ab48468 – IFNγ + Granzyme B Human FLUOROSPOT Kit (with plates)

Instructions for Use

For the qualitative measurement of IFNγ and Granzyme B production and secretion in a single cell suspension.

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

Version 3b Last Updated 26 August 2021

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1. BACKGROUND

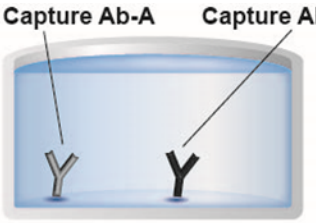
Abcam's Interferon gamma (IFNγ) and Granzyme B Human FLUOROSPOT Kit is an *in vitro* fluorescent ELISPOT assay designed for the qualitative measurement of IFNγ and Granzyme B production and secretion in a single cell suspension.

After cell stimulation, locally produced cytokines are captured by IFNγ and Granzyme B specific monoclonal antibodies. After cell lysis, trapped cytokine molecules are revealed by a secondary anti-IFNγ FITC conjugated antibody and a biotinylated anti-Granzyme B antibody. Those are in turn recognised by anti-FITC green fluorescent dye and streptavidin-phycoerythrin conjugates. PVDF-bottomed-well plates are then read under a UV light beam. Green fluorescent spots indicate IFNγ production while Granzyme B is revealed by red spots. Yellow spots will indicate dual cytokine producing cells.

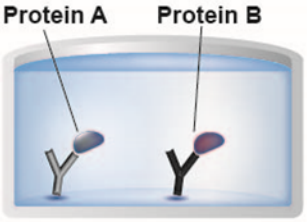
The ELISPOT assay is designed to enumerate cytokine producing cells in a single cell suspension. This method has the advantage of requiring minimal *in vitro* manipulations allowing cytokine production analysis as close as possible to *in vivo* conditions in a highly specific way. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation, and the follow-up of such frequency during a treatment and/or a pathological state. The ELISPOT assay constitutes an ideal tool in the Th1 / Th2 response, vaccine development, viral infection monitoring and treatment, cancerology, infectious diseases, autoimmune diseases and transplantation.

Using sandwich immuno-enzyme technology, Abcam ELISPOT assays can detect secreted cytokines or soluble molecules 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.

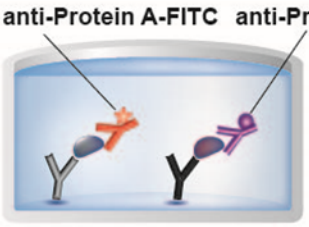
2. ASSAY SUMMARY



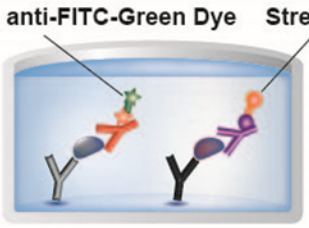
Equilibrate all reagents to room temperature. Prepare all the reagents and samples as instructed. 96 well PVDF bottomed plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-Granzyme B capture antibody.



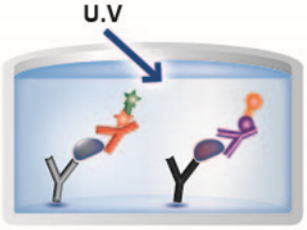
Add sample (Cells) to appropriate wells. Incubate at 37°C. Upon stimulation the cells produce cytokines which bind to the capture antibodies.



Lyse cells and wash each well. Add prepared FITC conjugated anti-IFNγ and Biotinylated anti-Granzyme B detection antibodies. Incubate at room temperature.



To each well add prepared Streptavidin-phycoethrin (for Granzyme B) and anti-FITC-green fluorescent dye (for IFNγ) conjugates. Incubate at room temperature.



Fluorescent spots are visualized under a UV light beam. Cells producing IFNγ give green spots while those producing

Granzyme B give red spots. Dual cytokine producing cells give yellow 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 (except uncoated 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 IFNγ	1 x 500 µL	2 x 500 µL	+2-8ºC
Capture Antibody for Granzyme B	1 x 500 µL	2 x 500 µL	+2-8ºC
FITC conjugated detection antibody for IFNγ	1 x 1 vial	2 x 1 vial	+2-8ºC
Biotinylated Detection antibody for Granzyme B	1 x 1 vial	2 x 1 vial	+2-8ºC
Anti-FITC antibody green fluorescence conjugate.	1 x 1 vial	2 x 1 vial	+2-8ºC
Streptavidin-phycoerythrin conjugate	1 x 1 vial	2 x 1 vial	+2-8ºC
Bovine Serum Albumin (BSA)	1 x 1 g	2 x 1 g	+2-8ºC
Blocking reagent (dry skimmed milk)	1 x 1 vial	2 x 1 vial	Room temperature

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- 35% Ethanol in water.

  For one plate mix 3.5 mL of ethanol with 6.5 mL of distilled water.

- Cell culture media (RPMI 10% FCS).

  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.
- Phosphate buffered saline (10X Concentrate solution).

  For 1 Liter weigh:

  80 g NaCl

  2 g KH_2PO_4

  14.4 g $Na_2HPO_4 \cdot 2H_2O$

  Add distilled water to 1 Liter. Check that the pH is between 7.4 +/- 0.1. This solution should be diluted to 1X before use.

- 0.05% Tween 20 in PBS.

  For one plate dissolve 50 µL of Tween 20 in 100 mL of 1X diluted PBS.

- Miscellaneous laboratory plastic and/or glass, if possible sterile.
- ELISPOT CO_2 incubator.
- ELISPOT reading system.

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 in PBS (Dilution buffer)**

For one plate dissolve 0.2 g of BSA in 20 mL of 1X diluted PBS.

9.2 **Detection antibodies**

Reconstitute each vial with 550 µL of distilled water. Gently mix the solution and wait until all the lyophilised material is reconstituted.

For 1 plate dilute 100 µL of reconstituted FITC conjugated IFNγ detection antibody and 100 µL of reconstituted Biotinylated Granzyme B detection antibody into 10 mL of 1% BSA in PBS.

If not used within a short period of time, reconstitute detection antibody should be aliquoted and stored at -20°C. In these conditions the reagent is stable for at least one year.

9.3 **Anti FITC-green fluorescence conjugate / Steptavidin-phycoerythrin**

Add the volume indicated on each vial of Streptavidin-PE conjugate and anti-FITC antibody Green Fluorescence conjugate to 10 mL of Dilution Buffer.

10 mL of diluted conjugates is required for one plate. Mix well.

Preparation immediately before use is recommended.

Note: The quantity of anti FITC-green fluorescence and Streptavidin-PE conjugates may need adjustments depending on the cell types and on the stimulating antigen studied.

The balance of the 2 different cytokines secreted varies with the cells stimulation. Conjugates dilutions advised in this protocol have been optimised for best results in the suggested protocol (polyclonal activation).

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).

All the procedure beyond the stimulation step is the same regardless of the method (direct/indirect) chosen.

10.1 Positive Assay Control, IFNγ and Granzyme B production

We recommend to dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated foetal calf serum) containing 1 ng/mL PMA and 500ng/mL Ionomycin. Distribute from 1 x 10^5 to 2.5 x 10^4 cells in antibody coated PVDF-bottomed-wells and incubate for 15-20 hours in an incubator.

However, different conditions may be assayed with varying cells types and stimulating antigens or mitogens. The assay should be optimised in each application.

11. ASSAY PROCEDURE

11.1 Incubate PVDF-bottomed-well plates with 25 µL of 35% Ethanol for 30 seconds at room temperature.

11.2 Empty wells and wash three times with 100 µL/well of PBS.

11.3 Pipette 100 µL of IFNγ capture antibody and 100 µL of Granzyme B capture antibody in 10 mL of PBS. Mix and dispense 100 µL into each well, cover the plate and incubate overnight at +4°C.

11.4 Empty wells and wash once with 100 µL/well of PBS.

11.5 Dispense 100 µL/well of RPMI 10% FCS cell culture media into wells, cover and incubate for 2 hours at room temperature.

11.6 Empty wells by flicking the plate over a sink and tapping it on absorbent paper.

11.7 Wash plate once with PBS.

11.8 Dispense into wells 100 µL/well of cell suspension containing the appropriate number of cells and appropriate concentration of stimulator. Cells may have been previously *in vitro* stimulated (Indirect ELISPOT). Cover the plate with a standard 96-well plate plastic lid and incubate cells at 37°C in an ELISPOT CO_2 incubator for an appropriate length of time (15-20 hours).

Note: During this period do not disturb the plate. 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.

11.9 Empty wells by flicking the plate over a sink and tapping it on absorbent paper.

11.10 Dispense 100 µL of PBS-0.05% Tween 20 into wells and incubate for 10 minutes at +4°C.

11.11 Wash wells 3x with PBS-0.05% Tween 20.

11.12 Dispense 100 µL Detection Antibodies into wells, cover the plate and incubate 1 hour 30 minutes at room temperature.

11.13 Wash 3x with PBS-0.05% Tween 20.

11.14 Distribute 100 µL of anti FITC-green fluorescence conjugate/Streptavidin-phycoerythrin solution (for IFNγ/Granzyme B respectively) in each well. Seal the plate and incubate for 1 hour at room temperature away from light.

11.15 Wash three times with PBS-0.05% Tween 20.

11.16 Peel off the plate bottom and wash three times both sides of the membrane under running distilled water. Remove all residual buffer by repeated tapping on absorbent paper.

11.17 Dry wells away from light.

11.18 Read spots on an ELISPOT reader under a UV light source. Green fluorescent spots indicate IFNγ production while Granzyme B is revealed by red spots. Yellow spots will indicate dual cytokine producing cells.

Plates may be stored at +4°C away from light. Please note fluorescence may fade over prolonged periods so for best results read within 24 hours.

Optimal assay performances are observed between $0.5x10^5$ and $2x10^5$ 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. TROUBLESHOOTING

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

13. NOTES

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

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