ELISpot

Mouse TNF-α

Catalog Number EL410

For the quantitative determination of the frequency of cells releasing mouse TNF-α.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Mouse TNF-α is a pleiotrophic cytokine that plays a central role in inflammation and apoptosis (1-5). It is synthesized initially as a 26 kDa type II transmembrane (TM) glycoprotein that is 235 amino acids (aa) in length (6-8). It contains a 35 aa cytoplasmic domain, a 21 aa TM segment, and a 179 aa extracellular region that is divided into a 23 aa linker region, and a 156 aa mature molecule (6). TNF-α is assembled intra-cellularly to form a transmembrane, noncovalently linked homotrimeric protein. The 156 aa soluble form of trimeric TNF is released from the C-terminus of the TM protein through the activity of TACE, a membrane-bound disintegrin metalloprotease (9). Mouse TNF-α shares 80% aa identity with human TNF-α, and 95% aa identity with rat TNF-α (6, 10, 11). Mouse and human TNF-α show significant cross-species bioactivity (6). Mouse cells known to express TNF-α include neurons (12), $CD8^+$ T cells (13), mast cells (14), macrophages (15), astrocytes, monocytes and microglia (16) and dendritic cells (17). Rat cells known to express TNF-α include endothelial cells (18) and pancreatic acinar cells (19).

There are two mouse receptors for TNF-α. TNF RI/TNFRSF1A is a 55-60 kDa type I TM glycoprotein that contains a death domain and binds TNF- α with a high affinity (20-22). TNF RII/TNFRSF1B is a 75-80 kDa TM glycoprotein that also binds TNF-α with high affinity (20). Although TNF RI and TNF RII share a general structural design, TNF RII has little amino acid identity to TNF RI. Unlike TNF RI, which is species indifferent, TNF RII is partially species specific and prefers mouse TNF-α (20).

TNF-α is involved in a number of pathophysiological processes. It is considered one of the prototypical pro-inflammatory molecules and is induced in macrophages by gram-negative bacteria (LPS). Once expressed, TNF-α is reported to promote inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, serve as a chemotactic agent for monocytes, and activate phagocyte killing mechanisms (via increased NO_2^-/O_2^-/H_2O_2) (23). TNF-α also has documented antiviral activity, possibly acting through iNOS or IFN-β (24).

The Mouse TNF-α ELISpot assay is designed for the detection of mouse TNF-α secreting cells at the single cell level, and it can be used to quantitate the frequency of mouse TNF-α secreting cells. ELISpot assays are well suited for monitoring cytokine responses to various stimuli, treatments and therapies, and have been used for the quantitation of various TNF-α secreting cell types. Other methods used for the assessment of TNF-α secreting cells, such as the chromium release assays with quantitation by limiting dilution, are tedious and require previous *in vitro* expansion of TNF-α producing cells for several days. These assays typically are not suitable for measuring infrequent TNF-α responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of TNF-α producing cells, and thus are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research into areas as diverse as inflammation, vaccine development, and the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (25, 26). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (27, 28). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique.

A polyclonal antibody specific for mouse TNF-α has been pre-coated onto a polyvinylidene difluoride (PVDF)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37 °C CO_2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted TNF-α. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse TNF-α is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual TNF-α secreting cell. The spots can be counted with an ELISpot reader or using a stereomicroscope.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in the density of spots, intensity of specific staining, and background levels.

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid, and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT Substrate has been washed off.
- Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development. It may damage the PVDF membrane filter. The underdrain cover may be removed only after completing the incubation with the BCIP/NBT Substrate.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- Upon completing the experiment, do not dry the microplate at a temperature above 37 °C. It may cause the PVDF membrane filters to crack.
- The 96-well microplate provided in this kit is not sterile. Due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been shown to be an issue with this ELISpot procedure.
- This kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the provided microplate and reagents.
- The controls listed are recommended for each ELISpot experiment:

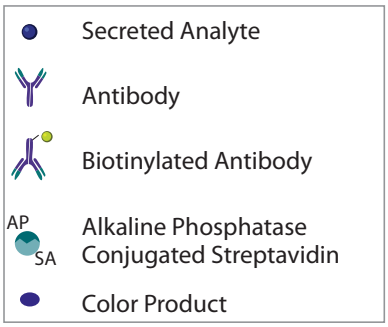
  Positive Control - Use recombinant mouse TNF-α.

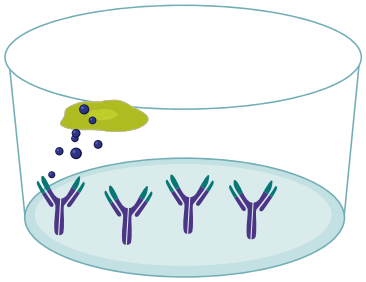
  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

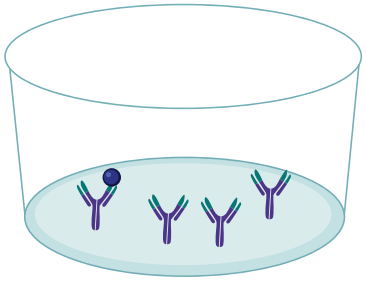
  Detection Antibody Control - Substitute phosphate buffered saline for detection antibody.

ELISPOT SCHEMATIC

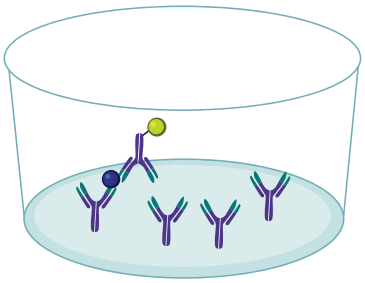




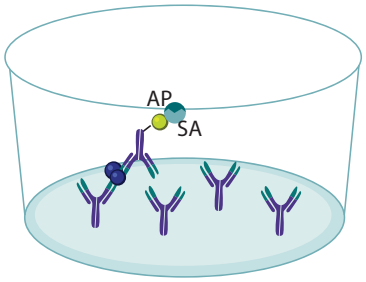
Incubate TNF-α secreting cells in an antibody-coated well.



Remove cells by washing. Secreted TNF-α is captured by the immobilized antibody.



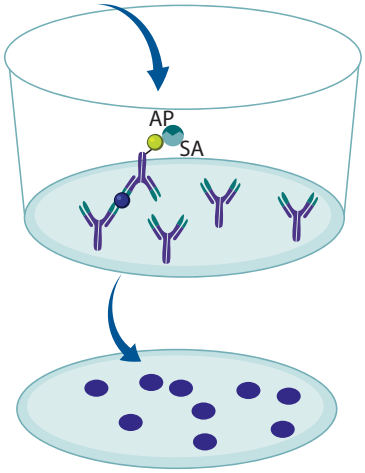
Incubate with biotinylated anti-TNF-α antibody.



Remove unbound biotinylated antibody by washing. Incubate with alkaline phosphatase conjugated streptavidin.

Wash to remove unbound enzyme.

Add BCIP/NBT Substrate



Add substrate and monitor the formation of colored spots. Analyze using either an ELISpot reader or dissection microscope.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

Note: *This kit is validated for single use only. Results obtained using previously opened or reconstituted reagents may not be reliable.*

PART	PART #	DESCRIPTION
Mouse TNF-α Microplate	890900	96-well PVDF-backed microplate coated with a polyclonal antibody specific for mouse TNF-α.
Mouse TNF-α Detection Antibody Concentrate	890901	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse TNF-α with preservatives.
Streptavidin-AP Concentrate A	895358	150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.
Dilution Buffer 1	895307	12 mL of a buffer for diluting Mouse TNF-α Detection Antibody Concentrate with preservatives.
Dilution Buffer 2	895354	12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative.
BCIP/NBT Substrate	895867	12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).
Mouse TNF-α Positive Control	890902	2 ng of recombinant mouse TNF-α with preservatives; lyophilized.

OTHER SUPPLIES REQUIRED

- Dissection microscope or an ELISpot reader.
- Pipettes and pipette tips.
- Deionized water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- 37 °C CO_2 incubator.
- Sterile culture media.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

BCIP/NBT is toxic if swallowed, in contact with skin, or if inhaled. It is a highly flammable liquid and vapor may cause serious irritation and damage to organs. Do not eat, drink, or smoke when using this product. Do not breathe fumes. Use only in a well-ventilated area. Keep away from heat, sparks, open flames, and hot surfaces. Keep the container tightly closed.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

REAGENT PREPARATION

Bring all reagents to room temperature, except the Mouse TNF-α Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2-8 °C.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Mouse TNF-α Positive Control - Reconstitute the lyophilized Mouse TNF-α Positive Control with 250 µL of culture medium that is used to incubate cells.

Detection Antibody Mixture- Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Mouse TNF-α Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare the Detection Antibody Mixture immediately before use.**

Streptavidin-AP Concentrate A - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare the Streptavidin-AP immediately before use.**

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator.

ASSAY PROCEDURE

Bring all reagents to room temperature, except the diluted Detection Antibody Mixture, which should remain at 2-8 °C. All samples and controls should be assayed at least in duplicate.

1. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37 °C CO_2 incubator. Optimal incubation time for each stimulus should be determined by the investigator. **Do not disturb the cells during the incubation period.**
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250-300 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
   Note: *Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.*
5. Add 100 µL of the diluted Detection Antibody Mixture into each well, and incubate overnight at 2-8 °C. Alternatively, incubation with detection antibodies can be done for 2 hours at room temperature on a rocking platform.
6. Repeat the wash procedure described in step 4.
7. Add 100 µL of the diluted Streptavidin-AP Concentrate A into each well, and incubate for 2 hours at room temperature.
8. Repeat the wash procedure described in step 4.
9. Add 100 µL of the BCIP/NBT Substrate into each well, and incubate for 1 hour at room temperature. **Protect from light.**
10. Decant the BCIP/NBT Substrate solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels and dry completely either at room temperature (60-90 minutes) or 37 °C (15-30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots using either a dissection microscope or an ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added to the well.

REPRODUCIBILITY DATA

Splenocytes from a C57BL mouse (1 x 10^6 cells/mL) were stimulated with 3.0 µg/mL anti-mouse CD3ε antibodies overnight at 37 °C in a 5% CO_2 incubator. The sample was assayed in seven wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	96
2	92
3	52
4	94
5	72
6	82
7	71

TROUBLESHOOTING GUIDE

OBSERVATION	PROBLEM	CORRECTIVE ACTION
Following the incubation with the BCIP/NBT Substrate and rinsing the microplate with deionized water, the dark blue background color of the filter membrane attenuates visualization and quantitation of spots.	The membrane is wet.	Microplates cannot be analyzed accurately until the PVDF filter membranes are completely dry. Wait until the membrane becomes dry (typically 15-30 minutes at 37 °C or 60-90 minutes at room temperature).
The number of spots in the wells that contained the cells is high, but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment; perhaps the result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been brought to room temperature.	Warm the appropriate reagents to room temperature before adding them to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned blue-black.	Cell stimulation problem.	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells were added to the wells.	Increase the number of cells added per well.
Following incubation with the BCIP/NBT Substrate and drying the microplate, the density of the spots makes them difficult to quantify.	Too many cells were added to the wells.	Make dilutions of cells (*i.e.* 1 x 10^6, 5 x 10^5, 1 x 10^5, 5 x 10^4, 1 x 10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.

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