

# **ab64034 – Rat TNF $\alpha$ ELISPOT Kit (with precoated plates)**

## Instructions for Use

For the qualitative measurement of TNF $\alpha$  production and secretion in a single cell suspension.

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

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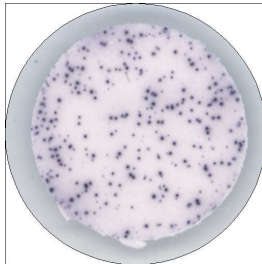
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## 1. BACKGROUND

Abcam's TNF $\alpha$  Rat ELISPOT kit is designed for the qualitative measurement of TNF $\alpha$  production and secretion in a single cell suspension.

The ELISPOT assay involves a capture antibody highly specific for the analyte of interest 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 minimize 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 colored spots which can be quantified using appropriate analysis software or manually using a microscope.



The ELISPOT assay 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,

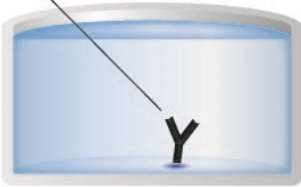
vaccine development, viral infection monitoring and treatment, cancerology, infectious disease, autoimmune diseases and transplantation.

Utilising sandwich immuno-enzyme technology, Abcam 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.

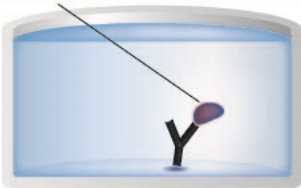
Tumor Necrosis Factor (TNF $\alpha$ ), also known as cachectin, is a polypeptide cytokine produced by monocytes and macrophages. It functions as a multipotent modulator of immune response and further acts as a potent pyrogen. TNF $\alpha$  circulates throughout the body responding to stimuli (infectious 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. Activation of B-cells by the Epstein Barr virus can be inhibited by TNF $\alpha$ . Due to its varied actions throughout the immune system, TNF $\alpha$  may play a role in the pathogenesis of many disease states.

## 2. ASSAY SUMMARY

Capture Antibody



Protein



anti-Protein-Biotin



Streptavidin-AP



Substrate **Colored Product**



Equilibrate all reagents to room temperature. Prepare all the reagents and samples as instructed. 96-well PVDF bottomed plates are supplied pre-coated with capture antibody.

Add sample (Cells) to appropriate wells. Incubate at 37 °C.

Aspirate and wash each well. Add prepared Biotinylated labeled detector antibody. Incubate at room temperature.

Add prepared Streptavidin-Alkaline Phosphatase mix to each well. Incubate at room temperature.

Add the substrate solution BCIP/NBT to each well and monitor spot formation.



### **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. Modifications to the kit components or procedures may result in loss of performance.

### **4. STORAGE AND STABILITY**

**Store kit at +2-8 °C immediately upon receipt.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Quantity		Storage Condition (Before Preparation)
	1 x 96 tests	5 x 96 tests	
Pre-coated 96 well PVDF bottomed Microplate	1 x 96 wells	5 x 96 wells	+2-8°C
Biotinylated Detection antibody	1 x 1 vial	1 x 1 vial	+2-8°C
Streptavidin-Alkaline Phosphatase Conjugate	1 x 10 µL	1 x 50 µL	+2-8°C
Bovine Serum Albumin (BSA)	1 x 200 mg	1 x 1 g	+2-8°C
Ready to use BCIP/NBT substrate buffer	1 x 10 mL	1 x 50 mL	+2-8°C



### **6. MATERIALS REQUIRED, NOT SUPPLIED**

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

- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS).
- Cell stimulation reagents (PMA, Ionomycin, LPS).
- 0.05% PBS-T Solution (Wash Buffer).

For one plate dissolve 50  $\mu$ L of Tween 20 in 100 mL of 1X PBS.

- 1X Phosphate Buffered Saline (PBS).

For 1L of 10X PBS weigh out:

80 g NaCl

2 g  $\text{KH}_2\text{PO}_4$

14.4 g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ .

Add distilled water to 1L. Adjust the pH of the solution to 7.4 +/- 0.1. Dilute the solution to 1X before use.

- Miscellaneous laboratory plastic and/or glass, if possible sterile.
- $\text{CO}_2$  incubator.

## 7. LIMITATIONS

- 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.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

## 8. TECHNICAL HINTS

- Kit components should be stored as indicated. All the reagents should be equilibrated to room temperature before use.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross-contamination; for the dispensing of the Substrate solution, avoid pipettes with metal parts.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. Please contact our Technical Support staff with any questions.**

## 9. REAGENT PREPARATION

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

### 9.1 1% BSA PBS Solution (Dilution Buffer)

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

### 9.2 Detection Antibody

Reconstitute the lyophilised antibody with 550 µL of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution.

Dilute 100 µL of resuspended antibody into 10 mL Dilution Buffer and mix well.

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.

*Please note for 1x96 demo kits, Biotinylated detection antibody is provided in liquid form.*

### 9.3 Streptavidin – AP conjugate

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

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

Do not keep this solution for further experiments.

## **10. CONTROL PREPARATION**

Cells can either be stimulated directly in the antibody coated wells (Direct) or, first stimulated in 24 well plates or a 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 regardless of the method (direct/indirect) chosen.

### **10.1 Positive Assay Control, 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 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1  $\mu$ g/mL LPS.

Distribute  $1 \times 10^5$  to  $2.5 \times 10^5$  cells per 100  $\mu$ L 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.

### **10.2 Negative Assay Control**

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

## 11. SAMPLE PREPARATION

- 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 100  $\mu\text{L}$ .
- Optimal assay performances are observed between  $1 \times 10^5$  and  $2.5 \times 10^5$  cells per 100  $\mu\text{L}$ .
- Stimulators and incubation times can be varied depending on the frequency of cytokine producing cells and therefore should be optimised by the testing laboratory.

## 12. ASSAY PROCEDURE

- 12.1 Add 100  $\mu$ L of 1X PBS to each well.
- 12.2 Incubate plate at room temperature for 10 minutes.
- 12.3 Empty the wells by flicking the plate over a sink and gently tapping on absorbent paper.
- 12.4 Add 100  $\mu$ L of sample, positive or negative controls cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated).
- 12.5 Cover the plate and incubate at 37°C in a CO<sub>2</sub> incubator for an appropriate length of time (10-15 hours).  
*Note: Do not agitate or move the plate during this incubation. The most appropriate incubation time for each experiment must be empirically determined by the end user as this can vary depending on the specific activation conditions, cell type and analyte of interest.*
- 12.6 Empty the wells and remove excess solution then add 100  $\mu$ L of PBS-T to each well.
- 12.7 Incubate the plate at 4°C for 10 minutes.
- 12.8 Empty the wells as previous and wash the plate 3x with 100  $\mu$ L of PBS-T.
- 12.9 Add 100  $\mu$ L of diluted detection antibody to each well.
- 12.10 Cover the plate and incubate at room temperature for 1 hour 30 minutes.
- 12.11 Empty the wells as previous and wash the plate 3x with 100  $\mu$ L of PBS-T.
- 12.12 Add 100  $\mu$ L of diluted Streptavidin-AP conjugate to each well.
- 12.13 Cover the plate and incubate at room temperature for 1 hour.
- 12.14 Empty the wells and wash the plate 3x with 100  $\mu$ L of PBS-T.

## ASSAY PROCEDURE

- 12.15 Peel off the plate bottom and wash both sides of the membrane 3 x under running distilled water. When washing is complete remove any excess solution by repeated tapping on absorbent paper.
- 12.16 Add 100  $\mu$ L of ready-to-use BCIP/NBT buffer to each well
- 12.17 Incubate the plate for 5-15 minutes monitoring spot formation visually throughout the incubation period to assess sufficient color development.
- 12.18 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.
- 12.19 Allow the wells to dry and then read results. The frequency of the resulting colored spots corresponding to the cytokine producing cells can be determined using an appropriate ELISPOT reader and analysis software or manually using a microscope.

*Note: spots may become sharper after overnight incubation at 4°C. Plate should be stored at room temperature away from direct light, but please note color may fade over prolonged periods so read results within 24 hours.*

## 13. TYPICAL SAMPLE VALUES

### Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (LPS) of 5 different mouse splenocyte 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	Minimum number of spots per well	Maximum number of spots per well	CV%
100,000 (recommended)	3	238	236	241	1.1
50,000	3	130	125	135	3.9
25,000	3	75	72	77	3.8
12,500	3	37	32	43	15.0
6,250	3	21	20	23	7.2

## 14. ASSAY SPECIFICITY

The assay recognizes native Rat TNF $\alpha$  and cross reacts with murine TNF $\alpha$ .



### **15. TROUBLESHOOTING**

Please refer to [www.abcam.com/ELISAandReagents](http://www.abcam.com/ELISAandReagents) for troubleshooting tips.

16. NOTES





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