Instructions for Use

For the qualitative measurement of IFNγ and IL-2 production and secretion in a single cell suspension.

This product is for research use only and is not intended for diagnostic use.
# Table of Contents

## INTRODUCTION
1. BACKGROUND 2
2. ASSAY SUMMARY 5

## GENERAL INFORMATION
3. PRECAUTIONS 6
4. STORAGE AND STABILITY 6
5. MATERIALS SUPPLIED 7
6. MATERIALS REQUIRED, NOT SUPPLIED 8
7. LIMITATIONS 9
8. TECHNICAL HINTS 9

## ASSAY PREPARATION
9. REAGENT PREPARATION 10
10. CONTROL PREPARATION 12
11. SAMPLE PREPARATION 13

## ASSAY PROCEDURE
12. ASSAY PROCEDURE 14

## RESOURCES
13. TROUBLESHOOTING 17
14. NOTES 18
INTRODUCTION

1. BACKGROUND

Abcam’s Interferon gamma (IFNy) and IL-2 Human Dual ELISPOT Kit is an in vitro ELISPOT assay designed for the qualitative measurement of IFNy and IL-2 production and secretion in a single cell suspension.

The ELISPOT assay involves capture antibodies highly specific for the analytes 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 minimise any non-antibody dependent unspecific binding and finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtitre plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated and FITC conjugated detection antibodies are then added which bind to the previously captured analyte. HRP conjugated anti-FITC antibodies and Streptavidin-Alkaline Phosphatase are added and bind to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. Colour substrate is then applied to the wells resulting in colored spots which can be quantified using appropriate analysis software or manually using a microscope.

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
INTRODUCTION

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. This Dual Colour ELISPOT kit allows you to analysis the production of two cytokines simultaneously in the same well.

IFNγ, also called Type II interferon, is a homodimeric glycoprotein containing approximately 21 to 24 kD subunits. The human IFNγ gene, situated on chromosome 12, contains three introns; the four exons code for a polypeptide of 166 amino acids, 20 of which constitute the signal peptide. In contrast to IFNα and IFNβ synthesis, which can occur in any cell, production of IFNγ is a function of T cells and NK cells. All IFNγ inducers activate T cells either in a polyclonal (mitogens or antibodies) or in a clonally restricted, antigen-specific, manner. IFNγ is produced during infection by T cells of the cytotoxic/suppressor phenotype (CD8) and by a subtype of helper T cells, the Th1 cells. Th1 cells secrete IL-2, IL-3, TNFα and IFNγ, whereas Th2 cells mainly produce IL-3, IL-4, IL-5, and IL-10, but little or no IFNγ. IFNγ preferentially inhibits the proliferation of Th2 but not Th1 cells, indicating that the presence of IFNγ during an immune response will result in the preferential proliferation of Th1 cells.

Type II IFN or IFNγ is a lymphokine that displays no molecular homology with type I IFN, but shares some important biologic activities.
Specifically, IFNγ induces an anti-viral state and is anti-proliferative. In addition, IFNγ has several properties related to immunoregulation. IFNγ is a potent activator of mononuclear phagocytes, e.g. IFNγ stimulates the expression of Mac-1, augments endocytosis and phagocytosis by monocytes, and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα. IFNγ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines. On T and B cells IFNγ promotes differentiation. It enhances proliferation of activated B cells and can act synergistically with IL-2 to increase immunoglobulin light-chain synthesis. IFNγ is one of the natural B-cell differentiation factors. Finally, IFNγ activates neutrophils, NK cells and vascular endothelial cells.

IL-2 is a powerful immunoregulatory lymphokine produced by T-Cells in response to antigenic or mitogenic activation. IL-2 stimulates growth and differentiation of B-Cells, most T-cells, NK cells, monocytes and macrophages. Mature IL-2 is a 15.4kDa globular glycoprotein containing 133 amino acid residues including one intrachain disulfide bond between residues 58 and 105.

Apart from its most important role to mediate antigen-specific T-lymphocyte proliferation, IL-2 modulates the expression of interferon-γ and major histocompatibility antigens. Alterations in the ability of T-cell to synthesize IL-2 have been observed in physiologic and pathologic states. Currently, IL-2 is used to enhance the immune system of patients for the treatment of cancer and infectious disease.
2. ASSAY SUMMARY

Equilibrate all reagents to room temperature. Prepare all the reagents and samples as instructed. 96-well PVDF bottomed plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-IL-2 capture antibodies.

Add sample (Cells) to appropriate wells. Incubate at 37 °C. Upon stimulation the cells produce cytokines which bind to the capture antibodies.

Lyse cells and wash each well. Add prepared FITC conjugated anti-IFNγ and Biotinylated anti-IL-2 detector antibodies. Incubate at room temperature.

Add prepared HRP conjugated anti-FITC (for IFNγ) and Streptavidin-Alkaline Phosphatase (for IL-2) mix to each well. Incubate at room temperature.

Add the substrate solutions AEC (for IFNγ) and BCIP/NBT (for IL-2) to each well and monitor spot formation. Cells producing IFNγ give red/brownish spots while those producing IL-2 give blue/purple spots.
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 (except non-precoated plates which should be stored at room temperature).

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

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage Condition (Before Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well PVDF bottomed Microplate (non-sterile)</td>
<td>5 x 96 wells</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Capture Antibody for IFNγ</td>
<td>1 x 500 μL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Capture Antibody for IL-2</td>
<td>1 x 500 μL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>FITC conjugated detection antibody for IFNγ</td>
<td>1 x 1 vial</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Biotinylated Detection antibody for IL-2</td>
<td>1 x 1 vial</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Anti-FITC antibody HRP conjugate.</td>
<td>1 x 120 μL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Streptavidin-Alkaline Phosphatase conjugate</td>
<td>1 x 50 μL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>1 x 1 g</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>50X Concentrated AEC substrate buffer</td>
<td>1 x 1 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>10X Concentrated dilution buffer for AEC substrate</td>
<td>1 x 5 mL</td>
<td>+2-8°C</td>
</tr>
<tr>
<td>Ready to use BCIP/NBT substrate buffer</td>
<td>2 x 27 mL</td>
<td>+2-8°C</td>
</tr>
</tbody>
</table>
6. MATERIALS REQUIRED, NOT SUPPLIED

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

- 35% Ethanol (PVDF Membrane Activation Buffer).
  For one plate, dilute 3.5 mL of Ethanol with 6.5 mL of distilled water.
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS).
- Cell culture media + 10% Serum (Blocking Buffer).
  For one plate add 1 mL Serum (e.g. FCS) to 9 mL of culture media (use same cell culture medium as used to derive the cell suspension).
- Cell stimulation reagents (e.g. PMA and Ionomycin).
- 0.05% PBS-T Solution (Wash Buffer).
  For one plate dilute 50 μL of Tween 20 in 100 mL of 1X PBS.
- 1X Phosphate Buffered Saline (PBS) (Coating Buffer).
  For 1L of 10X PBS weigh-out:
  80g NaCl
  2g KH$_2$PO$_4$
  14.4g Na$_2$HPO$_4$$\cdot$2H$_2$O
  Add distilled water to 1L. Adjust the pH of the solution to 7.4 +/- 0.1. Dilute the solution to 1X before use.
- Miscellaneous laboratory plastic and/or glass, if possible sterile.
- CO$_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 **Capture Antibodies**

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

These reagents are supplied sterile once opened keep the vials sterile or aliquot and store at -20°C. For optimal performance prepare the Capture Antibodies dilution immediately before use.

9.3 **Detection Antibodies**

For anti-IFNγ 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.

For anti-IL-2 detection antibody, reconstitute the lyophilised antibody with 750 μL of distilled water.

Gently mix the solution and wait until all the lyophilised material is back into solution.

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

If not used within a short period of time, reconstituted Detection Antibody should be aliquotted 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.
ASSAY PREPARATION

9.4 **Streptavidin-AP conjugate and Anti-FITC antibody-HRP conjugate (Diluted conjugates)**

For one plate, dilute 10 µL of Streptavidin-AP conjugate and 20 µL of anti-FITC antibody HRP conjugate in 10 mL of Dilution Buffer. Mix well.

For optimal performance prepare the dilution immediately prior to use. Do not keep the dilutions for further experiments.

9.5 **AEC Substrate**

For one plate, dilute 1 mL 10X Concentrated dilution buffer for AEC substrate with 9 mL of distilled water. Then add 200 µL of 50X Concentrated AEC substrate buffer.
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 the type of cell assayed and the expected cell frequency. When a low number of cytokine producing cells are expected it is 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.

10.1 Positive Assay Control, IFNγ / IL-2 production

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

Dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 ng/mL PMA and 500 ng/mL Ionomycin. Distribute $0.5 \times 10^4$ to $2 \times 10^4$ cells per 100 μL in required wells of an antibody coated 96-well PVDF plates 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.

10.2 Negative Assay Control

Dilute PBMC in culture media to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 μL with no stimulation.
11. **SAMPLE PREPARATION**

- Dilute PBMC 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 $1 \times 10^5$ and $2.5 \times 10^5$ 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.
12. **ASSAY PROCEDURE**

12.1 Add 25 μL of 35% Ethanol to each well.

12.2 Incubate plate at room temperature for 30 seconds.

12.3 Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100 μL of 1X PBS per well.

12.4 Add 100 μL of diluted anti-IFNγ and anti-IL-2 Capture Antibodies to each well.

12.5 Cover the plate and incubate at 4°C overnight.

12.6 Empty the wells as previous (Step 12.3) and wash the plate once with 100 μL of 1X PBS per well.

12.7 Add 100 μL of Blocking Buffer to each well.

12.8 Cover the plate and incubate at room temperature for 2 hours.

12.9 Empty the wells as previous (Step 12.3) and thoroughly wash 3x with 100 μL of 1X PBS per well.

12.10 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).

12.11 Cover the plate and incubate at 37°C in a CO₂ incubator for an appropriate length of time (15-20 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.*

12.12 Empty the wells and remove excess solution then add 100 μL of Wash Buffer to each well.

12.13 Incubate the plate at 4°C for 10 minutes.

12.14 Empty the wells as previous (Step 12.3) and wash the plate 3x with 100 μL of Wash Buffer.
ASSAY PROCEDURE

12.15 Add 100 μL of diluted FITC conjugated anti-IFNγ and biotinylated anti-IL-2 Detection Antibodies to each well.

12.16 Cover the plate and incubate at room temperature for 1 hour 30 minutes.

12.17 Empty the wells as previous (Step 12.3) and wash the plate 3x with 100 μL of Wash Buffer.

12.18 Add 100 μL per well of diluted conjugates: anti-FITC-HRP (for IFNγ detection) and streptavidin-AP (for IL-2 detection).

12.19 Cover the plate and incubate at room temperature for 1 hour.

12.20 Empty the wells and wash the plate 3x with 100 μL of Wash Buffer.

12.21 Peel of the plate bottom and wash both sides of the membrane 3x under running distilled water. When washing is complete remove any excess solution by repeated tapping on absorbent paper.

12.22 Add 100 μL of prepared AEC substrate to each well.

12.23 Incubate the plate for 5-20 min monitoring spot formation visually throughout the incubation period to assess sufficient color development.

12.24 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.

12.25 Add 100 μL of ready to use BCIP/NBT buffer to each well.

12.26 Incubate the plate for 5-15 min monitoring spot formation visually throughout the incubation period to assess sufficient color development.

12.27 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.
12.28 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. Cells producing IFNγ give red/brownish spots while those producing IL-2 give blue/purple spots.

*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. TROUBLESHOOTING

Please refer to www.abcam.com/ELISAandReagents for troubleshooting tips.
UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp

Copyright © 2014 Abcam, All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.