

Human Granzyme B ELISPOT Kit

Catalog No.: CDK112A	Quantity: 5 x 96 tests	Lot No.: TBD	Exp. Date: TBD
Catalog No.: CDK112B	Quantity: 10 x 96 tests	Lot No.: TBD	Exp. Date: TBD
Catalog No.: CDK112C	Quantity: 15 x 96 tests	Lot No.: TBD	Exp. Date: TBD
Catalog No.: CDK112D	Quantity: 20 x 96 tests	Lot No.: TBD	Exp. Date: TBD

NOTE: this is a sample protocol which is subject to variation by Lot Number. Refer to the protocol inserted in your package for the current lot number specifications and expiration date or contact our technical support at tech@cellsciences.com

1. Intended Use:

This ELISPOT assay is a highly specific immunoassay for the analysis of Granzyme B production and secretion at the single cell level in conditions closely comparable to the *in vivo* environment, with minimal cell manipulations. The assay is designed to determine the frequency of Granzyme B producing cells under a given stimulation, allowing the comparison of such frequency during a specific treatment or a pathological state. The ELISPOT assay provides a tool for research areas such as Th1 / Th2 responses, vaccine development, viral infection monitoring and treatment, oncology, 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 plate-coated antibodies to avoid diffusion in supernatant, protease degradation or binding to soluble membrane receptors. After cell removal, the captured cytokines are revealed by detection antibodies and appropriate conjugates.

2. Reagents provided:

Catalog No. / Item	Description	Preparation	Storage
CDK112-A. Capture Antibody for Granzyme B	Liquid, 500 µL	Use as per kit instructions	2-8 °C
CDK112-B. Biotinylated Detection Antibody	Lyophilized	Resuspend with 550 µL	2-8 °C
CDK112-C. Streptavidin-Alkaline Phosphatase Conjugate	Liquid, 55 µL	Use as per kit instructions	2-8 °C
CDK112-D. BCIP/NBT Substrate	Liquid, 27 mL	Ready to use	2-8 °C
CDK112-E. Bovine Serum Albumin (BSA)	Lyophilized, 2 g	Use to prepare Dilution Buffer as per kit instructions	2-8 °C
CDK112-F. Dry Skimmed Milk	Lyophilized, 2 g	Use to prepare Blocking Buffer as per kit instructions 1000-fold in Washing solution	2-8 °C

3. Materials/Reagents Not Provided:

- 96-well PVDF bottom plates
- Miscellaneous laboratory plastic and/or glass, sterile if possible
- Ethanol
- Cell culture reagents
- Cell stimulation reagents



- CO₂ incubator
- Tween 20
- Phosphate buffered saline (PBS)

4. Storage

Store kit reagents 2-8 °C. Immediately after use, remaining reagents should be returned to cold storage (2-8 °C). The expiration date of the kit is stated on the box front label and on page 1 of this protocol. 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

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures i.e. CDC/NIH Health manual: "Biosafety in Microbiological and Biomedical Laboratories" 2009.
- 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.

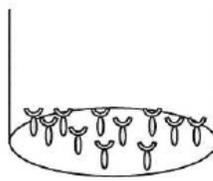
6. Principle of the Method:

A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96-well microtiter 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



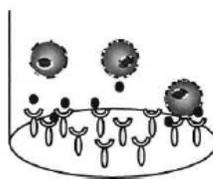
antibodies. Following incubation and washing, substrate is then applied to the wells resulting in colored spots which can be quantified using appropriate analysis software or manually using a microscope.

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



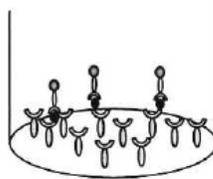
● Antigen / Mitogen

 Biotinylated detection antibody

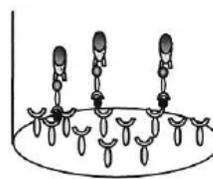
 Streptavidin - alkaline phosphatase conjugated

 Substrate product

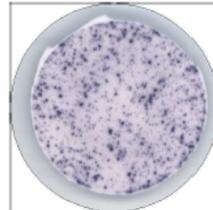
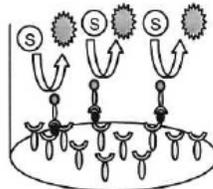
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatase conjugated



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



7. Reagent Preparation:

• Phosphate Buffered Saline (10X concentrate solution)

For 1 liter of 10X concentrate solution: 80.0 g NaCl
2.0 g KH_2PO_4
14.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$.

Add distilled water to 1 liter. Check that pH is 7.4 +/- 0.1.

Note: This solution should be diluted to 1X before use.

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

For one plate, dissolve 200 mg of powder in 10 mL of 1X diluted PBS.



- **1% BSA in PBS (Dilution Buffer)**

For one plate, dissolve 200 mg of BSA in 20 mL of 1x diluted PBS.

- **0.05% Tween in PBS (Washing Buffer, PBS-T)**

For one plate, dissolve 50 μ L 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.

For one plate, dilute 100 μ L of capture antibody in 10 mL of 1X PBS and mix well.

- **Detection antibody**

Reconstitute the lyophilized antibody with 550 μ L 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. To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2 μ m filter disc.*

For one plate, dilute 100 μ L 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 μ L Streptavidin alkaline phosphatase conjugate into 10 mL Dilution Buffer and mix well. ***DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS.***

- **BCIP/NBT**

The reagent is ready-to-use. It should be clear to pale yellow. If precipitates appear, filter using a disposable syringe and a 0.2 μ m filter disc.

8. Assay Control:

Positive 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 1ng/mL PMA and 500 ng/ml ionomycin (Sigma, Saint Louis, MO).

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 optimized in 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 100 μ L 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 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 vary depending on the frequency of cytokine producing cells and therefore should be optimized by the testing laboratory.

9. ELISPOT Procedure:

Prepare all reagents as instructed in sections 7 and 8.

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

Assay Step	Details
1. Addition	Add 25 μ L of 35% ethanol to every well
2. Incubation	Incubate plate at room temperature (RT) for 30 seconds
3. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Thoroughly wash the plate (being sure to wash each well) 3x with 100 μ L of 1X PBS.
4. Addition	Add 100 μ L of diluted capture antibody to each well
5. Incubation	Cover the plate and incubate at 4 $^{\circ}$ C overnight
6. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Thoroughly wash the plate (being sure to wash each well) with 100 μ L of 1X PBS.
7. Addition	Add 100 μ L of blocking buffer to each well
8. Incubation	Cover the plate and incubate at RT for 2 hours
9. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Thoroughly wash the plate (being sure to wash each well) 1x with 100 μ L of 1X PBS.
10. Addition	Add 100 μ L of sample, positive and negative control cell suspension to appropriate wells providing the required concentration of cell and stimulant (cells may have been previously stimulated see Section 8).
11. Incubation	Cover the plate and incubate at 37 $^{\circ}$ C in a CO ₂ incubator for 15-20 hours Note: do not agitate or move the plate during incubation
12. Addition	Empty the wells, remove excess solution and add 100 μ L of PBS-T to each well
13. Incubation	Incubate plate at 4 $^{\circ}$ C for 10 minutes
14. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Wash the plate (being sure to wash each well) 3x with 100 μ L of 1X PBS-T.
15. Addition	Add 100 μ L of diluted detection antibody to each well
16. Incubation	Cover the plate and incubate at 37 $^{\circ}$ C for 1 hour and 30 minutes
17. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Wash the plate (being sure to wash each well) 3x with 100 μ L of 1X PBS-T.



18. Addition	Add 100 μ L of diluted streptavidin-AP conjugate to each well
19. Incubation	Cover the plate and incubate at RT for 1 hour
20. Wash	Empty the wells by flicking the plate & gently tapping on absorbent paper. Wash the plate (being sure to wash each well) 3x with 100 μ L of 1X PBS-T.
21. Wash	Peel off the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing is complete remove and excess solution by repeated tapping on absorbent paper.
22. Addition	Add 100 μ L or ready to use BCIP/NBT buffer to each well
23. Development	Incubate the plate for 5-15 minutes monitoring spot formation visually throughout the incubation period to assess sufficient color development
24. Wash	Empty the wells and rinse both sides of the membrane 3x under running distilled water. Completely remove any excess solution by repeated tapping on absorbent paper.
<p>Read Spots: allow the wells to dry and then read results. The frequency of the resulting colored spots corresponds to the cytokine producing cell can be determined using an ELISpot reader and analysis software or manually using a microscope.</p> <p>Note: spots may become sharper after an overnight incubation at 4 °C.</p>	

Plate should be stored at RT away from direct light. Note that color may fade over prolonged periods so it is best to read results within 24 hours.

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%

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