

Human Granzyme B ELISPOT Kit

Catalog No: CDK112A	Quantity: 5 x 96 tests	Without Plates
Catalog No: CDK112B	Quantity: 10 x 96 tests	Without Plates
Catalog No: CDK112C	Quantity: 15 x 96 tests	Without Plates
Catalog No: CDK112D	Quantity: 20 x 96 tests	Without Plates

PRODUCT SPECIFICATIONS :

- Specificity :** Recognizes natural human Granzyme B
- Incubation :** 3 h after cell stimulation procedure
- Kit Content :** Capture antibody, Biotinylated Detection antibody, Streptavidin Alkaline phosphatase conjugate, BCIP/NBT ready-to-use substrate buffer, BSA, dry skimmed milk.

1. Intended Use:

This ELISPOT assay is designed to enumerate Granzyme B producing cells in a single cell suspension. This method has the advantage of requiring a minimum of *in-vitro* manipulations allowing Granzyme B production analysis as close as possible to *in-vivo* conditions in a highly specific way. This technique is designed to determine the frequency of Granzyme B producing cells under a given stimulation, and the follow-up of such frequency during a treatment and/or a pathological state. 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.

Cell Sciences® ELISPOT assay is based on sandwich immuno-enzyme technology. 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 kit has been configured for research use only and is not to be used in diagnostic procedures.

2. Reagents provided - Contents shown for 5 x 96-well test format:

- A. Capture Antibody for Granzyme B (0.5 ml supplied sterile)
- B. Biotinylated Detection Antibody (lyophilized, resuspend in 0.55 ml)
- C. Streptavidin-Alkaline Phosphatase conjugated (50 µl)
- D. Ready-to-use BCIP/NBT substrate buffer (50 ml)
- E. Bovine Serum Albumin (BSA)
- D. Dry Skimmed Milk (Blocking reagent)

Store all reagents at 4°C.



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3. Materials/Reagents Not Provided:

- 96-well PVDF bottom plates (We recommend Millipore plates catalogue # MSIPN4510, MSIPS4510 and M8IPS4510).
- Miscellaneous laboratory plastic and/or glass, sterile if possible
- Ethanol
- Cell culture media
- Cell stimulation reagents
- CO₂ incubator
- Tween 20
- Phosphate buffered saline (PBS)

4. Storage

Store kit reagents between 2 and 8°C. Immediately after use, remaining reagents should be returned to cold storage (2 to 8°C). The expiration date of the kit and reagents is stated on the box front labels. The expiration of the kit components can only be guaranteed if the components are stored properly, and in the case of repeated use of one component, if the reagent is not contaminated by the first handling.

5. Safety and Precautions for Use

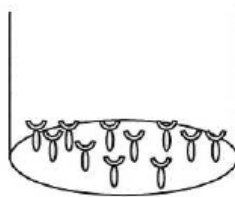
- **For Research Use Only. Not to be used as a diagnostic test.**
- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g. CDC/NIH Health manual: " Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on the vial or bottle labels.
- All reagents should be warmed to room temperature before use.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- **BCIP/NBT buffer** is potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling this reagent, always wear gloves.
- Follow incubation times described in the assay procedure.




6. Principle of the Method:

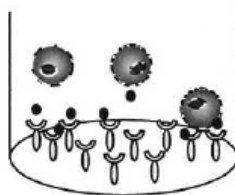
A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96-well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimize any non-antibody dependent unspecific binding and washed. Cell suspension and stimulant 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.


1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody




 Capture antibody

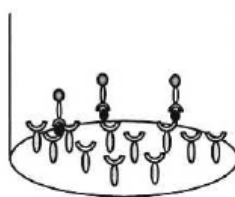
2. Incubation of cells in the coated microwell




 Antigen / Mitogen

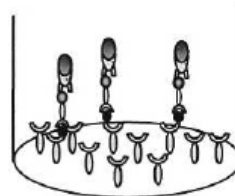
 Biotinylated detection antibody

3. Cell removal by washing. Incubation with biotinylated antibody



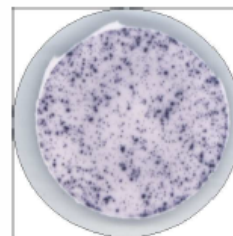
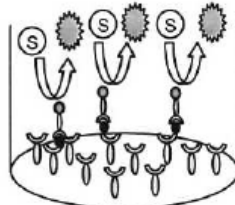
 Streptavidin - alkaline phosphatase conjugated

4. Incubation with streptavidin – alkaline phosphatase conjugated



 Substrate product

5. Addition of substrate BCIP/NBT and monitoring of spot formation.



7. Reagent Preparation:

• Phosphate Buffered Saline (10X concentrate solution)

For 1 liter: 80.0 g NaCl; 2.0 g KH₂PO₄; 14.4 g Na₂HPO₄·2H₂O. Add distilled water to 1 liter. Check that pH is 7.4 +/- 0.1. **This solution should be diluted to 1X before use.**

• 2% dry skimmed milk in PBS (Blocking Buffer)

For one plate, dissolve 0.2 g of powder in 10 ml of 1X diluted PBS.

• 1% BSA in PBS (Dilution Buffer)

For one plate, dissolve 0.2 g of BSA in 20 ml of 1x diluted PBS.

• 0.05% Tween in PBS (Washing Buffer)

For one plate, dissolve 50 µl of Tween 20 in 100 ml of 1X diluted PBS.

• 35% ethanol in water (PVDF Membrane Activation Reagent)

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

• Capture antibody

This reagent is supplied sterile. Once opened, keep the vial sterile or aliquot and store at -20°C. For optimal performance, dilute the capture antibody immediately before use.

Dilute 100 µl of capture antibody in 10 ml of 1X PBS and mix well.

• Detection antibody

Reconstitute the lyophilized antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilized material has dissolved.

If not used within a short period of time, reconstituted Detection Antibody should be aliquoted and stored at -20°C. Under these conditions, the reagent is stable for at least one year. For optimal performance, prepare the reconstituted antibody dilution immediately prior to use.

Dilute 100 µl of detection antibody into 10 ml of Dilution Buffer and mix well.

• Streptavidin alkaline phosphatase conjugate

For optimal performance, dilute the streptavidin-alkaline phosphatase immediately prior to use.

For 1 plate, dilute 10 µl Streptavidin alkaline phosphatase conjugate into 10 ml Dilution Buffer and mix well.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS.



8. Assay Control:

Positive Control: Granzyme B production by PBMC upon stimulation by PHA. This protocol is given as a suggestion.

Dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 10 µg/ml PHA (Sigma, Saint Louis, MO). Distribute 2×10^4 to 5×10^4 cells in antibody coated PVDF-bottomed-wells and incubate for 15-20 hours in an incubator. For other types of cell stimulation, incubation times may vary, depending on the frequency of cytokine producing cells, and should be optimized for each situation.

Negative Control

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

Samples

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 100µl. Optimal assay performances are observed between 1×10^5 and 2.5×10^5 cells per 100µl.

9. ELISPOT Procedure:

1. Add 25 µl of 35% ethanol to every well and incubate plate for 30 sec at room temperature.
2. Empty wells by flicking the plate over a sink and gently tapping on absorbent paper. Thoroughly wash the plate 3 times with 100 µl 1x PBS per well.
3. Add 100 µl diluted capture antibody to each well. Cover the plate and incubate overnight at +4°C.
4. Empty wells as in step 2 and wash once with 100 µl 1x PBS per well
5. Dispense 100 µl of Blocking Buffer into wells, cover and incubate for 2 hours at room temperature
6. Empty wells as in step 2 and thoroughly wash 3x with 100 µl 1x PBS per well.
7. Add 100 µl of cell suspensions containing the appropriate number of cells and the appropriate concentration of stimulants (test samples, positive controls, and negative controls).
8. Cover the plate with a standard 96-well plate plastic lid and incubate cells at 37°C in a CO₂ incubator for an appropriate length of time (15 – 20 hours). **During this time do not agitate or move the plate.**
9. Empty wells by flicking the plate over a sink and gently tapping it on absorbent paper.
10. Add 100 µl of PBS containing 0.1% Tween 20 to each well and let sit for 10 minutes at +4°C.
11. Empty wells as in step 2 and wash 3 times with 100 µl PBS containing 0.1% Tween 20.



12. Add 100 µl diluted detection antibody to each wells. Cover the plate and incubate 1 hour 30 minutes at 37°C.
13. Empty wells as in step 2 and wash 3 times with 100 µl PBS containing 0.1% Tween 20.
14. Add 100 µl of diluted streptavidin-alkaline phosphatase conjugate to each well. Seal the plate and incubate for 1 hour at 37°C.
15. Empty wells and wash three times with 100 µl PBS containing 0.1% Tween 20.
16. Peel off the plate bottom and wash both sides of the membrane 3 times with running distilled water. Remove any residual solution by repeated tapping on absorbent paper.
17. Add 100 µl ready-to-use BCIP/NBT buffer to every well.
18. Monitor the spot formation visually during the reaction time of around 5-15 minutes at room temperature.
19. Empty wells and rinse both sides of the membrane 3 times with running distilled water. Completely remove any excess solution by gentle repeated tapping on absorbent paper.
20. Dry wells. Read spots. Note that spots may become sharper after one night at +4°C.

Store the plate at room temperature away from direct light.

10. Performance Characteristics:

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 forming cells, range, and CV for the 6 concentrations.

Cells / well	n	Mean Number of Spots per Well	Min.	Max.	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%

FOR RESEARCH USE ONLY. NOT FOR USE IN HUMAN DIAGNOSTIC OR THERAPEUTIC PROCEDURES.



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