ab48722 – Human Interferon gamma + IL-10 ELISPOT Set

Instructions for Use

For the qualitative measurement of IFNγ and IL-10 production and secretion in a single cell suspension.

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

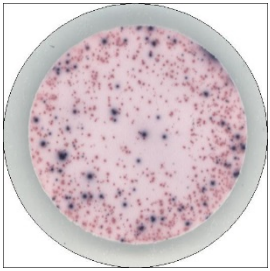
Version 1a Last Updated 12 March 2021

Table of Contents

1. BACKGROUND

Abcam's Human Interferon gamma + IL-10 ELISPOT Set is an *in vitro* ELISPOT assay designed for the qualitative measurement of IFNγ and IL-10 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 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.

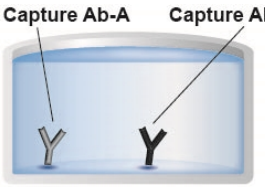
Utilising sandwich immuno-enzyme technology, Abcam ELISPOT assays can detect both secreted cytokines and single cells that simultaneously produce multiple cytokines. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation 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-

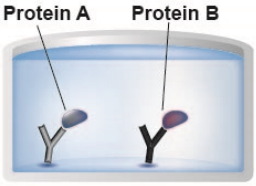
1, augments endocytosis and phagocytosis by monocytes, and activates macrophages to kill tumor cells by releasing reactive oxygen intermediates and TNFα. IFNγ induces or augments the expression of MHC antigens on macrophages, T and B cells and some tumor cell lines. On T and B cells IFNγ promotes differentiation. 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.

Interleukin-10 is a pleiotropic cytokine playing an important role as a regulator of lymphoid and myeloid cell function. Due to the ability of IL-10 to block cytokine synthesis and several accessory cell functions of macrophages this cytokine is a potent suppressor of the effector functions of macrophages, T-cells and NK cells. In addition, IL-10 participates in regulating proliferation and differentiation of B-cells, mast cells and thymocytes. The primary structure of human IL-10 has been determined by cloning the cDNA encoding the cytokine. The corresponding protein exists at 160 amino acids with a predicted molecular mass of 18.5 kDa. Based on its primary structure, IL-10 is a member of the four -helix bundle family of cytokines. In solution human IL-10 is a homodimer with an apparent molecular mass of 39 kDa. Although it contains an N-linked glycosylation site, it lacks detectable carbohydrates. Recombinant protein expressed in E. coli thus retains all known biological activities.The human IL-10 gene is located on chromosome 1 and is present as a single copy in the genome. The human IL-10 exhibits strong DNA and amino acid sequence homology to the murine IL-10 and an open reading frame in the Epstein- Barr virus genome, BCRF1 which shares many of the cellular cytokine's biological activities and may therefore play a role in the host- virus interaction. The immunosuppressive properties of IL-10 suggest a possible clinical use of IL-10 in suppressing rejections of grafts after organ transplantations. IL-10 can furthermore exert strong anti-inflammatory activities.

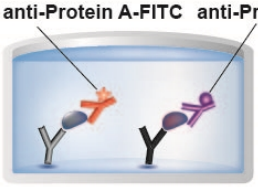
2. ASSAY SUMMARY



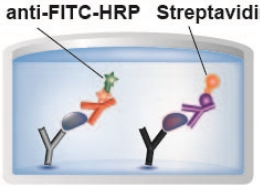
Equilibrate all reagents to room temperature. Prepare all the reagents and samples as instructed. 96-well PVDF bottomed plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-IL-10 capture antibodies.



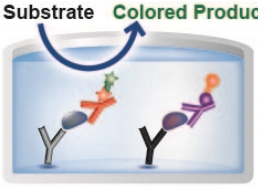
Add sample (Cells) to appropriate wells. Incubate at 37 ºC. Upon stimulation the cells produce cytokines which bind to the capture antibodies.



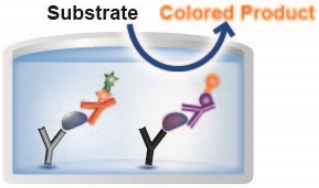
Lyse cells and wash each well. Add prepared FITC conjugated anti-IFNγ and Biotinylated anti-IL-10 detector antibodies. Incubate at room temperature.



Add prepared HRP conjugated anti-FITC (for IFNγ) and Streptavidin-Alkaline Phosphatase (for IL-10) mix to each well. Incubate at room temperature.



Add the substrate solutions AEC (for IFNγ) and BCIP/NBT (for IL-10) to each well and monitor spot formation. Cells producing IFNγ give red/brownish spots while those producing IL-10 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 +2-8ºC 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)
	20 x 96 tests	
Capture Antibody for IFNγ	4 x 500 µL	+2-8ºC
Capture Antibody for IL-10	4 x 500 µL	+2-8ºC
FITC conjugated detection antibody for IFNγ	4 x 1 vial	+2-8ºC
Biotinylated Detection antibody for IL-10	4 x 1 vial	+2-8ºC
Anti-FITC antibody HRP conjugate.	4 x 100 µL	+2-8ºC
Streptavidin-Alkaline Phosphatase conjugate	4 x 50 µL	+2-8ºC
Bovine Serum Albumin (BSA)	4 x 1 g	+2-8ºC
50X Concentrated AEC substrate buffer	4 x 1 mL	+2-8ºC
10X Concentrated dilution buffer for AEC substrate	4 x 5 mL	+2-8ºC
Ready to use BCIP/NBT substrate buffer	4 x 50 mL	+2-8ºC

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_2PO_4

  14.4g $Na_2HPO_4 \cdot 2H_2O$

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

- 96 well PVDF bottomed plates.
- 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**

For 1 plate, 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 -20ºC. For optimal performance prepare the Capture Antibodies dilution immediately before use.

9.3 **Detection Antibodies**

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

For 1 plate, 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 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.

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.

For optimal performance prepare the dilution immediately prior to use.

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-10 production

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

Dilute CD4+ cells 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 2.5 x 10^4 to 1 x 10^5 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 CD4+ cells 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 CD4+ cells 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 x 10^5 and 2.5 x 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-10 Capture Antibodies to each well.

12.5 Cover the plate and incubate at 4ºC 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 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. 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 4ºC for 10 minutes.

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

12.15 Add 100 µL of diluted FITC conjugated anti-IFNγ and biotinylated anti-IL-10 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-10 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-10 give blue/purple spots.

Note: Spots may become sharper after overnight incubation at 4ºC. 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.

14. NOTES

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)