TNFA/TNFR1 BINDING ASSAY KITS

PROTOCOL

Part # 64BDTNFPEG & 64BDTNFPEH

Test size: 500 tests (64BDTNFPEG), 10 000 tests (64BDTNFPEH) - assay volume: 20 µL

Revision: 02 - Aug. 2021

Store at: ≤-60°C

This product is intended for research purposes only. The product is not intended to be used for therapeutic or diagnostic purposes.

ASSAY PRINCIPLE

The HTRF TNFα/TNFR1 Binding Assay is designed to measure the interaction between TNFα and TNFR1. Utilizing HTRF (Homogeneous Time-resolved Fluorescence) technology, the assay enables simple and rapid characterization of compound and antibody blockers in a high throughput format.

As shown in Figure 1, the interaction between TNFα and TNFR1 is detected by using anti-Tag1 labeled with Europium (HTRF donor) and anti-Tag2 labeled with XL665 (HTRF acceptor). When the donor and acceptor antibodies are brought into close proximity due to TNFα and TNFR1 binding, excitation of the donor antibody triggers fluorescence resonance energy transfer (FRET) towards the acceptor antibody, which in turn emits specifically at 665 nm. This specific signal is directly proportional to the extent of TNFα/TNFR1 interaction. Thus, compound or antibody blocking TNFα/TNFR1 interaction will cause a reduction in HTRF signal.

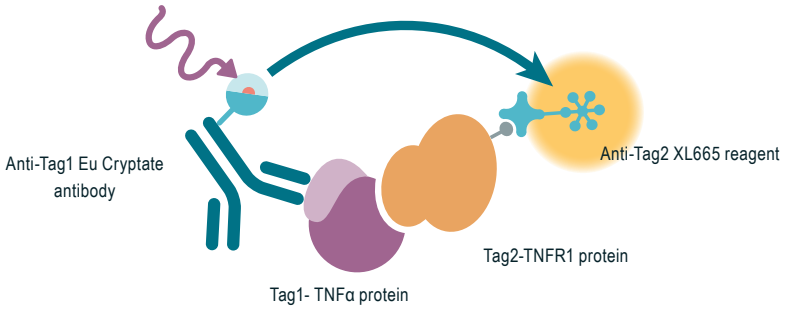
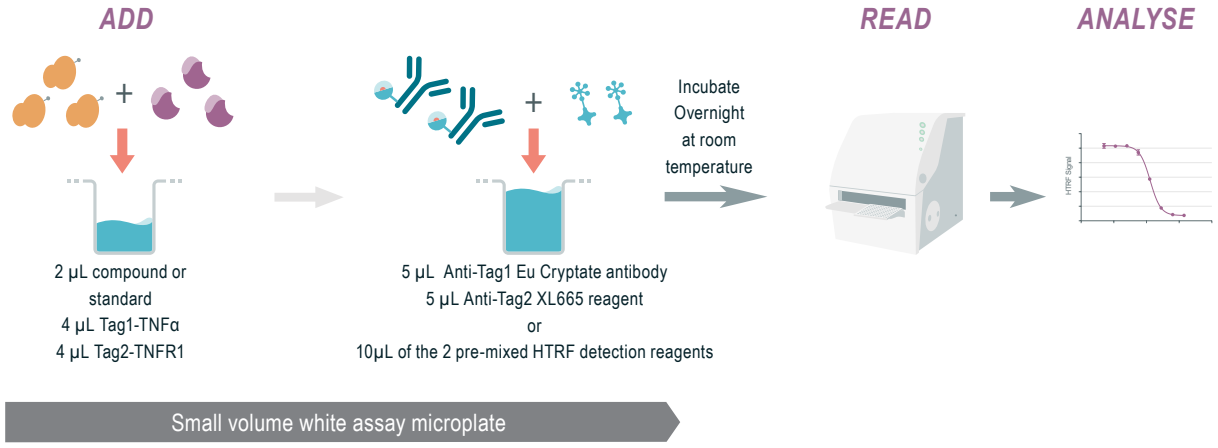


Figure 1: Principle of the HTRF TNFα/TNFR1 assay.

PROTOCOL AT A GLANCE



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MATERIALS:

KIT COMPONENTS	500 TESTS CAT # 64BDTNFPEG	10,000 TESTS CAT # 64BDTNFPEH
Tag1-TNFα Lyophilized	1 vial	2 vials
Tag2-TNFR1 Lyophilized	1 vial	2 vials
TNFα-TNFR1 standard Frozen	1 vial - 40 µL 1 µM	1 vial - 40 µL 1 µM
Anti-Tag1 Eu Cryptate antibody- Frozen	1 vial - 50 µL 50X	1 vial - 1 mL 50X
Anti-Tag2 XL665 reagent Frozen	1 vial - 50 µL 50X	1 vial - 1 mL 50X
PPI Europium Detection Buffer Frozen	1 vial - 20 mL	1 vial - 220 mL

For reading, an HTRF®-Certified Reader is needed. Make sure to use the set-up for Eu Cryptate. For a list of HTRF-compatible readers and setup recommendations, please visit our website at: www.cisbio.com/readers

For HTRF microplate recommendations, please visit:www.cisbio.com/microplate-recommendations

STORAGE AND STABILITY

Store the kit at ≤-60°C. Under appropriate storage conditions, reagents are stable until the expiry date indicated on the label.

Reagents

Once reconstituted, tagged TNFα & TNFR1 stock solution may be frozen, and can be thawed only once.
Once thawed (or reconstituted), anti-Tag 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 ≤-60°C.

Thawed PPI Europium Detection Buffer can be stored at 2-8°C on your premises.

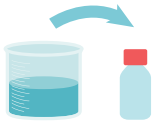
REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified PPI Europium detection buffer. The use of an incorrect buffer may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature.
- Before use, allow all reagents to warm up to room temperature then homogeneize buffer. It is recommended to filter buffers before use.
- The tagged protein solutions must be prepared in individual vials - DO NOT premix tagged solutions prior to dispensing.
- The anti-Tag solutions must be prepared in individual vials and can be premixed prior to dispensing.
- Compounds may be prepared in PPI Europium detection buffer.

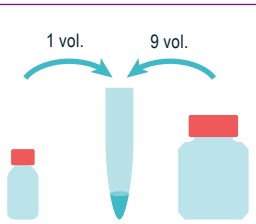
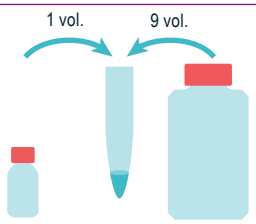
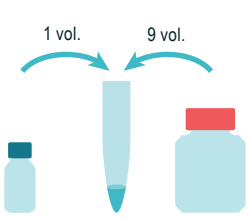
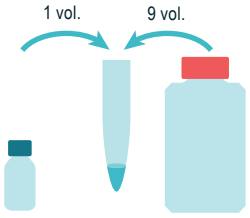
TO PREPARE STOCK SOLUTIONS:

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

500 TESTS		10,000 TESTS	
Tag1-TNFα			
Reconstitute the Tag1-TNFα with 200 µL of distilled water in order to obtain a 10X stock solution. Mix gently, DO NOT vortex ! This stock solution can be frozen and stored at ≤ 60°C.			Reconstitute the Tag1-TNFα with 2 mL of distilled water in order to obtain a 10X stock solution. Mix gently, DO NOT vortex ! This stock solution can be frozen and stored at ≤ 60°C.
Tag2-TNFR1			
Reconstitute the Tag2-TNFR1 with 200 µL of distilled water in order to obtain a 10X stock solution. Mix gently, DO NOT vortex ! This stock solution can be frozen and stored at ≤ 60°C.			Reconstitute the Tag2-TNFR1 with 2 mL of distilled water in order to obtain a 10X stock solution. Mix gently, DO NOT vortex ! This stock solution can be frozen and stored at ≤ 60°C.
TNFα-TNFR1 Standard			
Thaw the TNFα-TNFR1 standard. Mix gently. This 5X standard stock solution can be frozen and stored at ≤ 60°C.			Thaw the TNFα-TNFR1 standard. Mix gently. This 5X standard stock solution can be frozen and stored at ≤ 60°C.
Anti-Tag1 Eu Cryptate antibody			
Thaw the Anti-Tag1 Eu Cryptate antibody. Mix gently. This 50X Anti-Tag1 Eu Cryptate antibody stock solution can be frozen and stored at ≤ 60°C.			Thaw the Anti-Tag1 Eu Cryptate antibody. Mix gently. This 50X Anti-Tag1 Eu Cryptate antibody stock solution can be frozen and stored at ≤ 60°C.
Anti-Tag2 XL665 reagent			
Thaw the Anti-Tag2 XL665 reagent. Mix gently. This 50X Anti-Tag2 XL665 reagent stock solution can be frozen and stored at ≤ 60°C.			Thaw the Anti-Tag2 XL665 reagent. Mix gently. This 50X Anti-Tag2 XL665 reagent stock solution.can be frozen and stored at ≤ 60°C.
PPI Europium Detection Buffer			
Thaw the PPI Europium Detection Buffer The thawed buffer can be stored at 2-8°C on your premises.			Thaw the PPI Europium Detection Buffer The thawed buffer can be stored at 2-8°C on your premises..

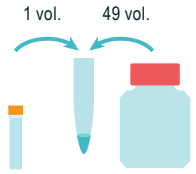
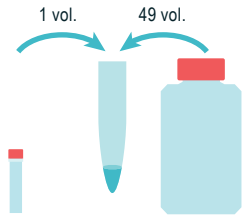
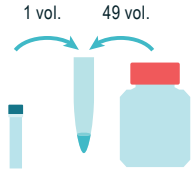
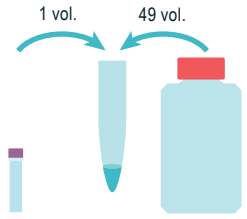
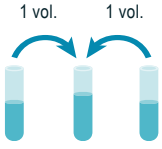
TO PREPARE TAG1-TNFA AND TAG2-TNFR1 WORKING SOLUTIONS:

Each well requires 4 µL of each Tag-protein.

500 TESTS		10,000 TESTS	
Tag1-TNFα			
Dilute 10-fold the 10X stock solution (reconstituted reagent) of Tag1-TNFα with PPI Europium Detection Buffer : e.g. 200 µL of reconstituted Tag1-TNFα stock solution + 1800 µL of PPI Europium Detection Buffer .			Dilute 10-fold the 10X stock solution (reconstituted reagent) of Tag1-TNFα with PPI Europium Detection Buffer : e.g. 2 mL of reconstituted Tag1-TNFα stock solution + 18 mL of PPI Europium Detection Buffer .
Tag2-TNFR1			
Dilute 10-fold the 10X stock solution (reconstituted reagent) of Tag2-TNFR1 with PPI Europium Detection Buffer : e.g. 200 µL of reconstituted Tag2-TNFR1 stock solution + 1800 µL of PPI Europium Detection Buffer .			Dilute 10-fold the 10X stock solution (reconstituted reagent) of Tag2-TNFR1 with PPI Europium Detection Buffer : e.g. 2 mL of reconstituted Tag2-TNFR1 stock solution + 18 mL of PPI Europium Detection Buffer .

TO PREPARE ANTI-TAG1 EU CRYPTATE ANTIBODY AND ANTI-TAG2 XL665 REAGENT WORKING SOLUTIONS:

Each well requires 5 µL of each anti-Tag donor & acceptor reagents.

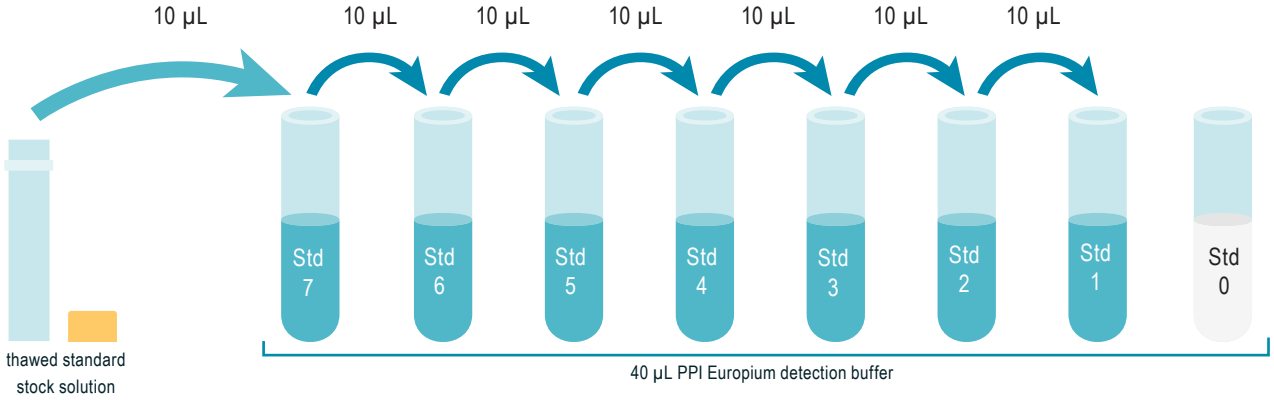
500 TESTS		10,000 TESTS	
Anti-Tag1 Eu Cryptate antibody			
Dilute 50-fold the 50X stock solution (thawed reagent) of Anti-Tag1 Eu Cryptate antibody with PPI Europium Detection Buffer : e.g. 50 µL of reconstituted Anti-Tag1 Eu Cryptate antibody stock solution + 2450 µL of PPI Europium Detection Buffer .			Dilute 50-fold the 50X stock solution (thawed reagent) of Anti-Tag1 Eu Cryptate antibody with PPI Europium Detection Buffer : e.g. 1 mL of reconstituted Anti-Tag1 Eu Cryptate antibody stock solution + 49 mL of PPI Europium Detection Buffer .
Anti-Tag2 XL665 reagent			
Dilute 50-fold the 50X stock solution (thawed reagent) of Anti-Tag2 XL665 reagent with PPI Europium Detection Buffer : e.g. 50 µL of reconstituted Anti-Tag2 XL665 reagent stock solution + 2450 µL of PPI Europium Detection Buffer .			Dilute 50-fold the 50X stock solution (thawed reagent) of Anti-Tag2 XL665 reagent with PPI Europium Detection Buffer : e.g. 1 mL of reconstituted Anti-Tag2 XL665 reagent stock solution + 49 mL of PPI Europium Detection Buffer .
anti-Tag HTRF detection solutions (pre-mixed)			
Pre-mix the two ready-to-use anti-Tag HTRF detection solutions just prior to dispensing the reagents: e.g. 2.5 mL of Anti-Tag1 Eu Cryptate antibody + 2.5 mL of Anti-Tag2 XL665 reagent			Pre-mix the two ready-to-use anti-Tag HTRF detection solutions just prior to dispensing the reagents: e.g. 20 mL of Anti-Tag1 Eu Cryptate antibody + 20 mL of Anti-Tag2 XL665 reagent

TO PREPARE WORKING TNFA-TNFR1 STANDARD SOLUTIONS:

- Each well requires 2 µL of standard.
- 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. Thaw the standard vial, the concentration of the TNFα-TNFR1 standard stock solution = 1 µM (1 000 000 pM)
2. Prepare the following dilutions:
   •Dilute the thawed standard stock solution 5-fold with PPI Europium detection buffer.
   In practice: take 10 µL of stock solution and add it to 40 µL of PPI Europium detection buffer. Mix gently. This yields the high standard (Std 7: 200 000 pM) for the top of the curve.
   •.Use the high standard (Std 7) to prepare the standard curve using 5-fold serial dilutions as follows:
   - Dispense 40 µL of PPI Europium detection buffer into each vial from Std 6 to Std 0
   - Add 10 µL of standard to 40 µL of PPI Europium detection buffer, 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 is PPI Europium detection buffer alone.



STANDARD	SERIAL DILUTIONS	WORKING SOLUTIONS	FINAL CONCENTRATIONS
Standard Stock solution	Thaw the TNFα-TNFR1 standard stock solution	1 µM (1 000 000 pM)	
Standard 7	10 µL standard stock solution + 40 µL PPI Europium detection buffer	200 000 pM	20 000 pM
Standard 6	10 µL Standard 7 + 40 µL PPI Europium detection buffer	40 000 pM	4 000 pM
Standard 5	10 µL Standard 6 + 40 µL PPI Europium detection buffer	8 000 pM	800 pM
Standard 4	10 µL Standard 5 + 40 µL PPI Europium detection buffer	1 600 pM	160 pM
Standard 3	10 µL Standard 4 + 40 µL PPI Europium detection buffer	320 pM	32 pM
Standard 2	10 µL Standard 3 + 40 µL PPI Europium detection buffer	64 pM	6.4 pM
Standard 1	10 µL Standard 2 + 40 µL PPI Europium detection buffer	12.8 pM	1.28 pM
Standard 0	40 µL PPI Europium detection buffer	0 pM	0 pM

ASSAY PROTOCOL

	Standard	Samples
STEP 1 	Dispense into each standard well 2 µL of standard 4 µL of Tag1-TNFα 4 µL of Tag2-TNFR1.	Dispense into each sample well 2 µL of compound/antibody or buffer 4 µL of Tag1-TNFα 4 µL of Tag2-TNFR1.
STEP 2 	Dispense into all standard & sample wells 10 µL of pre-mixed Anti-Tag1 Eu Cryptate antibody and Anti-Tag2 XL665 reagent	
STEP 3 	Seal the plate and incubate for Overnight.at room temperature	
STEP 4 	Remove the plate sealer and read on an HTRF® compatible reader.	

STANDARD PROTOCOL FOR INHIBITORY ASSAY IN 20 µL FINAL VOLUME

	Standard	Inhibitor	Tag1-TNFα	Tag2-TNFR1	Anti-Tag1 Eu Cryptate antibody	Anti-Tag2 XL665 reagent	PPI Europium detection buffer
Standard	2 µL	-	4 µL	4 µL	5 µL	5 µL	-
Sample	-	2 µL	4 µL	4 µL	5 µL	5 µL	-
Positive control	-	-	4 µL	4 µL	5 µL	5 µL	2 µL
Negative control	-	-	4 µL	-	5 µL	5 µL	6 µL
Buffer control	-	-	-	-	-	-	20 µL

EXAMPLE OF PLATE MAP

	1	2	3	4	5	6
A	Buffer control: 20 µL PPI Europium detection buffer	Repeat Well A1	Repeat Well A1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well A4	Repeat Well A4
B	Negative control: 6 µL PPI Europium detection buffer 4 µL Tag1-TNFα 10 µL pre-mix anti-Tag reagents	Repeat Well B1	Repeat Well B1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well B4	Repeat Well B4
C	Positive control: 2 µL PPI Europium detection buffer 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well C1	Repeat Well C1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well C4	Repeat Well C4
D	Std 0: 2 µL Standard 0 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well D1	Repeat Well D1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well D4	Repeat Well D4
E	Std 1: 2 µL Standard 1 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well E1	Repeat Well E1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well E4	Repeat Well E4
F	Std 2: 2 µL Standard 2 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well F1	Repeat Well F1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well F4	Repeat Well F4
G	Std 3: 2 µL Standard 3 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well G1	Repeat Well G1	Compound...: 2 µL compound... 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well G4	Repeat Well G4
H	Std 4: 2 µL Standard 4 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well H1	Repeat Well H1			
I	Std 5: 2 µL Standard 5 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well I1	Repeat Well I1			
J	Std 6: 2 µL Standard 6 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well J1	Repeat Well J1			
K	Std 7: 2 µL Standard 7 4 µL Tag1-TNFα 4 µL Tag2-TNFR1 10 µL pre-mix anti-Tag reagents	Repeat Well K1	Repeat Well K1			

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

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

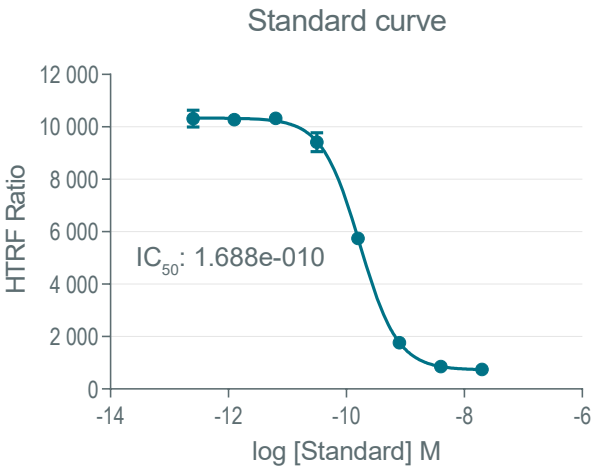
For more information about data reduction, please visit www.cisbio.com/data-reduction

RESULTS

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

Readouts on HTRF® compatible reader.

Note that results may vary from one HTRF® compatible reader to another.



ANALYTICAL CHARACTERISTICS

	Adalimumab	Human TNFα	Human TNFR1	Anti TNFR1	Anti TNFR2	SPD304
IC_{50}	83 pM	1.6 nM	1.6 nM	210 pM	No binding	> 6 µM

FOR MORE INFORMATION
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