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ab100785 – TNF alpha Rat SimpleStep ELISA® Kit

For the quantitative measurement of Rat TNF alpha in cell lysates and tissue lysates.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Abcam's TNF alpha ELSA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Rat TNF alpha in cell lysates and tissue lysates.

This assay employs an antibody specific for Rat TNF alpha coated on a 96-well plate. Standards and samples are pipetted into the wells and TNF alpha present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Rat TNF alpha antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TNF alpha bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed.

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Add standard or sample to each well used. Incubate at room temperature.

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Add prepared biotin antibody to each well. Incubate at room temperature.

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Add prepared Streptavidin solution. Incubate at room temperature.

⇩

Add TMB One-Step Development Solution to each well. Incubate at room temperature. Add Stop Solution to each well. Read at 450nm immediately.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Amount	Storage Condition (Before Preparation)
TNF alpha Microplate (12 x 8 wells)	96 wells	-20°C
20X Wash Buffer Concentrate	25 mL	-20°C
Recombinant Rat TNF alpha Standard	2 vials	-20°C
5X Sample Diluent Buffer	10 mL	-20°C
5X Assay Diluent	15 mL	-20°C
Biotinylated anti-Rat TNF alpha	2 vial	-20°C
200X HRP-Streptavidin Concentrate	200 µL	-20°C
TMB One-Step Substrate Reagent	12 mL	-20°C
Stop Solution	8 mL	-20°C
2X Cell Lysis Buffer	5mL	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 2 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.

- When preparing your standards, it is very critical to briefly spin down the vial first. The powder may drop off from the cap when opening it if you do not spin down. Be sure to dissolve the powder thoroughly when reconstituting. After adding Assay Diluent to the vial, we recommend inverting the tube a few times, then flick the tube a few times, and then spin it down; repeat this procedure 3-4 times. This is a technique we find very effective for thoroughly mixing the standard without too much mechanical force.
- Do not vortex the standard during reconstitution, as this will destabilize the protein.
- Once your standard has been reconstituted, it should be used right away or else frozen for later use.
- Keep the standard dilutions on ice while during preparation, but the ELISA procedure should be done at room temperature.
- Be sure to discard the working standard dilutions after use – they do not store well.
- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

9. Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare only as much reagent as is needed on the day of the experiment.

9.1 1X Wash Buffer PT

5X Sample Diluent Buffer should be diluted 5-fold with deionized or distilled water before use.

9.2 1X Assay Diluent

5X Assay Diluent should be diluted 5-fold with deionized or distilled water before use.

9.3 1X Cell Lysis

2X Cell Lysis Buffer should be diluted 2-fold with deionized or distilled water (for cell Lysate and tissue Lysate).

*When it comes to the actual use of this reagent for sample preparation, the methods vary depending on sample type/appearance. The choices of the method for lysis and homogenization include glass-bead "smash," douncing, freeze/thaw, sonication and crushing frozen tissue with a mortar and pestle, or even a combination of these. There is no best method for all sample types; your choice of method should be made following a brief search of the literature to see how samples similar to yours have been prepared in previous investigations.

9.4 1X Wash Solution

If the 20X Wash Concentrate contains visible crystals, equilibrate to room temperature and mix gently until dissolved. Dilute 20 mL of 20X Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1X Wash Solution.

9.5 1X Biotinylated TNF alpha Detection Antibody

Briefly spin the Biotinylated anti-Rat TNF alpha vial prior to use. Add 100µL of 1X Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can either be stored at 4°C for 5 days or aliquoted and frozen at -20°C for 2 months). The detection antibody concentrate must be diluted 80-fold with 1X Assay Diluent prior to use in the Assay Procedure.

9.6 1X HRP-Streptavidin Solution

Briefly spin the 200X HRP-Streptavidin concentrate vial and pipette up and down to mix gently before use. HRP-Streptavidin concentrate must be diluted 200-fold with 1X Assay Diluent prior to use in the Assay Procedure.
For example: Briefly spin the vial and pipette up and down to mix gently. Add 50 µL of 200X HRP-Streptavidin concentrate into a tube with 10 mL 1X Assay Diluent to prepare a final 200 fold diluted 1X HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

10. Standard Preparation

- Prepare serially diluted standards immediately prior to use.
- Always prepare a fresh set of standards for every use.
- Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution.

10.1 Briefly spin the vial of TNF alpha Standard. Prepare the 100 ng/mL TNF alpha **Stock Standard** by adding 400 µL 1X Sample Diluent Buffer into the vial (see table below).

10.2 Dissolve the powder thoroughly by a gentle mix.

10.3 Label tubes #1-7.

10.4 Prepare **Standard #**1 by adding 100 µL 100 ng/mL **Stock Standard** to 400 µL Sample Diluent Buffer into tube #1. Mix thoroughly and gently.

10.5 Pipette 400 µL 1X Sample Diluent Buffer into each tube.

10.6 Prepare **Standard #**2 by transferring 200 µL from tube #1 to #2, mix thoroughly.

10.7 Prepare **Standard #**3 by transferring 200 µL from tube #2 to #3, mix thoroughly.

10.8 Using the table below as a guide, prepare further serial dilutions

10.9 1X Sample Diluent Buffer serves as the zero standard (0 pg/mL).

Standard #	Volume to Dilute (µL)	Volume Diluent (µL)	Total Volume (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	100	400	500	100,000	20,000
2	200	400	600	20,000	6,667
3	200	400	600	6,667	2,222
4	200	400	600	2,222	740.7
5	200	400	600	740.7	246.9
6	200	400	600	246.9	82.30
7	0	400	400	0	0

11. Sample Preparation

General Sample Information:

- Tissue lysate and cell lysate sample should be diluted at least 5-fold with 1X Sample Diluent Buffer.
- Since different cells and tissues may contain different amounts of protein, as a starting point, we suggest using 500 µL of lysis buffer per $1x10^6$ cells or 10 mg tissue. You may have to adjust this based upon your results. Your target total protein concentration of the homogenate should be at least 1,000 µg/mL, but 2,000 µg/mL or more would be better.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

13. Assay Procedure

Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.

- It is recommended to assay all standards, controls and samples in duplicate.

13.1. Add 100 µL of each standard (see Standard Preparation section 10) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking. We recommend using 50-500 µg/mL of total protein for lysate sample. The amount of sample used depends on the abundance of target protein. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

13.2. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with 300 µL 1X Wash Solution using a multi-channel Pipette or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining liquid by aspirating or decanting. Invert the plate and blot it against clean paper towels.

13.3. Add 100 µL of 1X Biotinylated TNF alpha Detection Antibody (Reagent Preparation section 9) to each well. Incubate for 1 hour at room temperature with gentle shaking.

13.4. Discard the solution. Repeat the wash as in step 13.2.

13.5. Add 100 µL of 1X HRP-Streptavidin solution (see Reagent Preparation section 9) to each well. Incubate for 45 minutes at room temperature with gentle shaking.

13.6. Discard the solution. Repeat the wash as in step 13.2.

13.7. Add 100 µL of TMB One-Step Substrate Reagent to each well.

13.8. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

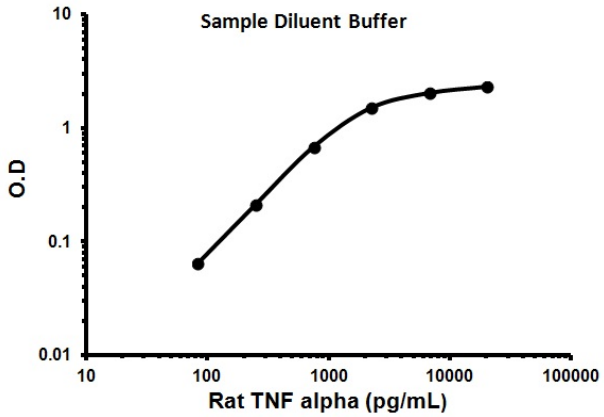
13.9. Add 50 µL of Stop Solution to each well. Read at 450 nm immediately.

14. Calculations

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

15. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.



Conc. (pg/mL)	O.D.
	Sample Diluent Buffer
82.3	0.064
246.9	0.21
740.7	0.673
2,222.2	1.489
6,666.7	2.012
20,000	2.287

Figure 1. Example of a typical rat TNF alpha dilution series.

16. Typical Sample Values

SENSITIVITY –

The minimum detectable dose of TNF alpha is typically less than 25 pg/mL.

RECOVERY –

Recovery was determined by spiking Rat TNF alpha into normal Rat tissue lysate and cell lysate. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Tissue Lysate	92.48	80-104
Cell Lysate	93.17	81-105

LINEARITY OF DILUTION-

Tissue Lysate	Average % Expected Value	Range (%)
1:2	90	80-103
1:4	94	84-106

Cell Lysate	Average % Expected Value	Range (%)
1:2	88	76-102
1:4	92	83-104

PRECISION –

	Intra-assay Precision	Inter-Assay Precision
CV (%)	<10%	<12%

17. Assay Specificity

Cross Reactivity: This ELISA kit shows no cross-reactivity with the following cytokines tested: (e.g., rat CINC-2, CINC-3, CNTF, Fractalkine, IL-1a, IL-1β, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, Leptin, Lix, MCP-1, MIP-3a, β-NGF, TIMP-1, VEGF).

18. Troubleshooting

Problem	Reason	Solution
Low Precision	Use of expired components	Check the expiration date listed before use. Do not interchange components from different lots
	Splashing of reagents while loading wells	Pipette properly in a controlled and careful manner
	Inconsistent volumes loaded into wells	Pipette properly in a controlled and careful manner. Check pipette calibration. Check pipette for proper performance
	Insufficient mixing of reagent dilutions	Thoroughly agitate the lyophilized components after reconstitution. Thoroughly mix dilutions
	Improperly sealed microplate	Check the microplate pouch for proper sealing. Check that the microplate pouch has no punctures. Check that three desiccants are inside the microplate pouch prior to sealing
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

Problem	Cause	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light.

19. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abcam.com | +64-(0)9-909-7829