cellsciences.com

Human CD178 Fas-Ligand Matched Antibody Pair for ELISPOT

Catalog No:	**Size:**
CDK110A	5 x 96 tests
CDK110B	10 x 96 tests
CDK110C	15 x 96 tests
CDK110D	20 x 96 tests

1. INTRODUCTION

The Cell Sciences® ELISPOT 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, oncology, infectious disease, autoimmune diseases and transplantation.

Utilizing sandwich immuno-enzyme technology, 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 receptors. After cell removal, the captured cytokines are revealed by detection antibodies and appropriate conjugates.

Background

Fas Ligand (FasL/CD95/CD178) is a member of the tumor necrosis factor superfamily which is expressed as a homo-trimeric transmembrane protein on the cell surface. It also exists in a soluble form, generated by cleavage of the membrane-bound form by matrix metalloproteinase 7 (MMP-7). The primary function of FasL is the induction of programmed cell death (apoptosis), triggered by binding to the Fas receptor (Fas/CD95).

Fas is a member of the death receptor family, which upon binding FasL, transmits a signal internally to initiate apoptosis. FasL can also be bound by DcR3, a soluble decoy receptor with no signal transduction capabilities. The Fas/FasL signaling pathway is essential for immune system regulation, including activation-induced cell death (AICD) of T cells and cytotoxic T lymphocyte (CTL)-induced cell death. It has also been implicated in the progression of several cancers. Defects in this gene may be related to some cases of systemic lupus erythematosus (SLE).

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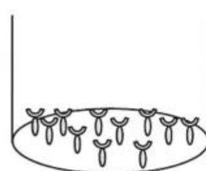
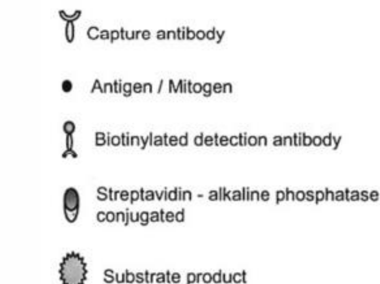
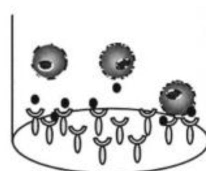
2. PRINCIPLE OF THE METHOD

1. 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 non-specific binding and washed.

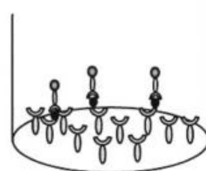




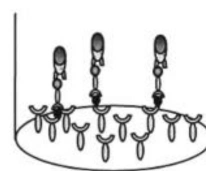
2. Cell suspension and stimulant are added and the plate is incubated, allowing the specific antibodies to bind any analytes produced.



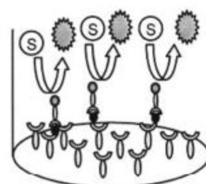
3. Cells are then removed by washing prior to the addition of biotinylated detection antibodies which bind to the previously captured analyte.



4. Enzyme-conjugated streptavidin is added, binding to the detection antibodies.



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



3. REAGENTS PROVIDED (suitable for 96 wells per plate)

REAGENTS (Store at 2-8 °C)	5 plates	10 plates	15 plates	20 plates
Anti-IL-5 Capture Antibody (0.5 mL/vial)	1 vial	2 vials	3 vials	4 vials
Biotinylated anti-IL-5 Detection Antibody (lyophilized)	1 vial	2 vials	3 vials	4 vials
Streptavidin-Alkaline Phosphatase (50 µL/vial)	1 vial	2 vials	3 vials	4 vials
Bovine Serum Albumin (BSA) (lyophilized; 2 g/vial)	1 vial	2 vials	3 vials	4 vials
Blocking Reagent (dry milk) (2 g/vial)	1 vial	2 vials	3 vials	4 vials
BCIP/NBT Substrate (25 mL/bottle)	2 bottles	4 bottles	6 bottles	8 bottles

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4. MATERIALS/ REAGENTS REQUIRED BUT NOT PROVIDED

- 96-well PVDF-bottomed microtiter plates
- Ethanol
- Cell culture reagents (e.g. RPMI-1640, L-glutamine, FCS)
- Cell stimulation reagents (PMA, Ionomycin, anti-CD3 antibody, IL-2)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- 10, 50, 100, 200 and 1,000 µL adjustable single channel micropipettes with disposable tips
- 50-300 mL multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Miscellaneous laboratory plastic and/or glass, if possible sterile.

5. STORAGE INSTRUCTIONS

Store the kit reagents 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 box front labels. 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.

6. SAFETY AND PRECAUTIONS FOR USE

- FOR RESEARCH ONLY. Not for diagnostic 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" 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.
- 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 wash 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.

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7. REAGENT PREPARATION

Coating Buffer - Phosphate Buffered Saline (PBS - 10X Concentrated Solution)

- For 1 liter of 10X PBS, weigh out: 80 g NaCl + 2 g KH_2PO_4 + 14.4 g $Na_2HPO_{4\ 2}H_2O$.
- Add distilled water to 1 L.
- Adjust the pH of the solution to 7.4 +/- 0.1 if necessary.
- **Dilute the solution to 1X before use.**

Blocking Buffer - Skimmed milk in 1X PBS

- For one non-sterile plate dissolve 200 µg of powder in 10 mL of 1X PBS
- For one sterile plate, dilute 5 mL of liquid milk in 5 mL of 1X PBS.

***Note**: 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 (UHT) if the one provided has expired.*

Dilution Buffer - 1% BSA-PBS Solution

- For one plate, dissolve 200 µg of BSA in 20 mL of 1X PBS.

Wash Buffer - 0.05% Tween PSS Solution

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

PVDF Membrane Activation Buffer - 35% Ethanol

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

Capture Antibody

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

***Note**: 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.*

Detection Antibody

- Reconstitute the lyophilized antibody with 550 µL of distilled water. Gently mix the solution and wait until all the lyophilized material dissolves.
- Dilute 100 µL of antibody into 10 mL Dilution Buffer and mix well.

***Note**: 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.*

Streptavidin-Alkaline Phosphatase Conjugate

- For 1 plate dilute 10 µl of Streptavidin-AP conjugate into 10 mL Dilution Buffer and mix well.

***Note**: For optimal performance prepare the Streptavidin-AP dilution immediately prior to use. DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS.*

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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 and 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 high, it is better to use the indirect ELISpot method.

All the steps in the method following stimulation of the cells are the same whether direct or indirect stimulation.

Positive Assay Control, FasL production

We suggest the following polyclonal activation as a positive control in your assay.

We recommend pre-stimulation of PBMC at $1x10^6$ cells/mL in culture media (e.g. RPMI 1640 + 2 mM L-glutamine + 10 % heat inactivated fetal calf serum) containing anti-CD3 antibody (we suggest using Cat. No. CDM127 at 1 µg/ mL) and IL-2 at 20 ng/mL for 3 days. Then wash the cells once to remove the stimulating antibody and dilute the cells in media containing IL-2 only for 24 hours. Harvest cells and re-suspend in culture media added with 5 ng/mL PMA and 500 ng/mL ionomycin. Distribute 3×10^5 to 4×10^5 cells/100mL in appropriate wells of an antibody-coated 96-well PVDF plate, and incubate for 15-20 hours in an incubator.

For antigen-specific stimulation, the optimal concentration of the antigen and the optimal concentration of number of cells must be determined experimentally, as it depends on the frequency of cytokine-producing cells.

Negative Assay Control

Dilute PBMC in culture medium with no stimulation, to give the same concentration of unstimulated cells in 100 µl as in the stimulated sample cells.

Samples

Dilute PBMC in culture medium containing stimulation (e.g., sample, vaccine, peptide, infected cells, etc.) to yield the appropriate concentration of cells in 100 µL.

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

Stimulators and incubation times can be varied depending on the frequency of cytokine producing cells, and should be optimized by the testing laboratory.

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9. METHOD

Prepare reagents and cells as shown in sections 7 & 8.
Note: For optimal performance dilute the Streptavidin-AP immediately prior to use.

Assay Step	Details
1. Addition	Add 25µL 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 over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100 µL of **1X PBS** per well.
4. Addition	Add 100 µL of diluted **Capture Antibody** to every well.
5. Incubation	Cover the plate and incubate at 4 ºC overnight.
6. Wash	Empty the wells as in step 3, and wash the plate once with 100 µL of **1X PBS** per well.
7. Addition	Add 100 µL of **Blocking Buffer** to every well.
8. Incubation	Cover the plate and incubate at RT for 2 hours.
9. Wash	Empty the wells as in step 3, and thoroughly wash 3x with 100 µL of **1X PBS** per well.
10. Addition	Add 100 µL of **sample, (+) and (–) controls** to appropriate wells, with the required concentration of cells and stimulant (if direct stimulation is appropriate - see Section 8).
11. Incubation	Cover the plate and incubate at 37 °C in a CO_2 incubator for an appropriate length of time (15-20 hours). ***Note**: do not agitate or move the plate during this incubation.*
12. Addition	Empty the wells and remove excess solution then add 100 µL of **PBS-T** to every well.
13. Incubation	Incubate the plate at 4 °C for 10 minutes.
14. Wash	Empty the wells as in step 3, and wash the plate 3x with 100 µL of **PBS-T**.
15. Addition	Add 100 µL of diluted **Detection Antibody** to every well.
16. Incubation	Cover the plate and incubate at RT for 1 hour and 30 minutes.
17. Wash	Empty the wells as in step 3, and wash the plate 3x with 100µL of **PBS-T**.
18. Addition	Add 100 µL of diluted **Streptavidin-AP conjugate** to every well.
19. Incubation	Cover the plate and incubate at RT for 1 hour.
20. Wash	Empty the wells and wash the plate 3x with 100µL of **PBS-T**.
21. Wash	Peel off the plate bottom, wash both sides of the membrane 3x under running distilled water, and remove any excess solution by repeated tapping on absorbent paper.
22. Addition	Add 100 µL of ready-to-use **BCIP/NBT Buffer** to every well.
23. Development	Incubate the plate for 5-20 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 gentle repeated tapping on absorbent paper.

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9. METHOD

Read Spots: Allow the wells to dry and then read results. The frequency of the resulting colored spots corresponds 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.

The developed plate should be stored at RT away from direct light, but please note 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 5 different PBMC concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the five cell concentrations.

Cells/well	n	Mean # of Spots/Well	Min	Max	CV%
25,000 (recommended)	12	871	837	908	3.1
12,500 (recommended)	12	833	796	891	4.4
6,250	12	723	647	825	9.7
3,125	12	517	461	577	8.7
1,560	12	294	258	320	7.5

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