FLISA-Dtech™

Mouse TNF-α Assay Kit

Ref: FLI-mTNFalpha

1 | PRESENTATION

BACKGROUND

Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine family produced mainly by activated macrophages, T-lymphocytes, and other immune cells. It plays an important role in the regulation of inflammatory and immune responses and is involved in the pathogenesis of several inflammatory and autoimmune diseases.

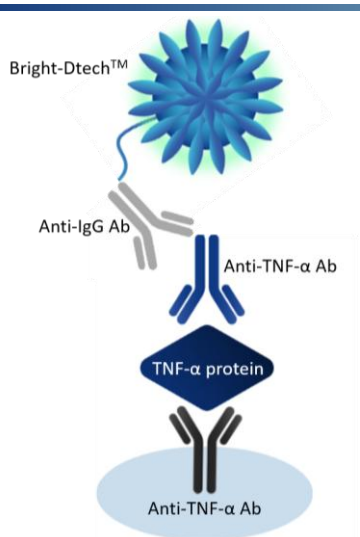
INTENDED USE

The Mouse TNF-α TR-FLISA kit is designed for the quantitative determination of TNF-α protein in mouse serum and plasma.

The assay uses Bright-Dtech™ fluorescent lanthanide nanoparticles coupled to an anti-IgG antibody for sensitive, time-resolved fluorescence (TRF) detection. The detection antibody is selected to minimize cross-reactivity with endogenous mouse IgG, supporting reliable detection of mouse TNF-α in mouse samples.

The assay range is 6.9 – 15 000 pg/mL leading to a very low limit of detection of 1.8 pg/mL.

TR-FLISA ASSAY PRINCIPLE



TR-FLISA (Time Resolved – Fluorescence Linked Immunosorbent Assay) is a fluorescence-based immunoassay derived from the conventional ELISA format that uses TRF detection instead of enzyme-based detection. The delayed fluorescence measurement reduces short-lived background fluorescence, supporting sensitive quantitative detection.

This assay uses a sandwich FLISA format. The microplate is pre-coated with an anti-TNF-α capture antibody, which captures TNF-α present in the standards or diluted mouse serum or plasma samples. After incubation and washing, a second anti-TNF-α detection antibody is added to bind the captured TNF-α, forming the sandwich complex. Following incubation and washing, Bright-Dtech™ nanoparticles coupled to an anti-IgG antibody are added for detection. The anti-IgG antibody binds to the detection antibody, while the Bright-Dtech™ nanoparticles provide the fluorescent signal. The signal is measured after excitation at 340 nm using a compatible time-resolved fluorescence (TRF) reader.

The fluorescence signal is proportional to the amount of TNF-α captured in the assay. The concentration of TNF-α in the samples is determined by comparison with the standard curve. The anti-IgG detection system is selected to minimize cross-reactivity with endogenous mouse IgG, thereby reducing potential interference from the sample matrix.

2 | MATERIALS

KIT COMPONENTS

Standard stock (67X)

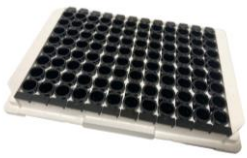
Secondary Antibody (1000X)

BDT Detection Antibody (200X)

Wash Buffer (10X)

Dilution Buffer

Assay Buffer



96-well black microplate (12 strips of 8 wells) coated with anti-TNF-α Ab

MATERIALS REQUIRED BUT NOT SUPPLIED

- Deionized or distilled water
- Adhesive sealing film for plates
- Precision single- and multichannel pipettes with disposable tips
- TRF plate reader with 340 nm excitation filter and 535 nm emission filter

3 | REAGENT AND SAMPLE PREPARATION

STORAGE AND PRECAUTIONS

- The kit must be stored at 2-8°C and used before the expiration date indicated on the box.
- The instructions described below are for testing all the assay points of the kit. Adjust the volume according to the test desired.
- Do not substitute or mix reagents with those from other lots or other source.
- We can not guarantee the performance of the product outside the condition described.
- Bring all reagents, plate, standard and samples to room temperature prior to use and homogenize them with a vortex.

PREPARATION OF WASH BUFFER (1X)

1. Prepare a 1X solution of 'Wash Buffer' by diluting **'Wash Buffer (10X)'** in deionized or distilled water. For 96 wells, prepare 500 mL of 1X Wash Buffer by adding **50 mL** of **'10X Wash Buffer'** to **450 mL** of deionized or distilled water.
2. Mix the 1X solution thoroughly by vortexing.

PREPARATION OF WORKING STANDARD DILUTIONS

1. Spin down the **'Standard stock (67X)'** tube briefly before use.
2. Prepare a 1X solution of **'Standard stock'** using **'Dilution Buffer'** as the diluent. Add 1 µL of **'Standard stock (67X)'** to a vial containing 99 µL of **'Dilution Buffer'** to prepare a 16X solution. Next, add 12 µL of '16X solution' to a vial containing 188 µL of **'Dilution Buffer'** to obtain the 1X solution.
3. Use this 1X solution to prepare the first point at 15 000 pg/mL, using **'Dilution Buffer'** as the diluent. Perform a 3-fold serial dilution to generate a 7-point standard curve, as shown in Figure 1. This procedure will yield sufficient volume to run the standard dilution series in triplicate.
4. For Blank points, perform triplicate using **'Dilution Buffer'**.

Note: *The diluted standards should be used immediately and must not be stored for future use.*

Standard	Serial dilutions	Working concentration (pg/mL)
Std 16X	1 µL Std stock (67X) + 99 µL Dilution Buffer	-
Std 1X	1 µL Std 16X + 188 µL Dilution Buffer	-
Std 1	150 µL Std 1X + 450 µL Dilution Buffer	15 000
Std 2	200 µL Std 1 + 400 µL Dilution Buffer	5 000
Std 3	200 µL Std 2 + 400 µL Dilution Buffer	1 666
Std 4	200 µL Std 3 + 400 µL Dilution Buffer	555
Std 5	200 µL Std 4 + 400 µL Dilution Buffer	62
Std 6	200 µL Std 5 + 400 µL Dilution Buffer	20.5
Std 7	200 µL Std 6 + 400 µL Dilution Buffer	6.8
Blank	400 µL Dilution Buffer	0

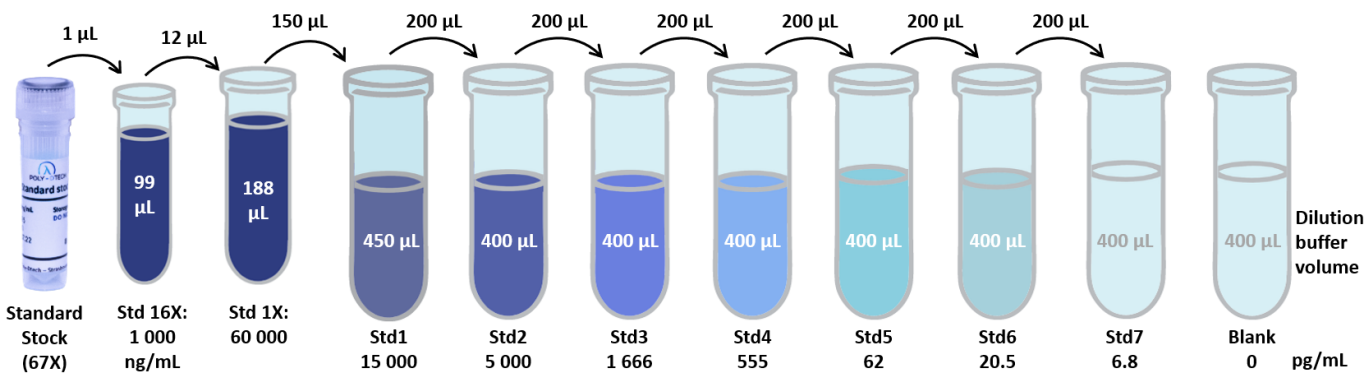
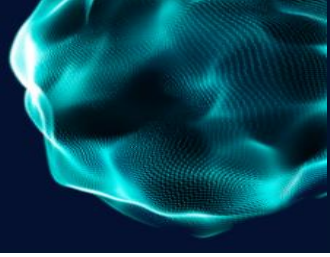


Figure 1: *Preparation of serail dilutions for the standard curve*



SAMPLE PREPARATION

- For dilution: use Dilution Buffer
- It is recommended that each investigator determine the optimal sample dilution to fall within the range of the standard curve.
- Mouse serum or plasma are suitable.
- Cell culture supernatant might be suitable too, but it has not been tested.
- Avoid repeated freeze-and-thaw cycle.

PREPARATION OF SECONDARY ANTIBODY (1X)

1. Vortex the **'Secondary Antibody'** tube briefly before use.
2. Prepare a 1X solution of **'Secondary Antibody'** by diluting **'Secondary Antibody (1000X)'** in **'Dilution Buffer'**.

   For 96 wells, prepare 10 mL of 1X Secondary Antibody by adding **10 µL** of **'Secondary Antibody (1000X)'** to vial containing **9 990 µL** of **'Dilution buffer'**.
3. Vortex the 1X solution thoroughly before use.

The 1X Secondary Antibody solution is ready to be distributed into the wells.

Note: *The 1X Secondary Antibody solution must not be stored for future use.*

PREPARATION OF BDT DETECTION ANTIBODY (1X)

1. Sonicate or vigorously vortex (sonication is strongly recommended instead of vortexing) the **'BDT Detection Antibody (200X)'** tube to ensure a homogeneous solution.
2. Prepare a 1X solution of **'BDT Detection Antibody'** by diluting **'BDT Detection Antibody (200X)'** in **'Assay Buffer'**.

   For 96 wells, prepare 10 mL of 1X BDT Detection Antibody by adding 50 µL of **'BDT Detection Antibody (200X)'** to a vial containing **9 950 µL** of **'Assay buffer'**.
3. Vortex the 1X solution thoroughly before use.

Note: *The 1X Detection Antibody solution should be prepared 30 minutes before use and must not be stored for future use.*

4 | ASSAY PROTOCOL

PROTOCOL

1. Prepare all reagents, standard dilutions, samples, and antibody solutions as indicated in ***'Section 3'***. It is recommended to test all standards in triplicate and samples at least in duplicate.
2. Remove the coated microplate from 4°C and allow it to equilibrate to room temperature for **20-30 minutes**.
3. Wash the plate **3 times** before adding standard, sample or blank. Wash by adding **250 µL** of Wash buffer (1X) to each well. Remove excess Wash Buffer by aspiration or by tapping the inverted plate on absorbent paper.
4. Add **100 µL** of standard dilutions (see '*Standard Preparation*' section), samples (see '*Sample Preparation*' section), or blank to the appropriate wells. Cover the plate with adhesive foil.
5. Incubate **1 hour** at 37°C with gentle shaking.
6. Remove the incubation solution and wash **3 times** as described in step 3.
7. Add **100 µL** of 1X Secondary Antibody solution to each well (see '*Preparation of Secondary Antibody (1X)*' section). Cover the plate with adhesive foil.
8. Incubate **1 hour** at 37°C with shaking.
9. Remove the incubation solution and wash **3 times** as described in step 3.
10. Add **100 µL** of 1X BDT Detection Antibody solution to each well (see '*Preparation of Detection Antibody (1X)*' section). Cover the plate with adhesive foil.
11. Incubate **1 hour** at 37°C with shaking.
12. Remove the incubation solution and wash **3-6 times**. Add **250 µL** of Wash buffer (1X) to each well. Allow each wash to shake for **5 minutes** for each wash. Remove excess of Wash Buffer by aspiration or by tapping the inverted plate on absorbent paper.
13. Add **200 µL** of Wash buffer (1X) to each well.
14. Measure the fluorescence at **535 nm** using a compatible TRF plate reader with excitation at **340 nm**. Refer to the '*TRF plate reader settings*' section.

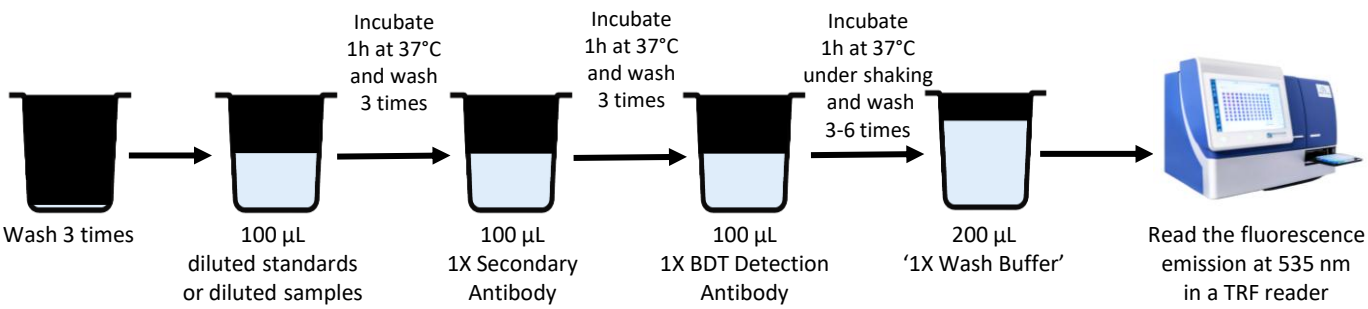
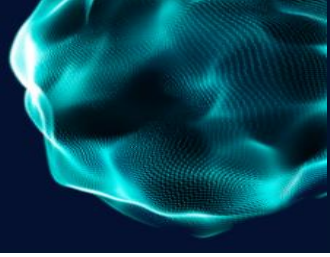


Figure 2: *Assay procedure summary*



TRF PLATE READER SETTINGS

- The instrument settings bellow are provided as guideline only.
- Settings must be optimized for each reader (*).

Parameter	Setting
Excitation filter	340 nm
Emission filter	535 nm
Number of flashes *	100
Delay time *	70 µs
Integration time *	2 ms
Well scan	Multi-points

Table 1: *Example of instrument settings for a TRF reader*

5 | ANALYSE

CALCULATIONS

- Create a standard curve by plotting the mean 535 nm RFU for each set of duplicate or triplicate standards on the Y-axis versus the corresponding TNFα concentration on the X-axis.
- The best-fit standard curve can be determined according to a nonlinear regression using a four or five-parameter logistic (4PL or 5PL) curve-fit (sigmoidal dose-response curve with variable slope). For that, we recommend to use a commercial software program.
- To determine the unknown TNF-α concentrations in samples, find the unknown's mean 535 nm RFU values, insert them in the standard curve equation and find out the expected concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- The intensity of the resulting signal is directly proportional to the concentration of the antigen present in the sample.
- Any undiluted sample reading greater than the highest standard should be diluted appropriately with 'Dilution Buffer' and retested.
- Any diluted sample reading greater than the highest standard should be further diluted appropriately with 'Dilution Buffer' and retested.
- Any diluted sample reading lower than the lowest standard should be less diluted appropriately with 'Dilution Buffer' and retested.

TYPICAL STANDARD CURVE

The standard curve below is an example only and should not be used to calculate results for unknown samples. Results may vary between different TRF readers.

A new standard curve must be generated with each assay.

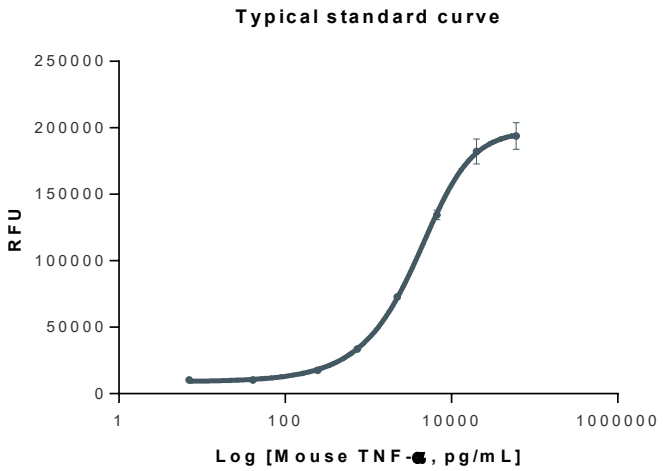


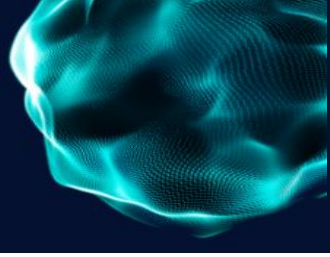
Figure 3: *A representative standard curve from the Mouse TNF-α TR-FLISA kit (Ref. FLI-mTNFalpha)*

6 | PERFORMANCE CHARACTERISTICS

VALIDATION

Assay Type	TR-FLISA
Format	96-well plate
Hands-on time	20-30 minutes
Incubation time	3 h
Sample type (volume)	Diluted serum or plasma (100 µL)
Specificity	TNF-α from mice samples
Limit of Detection (LOD)	1.8 pg/mL
EC_{50}	3.4 ng/mL
Dynamic range	6.8 - 15 000 pg/mL

Table 2: *Validation parameters resume*



SENSITIVITY

The Limit of Detection (LOD) for mouse TNF-α is **1.8 pg/mL**. This was determined by adding three standard deviations to the mean value of 40 blank (zero) standard replicates, run across four independent assays, and calculating the corresponding concentration.

PRECISION

Intra-assay precision

Intra-assay precision was evaluated by measuring three concentrations (high, medium, and low) of TNF-α in Dilution Buffer, with 9 replicate determinations. The intra-assay percentage coefficient of variation (CV%) was < 11%.

Spike	Number of replicates	CV (%)
High	9	11
Medium	9	2
Low	9	5

Table 3: *Intra-assay precision performance*

Inter-assay precision

Inter-assay precision was evaluated by measuring three concentrations (high, medium, and low)) of TNF-α in Dilution Buffer, with 9 replicate determinations in 3 independent assays. The inter-assay percentage coefficient of variation (%CV) was < 2%.

Spike	Number of replicates	CV (%)
High	3 x 9	1.3
Medium	3 x 9	1.6
Low	3 x 9	0.5

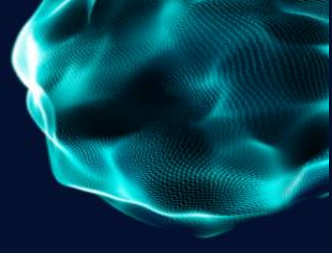
Table 4: *Inter-assay precision performance*

LINEARITY

To evaluate the linearity of the assay, a high concentration of TNF-α was spiked into three different undiluted mouse sera. The samples were then serially diluted 2-fold to 1/128 using Dilution Buffer.

Dilution factor	% Linearity
1/2	102
1/4	104
1/8	86
1/16	91
1/32	108
1/64	83
1/128	101

Table 5: *Linearity performance*



RECOVERY

To determine whether Mouse TNF-α detection is affected by differences in the standard curve diluent and biological sample matrix, three different concentrations (high, medium, and low) of) of TNF-α were spiked into three different undiluted mouse sera.

Spike	Number of samples	% Recovery
High	3	100
Medium	3	100
Low	3	100

Table 6: *Recovery performance*

7 | TROUBLESHOOTING GUIDE

Problem	Possible cause	Solution
High background	Insufficient washing.	Review manual for proper wash technique. Increase number of washes. If using a plate washer, check all ports for obstructions.
	BDT Detection Antibody not mixed properly1;	Sonicate or mix vigorously the BDT Detection Antibody (200X) tube and the 1X solution.
Low or poor standard curve	Inaccurate pipetting.	Check pipettes.
	Improper standard dilution.	Before opening, briefly spin the standard stock tube. Check calculations and create a new standard curve.
	Incorrect procedure.	Check the protocol (ensure reagents were added in the correct order).
No or weak signal	Incubation times too short.	Ensure sufficient incubation times; switch to overnight standard/sample incubation.
	Inadequate reagent volumes or improper dilution.	Check pipettes and ensure correct preparation (review protocol).
	Incorrect procedure.	Check the protocol (ensure reagents were added in the correct order).
	Incorrect reader settings.	Check filters/reader settings (ensure the plate reader is set accurately for the type of detection being used).
High CV%	Insufficient washing.	Review manual for proper wash technique. Increase number of washes. If using a plate washer, check all ports for obstructions.
	Contaminated washing buffer.	Prepare fresh 1X washing buffer.
	BDT Detection Antibody not mixed properly.	Sonicate or mix vigorously the BDT Detection Antibody (200X) tube and the 1X solution.
Low sensitivity	Improper storage of the FLISA kit.	All reagents should be stored according to the instructions.