Murine TNF-αELISA Kit

Instructions for use

Catalogue numbers:

1x48 tests: EA101693
1x96 tests: EA101694
2x96 tests: EA101695

For research use only

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MurineTNF-α ELISA KIT

1. Intended use

The OriGeneMurine TNF-α ELISA is to be used for the in-vitro quantitative determination of murine tumor necrosis factor-α (mTNFα) in murine serum, buffered solutions or cell culture medium. The assay will recognize both natural and recombinant murine TNF-α.

This kit has been configured for research use only.

2. Principle of the method

The murine TNF-α Kit is a solid phase sandwich Enzyme Linked-Immuno- Sorbent Assay (ELISA). A monoclonal antibody specific for mTNFα has been coated onto the wells of the microtiter strips provided. The antigen and a biotinylated polyclonal antibody specific for mTNFα are simultaneously incubated. Revelation step includes Streptavidin-Horse Radish peroxidase and TMB as chromogen.

3. Reagents provided and reconstitution

Reagents (Store@2-8^{o}C)	**Quantity 1x48 well kit** Cat no. EA101693	**Quantity 1x96 well kit** Cat no. EA101694	**Quantity 2x96 well kit** Cat no. EA101695	**Reconstitution**
96 well microtitrer strip plate	1/2	1	2	Ready to use (Pre-coated)
Plastic plate covers	2	2	4	n/a
Standard: 1000 pg/ml	1 vial	2 vials	4 vials	Reconstitute as directed on the vial (see Assay preparation, section 8)
Standard Diluent (Buffer)	1 vial (25ml)	1 vial (25ml)	1 vial (25ml)	10x Concentrate, dilute in distilled water (see reagent preparation, section 8)
Biotinylated anti-mTNFα	1 vial (0.4ml)	1 vial (0.4ml)	2 vials (0.4ml)	Dilute in Biotinylated Antibody Diluent (see Assay preparation, section 8)
Biotinylated Antibody Diluent	1 vial (7ml)	1 vial (7ml)	1 vial (13ml)	Ready to use
Streptavidin-HRP	1 vial (5μl)	2 vials (5μl)	4 vials (5μl)	Add 0.5ml of HRP diluent prior to use (see Assay preparation, section 8)
HRP Diluent	1 vial (23ml)	1 vial (23ml)	1 vial (23ml)	Ready to use
Wash Buffer	1 vial (10ml)	1 vial (10ml)	2 vials (10ml)	200x Concentrate dilute in distilled water (see Assay preparation, section 8)
TMB Substrate	1 vial (11ml)	1 vial (11ml)	1 vial (24ml)	Ready to use
H_2SO_4 stop reagent	1 vial (11ml)	1 vial (11ml)	2 vials (11ml)	Ready to use

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000µl adjustable single channel micropipettes with disposable tips
- 50-300µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2 and 8°C. Immediately after use remaining reagents should be returned to cold storage (2-8°C). Expiry of the kit and reagents is stated on box front labels. The 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, the reagent is not contaminated by the first handling.

Wash Buffer: Once prepared store at 2-8°C for up to 1 week
Standard Diluent Buffer: Once prepared store at 2-8°C for up to 1 week
Standards : Once prepared use immediately and do not store
Biotinylated Secondary Antibody: Once prepared use immediately and do not store
Streptavidin-HRP: Once prepared use immediately and do not store

6. Specimen collection, processing & storage

Cell culture supernatants, serum, plasma or other biological samples will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.

Cell culture supernatants: Remove particulates and aggregates by spinning at approximately 1000 x g for 10 min.

Storage: If not analyzed shortly after collection, samples should be aliquoted (250-500µl) to avoid repeated freeze-thaw cycles and stored frozen at –70°C. Avoid multiple freeze-thaw cycles of frozen specimens.

Recommendation: Do not thaw by heating at 37°C or 56°C. Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984
- Laboratory gloves should be worn at all times
- Avoid any skin contact with H_2SO_4 and TMB. In case of contact, wash thoroughly with water
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used
- Do not pipette by mouth
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration
- Cover or cap all reagents when not in use
- Do not mix or interchange reagents between different lots
- Do not use reagents beyond the expiration date of the kit
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of H_2SO_4 and substrate solution, avoid pipettes with metal parts
- Use a clean plastic container to prepare the washing solution
- Thoroughly mix the reagents and samples before use by agitation or swirling
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent colour development. Warning TMB is toxic avoid direct contact with hands. Dispose off properly
- If a dark blue colour develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- Follow incubation times described in the assay procedure
- Dispense the TMB solution within 15 min of the washing of the microtitre plate

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8^{o}C storage.

Example plate layout(example shown for a 6 point standard curve)

	Standards		Sample Wells									
	1	2	3	4	5	6	7	8	9	10	11	12
A	1000	1000										
B	500	500										
C	250	250										
D	125	125										
E	62.5	62.5										
F	31.25	31.25										
G	Blank	Blank										
H												

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of Wash Buffer

Dilute the (200x) wash buffer concentrate 200 fold with distilled water to give a 1x working solution. Pour entire contents (10 ml) of the Washing Buffer Concentrate into a clean 2,000 ml graduated cylinder.Bring final volume to 2,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2˚-8˚C for up to 1 week.

8.3. Preparation of Standard Diluent Buffer

Add the contents of the vial (10x concentrate) to 225ml of distilled water before use.
This solution can be stored at 2-8^{o}C for up to 1 week.

8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of Standard Diluent shown on the vial immediately prior to use. This reconstitution gives a stock solution of 1000 pg/ml of mTNFα. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 1000 to 31.25 pg/ml. A fresh standard curve should be produced for each new assay.

- Immediately after reconstitution add 200µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 1000pg/ml
- Add 100µl of Standard Diluent to the remaining standard wells B1 and B2 to F1 and F2
- Transfer 100µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells
- Continue this 1:1 dilution using 100µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 1000pg/ml to 31.25 pg/ml
- Discard 100µl from the final wells of the standard curve (F1 and F2)

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of samples

Before testing, serum or plasmas samples have to be diluted 1:2 in standard buffer diluent.

8.6. Preparation of Biotinylated anti-mTNFα

It is recommended this reagent is prepared immediately before use. Dilute the biotinylated anti-mTNFα with the biotinylated antibody diluent in an appropriate clean glass vial using volumes appropriate to the number of required wells. Please see example volumes below:

Number of wells required	Biotinylated Antibody (µl)	Biotinylated Antibody Diluent (µl)
16	40	1060
24	60	1590
32	80	2120
48	120	3180
96	240	6360

8.7. Preparation of Streptavidin-HRP

It is recommended to centrifuge vial for a few seconds in a microcentrifuge to collect all the volume at the bottom.

Dilute the 5µl vial with 0.5ml of HRP diluent **immediately before use.** Do-not keep this diluted vial for future experiments. Further dilute the HRP solution to volumes appropriate for the number of required wells in a clean glass vial. Please see example volumes below:

Number of wells required	Streptavidin-HRP (µl)	Streptavidin-HRP Diluent (ml)
16	30	2
24	45	3
32	60	4
48	75	5
96	150	10

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 8.
Note: Final preparation of Biotinylated anti-mTNFα (section 8.5) and Streptavidin-HRP (section 8.6) should occur immediately before use.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.4
2.	Addition	Add 100µl of each **Standard, Sample and zero** in duplicate to appropriate number of wells
3.	Addition	Add 50µl of diluted **biotinylated anti-mTNFα** to all wells
4.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **3hours**
5.	Wash	Remove the cover and wash the plate as follows: a) Aspirate the liquid from each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step b and c another two times
6.	Addition	Add 100µl of **Streptavidin-HRP** solution into all wells
7.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **30 min**
8.	Wash	Repeat wash step 4.
9.	Addition	Add 100µl of ready-to-use **TMB Substrate Solution** into all wells
10.	Incubation	Incubate at room temperature (18 to 25°C) in dark for **10-20 min**
11.	Addition	Add 100µl of **H_2SO_4:Stop Reagent** into all wells
Read the absorbance value of each well (immediately after step 14.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm isacceptable).		

***Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range*

10. Data Analysis

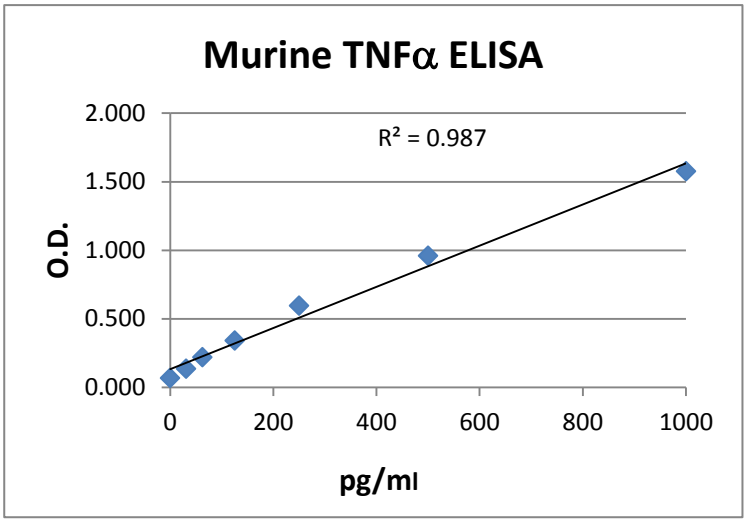
Calculate the average absorbance values for each set of duplicate standards and samples**.** Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding murine TNFα standard concentration on the horizontal axis.

The amount of mTNFα in each sample is determined by extrapolating OD values against mTNFα standard concentrations using the standard curve**.**

ExamplemTNFα Standard curve

Standard	TNFα Conc	OD (450nm) mean	CV (%)
1	1000	1,775	1,0
2	500	0,930	5,9
3	250	0,514	1,4
4	125	0,275	0,8
5	62.5	0,155	2,3
6	31.25	0,088	2,4
Zero	0	0,018	0,0



Note; curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **afresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The minimum detectable dose of mTNFα is **< 10.7 pg/ml**. This has been determined by adding 3 standard deviations to the mean optical density obtained when the zero standard was assayed 36 times.

12.2. Precision

Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates (3 duplicates) of 6 different samples containing different concentrations of TNFα. Data below show the mean concentration and the coefficient of variation for each sample. The overall **intra-assay coefficient of variation has been calculated to be 5%**.

Session	***Sample***	***Mean TNFa*** *pg/ml*	***SD***	***CV%***
Session 1	***Sample 1***	409.5	16.22	**4.0**
	Sample 2	234.1	22.7	**9.7**
	Sample 3	433.9	41.46	**9.6**
	Sample 4	198.9	1.42	**0.7**
	Sample 5	322.7	9.71	**3.0**
	Sample 6	163.3	5.62	**3.4**
Session 2	***Sample 1***	384.9	23.92	**6.2**
	Sample 2	217.3	15.30	**7.0**
	Sample 3	353.7	22.72	**6.4**
	Sample 4	177.4	5.34	**3.0**
	Sample 5	278.3	15.36	**5.5**
	Sample 6	129.5	4.36	**3.4**
Session 3	***Sample 1***	332.9	29.84	**9.0**
	Sample 2	227.7	0.68	**0.3**
	Sample 3	337.9	12.82	**3.8**
	Sample 4	175.5	8.62	**4.9**
	Sample 5	275.3	15.06	**5.5**
	Sample 6	142.2	6.26	**4.4**

Inter-assay

Assay to assay reproducibility within one laboratory will be evaluated in three independent experiments. Each assay will be carried out with 6 replicates (3 duplicates) in 2 murine pooled serum, 2 in RPMI and 2 in standard diluent with samples containing different concentrations of TNFα. 2 standard curves were run on each plate. **The calculated overall coefficient of variation was 9.4%.**

	Sample 1	***Sample2***	***Sample 3***	***Sample 4***	***Sample 5***	***Sample 6***
Mean TNFα *pg/ml*	375.77	226.2	367.86	183.93	292.08	146.93
SD	39.75	16.61	46.44	12.43	25.83	15.29
CV%	**10.6**	**7.3**	**12.6**	**6.8**	**8.8**	**10.4**

12.3. Specificity

The assay recognizes natural and recombinant murine TNFα and rat TNFα. To define specificity of this ELISA, several proteins were tested for cross reactivity. There was no cross reactivity observed for the proteins tested human TNFα, murine IL-2, murine IL-6, murine IL-10, murine IL-23, murine IFNγ, murine GM-CSF, murine CD138.

12.4. Stability

Storage Stability

Aliquots of spiked serum or culture media samples were stored at –20°C, 2-8°C, room temperature (RT) and at 37°C and the mTNFα level determined after 24h. For sample spiked in serum, we observe 30% loss at 2-8°C and RT and more than 50% if stored at 37°C. For sample spiked in culture media, we observe 30% loss when stored 24h at 2-8°C,RT or 37°C

Freeze-thaw Stability

Aliquots of spiked serum or culture media were stored frozen at –20°C and thawed up to 5 times and mTNFα level was determined. We observed 20% loss after 5 times.

12.5. Spike Recovery

The spike recovery was evaluated by spiking two concentrations of recombinant murine TNFa in two murine serum and one culture media in three experiments. Recoveries ranged from 60% to 106% with an overall **mean recovery of 68%.**

12.6. Dilution Parallelism

Two spiked murine serum with different levels of recombinant murine TNFa were analysed at four serial two fold dilutions (1:2-1:16) with two replicates each. Recoveries ranged from 96% to 145% with an overall **mean recovery of 113%.**

13. References

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14. Assay Summary

Total procedure length : 3h45mn

Add 100µl ofsample or diluted standard
↓
Add 50µl Biotinylated anti-mTNFα
↓
Incubate 3 hours at room temperature
↓
Wash three times
↓
Add 100µl of Streptavidin-HRP
↓
Incubate 30min at room temperature
↓
Wash three times
↓
Add 100 µl of ready-to-use TMB
Protect from light. Let the colour develop for 10-20 min.
↓
Add 100µl of H_2SO_4
↓
Read Absorbance at 450 nm

TECHNICAL CONSULTATION

OriGene Technologies, Inc.
9620 Medical Center Dr., Suite 200
Rockville, MD 20850

Phone: 1.888.267.4436
Fax: 301-340-9254
Email: techsupport@OriGene.com
Web: www.OriGene.com