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Human CD178 / Fas-Ligand ELISPOT Set, with Plates

Catalog No.: CDK111A
CDK111B
CDK111C
CDK111D

Quantity: 5 plates (5 x 96 tests)
10 plates (10 x 96 tests)
15 plates (15 x 96 tests)
20 plates (20 x 96 tests)

Lot No.: TBD **Exp. Date:** TBD

NOTE: This sample protocol 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

The Cell Sciences® ELISPOT assay 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. 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 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.

CD178, also known as Fas ligand (FasL), is a homotrimeric type-II transmembrane protein that is part of the tumor necrosis factor family, expressed on cytotoxic T lymphocytes. It signals through trimerization of FasR which spans the membrane of the "target" cell. This trimerization usually leads to apoptosis, or cell death. Soluble Fas ligand is generated by cleaving membrane-bound FasL at a conserved cleavage site by the external matrix metalloproteinase MMP-7.

CD178 is essentially expressed on activated T cells, testis and eye. CD178 has been shown to induce autocrine and paracrine T cells death.

2. REAGENTS PROVIDED

Part No.	Quantity				Preparation
CDK111-P. 96-well PVDF-bottom plates	5	10	15	20	Ethanol treatment as per kit instructions
CDK111-A. Capture Antibody, 500 µL vial	1	2	3	4	Sterile, dilute prior to use as per instructions
CDK111-B. Biotin Detection Antibody (lyophilized)	1	2	3	4	Reconstitute with 0.55 ml water prior to use as per instructions
CDK111-C. Streptavidin-Alkaline Phosphatase Conjugate, 50 µL	1	2	3	4	Dilute prior to use as per instructions
CDK111-D. Bovine Serum Albumin (BSA) - 2 g	1	2	3	4	Dissolve to prepare Dilution Buffer as per instructions
CDK111-E. Blocking Reagent, dry milk 2 g, OR liquid skim milk – 25 ml	1	2	3	4	Prepare for Dilution Buffer as per instructions
CDK111-F. BCIP/NBT Substrate, 25 ml bottle	2	4	6	8	Ready to use

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3. MATERIALS & REAGENTS REQUIRED BUT 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, anti-CD3 antibody cat.# CDM127, IL-2 cat.# CRI100)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)

4. STORAGE INSTRUCTIONS

Store the kit reagents between 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 (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 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 clean plastic containers 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.

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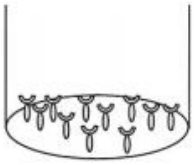
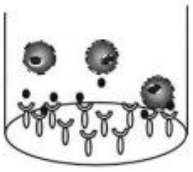
6. PRINCIPLE OF THE METHOD

A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtiter plate during kit manufacture. The plate is then blocked to minimize any non-antibody dependent nonspecific binding and washed. Cell suspension and stimulant are added and the plate is 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 added, binding to the detection antibodies. Following incubation and washing, substrate is applied to the wells resulting in colored spots which can be quantified using propriate analysis software or manually using a microscope.

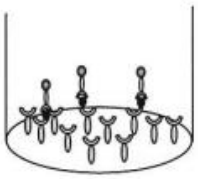
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody.

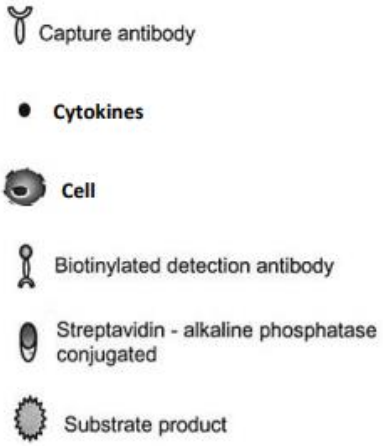


2. Cells are incubated in the presence of the stimulating agent. Upon stimulation they release cytokines which bind to the capture antibodies.



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



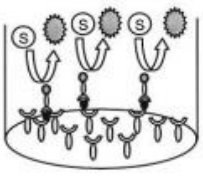


4. Any excess unbound detection antibodies is removed by washing. Incubation with streptavidin – alkaline phosphatase conjugate.

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.



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7. REAGENT PREPARATION

7.1. Phosphate Buffered Saline (10X concentrate solution)

For 1 liter of 10X PBS, 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. This solution should be diluted to 1X before use.

7.2. PVDF Membrane Activation Reagent – 35% ethanol in water

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

7.3. 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 skimmed milk (UHT) if this is expired.*

7.4. Wash Buffer (PBST) – 0.05% Tween in PBS

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

7.5. Dilution Buffer – 1% BSA (CDK111-D) in PBS

For one plate, dissolve 0.2 g of BSA in 20 mL of 1X PBS.

7.6. CDK111-A - Capture Antibody

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 prepare the Capture Antibody dilution immediately before use.

7.7. CDK111-B - 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. To avoid nonspecific background, it is recommended to filter the working solution using a disposable syringe and a 0.2 µm filter disc.

For one plate, dilute 100 µL of capture antibody in 10 mL of Dilution Buffer and mix well.
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 one year. For optimal performance, prepare the reconstituted antibody dilution immediately prior to use.

7.8. CDK111-C - 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

7.9. CDK111-E - BCIP/NBT

The reagent is ready-to-use. It should be clear to pale yellow. If precipitates appear, filter using a 0.2 µm syringe filter.

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8. SAMPLE AND CONTROL PREPARATION

8.1. 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).

8.2. Positive Assay Control, FasL production

Suggested polyclonal activation as a positive control in your assay:

It is recommended to first pre-stimulate PBMC at $1x10^6$ cells per ml in culture medium (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing anti CD3 antibody (use cat.# CDM127 at 1 µg/ml) and IL-2 (cat.# CRI100) at 20 ng/ml for 3 days. Then wash the cells once to remove the stimulating antibody and dilute the cells in medium containing IL-2 only for 24 hours. Harvest cells and resuspend in culture medium added with 5 ng/ml PMA and 500 ng/ml ionomycin (Millipore Sigma). Distribute $3x10^5$ to $4x10^5$ cells per 100 µl in required wells of an antibody coated 96-well PVDF plate 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.

8.3. Negative Assay Control

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

8.4. Samples

Dilute PBMC in culture medium and chosen stimulator (e.g., Sample, Vaccine, Peptide pool or infected cells) to give an appropriate cell number per 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 5x with 250 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 by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate ONCE with 100 µL of 1X PBS per well.
7.	Addition	Add 100 µL of **culture media with 10% serum** to every well.
8.	Incubation	Cover the plate and incubate at RT for 2 hours.
9.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate ONCE with 100 µL of 1X PBS per well.
10.	Addition	Add 100 µl of **sample, positive and negative controls** cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)
11.	Incubation	Cover the plate and incubate at 37 ºC 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, and then add 100 µL of Wash Buffer to every well.
13.	Incubation	Incubate the plate at 4 ºC for 10 minutes.
14.	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 Wash Buffer per well.
15.	Addition	Add 100 µL of diluted **Detection Antibody** to every well.
16.	Incubation	Cover the plate and incubate at RT for 1 hour 30 minutes
17.	Wash	Empty the wells as previously done and wash the plate 3x with 100µL of Wash Buffer.
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 by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100 µL of Wash Buffer per well.
21.	Wash	Peel off the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing is complete, 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 5-15 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 gentle repeated tapping on absorbent paper.

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9. METHOD (continued)

Reading Spots:

Allow the wells to dry and then read results. The frequency of the resulting colored spots corresponding 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 2-8ºC. Plate should be stored at RT away from direct light, but please note color may fade over prolonged periods, so read results within 24 hours.

10. PERFORMANCE CHARACTERISTICS

10.1. Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (PMA / Ionomycin) of 5 different PBMC cell 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 per well	Min.	Max	CV%
25000 (recommended)	12	871	837	908	3.1
12500 (recommended)	12	833	796	891	4.4
6250	12	723	2647	825	9.7
3125	12	517	461	1577	8.7
1560	12	294	258	320	7.5

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