

ab46617 – Human Granzyme B ELISPOT Kit (with un-coated plates)

Instructions for Use

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

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

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1. BACKGROUND

Abcam's Granzyme B Human ELISPOT kit is an *in vitro* ELISPOT assay designed for the qualitative measurement of Granzyme B 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.

INTRODUCTION

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.

Granzyme B belongs to the granzymes family, which are exogenous serine proteinases. Granzymes are produced by cytotoxic lymphocytes (CTLs) and NK cells. Activated cytotoxic T lymphocytes and NK cells are important effectors in immunologic defenses against tumor cell proliferation, viral infection and transplant tissue. They induce apoptosis via multiple mechanism thanks to granules exocytosis. Following adhesion of the CTL on the target cell (via CTL-receptor and antigen-presenting MHC molecules), CTLs secrete cytoplasmic granules containing granules and enzymes including granzymes and perforine (pore-forming protein). Granules are then released in the intercellular space, perforine forms a transmembrane pore .Through these pores granzymes enter the cytosol of the target cell.

Granzyme B is the most powerful pro-apoptotic member of granzyme family. Granzyme B activates the intracellular caspases inducing the death of the cell.

Granzymes are diagnostics markers for many disorders (viral infection, lymphomas and carcinoma's, auto-immune disease and rejection of transplant).

2. ASSAY SUMMARY

Capture Antibody



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.

Protein



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

anti-Protein-Biotin



Aspirate and wash each well. Add prepared Biotinylated detector antibody. Incubate at room temperature.

Streptavidin-AP



Add prepared Streptavidin-Alkaline Phosphatase mix to each well. Incubate at room temperature.

Substrate Colored Product



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 +2-8°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

Item	Quantity		Storage Condition (Before Preparation)
	5 x 96 tests	10 x 96 tests	
96 well PVDF bottomed Microplate	5 x 96 wells (non-Sterile)	10 x 96 wells (non-Sterile)	RT
Capture Antibody for Human Granzyme B	1 x 500 µL	2 x 500 µL	+2-8°C
Biotinylated Detection antibody (lyophilised)	1 x 1 vial	2 x 1 vial	+2-8°C
Streptavidin-Alkaline Phosphatase Conjugate	1 x 50 µL	2 x 50 µL	+2-8°C
Bovine Serum Albumin (BSA)	1 x 1 g	2 x 1 g	+2-8°C
Ready to use BCIP/NBT substrate buffer	2 x 27 mL	4 x 27 mL	+2-8°C
Dry skimmed milk	1 x 1 vial	2 x 1 vial	+2-8°C

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).
- 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).
- 1X Phosphate Buffered Saline (PBS) (coating and wash step).
For 1L of 10X PBS weigh out:
80 g NaCl
2 g KH_2PO_4
14.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.
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 (18-25°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 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 100 µL of capture antibody in 10 mL of 1X PBS and mix well.

9.3 **Skimmed milk in 1X PBS (Blocking buffer)**

For one non sterile plate dissolve 0.2 g of milk 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.

9.4 **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 -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.

9.5 Streptavidin – AP conjugate

For optimal performance prepare the Streptavidin-AP dilution immediately prior to use.

For 1 plate dilute 10 μ L 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 - Granzyme B 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 1×10^5 to 2.5×10^5 cells per 100 μ L 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 μ L 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 μ L.
- Optimal assay performances are observed between 1×10^5 and 2.5×10^5 cells per 100 μ L.
- 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 200 μL of PBS 1X per well.
- 12.4 Add 100 μL of diluted Capture Antibody 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 1x with 200 μL of PBS 1X 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 200 μL of PBS 1X 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 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 200 μL of PBS 1X to well.
- 12.13 Incubate the plate at 4°C for 10 minutes.
- 12.14 Empty the wells as previous and wash the plate 3x with 200 μL of PBS 1X.
- 12.15 Add 100 μL of diluted Detection Antibody (Biotinylated anti-Granzyme B) to every well.

ASSAY PROCEDURE

- 12.16 Cover the plate and incubate at 37 °C for 1 hour 30 minutes.
- 12.17 Empty the wells as previous and wash the plate 3x with 200 µL of PBS 1X.
- 12.18 Add 100 µL of diluted Streptavidin-AP conjugate to every well.
- 12.19 Cover the plate and incubate at 37 °C for 1 hour.
- 12.20 Empty the wells and wash the plate 3x with 200 µL of PBS 1X.
- 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 5-15 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 +4°C. 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 6 different PBMC concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the six 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	835	796	864	2.6
50,000	12	668	638	708	2.9
25,000	12	474	425	499	4.2
12,500	12	304	277	334	5.6
6,250	12	155	106	193	16.8
3,125	12	91	63	101	11.1

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)