

# Rat TNF $\alpha$ ELISpot

Instructions for use

Catalogue Numbers:

	Without Plates	With non-Sterile Plates	With sterile Plates
1x96 tests	867.021.001	867.021.001P	867.021.001S
5x96 tests	867.021.005	867.021.005P	867.021.005S
10x96 tests	867.021.010	867.021.010P	867.021.010S
15x96 tests	867.021.015	867.021.015P	867.021.015S
20x96 tests	867.021.020	867.021.020P	867.021.020S

**For research use only**

Fast Track Your Research.....

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## 1. Intended use

Diaclone **ELISpot** is a highly specific immunoassay for the analysis of cytokine and other soluble molecule production and secretion from T-cells at a single cell level in conditions closely comparable to the *in-vivo* environment with minimal cell manipulation. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation and the comparison of such frequency against a specific treatment or pathological state. The ELISpot assay constitutes an ideal tool in the investigation of Th1 / Th2 responses, vaccine development, viral infection monitoring and treatment, cancerology, infectious disease, autoimmune diseases and transplantation.

Utilising sandwich immuno-enzyme technology, Diaclone ELISpot assays can detect both secreted cytokines and single cells that simultaneously produce multiple cytokines. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation or binding on soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates.

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

## 2. Introduction

### 2.1. Summary

Tumor Necrosis Factor (TNF $\alpha$ ), also known as cachectin, is a polypeptide cytokine produced by monocytes and macrophages. It functions as a multipotent modulator of immune response and further acts as a potent pyrogen (4, 17). TNF $\alpha$  circulates throughout the body responding to stimuli (infectious agents or tissue injury), activating neutrophils, altering the properties of vascular endothelial cells, regulating metabolic activities of other tissues, as well as exhibiting tumoricidal activity by inducing localized blood clotting. TNF $\alpha$  also inhibits lipoprotein lipase activity resulting in cachexia, a physical wasting condition (4, 17). Activation of B-cells by the Epstein Barr virus can be inhibited by TNF $\alpha$  (15). Due to its varied actions throughout the immune system, TNF $\alpha$  may play a role in the pathogenesis of many disease states.

TNF- $\alpha$  production is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNF $\alpha$  within four hours of stimulation by recombinant IL-2 (9) and there is some *in vitro* evidence to suggest that TNF $\alpha$  is expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNF $\alpha$  is enhanced by gamma interferon. TNF $\alpha$  then induces or enhances the specific production of Class I MHC antigen, GM-CSF, and IL-1. Recent evidence has suggested an intracellular role for this peptide (23).

TNF $\alpha$  may play a significant role in the pathogenesis of inflammatory disease of the joints and other tissues. Chin et al. (6) found that TNF $\alpha$ , along with gamma interferon and IL-1 increased cell surface expression of ICAM -1 on synovial fibroblasts. Alvaro-Garcia et al. (3) reported that TNF $\alpha$  stimulates synovial proliferation.

Waage et al. (25) found that increased levels of TNF $\alpha$  in patients with septicemia and meningococcal disease correlated with fatal outcome. Scuderi et al. (22) suggest that increased levels of this cytokine may play a role in the host defense mechanism against parasitic infections. Girardin et al. (12) reported that increased serum TNF $\alpha$  levels correlated with the number of risk factors involved in children with gram-negative sepsis and purpura fulminans. Elevated levels of TNF $\alpha$  were also found in individuals suffering from myocarditis (11).

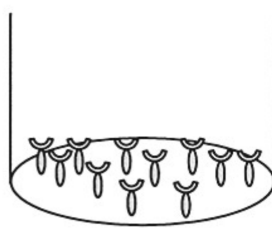
Recently, a growing body of information has pointed to a role for TNF $\alpha$  in the pathogenesis of AIDS. Alveolar macrophages (AM) from HIV positive individuals with opportunistic lung infections have been shown to spontaneously produce higher levels of TNF $\alpha$  in vitro than those HIV positive individuals without infection and HIV negative controls (14, 16). Krishnan et al. (16) report that higher TNF $\alpha$  production by AM was associated with lower counts of pneumocystis carinii in bronchoalveolar lavage fluid, indicating that TNF $\alpha$  may play a role in the control of this infection in AIDS. Israel-Biet et al. (14) also reported in in-vitro studies, that AM that express HIV (p24+) released significantly higher levels of TNF $\alpha$  than p24- alveolar macrophages and controls. Reddy et al. (20) found persistently elevated levels of circulating TNF $\alpha$  in HIV seropositive individuals and suggest a possible involvement of this cytokine in the development of AIDS.


Measurement of TNF $\alpha$  levels has also been shown to be useful in transplant research, where Maury et al. (18) and McLaughlin et al. (19). Both reported TNF $\alpha$  to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNF $\alpha$  levels in Bone Marrow Transplant (BMT) (13, 21). BMT patients with major transplant related complications such as interstitial pneumonitis and severe acute graft-versus - host disease had TNF $\alpha$  levels significantly increase over controls (13).

## 2.2. Principle of the method

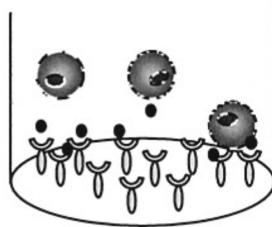
A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimise any non-antibody dependent unspecific binding and washed. Cell suspension and stimulant are added and the plate incubated allowing the specific antibodies to bind any analytes produced. Cells are then removed by washing prior to the addition of Biotinylated detection antibodies which bind to the previously captured analyte. Enzyme conjugated streptavidin is then added binding to the detection antibodies. Following incubation and washing substrate is then applied to the wells resulting in coloured spots which can be quantified using appropriate analysis software or manually using a microscope.

1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody





 Capture antibody

2. Incubation of cells in the coated microwell



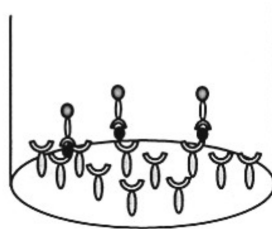
 Antigen / Mitogen

 Biotinylated detection antibody

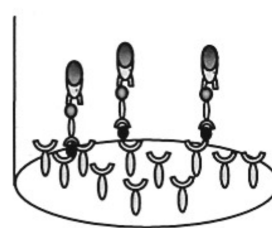
 Streptavidin - alkaline phosphatase conjugated

 Substrate product

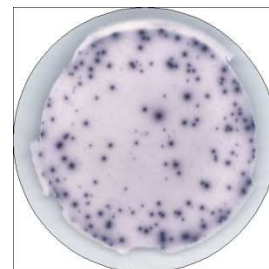
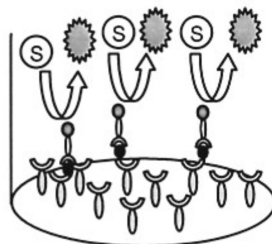
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatase conjugated



5. Addition of substrate BCIP/NBT and monitoring of spot formation.



### 3. Reagents provided (Contents shown for 5x96 test format)

- 96 well PDVF bottomed plates (5 if ordered)
- Capture Antibody for Rat TNF $\alpha$  (0.5ml supplied sterile)
- Biotinylated detection antibody (lyophilised, resuspend in 0.55ml)
- Streptavidin-Alkaline Phosphatase conjugate (50 $\mu$ l)
- Bovine Serum Albumin (BSA)
- Blocking reagent
- Ready to use BCIP/NBT substrate buffer (50ml)

Please note for 1 x 96 demo kits Biotinylated detection antibody is provided in liquid form.

### 4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin, LPS)
- CO<sub>2</sub> incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- 96 well PVDF bottomed plates if not ordered (we recommended Millipore plates catalogue # MSIPN4510, MSIPS4510 and M8IPS4510)

### 5. Storage Instructions

Store kit reagents between 2 and 8°C except uncoated plates which should be stored at RT. Immediately after use remaining reagents should be returned to cold storage (2 to 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 the case of repeated use of one component, the reagent is not contaminated by the first handling.

## 6. Safety & Precautions for use

- For **research use only** not to be used as a diagnostic test
- 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
- 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.
- 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
- 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
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells
- **BCIP/NBT buffer** is potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling this reagent, always wear gloves
- Follow incubation times described in the assay procedure

## 7. Reagent Preparation

### 7.1. 1X Phosphate Buffered Saline (PBS) (Coating Buffer)

For 1 litre of 10X PBS weigh-out:   80g NaCl  
  2g KH<sub>2</sub>PO<sub>4</sub>  
  14.4g Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O.

Add distilled water to 1 litre. Adjust the pH of the solution to 7.4 +/- 0.1 were required.

**Dilute the solution to 1X before use.**

### 7.2. Skimmed milk in 1X PBS (Blocking Buffer)

For one non-sterile plate dissolve 0.2g of powder in 10mL of 1X PBS

For one sterile plate dilute 5ml of liquid milk in 5ml of 1X PBS

Please note liquid milk has a shorter expiration date than the other reagents of the kit (indicated on the vial).  
The use of expired milk can lead to unspecific stimulation.  
Use any fresh semi skimmed milk (UHT) if the one provided has expired.

### 7.3. 1% BSA PBS Solution (Dilution Buffer)

For one plate dissolve 0.2 g of BSA in 20 ml of 1X PBS.

### 7.4. 0.05% PBS-T Solution (Wash Buffer)

For one plate dissolve 50µl of Tween 20 in 100mL of 1X PBS.

### 7.5. 35% Ethanol (PVDF Membrane Activation Buffer)

For one plate mix 3.5 ml of ethanol with 6.5 ml of distilled water.

### 7.6. Capture Antibody

*This reagent is supplied sterile once opened keep the vial sterile or aliquot and store at -20°C. For optimal performance prepare the Capture Antibody dilution immediately before use.*

Dilute 100µl of capture antibody in 10 mL of 1X PBS and mix well.

### 7.7. Detection Antibody

Reconstitute the lyophilised antibody with 0.55mL of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution.

*If not used within a short period of time, reconstituted Detection Antibody should be aliquoted and stored at -20°C. In these conditions the reagent is stable for at least one year. For optimal performance prepare the reconstituted antibody dilution immediately prior to use.*

Dilute 100µl of antibody into 10ml Dilution Buffer and mix well.

Please note for 1 x 96 demo kits Biotinylated detection antibody is provided in liquid form.



## **7.8. Streptavidin – AP conjugate**

*For optimal performance prepare the Streptavidin-AP dilution immediately prior to use*

For 1 plate dilute 10µl of Streptavidin-AP conjugate into 10 mL Dilution Buffer and mix well.

*Do not keep this solution for further experiments.*

## **8. Sample and Control Preparation**

### **8.1. Cell Stimulation**

Cells can either be stimulated directly in the antibody coated wells (Direct) or, first stimulated in 24 well plates or flask, harvested, and then plated into the coated wells (Indirect).

The method used is dependent on 1) the type of cell assayed 2) the expected cell frequency. When a low number of cytokine producing cells are expected it is also advised to test them with the direct method, however, when this number is particularly high it is better to use the indirect ELISpot method.

All the method steps following stimulation of the cells are the same whatever the method (direct/indirect) chosen.

### **8.2. Positive Assay Control, Rat TNF $\alpha$ production**

We recommend using the following polyclonal activation as a positive control in your assay.

Isolate splenocytes in culture media (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing containing 1µg/ml LPS (Sigma, Saint Louis, MO). Distribute  $1 \times 10^5$  to  $2.5 \times 10^5$  cells per 100µl in required wells of an antibody coated 96-well PVDF plates and incubate for 10-15 hours in an incubator.

For antigen specific stimulation, the optimal concentration of the antigen and the optimal concentration of number of cells have to be determined experimentally, as it is depending on the frequency of cytokine producing cells

### **8.3. Negative Assay Control**

Dilute splenocytes in culture media to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100µl with no stimulation.

### **8.4. Sample**

Dilute splenocytes in culture medium and stimulator of interest (i.e. Sample, Vaccine, Peptide pool or infected cells) to give an appropriate cell number per 100µl.

Optimal assay performances are observed between  $1 \times 10^5$  and  $2.5 \times 10^5$  cells per 100µl.

Stimulators and incubation times can be varied depending on the frequency of cytokine producing cells and therefore should be optimised by the testing laboratory.

## 9. Method

Prepare all reagents as shown in section 7 and 8.

**Note:** For optimal performance prepare the **Streptavidin-AP** dilution immediately prior to use

Assay Step		Details
1.	Addition	Add 25µl of 35% ethanol to every well
2.	Incubation	Incubate plate at room temperature (RT) for 30 seconds
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100µl of 1X PBS per well
4.	Addition	Add 100µl of diluted <b>capture antibody</b> to every well
5.	Incubation	Cover the plate and incubate at 4°C overnight
6.	Wash	Empty the wells as previous and wash the plate once with 100µl of 1X PBS per well
7.	Addition	Add 100µl of <b>Blocking buffer</b> to every well
8.	Incubation	Cover the plate and incubate at RT for 2 hours
9.	Wash	Empty the wells as previous and thoroughly wash 3x with 100µl of 1X PBS per well
10.	Addition	Add 100µl of <b>sample, positive and negative controls</b> cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)
11.	Incubation	Cover the plate and incubate at 37°C in a CO <sub>2</sub> incubator for an appropriate length of time (15-20 hours). <b>Note: do not agitate or move the plate during this incubation</b>
12.	Addition	Empty the wells and remove excess solution then add 100µl of PBS-T to every well
13.	Incubation	Incubate the plate at 4°C for 10 min
14.	Wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
15.	Addition	Add 100µl of diluted <b>detection antibody</b> to every well
16.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
17.	wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
18.	Addition	Add 100µl of diluted <b>Streptavidin-AP conjugate</b> to every well
19.	Incubation	Cover the plate and incubate at RT for 1 hour
20.	Wash	Empty the wells and wash the plate 3x with 100µl of PBS-T
21.	Wash	Peel of the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing complete remove any excess solution by repeated tapping on absorbent paper.
22.	Addition	Add 100µl of ready-to-use <b>BCIP/NBT buffer</b> to every well
23.	Development	Incubate the plate for <b>5-20 min</b> monitoring spot formation visually throughout the incubation period to assess sufficient colour development
24.	Wash	Empty the wells and rinse both sides of the membrane 3x under running distilled water. Completely remove any excess solution by gentle repeated tapping on absorbent paper
<p><b>Read Spots:</b> allow the wells to dry and then read results. The frequency of the resulting coloured spots corresponding to the cytokine producing cells can be determined using an appropriate ELISpot reader and analysis software or manually using a microscope.</p> <p><i>Note: spots may become sharper after overnight incubation at 4°C</i></p>		

Plate should be stored at RT away from direct light, but please note colour may fade over prolonged periods so read results within 24 hours.

## 10. Performance Characteristics

### 10.1. Specificity

The assay recognizes natural Rat TNF $\alpha$  and cross react with murine TNF $\alpha$ .

### 10.2. Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (LPS) of 5 different mouse splenocytes concentrations, 3 repetitions in 1 batch. The data shows the mean spot number, range and CV for the five cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
100000 recommended	3	238	236	241	1.1%
50000	3	130	125	135	3.9%
25000	3	75	72	77	3.8%
12500	3	37	32	43	15.0%
6250	3	21	20	23	7.2%

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