Rat TNF-alpha ELISpot Kit – Pre-coated

SKU: RTDC0182

Instructions for use

	Pre-coated Plates
1x96 tests	RTDC0182
2x96 tests	RTDC0182-2
5x96 tests	RTDC0182-5

For research use only

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1. Intended use

Assay Genie **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, Assay Genie 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.

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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 gramnegative sepsis and purpura fulminians. 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 broncheoalveolar 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 microtiter 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.

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

2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.

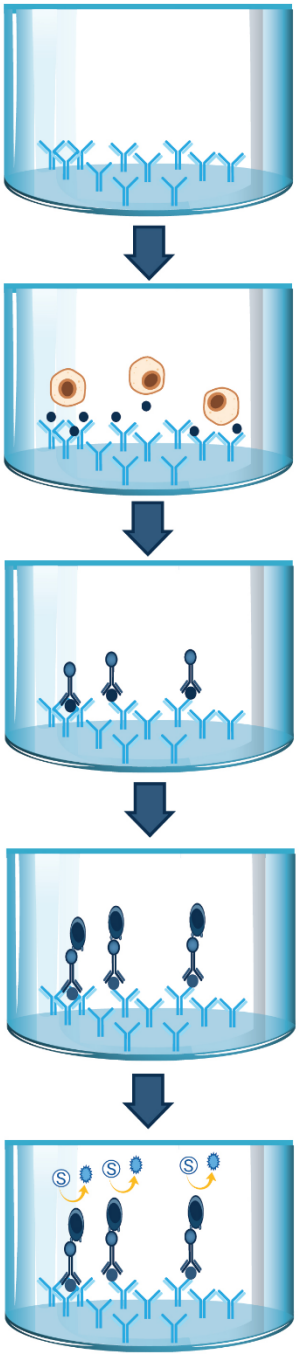
3. Cells are removed by washing. Detection antibody is added.

4. Any excess unbound detection antibodies are removed by washing. Streptavidin-alkaline phosphatase is added.

5. Any excess unbound Strep-AP is removed by washing. BCIP/NBT is added.

BCIP/NBT is reduced by alkaline phosphatase to produce a precipitate observed as blue/purple spots.

One spot corresponds to one single producing cell.



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3. Reagents provided

Reagents	RTDC0182	RTDC0182-2	RTDC0182-5	Reconstitution
Pre-coated 96-well PVDF bottom plates	2	2	5	Rehydrate with 100 µl of PBS1X (see section 9)
Biotinylated Detection antibody	1 (100 µl)	1 (200 µl)	1	Reconstitute with 0.55 ml of distilled water Dilute prior to use (see Detection Antibody, section 7.4)
Streptavidin-Alkaline Phosphatase conjugate	1 (10 µl)	1 (20 µl)	1 (50 µl)	Dilute prior to use (see Streptavidin-AP conjugate, section 7.5)
Bovine Serum Albumin (BSA) – 2 g	1	1	1	Dissolve to prepare dilution buffer (see 1%BSA PBS solution, section 7.3)
Ready to use BCIP/NBT - (Substrate buffer)	1 (11 ml)	1 (25 ml)	2 (25 ml)	Ready to use

***Please note for RTDC0182 and RTDC0182-2: detection antibody is provided in liquid form.**
For RTDC0182: Volume of reagents are sufficient for a total of 96 tests but 2 plates are provided to allow to run 2*48 tests.

4. Materials/Reagents required but not provided

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (e.g. PMA, Ionomycin, LPS)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)

5. Storage Instructions

Store kit reagents between 2 and 8°C. 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.

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6. Safety & Precautions for use

- For **research use only** not to be used as a diagnostic test.
- Handling of reagents, blood specimens, PBMC, human cell lines 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 substrate** may cause an allergic skin reaction, caution should be taken when handling this reagent, always wear gloves.
- Follow incubation times described in the assay procedure.

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7. Reagent Preparation

7.1. 1X Phosphate Buffered Saline (PBS)

For 1 litre of 10X PBS, weigh-out: 80g NaCl
2g KH_2PO_4
14.4g Na_2HPO_4 ; $2H_2O$.
Add distilled water to 1 litre.

Dilute the solution to 1X before use.
Check the pH of the 1X solution and adjust to required pH : 7.4 +/- 0.1.

7.2. 0.05% Tween PBS Solution (Wash Buffer)

For one plate, dilute 50 µl of Tween 20 in 100 ml of PBS 1X.

7.3. 1% BSA PBS Solution (Dilution Buffer)

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

7.4. Detection Antibody

Reconstitute the lyophilised antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilised material is back into solution. **Please note for RTDC0182 and RTDC0182-2 kits, detection antibody is provided in liquid form.**

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.

For one plate, dilute 100 µl of antibody into 10 ml of Dilution Buffer and mix well.

To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2µm filter disc.

7.5. Streptavidin – AP conjugate

For optimal performance, prepare the Streptavidin-AP dilution immediately prior to use.
It is recommended to centrifuge the vial for a few seconds to collect all the volume at the bottom.

For one plate, dilute 10 µl of Streptavidin-AP conjugate into 10 ml of Dilution Buffer and mix well.

Do not keep this solution for further experiments.
To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2µm filter disc.

7.6. BCIP/NBT

The reagent is ready-to-use.

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It should be clear to pale yellow. If precipitates occur, filter the solution using a disposable syringe and a 0.2µm filter disc.

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 medium (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1µg/ml LPS (Sigma, Saint Louis, MO). Distribute $1x10^5$ to $2.5x10^5$ cells per 100 µl in required wells of an antibody coated 96-well PVDF plate and incubate for 15-20 hours in an incubator.

For other stimulators incubation times may vary, depending on the frequency of cytokine producing cells, and should be optimised in each situation.

8.3. Negative Assay Control

Dilute splenocytes in culture medium to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 µl 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 100 µl.

Optimal assay performances are observed between $1x10^5$ and $2.5x10^5$ cells per 100 µl.

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

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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 100 µl of PBS 1X to every well
2.	Incubation	Incubate plate at room temperature (RT) for 10 min
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper
4.	Addition	Add 100 µl of **sample, positive and negative controls** cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)
5.	Incubation	Cover the plate and incubate at 37ºC in a CO_2 incubator for an appropriate length of time (15-20 hours) ***Note: do not agitate or move the plate during this incubation***
6.	Addition	Empty the wells and remove excess solution then add 100 µl of Wash Buffer to every well
7.	Incubation	Incubate the plate at 4ºC for 10 min
8.	Wash	Empty the wells as previous and wash the plate 3x with 100 µl of Wash Buffer
9.	Addition	Add 100 µl of diluted **detection antibody** to every well
10.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
11.	Wash	Empty the wells as previous and wash the plate 3x with 100 µl of Wash Buffer
12.	Addition	Add 100 µl of diluted **Streptavidin-AP conjugate** to every well
13.	Incubation	Cover the plate and incubate at RT for 1 hour
14.	Wash	Empty the wells and wash the plate 3x with 100 µl of Wash Buffer
15.	Wash	Peel off 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
16.	Addition	Add 100 µl of ready-to-use **BCIP/NBT buffer** to every well
17.	Development	Incubate the plate for **5-15 min** monitoring spot formation visually throughout the incubation period to assess sufficient colour development
18.	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
Read Spots: 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. *Note: spots may become sharper after overnight incubation at 4ºC in the dark*		

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

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10. Performance Characteristics

10.1. Specificity

The assay recognizes natural rat TNF-alpha and cross reacts 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. The data show 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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