

Dual-Color ELISpot

Human IFN- γ /Granzyme B Kit

Catalog Number ELD5818

For the quantitative determination of the frequency of cells releasing human Interferon gamma (IFN- γ) and/or Granzyme B.

This package insert must be read in its entirety before using this product.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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INTRODUCTION

The Dual-Color Human IFN- γ /Granzyme B ELISpot assay is designed for the simultaneous detection of IFN- γ and Granzyme B secreting cells at the single cell level and can be used to simultaneously quantitate the frequency of human IFN- γ and Granzyme B secreting cells. ELISpot assays are well suited for monitoring immune responses to various treatments and therapies and have been used for the quantitation of antigen-specific CD4⁺ and/or CD8⁺ T cell responses. Other methods for assessment of antigen-specific T cell responses, such as chromium release assays with quantitation by limiting dilution, are tedious and require previous *in vitro* expansion of T cells for several days. These assays typically are not suitable for measuring infrequent T cell responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of T cells and are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in vaccine development and for the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (1, 2). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (3, 4). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique.

A monoclonal antibody specific for human IFN- γ and a polyclonal antibody specific for human Granzyme B have been pre-coated onto a PVDF (polyvinylidene difluoride)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37° C CO₂ incubator for a specified period of time. During this incubation period, the immobilized antibodies in the immediate vicinity of the secreting cells bind secreted IFN- γ and Granzyme B. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for Granzyme B and a horseradish peroxidase-conjugated polyclonal antibody specific for IFN- γ are added to the wells. Following a wash to remove any unbound antibodies, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. After washing the BCIP/NBT from the wells with tap water, an AEC chromogen solution is then added to the wells. A blue-black colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual Granzyme B secreting cell. A red precipitate also forms and appears as spots, with each red spot representing an individual IFN- γ secreting cell. The spots can be counted with an ELISpot reader system or using a stereomicroscope.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in pipetting and washing techniques, incubation time or temperature, or kit age can cause variation in density of spots, intensity of specific staining and background levels.

MATERIALS PROVIDED

Human IFN- γ /Granzyme B Microplate (Part 893792) - One 96-well PVDF-backed microplate coated with a monoclonal antibody specific for human IFN- γ and a polyclonal antibody specific for human Granzyme B.

Detection Antibody Concentrate A (Part 893002) - 150 μ L of a 120X concentrated solution of biotinylated polyclonal antibody specific for human Granzyme B with preservatives.

Detection Antibody Concentrate B (Part 893345) - 250 μ L of a 60X concentrated solution of horseradish peroxidase-conjugated polyclonal antibody specific for human IFN- γ with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 μ L of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a buffer for diluting Detection Antibody Concentrates with preservatives.

Dilution Buffer 2 (Part 895354) - 12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.

Wash Buffer Concentrate (Part 895308) - 50 mL of a 10X concentrated solution of a buffered surfactant with preservative.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Human IFN- γ Positive Control (Part 890893) - 2 ng of recombinant human IFN- γ ; lyophilized.

Human Granzyme B Positive Control (Part 893202) - 3 ng of recombinant human Granzyme B; lyophilized.

AEC Chromogen (Part 895922) - 300 μ L of 2% 3-Amino-9-Ethyl-Carbazole (AEC) in stabilizing buffer.

AEC Chromogen Buffer (Part 895923) - 12 mL of 0.1% H₂O₂ in Acetate Buffer for diluting AEC Chromogen.

Dual-Color ELISpot Schematic (Part 749057) - A full-color diagram of the assay principle.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond the kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO₂ incubator
- Sterile culture media
- Dissection microscope or an ELISpot reader capable of detecting blue and red spots

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Do not use reagents from this kit with components from other R&D Systems' ELISpot or ELISA kits and/or components manufactured by other vendors.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with AEC chromogen.

Although the toxicity of the chromogenic substrates BCIP/NBT and AEC is not currently known, wear gloves to avoid contact with skin. Follow local, state, and federal regulations to dispose of BCIP/NBT and AEC.

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of soft aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the AEC has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- After completion of the experiment, do not dry the microplate at a temperature higher than 37° C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment.
 - Positive Control - Use recombinant human IFN- γ and recombinant human Granzyme B in separate wells.
 - Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.
 - Background Control - Use sterile culture media.
 - Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

REAGENT PREPARATION

1X Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Positive Controls - Reconstitute the lyophilized human IFN- γ and human Granzyme B with 250 μ L of culture medium that is used to incubate cells.

Detection Antibody Mixture (Concentrate A + Concentrate B) - Tap or vortex each vial to release reagent collected in the cap. Transfer 100 μ L of Detection Antibody Concentrate A and 200 μ L of Detection Antibody Concentrate B into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare the Detection Antibody Mixture immediately before use.**

Streptavidin-AP - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 μ L of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare the Streptavidin-AP immediately before use.**

AEC Chromogen Solution - Transfer 250 μ L of AEC Chromogen to the vial labeled AEC Chromogen Buffer and mix well. **For optimal performance, prepare the AEC Chromogen immediately before use.**

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator. R&D Systems' cell selection products are suitable for the purification of effector and responder cells. For a complete product listing of human, mouse, and rat cell selection products, see the R&D Systems catalog or visit our website at www.RnDSystems.com/go/CellSelection.

ASSAY PROCEDURE

Bring all reagents to room temperature, except the Detection Antibody Concentrates and Dilution Buffer 1, which should remain at 2 - 8° C. All samples and controls should be assayed at least in duplicate. An Assay Record Template is provided at the back of this insert to record controls and samples assayed.

1. Fill all wells in the microplate with 200 μ L of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 μ L of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37° C CO₂ incubator. Optimal incubation time for each stimuli should be determined by the investigator. **Do not disturb the cells during the incubation period.**
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250 - 300 μ L) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels. **Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.**
5. Add 100 μ L of diluted Detection Antibody Mixture (A + B) into each well and incubate at 2 - 8° C overnight.
6. Repeat step 4.
7. Add 100 μ L of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat step 4.
9. Add 100 μ L of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light.**
10. Decant the BCIP/NBT chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water.
11. Add 100 μ L of AEC Chromogen/Buffer Solution into each well and incubate for 20 minutes at room temperature. **Protect from light.**
12. Decant the AEC Chromogen Solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels, and dry completely either at room temperature (60 - 90 minutes) or 37° C (15 - 30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots by using either a dissection microscope or an ELISpot reader capable of detecting blue and red spots. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

Peripheral blood mononuclear cells (2.5×10^5 cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μ g/mL calcium ionomycin overnight at 37° C in a 5% CO₂ incubator. The sample was assayed in eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Blue-Black (Granzyme B) Spots Counted	Number of Red (IFN- γ) Spots Counted
1	183	152
2	201	138
3	168	150
4	201	134
5	182	130
6	169	136
7	177	132
8	204	126

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT (or AEC) chromogen and rinsing the microplate with deionized water, the dark blue (or red) background color of filter membrane attenuates visualization and quantitation of spots.	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry, usually 15 - 30 minutes at 37° C or 60 - 90 minutes at room temperature.
The number of spots in the wells that contained the cells is high but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment - perhaps the result of using Streptavidin-AP, BCIP/NBT, and/or AEC solutions that have not been brought to room temperature	Warm the reagents to room temperature before adding to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue (or red).	Cell stimulation problem	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells added to the wells	Increase the number of cells added per well.
Following incubation with AEC and drying the microplate, the density of the spots makes it difficult to quantify them.	Too many cells were added to the wells	Make dilutions of cells (<i>i.e.</i> , 1×10^6 , 5×10^5 , 1×10^5 , 5×10^4 , 1×10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.

REFERENCES

1. Czerkinsky, C.C. *et al.* (1983) *J. Immunol. Methods* **65**:109.
2. Sedgwick, J.D. and P.G. Holt (1983) *J. Immunol. Methods* **57**:301.
3. Czerkinsky, C.C. *et al.* (1984) *J. Immunol. Methods* **72**:489.
4. Helms, T. *et al.* (2000) *J. Immunol.* **164**:3723.

ASSAY RECORD TEMPLATE

This template may be used as a record of samples and controls run in an assay.

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