ab46587 – Mouse Interferon gamma ELISPOT Kit (with un-coated plates)

Instructions for Use

For the qualitative measurement of Interferon gamma (IFNγ) production and secretion in a single cell suspension.

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

Version 1b Last Updated 12 March 2021

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1. BACKGROUND

Abcam's Mouse Interferon gamma ELISPOT Kit (with un-coated plates) is an *in vitro* ELISPOT assay designed for the qualitative measurement of IFNγ 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,

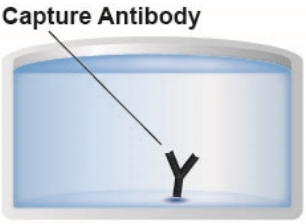
cancerology, infectious disease, autoimmune diseases and transplantation.

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.

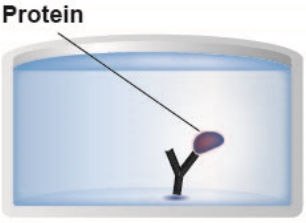
IFNγ production is a key function of Th1, $CD8^+$ CTLs and also NK cells. IFNγ is a cytokine critical for cell mediated immunity against viral and intracellular bacterial infections and is involved in the inflammatory response following secretion via macrophage activation and stimulation of antibody secretion. IFNγ is the hallmark effector cytokine of Th1 and therefore is an excellent marker for identifying a host response to intracellular pathogens. 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.

In addition, IFNγ has several properties related to immunoregulation. IFNγ is a potent activator of mononuclear phagocytes, 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.

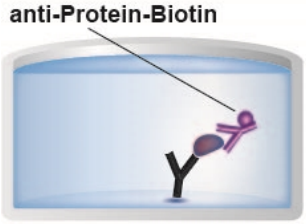
2. ASSAY SUMMARY



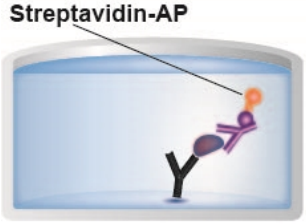
Equilibrate all reagents to room temperature. Prepare all the reagents and samples as instructed. 96-well PVDF bottomed plates are first treated with 35% ethanol and then coated with capture antibody.



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



Aspirate and wash each well. Add prepared Biotinylated 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 +2-8ºC 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

Item	Quantity		Storage Condition (Before Preparation)
	5 x 96 tests	10 x 96 tests	
96 well PVDF bottomed Microplate (non-sterile)	5 x 96 wells	10 x 96 wells	Room temperature
Capture Antibody for Murine IFNγ	1 x 500 µL	2 x 500 µL	+2-8ºC
Biotinylated Detection antibody (lyphophilised)	1 x 1 vial	2 x 1 vial	+2-8ºC
Streptavidin-Alkaline Phosphatase Conjugate	1 x 50 µL	2 x 50 µL	+2-8ºC
Bovine Serum Albumin (BSA)	1 x 1 g	2 x 1 g	+2-8ºC
Ready to use BCIP/NBT substrate buffer	1 x 50 mL	2 x 50 mL	+2-8ºC
Dry skimmed milk	1 x 1 vial	2 x 1 vial	+2-8ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Cell stimulation reagents (PMA, Ionomycin, Concalvin A, mIL-2).
- 35% Ethanol (PVDF Membrane Activation Buffer).

  For one plate mix 3.5 mL of Ethanol with 6.5 mL of distilled water.
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS).
- 0.05% PBS-T Solution (Wash Buffer).

  For one plate dissolve 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:

  80 g NaCl

  2 g KH_2PO_4

  14.4 g Na_2HPO_4 $_2H_2O$.

  Add distilled water to 1L. Adjust the pH of the solution to 7.4 +/- 0.1. Dilute the solution to 1x before use.
- CO_2 incubator.
- Miscellaneous laboratory plastic and/or glass, if possible sterile.

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 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 (18-25ºC) 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 **Skimmed milk in 1X PBS (Blocking buffer)**

For one non sterile plate dissolve 0.2 g of powder in 10 mL of 1X PBS.

For one sterile plate dilute 5 mL of liquid milk in 5 mL of 1X PBS.

Please note liquid milk has a shorter expiration date than 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.

9.3 **Capture Antibody**

This reagent is supplied sterile once opened keep the vial sterile or aliquot and store at -20ºC. For optimal performance prepare the Capture Antibody dilution immediately before use.

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

9.4 **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 -20ºC. In these conditions the reagent is stable for at least one year. For optimal performance prepare the reconstituted antibody dilution immediately prior to use.

9.5 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 - IFNγ production

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

Dilute mouse splenocytes in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 0.5 µg/mL Concavalin A and 2 ng/mL mIL-2. Incubate 2 days. Take off non adherent materials and harvest adherent cells with a cell scraper. Wash cells once. Dilute cells in culture media supplemented with 1 ng/mL PMA and 500 ng/mL Ionomycin.

Distribute 1 x 10^5 to 2.5 x 10^5 cells per 100 µL 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 cells has to be determined experimentally, depending on the frequency of cytokine producing cells.

10.2 **Negative Assay Control**

Dilute mouse splenocytes 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 mouse 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 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 and 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 Capture Antibody 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 1x 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 or 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 PBS-T to well.

12.13 Incubate the plate at 4ºC for 10 minutes.

12.14 Empty the wells as previous and wash the plate 3x with 100 µL of PBS-T.

12.15 Add 100 µL of diluted Detection Antibody (Biotinylated anti-IFNγ) to every well.

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

12.17 Empty the wells as previous and wash the plate 3x with 100 µL of PBS-T.

12.18 Add 100 µL of diluted Streptavidin-AP conjugate to every well.

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 PBS-T.

12.21 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.

12.22 Add 100 µL of ready-to-use BCIP/NBT buffer to every well

12.23 Incubate the plate for 5-20 minutes 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 Read Spots: 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 +4ºC Plate should be stored at room temperature away from direct light, but 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 (PMA / Ionomycin) of 5 different mouse splenocyte concentrations, 12 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	12	462	406	495	5.5
50,000 recommended	12	494	457	518	3.7
25,000 recommended	12	391	355	411	4.8
12,500	12	244	202	267	7.8
6,250	12	143	120	190	12.5

14. TROUBLESHOOTING

Please refer to **www.abcam.com/ELISAandReagents** for troubleshooting tips.

15. 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)