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Human Perforin ELISPOT Kit

Catalog No: CDK138A	**Quantity:**	5 x 96 tests	Without Plates
Catalog No: CDK138B	**Quantity:**	10 x 96 tests	Without Plates
Catalog No: CDK138C	**Quantity:**	15 x 96 tests	Without Plates
Catalog No: CDK138D	**Quantity:**	20 x 96 tests	Without Plates

PRODUCT SPECIFICATIONS :

Specificity : Recognizes natural human Perforin

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.

Intended Use:

This ELISPOT assay is designed to enumerate Perforin producing cells in a single cell suspension. This method has the advantage of requiring a minimum of *in-vitro* manipulations allowing Perforin production analysis as close as possible to *in-vivo* conditions in a highly specific way. This technique is designed to determine the frequency of Perforin 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.

Reagents provided - Contents of the Kit for 5 x 96 Wells:

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

Store all reagents at 4°C.

Materials/Reagents Not Provided:

- Sterile 96-well PVDF bottom plate.
- Miscellaneous laboratory plastic and/or glass, sterile if possible
- Ethanol

- Cell culture media
- Cell stimulation reagents
- CO_2 incubator
- Tween 20
- Phosphate buffered saline (PBS)

Storage Instructions:

Store kit reagents between 2-8°C. Immediately after use, remaining reagents should be returned to cold storage (2-8°C). Expiration date of the kit and reagents is stated on the box front labels. The stability of the kit components can only be guaranteed if the components are stored properly and if the reagents are not contaminated during the first handling when they are used more than once.

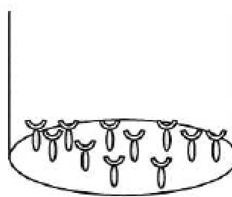
SAFETY & PRECAUTIONS FOR USE

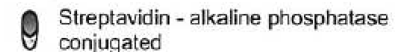
- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual: "Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles 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. Use a clean disposable plastic pipette tip for each reagent, standard, or sample addition in order to avoid cross contamination.
- Do not use reagents beyond the expiration date of the kit.
- 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.
- Follow incubation times described in the assay procedure.
- BCIP/NBT buffer is potentially carcinogenic and should be disposed of appropriately. Caution should be taken while handling this reagent. Always wear gloves.

Principle of the Method:

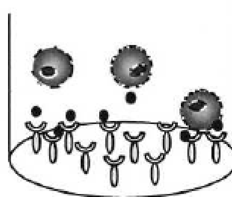
A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtitre plate either during kit manufacture or in the laboratory. The plate is then blocked to minimize any non-antibody dependent unspecific binding and washed. Cell suspension and stimulant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

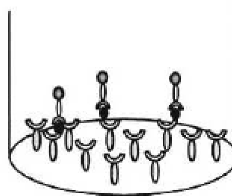




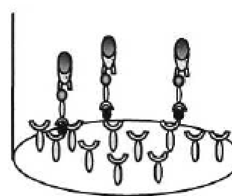
2. Incubation of cells in the coated microwell



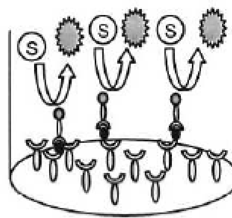
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatase conjugated



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



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Positive Control:

Perforin 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 $2x10^4$ to $5x10^4$ cells in antibody coated PVDF-bottomed-wells 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 for each situation.

Negative Control:

Dilute PBMC in culture medium at the same volume and number of cells as stimulated cells but without any stimulatory agent.

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 $1x10^5$ and $2.5x10^5$ cells per 100µl.

Reagent Preparation:

Phosphate Buffered Saline (10X concentrate solution)
For 1 liter: 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. **This solution should be diluted to 1X before use.**

Cell Culture Medium + 10% Serum (Blocking Buffer)
For one plate, add 1 ml serum (e.g., fetal calf serum, FCS) to 9 ml of culture medium (use the same cell culture medium as used to derive the cell suspension).

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

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 into 10 ml Dilution Buffer and mix well.

Do not keep this solution for further experiments.

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 Cell Culture Medium with 10% Serum (Blocking Buffer) into wells, cover and incubate for 2 hours at room temperature
6. Empty wells as in step 2 and thoroughly wash once 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). Cells may have been previously stimulated *in-vitro* (indirect ELISPOT).
8. Cover the plate with a standard 96-well plate plastic lid and incubate cells at 37°C in a CO_2 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 room temperature.

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 5-20 minutes at room temperature to assess sufficient color development.

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. Please note that color may fade after prolonged periods of time so read results as soon as possible.

Performance Characteristics:

Specificity

The assay recognizes natural human Perforin.

To define the specificity, several proteins were tested from cross reactivity. There was no cross reactivity observed for any protein tested (Granzyme B, Fas, Fas L, IL-1β, IL-2, IFNγ, TNFα, and TRAIL). This testing was performed using the equivalent human Perforin antibody pair in an ELISA assay.

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%
50,000	12	800	724	851	5.2%
25,000	12	580	361	668	16.8%
12,500	12	241	291	176	16.1%
6,250	12	89	58	105	18.2%
3,125	12	19	14	21	16.3%

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