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Mouse IFNγ ELISPOT Set

Catalog No. CDK158A
CDK158B
CDK158C
CDK158D

Quantity: 5 x 96 tests Without Plates
10 x 96 tests Without Plates
15 x 96 tests Without Plates
20 x 96 tests Without Plates

PRODUCT SPECIFICATIONS :

Specificity : Recognizes natural mouse IFN-gamma

Incubation : 3 h after cell stimulation procedure

Cross Reactivity: No cross reactivity with other mouse cytokines

Kit Content : Capture antibody, Biotinylated Detection antibody, Streptavidin Alkaline phosphatase conjugate, BCIP/NBT ready-to-use substrate buffer, BSA, dry skimmed milk.

1. INTENDED USE

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 membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates.

This kit has been configured for research use only.

2. PRINCIPLE OF THE METHOD

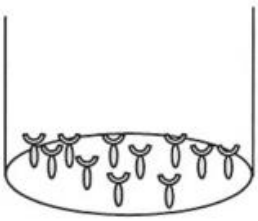
Capture antibodies highly specific for the analyte of interest are coated to the wells of a PVDF bottomed 96 well microtiter plate either during kit manufacture or in the laboratory. The plate is then blocked to minimize any non-antibody dependent unspecific binding and finally washed before adding the cells to be investigated. Cell suspension and stimulant are added to the coated and blocked microtiter plate and the plate incubated allowing the specific antibodies to bind any analytes produced. Biotinylated detection antibodies are then added which bind to the previously captured analyte. Enzyme conjugated Streptavidin is added binding to the detection antibodies. Any excess unbound analyte and antibodies are removed by careful washing. Color substrate is then applied to the wells resulting in colored spots which can be quantified using appropriate analysis software or manually using microscopes.

Cell Sciences, Inc.®
65 Parker Street
Unit 11
Newburyport, MA 01950

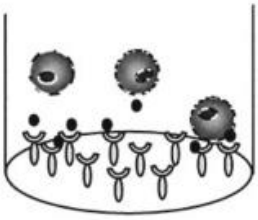
Toll Free: 888-769-1246
Phone: 978-572-1070
Fax: 978-992-0298

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

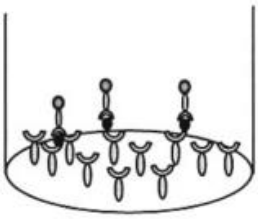
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody



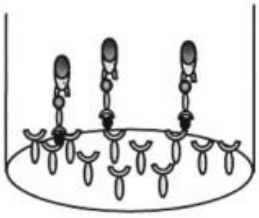
2. Incubation of cells in the coated microwell



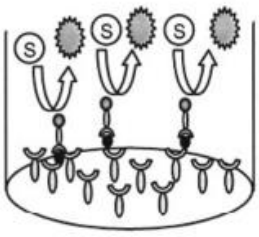
3. Cell removal by washing. Incubation with biotinylated antibody

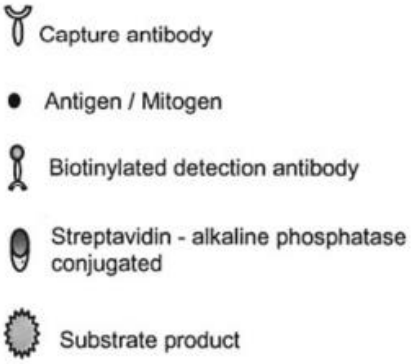


4. Incubation with streptavidin – alkaline phosphatase conjugated



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





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3. REAGENTS PROVIDED (for 5 x 96 tests)

- Capture Antibody for mouse IFNγ : 0.5 ml vial (supplied sterile)
- Biotinylated detection antibody: (lyophilized, resuspend in 0.55 ml)
- Streptavidin-Alkaline Phosphatase conjugate: 50 µl vial
- Bovine Serum Albumin (BSA): 1 vial
- Blocking Reagent
- Ready to use BCIP/NBT substrate buffer: 2 x 25 ml bottles

4. 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)
- CO_2 incubator
- Tween 20
- Phosphate Buffered Saline (PBS)
- 96 well PVDF bottomed plates if not ordered (we recommended Millipore plates catalog # MSIPN4510, MSIPS4510 and M8IPS4510).

5. STORAGE INSTRUCTIONS

Store the kit reagents between 2 and 8°C. Uncoated plates can be stored at room temperature. 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 by the first handling.

6. 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" 1984.
- 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 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 for the dispensing of H_2SO_4 and substrate solution. Avoid pipettes with metal parts.
- 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.

7. REAGENT PREPARATION

7.1. 1X Phosphate Buffered Saline (PBS) (Coating Buffer)

For 1 liter of 10X PBS weigh-out: 80 g NaCl
2 g KH_2PO_4
14.4 g Na_2HPO_4 $_2H_2O$.

Add distilled water to 1 liter. Adjust the pH of the solution to 7.4 +/- 0.1 where required.

Dilute the solution to 1X before use.

7.2. Skimmed milk in 1X PBS (Blocking Buffer)

For one non-sterile plate, dissolve 0.2 g of powder in 10 ml of 1X PBS.

For one sterile plate, dilute 5 ml of liquid milk in 5 ml of 1X PBS.

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.

7.3. 1% BSA PBS Solution (Dilution Buffer)

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

7.4. 0.05% PBS-T Solution (Wash Buffer)

For one plate, dissolve 50 µl of Tween 20 in 100 ml of 1X PBS.

7.5. 35% Ethanol (PVDF Membrane Activation Buffer)

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

7.6. Capture Antibody

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.

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

7.7. Detection Antibody

Reconstitute the lyophilized antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilized material is back into solution.

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.

Dilute 100 µl of antibody into 10 ml Dilution Buffer and mix well.

7.8. Streptavidin – AP conjugate

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

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

Do not keep this solution for further experiments.

8. SAMPLE AND CONTROL PREPARATION

8.1 Cell Stimulation

Cells can either be stimulated directly in the antibody coated wells (Direct) or, first stimulated in 24 well plates or flask, harvested, and then plated into the coated wells (Indirect).

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 also advised to test them with the direct method, however, when this 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 (direct/indirect) chosen.

8.2 Positive Assay Control, mouse IFNγ production

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

Dilute mouse splenocytes in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 0.5 µg/ml Concavalin A and 2 ng/ml mouse IL-2. Incubate 2 days. Take off non adherent materials and harvest adherent cells with a cell scraper. Wash cells once.
Dilute cells in culture media supplemented with 1 ng/ml PMA and 500 ng/ml ionomycin (Sigma, Saint Louis, MO). Distribute 1 x 10^5 to 2.5 x 10^5 cells per 100 µl in required wells of an antibody coated 96-well PVDF plates and incubate for 10-15 hours in an incubator.

For antigen specific stimulation, the optimal concentration of the antigen and the optimal concentration of number of cells have to be determined experimentally, as it is depending on the frequency of cytokine producing cells.

8.3 Negative Assay Control

Dilute mouse splenocytes in culture media 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 mouse splenocytes 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 1 x 10^5 and 2.5 x 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.

9. METHOD

Prepare all reagents as shown in section 7 and 8.
Note: For optimal performance prepare the Streptavidin-AP dilution 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 4ºC overnight.
6.	Wash	Empty the wells as previously done, 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 previously done, and thoroughly wash 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 37ºC in a CO_2 incubator for an appropriate length of time (10-15 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 4ºC for 10 min.
14.	Wash	Empty the wells as previously done, 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 30 min.
17.	Wash	Empty the wells as previously done, 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 of the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing 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 5-20 min, 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.
Read 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 4ºC.		

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 mouse splenocytes 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 number of Spots per well	Min.	Max.	CV%
100,000	12	462	406	495	5.5
50,000 Recommended	12	494	457	518	3.7
25,000 recommended	12	391	355	411	4.8
12,500	12	244	202	267	7.8
6,250	12	143	120	190	12.5

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