7) to prepare the standard curve using serial dilutions as follows:
  - Dispense 102 µL of diluent or cell culture medium into each vial from Std 6 to Std 0
  - Add 78 µL of standard to 102 µL of diluent or cell culture medium, mix gently and repeat the serial dilution to make standard solutions: std6, std5, std4, std3, std2, std1

This will create 7 standards for the analyte. Std 0 (Negative control) is diluent or appropriate culture medium alone




| STANDARD | SERIAL DILUTIONS | WORKING SOLUTIONS |
|---|---|---|
| Standard Stock solution | Reconstituted lyophilisate | 16.5 ng/mL |
| Standard 7 | 60 µL Standard stock Solution + 120 µL diluent #1 | 5 500 pg/mL |
| Standard 6 | 78 µL standard 7 + 102 µL diluent #1 | 2391 pg/mL |
| Standard 5 | 78 µL standard 6 + 102 µL diluent #1 | 1040 pg/mL |
| Standard 4 | 78 µL standard 5 + 102 µL diluent #1 | 452 pg/mL |
| Standard 3 | 78 µL standard 4 + 102 µL diluent #1 | 196 pg/mL |
| Standard 2 | 78 µL standard 3 + 102 µL diluent #1 | 85 pg/mL |
| Standard 1 | 78 µL standard 2 + 102 µL diluent #1 | 37 pg/mL |
| Standard 0 | 102 µL diluent #1 | 0 |

To prepare samples

- Each well requires 16 µL of sample.
- Just after their collection, put the samples at 4°C and test them immediately. For later use, samples should be dispensed into disposable plastic vials and stored at -60°C or below. Avoid multiple freeze/thaw cycles.
- Samples with a concentration above the highest standard (Std 7) must be diluted diluent #1 or in your appropriate sample medium.

## ASSAY PROTOCOL

| | | STANDARD (STD 0 – STD 7) | SAMPLES |
|---|---|---|---|
| Step 1 |  | Dispense 16 µL of each TNFα standard (Std 0 - Std 7) into each standard well | Dispense 16 µL of each sample into each sample well |
| Step 2 |  | Add 2 µL of TNFα d2 antibody working solution to all wells | |
| Step 3 |  | Add 2 µL of TNFα Eu Cryptate antibody working solution to all wells. | |
| Step 4 |  | Seal the plate and incubate at RT<br>*Reading possible after 2h | |
| Step 5 |  | Remove the plate sealer and read on an HTRF™ compatible reader | |

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| A | 16 µL Std 0 (Negative control)<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well A1 | Repeat Well A1 | 16 µL sample 1<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well A4 | Repeat Well A4 |
| B | 16 µL Std 1<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well B1 | Repeat Well B1 | 16 µL sample 2<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well B4 | Repeat Well B4 |
| C | 16 µL Std 2<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well C1 | Repeat Well C1 | 16 µL sample 3<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well C4 | Repeat Well C4 |
| D | 16 µL Std 3<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well D1 | Repeat Well D1 | 16 µL sample...<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well D4 | Repeat Well D4 |
| E | 16 µL Std 4<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well E1 | Repeat Well E1 | 16 µL sample...<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well E4 | Repeat Well E4 |
| F | 16 µL Std 5<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well F1 | Repeat Well F1 | 16 µL sample...<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well F4 | Repeat Well F4 |
| G | 16 µL Std 6<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well G1 | Repeat Well G1 | 16 µL sample...<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well G4 | Repeat Well G4 |
| H | 16 µL Std 7<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well H1 | Repeat Well H1 | 16 µL sample...<br>2 µL TNFa-d2<br>2 µL TNFa-Eu Cryptate | Repeat Well H4 | Repeat Well H4 |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | | | | | |
| C | | | | | | | | | | | | | | | | | | | | | | | | |
| D | | | | | | | | | | | | | | | | | | | | | | | | |
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| F | | | | | | | | | | | | | | | | | | | | | | | | |
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| J | | | | | | | | | | | | | | | | | | | | | | | | |
| K | | | | | | | | | | | | | | | | | | | | | | | | |

## DATA REDUCTION & INTERPRETATION

1) Calculate the 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 delta ratio of the acceptor and donor emission signals for each individual well. The Standard 0 (Negative control) plays the role of an internal assay control.

$$\text{Delta Ratio} = \text{Ratio Standard or sample} - \text{Ratio Standard 0}$$

3) Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

For more information about data reduction, please visit our website.

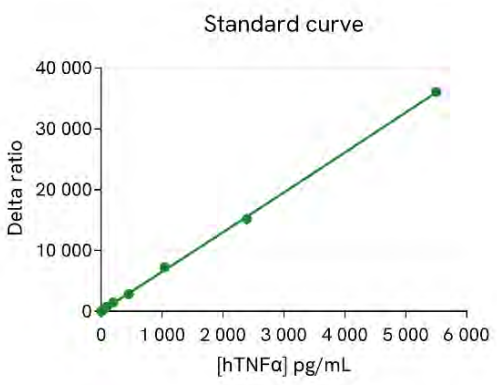
## RESULTS

This data must not be substituted for the data obtained in the laboratory, and should be considered only as an example.

Results may vary from one HTRF™ compatible reader to another.

Standard curve fitting with the 4 Parameter Logistic (4PL with $1/Y^2$) model

| | | Ratio (1) | CV% (2) |
|---|---|---|---|
| **Standard 0** | **Negative control** | 1171 | 3,1% |
| Standard 1 | 37 pg/mL | 1407 | 3,4% |
| Standard 2 | 85 pg/mL | 1878 | 8,0% |
| Standard 3 | 196 pg/mL | 2610 | 6,7% |
| Standard 4 | 452 pg/mL | 4273 | 3,6% |
| Standard 5 | 1 040 pg/mL | 8438 | 2,6% |
| Standard 6 | 2391 pg/mL | 16353 | 2,9% |
| Standard 7 | 5 500 pg/mL | 37235 | 0,8% |





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