Mouse TNF alpha Instant ELISA

Enzyme-linked Immunosorbent Assay for quantitative detection of mouse TNF α

Catalog Number BMS607-2INST

Pub. No. MAN0017838 **Rev.** A.0 (30)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Mouse TNF alpha Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse TNF-α. Cell culture supernatant, serum and plasma (citrate) were tested with this assay.

TNF alpha is a multifunctional cytokine involved in many different pathways, in homeostasis and pathophysiology of mammals. It can show opposing biological effects suggesting complex regulatory mechanisms. TNF alpha, also known as cachectin, was first detected as a cytotoxic factor inducing lysis of certain tumor cells. The TNF alpha gene is member 2 of the TNF-superfamily (consisting of at least 20 distinct members).

Principles of the test

An anti-mouse TNF-α coating antibody is adsorbed onto microwells. Mouse TNF-α present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated anti-mouse TNF-α antibody binds to mouse TNF-α captured by the first antibody. Streptavidin-HRP binds to the biotin conjugated anti-mouse TNF-α.

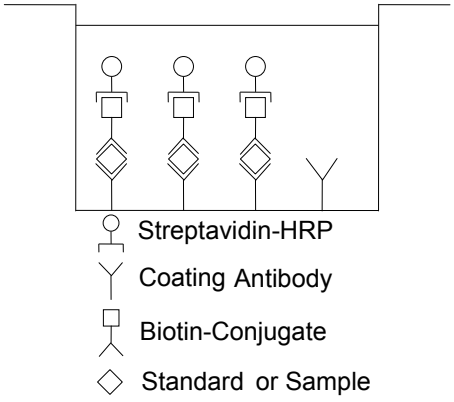

Fig. 1 First incubation

Following incubation unbound biotin conjugated antimouse TNF-α and Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

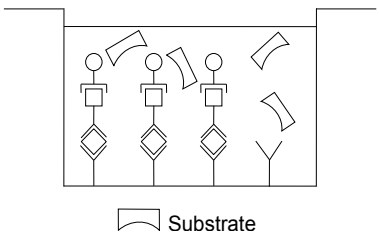

Fig. 2 Second incubation

A coloured product is formed in proportion to the amount of soluble mouse TNF-α present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 mouse TNF-α standard dilutions and mouse TNF-α sample concentration determined.

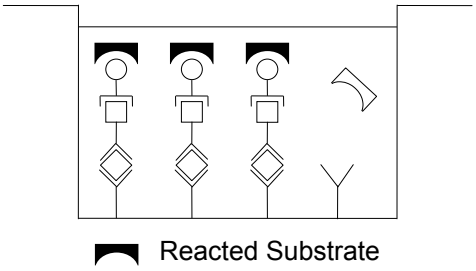

Fig. 3 Stop reaction

Reagents provided

Reagents for mouse TNF-α ELISA BMS607-2INST (96 tests)

1 aluminum pouch with a Microwell Plate coated with polyclonal antibody to mouse TNF-α, Biotin-Conjugate (anti-mouse TNF-α polyclonal antibody), Streptavidin-HRP and Sample Diluent, lyophilized.

2 aluminum pouches with a mouse TNF-α Standard curve (coloured)

1 bottle (25 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 bottle (12 mL) Sample Diluent (use when an external predilution of the samples is needed)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

2 Adhesive Films

Storage instructions – ELISA kit

Store ELISA plate and Standard curves or whole kit at -20°C. The plate and the standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 8°C. Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instructions

Cell culture supernatant, serum and plasma (citrate) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive mouse TNF-α. If samples are to be run within 24 hours, they may be stored at 2–8°C

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

Precautions for use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Preparation of reagents

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash buffer (1x)

1. Pour entire contents (25 mL) of the Wash Buffer Concentrate (20x) into a clean 500 mL graduated cylinder. Bring to final volume of 500 mL with glass-distilled or deionized water. Mix gently to avoid foaming.
2. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.

Test protocol

Note:

- Use plate immediately after removal from -20°C!
- Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!
- Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results.
- Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely - otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results.
- Allow the washing buffer to sit in the wells for a few seconds before aspiration.
- Remove covers of the standard strips carefully so that all the lyophilized pellets remain in the wells.

1. Determine the number of Microwell Strips required to test the desired number of samples plus Microwell Strips for blanks and standards (coloured). Each sample, standard and blank should be assayed in duplicate. Remove extra Microwell Strips from holder and store in foil bag with the desiccant provided at -20°C sealed tightly. Place Microwell Strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).
2. Add distilled water to all standard and blank wells as indicated on the label of the standard strips (A1, A2 to H1, H2).
3. Add 100 µl of distilled water to the sample wells.

Table 1 Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (2000 pg/mL)	Standard 1 (2000 pg/mL)	Sample 1	Sample 1
B	Standard 2 (1000 pg/mL)	Standard 2 (1000 pg/mL)	Sample 2	Sample 2
C	Standard 3 (500 pg/mL)	Standard 3 (500 pg/mL)	Sample 3	Sample 3
D	Standard 4 (250 pg/mL)	Standard 4 (250 pg/mL)	Sample 4	Sample 4
E	Standard 5 (125 pg/mL)	Standard 5 (125 pg/mL)	Sample 5	Sample 5
F	Standard 6 (62.5 pg/mL)	Standard 6 (62.5 pg/mL)	Sample 6	Sample 6
G	Standard 7 (31.3 pg/mL)	Standard 7 (31.3 pg/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

4. Add 50 µl of each sample, in duplicate, to the designated wells and mix the contents.
5. Cover with an adhesive film and incubate at room temperature (18ºC to 25ºC) for 3 hours, if available on a microplate shaker.

6. Remove adhesive film and empty wells. Wash the microwell strips 6 times with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
7. Pipette 100 µL of TMB Substrate Solution to all wells.
8. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

   The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

   It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.
9. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
10. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mouse TNF-α concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating mouse TNF-α for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding mouse TNF-α concentration.
- Samples have been diluted 1:2, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 2).
- Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low mouse TNF-α levels. Such samples require further external predilution according to expected mouse TNF-α values with Sample Diluent in order to precisely quantitate the actual mouse TNF-α level.
- If the sample is prediluted the value read from the standard curve must be multiplied by the dilution factor. For example, if the sample is prediluted 1:25 the value read from the standard curve must be multiplied by a factor of 25 to give the final result.
- It is suggested that each testing facility establishes a control sample of known mouse TNF-α concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

  Note: Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

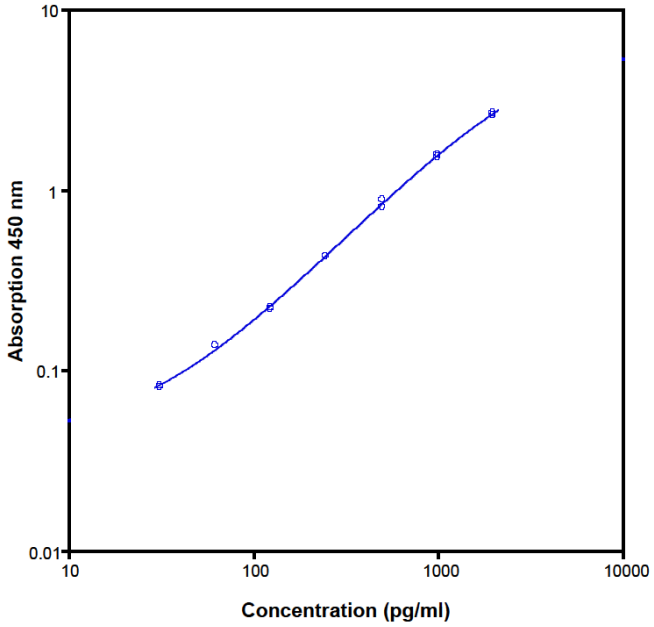

Fig. 4 Representative standard curve for mouse TNF-α ELISA. Mouse TNF-α was diluted in serial 2-fold steps in Sample Diluent.

Table 2 Typical data using the mouse TNF-α ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Mouse TNF-α Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2000	2.576 2.652	2.614	1.5
2	1000	1.549 1.515	1.532	1.1
3	500	0.876 0.799	0.837	4.5
4	250	0.423 0.423	0.423	0.1
5	125	0.218 0.222	0.220	0.8
6	62.5	0.137 0.137	0.137	0.1
7	31.3	0.081 0.080	0.080	0.8
Blank	0	0.031 0.035	0.033	6.9

The OD values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique, or temperature effects). Furthermore, shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.

- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Performance characteristics

Sensitivity

The limit of detection of mouse TNF-α defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 4 pg/ml (mean of 6 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of mouse TNF-α. 2 standard curves were run on each plate. Data below show the mean mouse TNF-α concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 7.2%.

Table 3 The mean mouse TNF-α concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Mouse TNF-α Concentration (pg/mL)	Coefficient of Variation (%)
1	1	2069	7.6
	2	2456	7.7
	3	2346	6.1
2	1	661	3.4
	2	653	14.8
	3	749	10.3
3	1	56	6.4
	2	47	6.0
	3	59	13.5
4	1	1889	4.0
	2	2150	3.7
	3	2100	4.7
5	1	643	3.8
	2	681	3.8
	3	767	12.4
6	1	55	8.4
	2	49	7.5
	3	65	6.0

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of mouse TNF-α. 2 standard curves were run on each plate. Data below show the mean mouse TNF-α concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 9.8%.

Table 4 The mean mouse TNF-α concentration and the coefficient of variation of each sample

Sample	Mean Mouse TNF-α Concentration (pg/mL)	Coefficient of Variation (%)
1	2290	8.7
2	688	7.7
3	54	12.2
4	2046	6.8
5	697	9.1
6	56	14.3

Spike recovery

The spike recovery was evaluated by spiking 4 levels of mouse TNF-α into pools of sera. Recoveries were determined in 3 independent experiments with 4 replicates each. The unspiked sera pools were used as blank in these experiments. Recoveries were shown to depend on the pools of sera used. Average recovery ranged from 23% to 102% with an overall mean recovery of 65%.

Dilution parallelism

Serum samples with different levels of mouse TNF-α were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 85.8% and 132.1% with an overall recovery of 108.5%.

Sample matrix	Dilution	Mean mouse TNF-α concentration (pg/mL)		
		Expected value	Observed value	% Recovery of Expected Values
1	1:2	—	1425	—
	1:4	713	845	118.6
	1:8	422	558	132.1
	1:16	279	310	111.2
2	1:2	—	516	—
	1:4	258	271	105.2
	1:8	136	149	110.1
	1:16	75	72	96.6
3	1:2	—	570	—
	1:4	285	314	110.3
	1:8	157	168	106.8
	1:16	84	72	85.8

Sample stability

Freeze-Thaw stability

Aliquots of serum samples (spiked) were stored at -20°C and thawed 5 times, and the mouse TNF-α levels determined. There was no significant loss of mouse TNF-α immunoreactivity detected by freezing and thawing.

Storage stability

Aliquots of serum samples (spiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mouse TNF-α level determined after 24 h. There was no significant loss of mouse TNF-α immunoreactivity detected during storage at -20°C and 4°C. Storage at room temperature and 37°C gave rise to loss of mouse TNF-α immunoreactivity.

Specificity

The assay detects both natural and recombinant mouse TNF-α. The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into mouse TNF-α positive serum. There was no crossreactivity detected.

Expected values

A panel of sera samples from randomly selected apparently healthy mice was tested for mouse TNF-α. There were no detectable mouse TNF-® levels found. Elevated mouse TNF-α levels depend on the type of immunological disorder.

Reagent preparation summary

Wash buffer (1x)

Add Wash Buffer Concentrate 20 x (25 ml) to 475 ml distilled water

Test protocol summary

1. Place standard strips in position A1/A2 to H1/H2.
2. Add distilled water, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
3. Add 100 µl distilled water to sample wells.
4. Add 50 µl sample to designated wells.
5. Cover microwell strips and incubate 3 hours at room temperature (18° to 25°C) if available on a microplate shaker at 400 rpm.
6. Empty and wash microwell strips 6 times with 400 µl Wash Buffer.
7. Add 100 µl of TMB Substrate Solution to all wells including blank wells.
8. Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
9. Add 100 µl Stop Solution to all wells including blank wells.
10. Blank microwell reader and measure colour intensity at 450 nm.

Note: Samples have been diluted 1:2, thus the concentration read from the standard curve must be multiplied by the dilution factor (x2).

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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