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Human IFN-γ / IL-5 Dual ELISPOT Set, with Plates

Catalog No.: CDK179A
CDK179B
CDK179C
CDK179D

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.

This Dual Color ELISpot kit allows you to analyze the production of two cytokines simultaneously in the same well. Recognizes both native and recombinant Interferon gamma (IFN-g) and Interleukin 5 (IL-5) and reacts with simian IFN-g.

2. REAGENTS PROVIDED

Part No.	Quantity				Preparation
CDK179-P. 96-well PVDF bottomed plates	5	10	15	20	Ethanol treatment
CDK179-A. Capture anti IFN-g, 500 µL vial	1	2	3	4	Sterile, dilute prior to use as per instructions
CDK179-B. Capture anti IL-5, 500 µL vial	1	2	3	4	
CDK179-C. FITC Detection anti IFN-g (lyophilized)	1	2	3	4	Reconstitute with 0.55 ml water prior to use as per instructions
CDK179-D. Biotin Detection anti IL-5 (lyophilized)	1	2	3	4	
CDK179-E. Anti-FITC HRP Conjugate	1	2	3	4	Dilute prior to use as per instructions
CDK179-F. Streptavidin-Alkaline Phosphatase Conjugate, 50 µL	1	2	3	4	
CDK179-G. Bovine Serum Albumin (BSA) - 2 g	1	2	3	4	Dissolve to prepare Dilution Buffer as per instructions
CDK179-H AEC Buffer A 10X 5 ml	1	2	3	4	Dilute prior to use as per instructions
CDK179-I AEC Buffer B 50X 1 ml	1	2	3	4	
CDK179-J. BCIP/NBT Substrate, 25 ml bottle	2	4	6	8	Ready to use

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65 Parker Street
Unit 11
Newburyport, MA 01950

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

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Web Site: www.cellsciences.com

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)
- 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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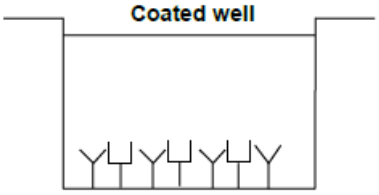
6. PRINCIPLE OF THE METHOD

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. Biotinylated and FITC detection antibodies are added which bind to the previously captured analyte. HRP-labelled anti-FITC antibodies and Streptavidin Alkaline Phosphatase are 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 appropriate analysis software or manually using a microscope.

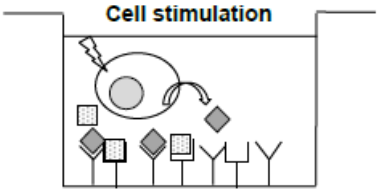
1. 96 PVDF-bottomed-well plates are first treated with 35% ethanol and then coated with anti-IFNγ and anti-IL-5 capture antibodies.

Figure 1



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

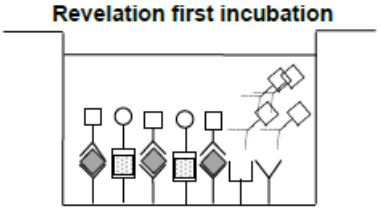
Figure 2



3. Cells are removed by washing.

Anti-IFNγ-FITC and anti-IL-5-biotin detection antibodies are added and bind to the captured cytokines

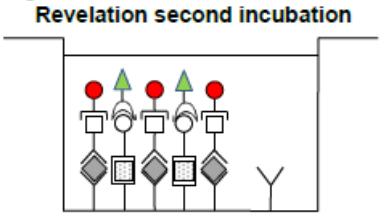
Figure 3

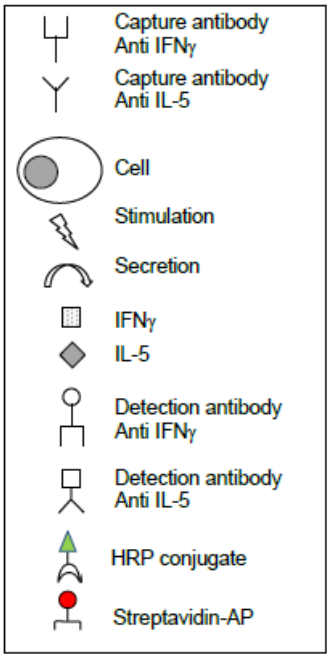


4. Any excess unbound detection antibodies is removed by washing.

Detection antibodies are in turn bound by anti-FITC-HRP for IFNγ and Streptavidin-AP for IL-5.

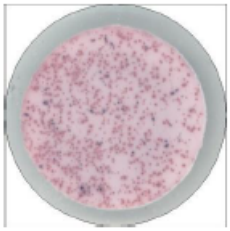
Figure 4





5. Any excess of AP and HRP conjugate is removed and wells are washed.

Finally coloured spots are developed by separate incubations with first AEC and then BCIP/NBT substrate buffers. Cells producing IFNγ give red/brownish spots while those producing IL-5 give blue/purple spots. Double producing cells corresponding to violet spots (preferably identified by a computerised overlay of blue and red spots).



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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 – Cell culture medium + 10% Serum

For one plate, add 1 ml of Serum (Fetal Calf Serum) to 9 ml of culture medium.

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 (CDK179-F) in PBS

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

7.6. CDK179-A + CDK179-B - Capture Antibodies

Dilute 100 µL of each capture antibody in the same tube containing 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 Antibodies dilution immediately before use.

7.7. CDK179-C + CDK179-D - Detection Antibodies

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 each detection antibodies in the same tube containing 10 mL of Dilution Buffer, mix well.
If not used within a short period of time, reconstituted Detection Antibodies 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. CDK179-E + CDK179-F - Streptavidin-Alkaline Phosphatase (AP) Conjugate + HRP Conjugate anti-FITC

Centrifuge vial for a few seconds to collect material in bottom of vial. For 1 plate, dilute 10 µL Streptavidin AP Conjugate and anti-FITC HRP Conjugate into the same tube containing 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 enzyme conjugates immediately prior to use. DO NOT KEEP THE DILUTIONS.*

7.9. CDK179-H + CDK179-I – AEC Substrate

For optimal performance, prepare the dilution immediately prior to use.
For one plate, dilute 1 ml of AEC buffer A 10X with 9 ml of distilled water. Then add 200 µl of AEC buffer B 50X.

7.10. CDK179-J - BCIP/NBT

The reagent is ready-to-use. It should be clear to pale yellow. If precipitates appear, filter with 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, IFN-gamma / IL-5 production

We recommend using the following polyclonal activation as a positive control in your assay:

Dilute PBMC in culture medium (e.g. RPMI 1640 supplemented with 2mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 ng/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 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.

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 mixture of **Capture Antibodies** 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 **Blocking Buffer** 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 mixture of **Detection Antibodies** 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 **HRP and AP conjugates** 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 **prepared AEC substrate** 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.
25.	Addition	Add 100 µL of **ready to use BCIP/NBT** buffer to every well.

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26.	Development	Incubate the plate for 5-15 minutes, monitoring spot formation visually throughout the incubation period to assess sufficient color development.
27.	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.

Reading Spots:

Allow the wells to dry and then read results. The frequency of the 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.

NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.

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