virax biolabs

ELISpot Human TNF-α Kit

UNCOATED PLATES

Instructions for use

Catalogue Numbers:

2148-01	Uncoated 96-well plate – 1 plate
2148-05	Uncoated 96-well plates – 5 plates

Note: This protocol (version 3) is given as a general procedure to assist when using **Virax Biolabs** Capture and Detection antibodies for ELISpot testing. Optimal dilutions of all reagents, samples and controls as well as the incubation times should be determined by each laboratory for every application.

For research use only

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INTENDED USE

The Human TNF-α Enzyme-Linked ImmunoSpot (ELISpot) assay is a highly sensitive immunological technique that detects cytokine secretion at the single-cell level. Unlike methods such as ELISA or RT-PCR, which measure cytokine or cytokine-related signals at the population level, ELISpot enables identification and quantification of the frequency of individual cytokine-secreting cells. This assay can be used assess cellular immune responses by determining the frequency of stimulated effector cells that secrete TNF-α.

INTRODUCTION

Description

TNF-α is a multifunctional cytokine that plays a central role in coordinating innate and adaptive immune responses. It is produced predominantly by activated monocytes and macrophages, but may also be released by lymphocytes, NK cells, neutrophils, mast cells, endothelial cells, and other cell types following infection, tissue injury, or immune stimulation. As a potent pro-inflammatory mediator and pyrogen, TNF-α promotes endothelial activation, adhesion molecule expression, vascular permeability, and the recruitment of immune cells to affected tissues. It also enhances the antimicrobial and cytotoxic functions of immune cells and contributes to the elimination of infected or damaged cells through the induction of apoptosis and other cell-death pathways. In addition to its local inflammatory effects, TNF-α influences antigen presentation, leukocyte activation, tissue metabolism, acute-phase responses, and the production of other immune mediators, including interleukin-1, granulocyte-macrophage colony-stimulating factor, and major histocompatibility complex molecules.

However, excessive or prolonged TNF-α production can cause systemic inflammation, tissue damage, metabolic dysfunction, and cachexia. Elevated TNF-α levels have been associated with severe infection and sepsis, chronic inflammatory and autoimmune disorders, transplant rejection, graft-versus-host disease, and complications arising during immunodeficiency. Due to its varied functions throughout the immune system, TNF-α is a valuable indicator of inflammatory activity.

This kit is for research use only and is not to be used in diagnostic procedures.

STORAGE

The ELISpot kit is shipped at 4°C. Store product contents; antibodies, enzyme conjugate and substrate at 2-8°C upon receipt. Plates can be kept at room temperature. Expiry of the kit and reagents is stated on the box label. Required storage conditions should be maintained to ensure the stated expiry of the kit components. Return reagents immediately to 2-8°C after use. Handle reagents under sterile conditions. Any contamination of the reagents or wrong storage conditions will invalidate the shelf life of the product.

CONTENTS

Plates	96 well uncoated ELISpot plate(s) / strips
Capture mAb	TNF-α capture antibody
Detection mAb	Biotinylated TNF-α antibody
Enzyme conjugate	Streptavidin-ALP (Alkaline Phosphatase)
Substrate	BCIP/NBT Ready-to-use solution
Assay diluent	Bovine Serum Albumin (BSA)

REAGENTS AND MATERIALS REQUIRED BUT NOT SUPPLIED

Please ensure you have these materials and reagents prior to starting assay.

- Cell culture reagents (e.g. RPMI-1640, L-glutamine, Foetal Calf Serum (FCS))
- Cell stimulation reagents (e.g. PMA, Ionomycin, peptide pool, CD3, CD28, lipopolysaccharide (LPS))
- CO_2 incubator
- Phosphate Buffered Saline (1x, PBS) / Dulbecco's phosphate buffer saline (DPBS)

REAGENTS PREPARATION

a) Blocking Buffer

Use the same buffer to block the plate membrane as is used for cell culture medium. We recommend 10% FCS in RPMI-1640 supplemented with L-glutamine. *Note: This buffer should be kept sterile.*

b) Dilution Buffer

Prepare 1% BSA in PBS (1x) solution e.g. 1g in 100mL PBS (1x). It is recommended to filter 1% BSA solution through a 0.2µm filter.

c) Capture Antibody

⚠️ **Prepare this reagent immediately prior to use.**

Capture antibody shipped at 1mg/mL, dilute prior to use. Dilute the capture mAb to 15µg/mL in sterile PBS and mix well. For one plate, dilute 15µl of antibody in 10mL of sterile PBS and mix well. It is recommended to filter the capture antibody through a 0.2µm filter prior to use to remove any antibody aggregates that may have formed. Any unused stock antibody can be kept at -20°C for long term storage or 4°C for 1 month.

d) Detection Antibody

⚠️ **Prepare this reagent immediately prior to use.**

Detection antibody shipped at x100, dilute prior to use. For one plate, dilute 100µl of antibody into 10mL of Dilution Buffer and mix well. It is recommended to filter the detection antibody through a 0.2µm filter prior to use to remove any antibody aggregates that may have formed. Any unused stock antibody can be kept at -20°C for long term storage or 4°C for 1 month.

e) Streptavidin-Alkaline Phosphatase (ALP) conjugate

⚠️ **Prepare this reagent immediately prior to use.**

Streptavidin-ALP is shipped at x1000, dilute prior to use. For one plate, dilute 10µL of Streptavidin-ALP conjugate into 10mL of Dilution Buffer and mix well.

ELISPOT PROTOCOL

a) Plate Activation and Blocking

⚠️ **Work under sterile conditions.**

1. Remove plate from its sealed package.
2. To activate the plate, add 15µL 35% ethanol (EtOH) per well and incubate for 30-60 seconds.
   Note: MSIPS4W10 (white plates), MSIPS4510 (clear plates) and M8IPS4510 (strip plates) all require this activation step. Do not leave ethanol on for longer than 1 minute.
3. Wash the plate 5 times with sterile PBS (1x), 200µL per well.
4. Add diluted capture antibody solution, 100µL per well.
5. Incubate for 3 hours or overnight at 2-8°C.

6. Following incubation; wash plate 5 times with 200µL sterile PBS per well to remove the excess capture antibody.
7. Add 100µL of blocking buffer per well.
8. Cover the plate and incubate at room temperature for at least 30 minutes.

Do not tap the plate. Empty the wells by flicking. *Note: If pipetting be careful to avoid contact with the membrane.*

b) Cell Incubation

⚠️ **Work under sterile conditions.**

At this step, either frozen or freshly isolated cells can be used. While working with frozen samples, it is suggested that to leave thawed cells at 37°C for at least one hour in order to remove cell debris.

1. Remove blocking buffer.
2. Pipette 50µL of stimulant and 50µL of cell suspension per well. Preferably stimulant and cell suspension can be mixed prior to plating. Cell suspension should not be added first.

Note: We recommend adding the following controls to your plate: negative control - cell suspension media without stimulant; Positive control – PMA/ionomycin or CD3/CD28 for adaptive immunity or LPS for innate immunity.

3. Place the plate in a CO_2 and temperature controlled humidified incubator (37°C and 5% CO_2) with lid on.
4. Incubate the plate for desire length of time depending on the stimulant and cell type i.e. 18-48 hours.

At this step, plate can also be wrapped using aluminium foil to prevent evaporation and allow equal heat distribution. Do not stack plates in order to maintain continuous and equal flow. Do not move plate during incubation to avoid cytokine "trailing" or streaks and ensure accurate spot formation.

c) Detection and Development

1. Remove cells and wash the plate 5 times with 200µL of PBS (1x). Tap dry on lint-free tissue.
2. Pipette 100µL of diluted detection antibody to every well.
3. Cover the plate and incubate at room temperature in the dark for 1 hour 30 minutes.
4. Empty the wells and wash the plate 5 times with 200µL of PBS (1x).
5. Pipette 100µL of diluted streptavidin-ALP conjugate to every well.
6. Cover the plate and incubate at room temperature in the dark for 1 hour.
7. Empty the wells and wash the plate 5 times with 200µL of PBS (1x).

Recommended extra step: Gently detach underdrain and rinse membrane with distilled water to remove excess PBS prior to BCIP/NBT addition. Tap plate dry using lint-free tissue.

8. Add 100µl of ready-to-use BCIP/NBT substrate to every well, and incubate in the dark for 3-10 minutes. Observe coloured spot development until distinct spots form.
9. To stop the spot development, wash the wells thoroughly with distilled water.
10. Tap dry the plate on lint free absorbent paper.
11. Air-dry the plate at room temperature for minimum 1 hour then read the wells using an ELISpot reader. *Hint: dry plate in BSC hood for quicker drying.*

⚠ **Make sure wells are completely dry before spot counting.**
Wet membrane increases background noise and interferes with spot detection and clarity.

Store plate in the dark at room temperature.

USEFUL INFORMATION

a) Plate Handling and Washing

ELISpot membrane is delicate. Handle the plate with extra care during pipetting and washing as the PVDF membrane may easily be damaged, i.e. puncture of the membrane.

It is also important not to allow membrane to dry for the duration of the assay.

Automated plate washers or squirt bottles with a wide spout can be used for the washing steps that do not require sterile conditions. However, vigorous dispensing for both manual and automated washing should be avoided to prevent damaging the PVDF membrane and dislodging antibodies from the well surface as this could lead to inaccurate results and poor reproducibility.

b) Buffer

To ensure sterile conditions during washing and blocking steps, use sterilised PBS (1x) or filter through a 0.2µm filter to avoid background.

c) Serum

Foetal calf serum is recommended to use for conditioning the wells. Prepare 10% FCS in cell culture medium for cell blocking. However, some batches of FCS may non-specifically activate the cells and can therefore be the cause of non-specific background. Test different batches of FCS to assess for low background reactivity and high antigen-specific responses before use.

d) Sample

Fresh or frozen human PBMCs can be used as samples. While working with frozen cells, it is recommended to rest the cells at 37°C for at least an hour to remove any cell debris. Excess cell debris in samples can lead to false positives and clumping effect during spot formation.

Please see table below for our recommended cell concentrations. However, cell number should be optimised while using polyclonal activators as higher cell concentration can lead to confluent spot formation and overlapping spots.

STIMULATION	NUMBER OF CELLS PER WELL
PMA/Ionomycin	5,000
LPS	5,000
Anti-CD3/CD28	100,000
Peptide	150,000 – 250,000
Negative	100,000

e) Stimuli

Samples can be stimulated using the antigen of interest, i.e. infected cells, vaccine, virus, and peptide pool. If the cell type and/or cytokine/protein of interest requires longer incubation, then a pre-incubation step can be added, however, prolonged cell incubation can lead to excessive cytokine/protein secretion. This will cause the larger spots to start merging and become indistinguishable. Therefore, incubation time and the amount of stimulant should be optimized depending on the cell type and antigen of interest.

f) Assay Controls

For positive control wells, anti-CD3/CD28 antibodies, PMA and Ionomycin and LPS are the commonly used T cell activators. Negative control wells should contain only unstimulated cell and cell culture medium. However, if anti-CD28 is used as a stimulant in the other wells, it should also be added to the negative control to help identifying the response is solely due to the stimulant i.e. peptide pool.

CYTOKINE	RECOMMENDED STIMULATIONS
IL-2	PMA/ionomycin; CD3/CD28
IL-6	PMA/ionomycin; CD3/CD28; LPS
IL-10	PMA/ionomycin; CD3/CD28
IFN-γ	PMA/ionomycin; CD3/CD28
TNF-α	CD3/CD28; LPS
IL-1β	LPS
IL-8	LPS

virax biolabs

BioCity Glasgow
Bo'Ness Road, Newhouse
Lanarkshire ML1 5UH
United Kingdom
www.viraxbiolabs.com
support@viraxbiolabs.com