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# Human Granzyme B ELISPOT Kit

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|---|---|---|---|
| **Catalog No.:** CDK113A | **Quantity:** 5 Plates (5 x 96 tests) | **Lot No.:** TBD | **Exp. Date:** TBD |
| **Catalog No.:** CDK113B | **Quantity:** 10 Plates (10 x 96 tests) | **Lot No.:** TBD | **Exp. Date:** TBD |
| **Catalog No.:** CDK113C | **Quantity:** 15 Plates (15 x 96 tests) | **Lot No.:** TBD | **Exp. Date:** TBD |
| **Catalog No.:** CDK113D | **Quantity:** 20 Plates (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 |
|---|---|---|---|
| **CDK113-P**. 96 well PVDF-bottom plates | 96 well plates, set of 10 | Use as per kit instructions | 2-8 °C |
| **CDK113-A**. Blocking Reagent, skim milk in PBS | Liquid, 25 mL bottle | Dilute prior to use as per instructions | 2-8 °C |
| **CDK113-B**. Capture Antibody | Liquid, 500 µL vial | Sterile, dilute prior to use as per instructions | 2-8 °C |
| **CDK113-C**. Biotinylated Detection Antibody | Lyophilized, 100 µL vial | Reconstitute prior to use as per instructions | 2-8 °C |
| **CDK113-D**. Streptavidin-Alkaline Phosphatase Conjugate | Liquid, 50 µL vial | Dilute prior to use as per instructions | 2-8 °C |
| **CDK113-E**. Bovine Serum Albumin (BSA) | Lyophilized, 2 g vial | Dissolve to prepare Dilution Buffer as per instructions | 2-8 °C |
| **CDK113-F**. BCIP/NBT Substrate | Liquid, 2 x 27 mL bottles | Ready to use | 2-8 °C |

**Note:** liquid milk Blocking Reagent has a shorter expiration date than other reagents in kit. The use of expired milk can lead to unspecific stimulation. Use any fresh skimmed milk if the one provided has expired.

## 3. Materials/Reagents Not Provided:

- Miscellaneous laboratory plastic and/or glass, if possible sterile
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin)



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- $CO_2$ incubator
- Tween 20
- Phosphate Buffered Saline (PBS)

**4. Storage**

Store kit reagents 2-8 °C, except uncoated plates, which can be stored at room temperature. Immediately after use, remaining reagents should be returned to cold storage (2-8 °C). The expiration date of the kit and reagents is stated on the kit box label. The expiration date of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated during 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,



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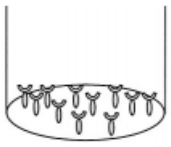
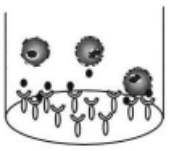
substrate is then applied to the wells resulting in colored spots which can be quantified using appropriate analysis software or manually using a microscope.

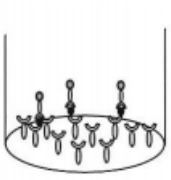
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.

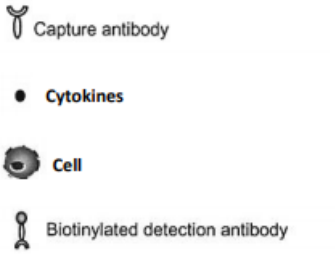


2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.

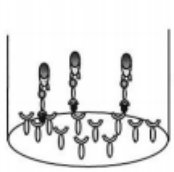


3. Cell removal by washing. Incubation with biotinylated detection antibody.





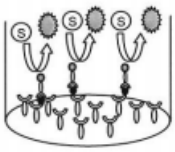

4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.

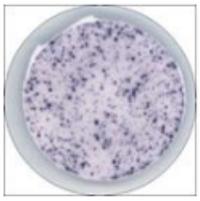


5. Any excess unbound Strep-AP is removed by washing. Incubation with BCIP/NBT.

Finally BCIP/NBT reduction by alkaline phosphatase give a precipitated product which give blue/purple spots.

One spot correspond to one single producing cell.





**7. Reagent Preparation:**

**Phosphate Buffered Saline (10X concentrate solution)**
For 1 liter of 10X concentrate solution, weigh out: 80.0 g NaCl
2.0 g $KH_2PO_4$
14.4 g $Na_2HPO_4$-$2H_2O$
Add distilled water to 1 liter. Check that pH is 7.4 +/- 0.1.
**NOTE: This is a 10X stock solution and should be diluted to 1X before use.**

**PVDF Membrane Activation Reagent – 35% ethanol in water**
For one plate, mix 3.5 mL of ethanol with 6.5 mL of distilled water.

**Blocking Buffer - 2% skimmed milk in PBS (CDK113-A)**
For one plate, dilute 5 mL of liquid milk with 5 mL of 1X PBS.



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*Please note liquid milk has a shorter expiration date than the 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 if the one provided has expired.*

**Dilution Buffer – 1% BSA (CDK113-E) in PBS**

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

**Wash Buffer (PBST) – 0.05% Tween in PBS**

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

**CDK113-B - Capture antibody**

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

*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.*

**CDK113-C - Detection antibody**

Reconstitute the lyophilized antibody with 550 µL 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.*

For one plate, dilute 100 µL of detection antibody into 10 mL of Dilution Buffer and mix well.

*To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2 µm filter disc.*

**CDK113-D- Streptavidin-Alkaline Phosphatase (AP) Conjugate**

Centrifuge vial for a few seconds to collect material in bottom of vial.

For 1 plate, dilute 10 µL Streptavidin AP Conjugate into 10 mL Dilution Buffer and mix well. To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2 µm filter disc.

*For optimal performance, dilute the streptavidin-alkaline phosphatase immediately prior to use.* ***DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS***

**CDK113-F - BCIP/NBT Substrate Solution**

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 µm filter disc.

**8. Sample and Control Preparation:**

**Cell Stimulation**

Cells can either be stimulated in the antibody-coated wells (direct stimulation) or can be first stimulated in separate plates or in flasks, harvested, and then plated into the coated wells (indirect stimulation).

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 suggested to stimulate with the direct method. When the expected 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 whatever the method of stimulation chosen (direct or indirect).



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**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 $1x10^5$ to $2.5x10^5$ cells per 100 µL 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 µL 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 µL. Optimal assay performances are observed between $1x10^5$ and $2.5x10^5$ cells per 100 µL.

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 µL 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 µL of 1X PBS. |
| 4. Addition | Add 100 µL of diluted **capture antibody** to each well |
| 5. Incubation | Cover the plate and incubate at 4 °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 µL of 1X PBS. |
| 7. Addition | Add 100 µL 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 µL of 1X PBS. |
| 10. Addition | Add 100 µL 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 °C in a $CO_2$ incubator for 15-20 hours<br>Note: do not agitate or move the plate during incubation |
| 12. Addition | Empty the wells, remove excess solution and add 100 µL of PBS-T to each well |



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| | |
|---|---|
| 13. Incubation | Incubate plate at 4 °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 µL of 1X PBS-T. |
| 15. Addition | Add 100 µL of **diluted detection antibody** to each well |
| 16. Incubation | Cover the plate and incubate at 37 °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 µL of 1X PBS-T. |
| 18. Addition | Add 100 µL 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 µL 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 µL 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. |
| 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.<br>Note: spots may become sharper after an overnight incubation at 4 °C. | |

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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