RatTNFα ELISpot

Instructions for use

Catalogue Numbers:

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<tr>
<th></th>
<th>Without Plates</th>
<th>With non-Sterile Plates</th>
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<tr>
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For research use only

Fast Track Your Research.............
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RatTNFα ELISpot

1. Intended use

OriGeneELISpot 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, OriGene 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 (TNFα), 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). TNFαcirculates 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. TNFαalso 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 TNFα(15). Due to its varied actions throughout the immune system, TNFα may play a role in the pathogenesis of many disease states.

TNF-αproduction is mediated by the action of lymphokines and endotoxins on the macrophage. Purified monocytes produce TNFαwithin four hours of stimulation by recombinant IL-2 (9) and there is some in vitro evidence to suggest that TNFαis expressed at high levels and with prolonged kinetics in T cells stimulated by both CD2 and CD28 (5). Secretion of TNFαenhanced by gamma interferon. TNFα 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).

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

Waage et al. (25) found that increased levels of TNFαin 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 TNFαlevels correlated with the number of risk factors involved in children with gram-negative sepsis and purpura fulminans. Elevated levels of TNFαwere also found in individuals suffering from myocarditis (11).

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

Measurement of TNFα levels has also been shown to be useful in transplant research, where Maury et al. (18) and McLaughlin et al. (19). Both reported TNFα to be markedly elevated in renal allograft rejection episodes. Recent evidence has been presented on increased TNFα 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 TNFα 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

2. Incubation of cells in the coated microwell

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 TNFα (0.5ml supplied sterile)
- Biotinylated detection antibody (lyophilised, resuspend in 0.55ml)
- Streptavidin-Alkaline Phosphatase conjugate (50µl)
- 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)
- CO₂ 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 KH₂PO₄
14.4g Na₂HPO₄·2H₂O.
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 TNFα 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 1x10⁵ to 2.5x10⁵ 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 1x10⁵ and 2.5x10⁵ 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

<table>
<thead>
<tr>
<th>Assay Step</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Addition</td>
<td>Add 25μl of 35% ethanol to every well</td>
</tr>
<tr>
<td>2. Incubation</td>
<td>Incubate plate at room temperature (RT) for 30 seconds</td>
</tr>
<tr>
<td>3. Wash</td>
<td>Empty the wells by flicking the plate over a sink &amp; gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100μl of 1X PBS per well</td>
</tr>
<tr>
<td>4. Addition</td>
<td>Add 100μl of diluted <strong>capture antibody</strong> to every well</td>
</tr>
<tr>
<td>5. Incubation</td>
<td>Cover the plate and incubate at 4°C overnight</td>
</tr>
<tr>
<td>6. Wash</td>
<td>Empty the wells as previous and wash the plate once with 100μl of 1X PBS per well</td>
</tr>
<tr>
<td>7. Addition</td>
<td>Add 100μl of <strong>Blocking buffer</strong> to every well</td>
</tr>
<tr>
<td>8. Incubation</td>
<td>Cover the plate and incubate at RT for 2 hours</td>
</tr>
<tr>
<td>9. Wash</td>
<td>Empty the wells as previous and thoroughly wash 3x with 100μl of 1X PBS per well</td>
</tr>
<tr>
<td>10. Addition</td>
<td>Add 100μl of <strong>sample, positive and negative controls</strong> cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)</td>
</tr>
<tr>
<td>11. Incubation</td>
<td>Cover the plate and incubate at 37°C in a CO₂ incubator for an appropriate length of time (15-20 hours). <strong>Note: do not agitate or move the plate during this incubation</strong></td>
</tr>
<tr>
<td>12. Addition</td>
<td>Empty the wells and remove excess solution then add 100μl of PBS-T to every well</td>
</tr>
<tr>
<td>13. Incubation</td>
<td>Incubate the plate at 4°C for 10 min</td>
</tr>
<tr>
<td>14. Wash</td>
<td>Empty the wells as previous and wash the plate 3x with 100μl of PBS-T</td>
</tr>
<tr>
<td>15. Addition</td>
<td>Add 100μl of diluted <strong>detection antibody</strong> to every well</td>
</tr>
<tr>
<td>16. Incubation</td>
<td>Cover the plate and incubate at RT for 1 hour 30 min</td>
</tr>
<tr>
<td>17. Wash</td>
<td>Empty the wells as previous and wash the plate 3x with 100μl of PBS-T</td>
</tr>
<tr>
<td>18. Addition</td>
<td>Add 100μl of diluted <strong>Streptavidin-AP conjugate</strong> to every well</td>
</tr>
<tr>
<td>19. Incubation</td>
<td>Cover the plate and incubate at RT for 1 hour</td>
</tr>
<tr>
<td>20. Wash</td>
<td>Empty the wells and wash the plate 3x with 100μl of PBS-T</td>
</tr>
<tr>
<td>21. Wash</td>
<td>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.</td>
</tr>
<tr>
<td>22. Addition</td>
<td>Add 100μl of ready-to-use <strong>BCIP/NBT buffer</strong> to every well</td>
</tr>
<tr>
<td>23. Development</td>
<td>Incubate the plate for <strong>5-20 min</strong> monitoring spot formation visually throughout the incubation period to assess sufficient colour development</td>
</tr>
<tr>
<td>24. Wash</td>
<td>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</td>
</tr>
</tbody>
</table>

**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 4°C**

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 TNFα and cross react with murine TNFα.

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.

<table>
<thead>
<tr>
<th>Cells / well</th>
<th>n</th>
<th>Mean number of spots per well</th>
<th>Min</th>
<th>Max</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 recommended</td>
<td>3</td>
<td>238</td>
<td>236</td>
<td>241</td>
<td>1.1%</td>
</tr>
<tr>
<td>50,000</td>
<td>3</td>
<td>130</td>
<td>125</td>
<td>135</td>
<td>3.9%</td>
</tr>
<tr>
<td>25,000</td>
<td>3</td>
<td>75</td>
<td>72</td>
<td>77</td>
<td>3.8%</td>
</tr>
<tr>
<td>12,500</td>
<td>3</td>
<td>37</td>
<td>32</td>
<td>43</td>
<td>15.0%</td>
</tr>
<tr>
<td>6,250</td>
<td>3</td>
<td>21</td>
<td>20</td>
<td>23</td>
<td>7.2%</td>
</tr>
</tbody>
</table>
11. Bibliography


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