

# Dual-Color FluoroSpot

## Human IFN- $\gamma$ /Granzyme B Kit

Catalog Number ELD5818NL

For the quantitative determination of the frequency of cells releasing human Interferon gamma (IFN- $\gamma$ ) 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- $\gamma$ /Granzyme B FluoroSpot assay is designed for the simultaneous detection of IFN- $\gamma$  and Granzyme B secreting cells at the single cell level and can be used to simultaneously quantitate the frequency of human IFN- $\gamma$  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<sup>+</sup> and/or CD8<sup>+</sup> 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.

The Dual-Color FluoroSpot assay provides a method for accurate detection of co-secreted cytokines by the same cell. Instead of enzyme conjugates as in an ELISpot assay, R&D Systems' novel Dual-Color FluoroSpot assay utilizes NothernLights™ fluorescent probes for the detection of secreted cytokines.

A monoclonal antibody specific for human IFN- $\gamma$  and a polyclonal antibody specific for human Granzyme B are coated onto a polyvinylidene difluoride (PVDF)-backed microplate. The coated microplate is then blocked and appropriately stimulated cells are pipetted into the wells. The microplate is then placed into a humidified 37°C CO<sub>2</sub> 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- $\gamma$  and Granzyme B. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for human Granzyme B is added to the wells. Following a wash to remove any unbound antibody, anti-IFN- $\gamma$  polyclonal antibody conjugated to a green fluorophore and streptavidin conjugated to a red fluorophore (for the detection of Granzyme B) are added to the well. Unbound fluorescent conjugates are subsequently removed by washing and a fluorescence enhancer is added. After incubation with this enhancer, it is removed from the wells, and the microplate is allowed to dry. Green fluorescent spots develop at the sites of IFN- $\gamma$  secreting cells, and red fluorescent spots appear at the sites of Granzyme B secreting cells. The spots can be visualized and counted using either a fluorescent ELISpot reader or using a conventional epifluorescence microscope.

## 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.
- Do not mix or substitute reagents with those from other lots or sources.
- 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.

## TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of 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 until after the NorthernLights Fluorescence Enhancer has been aspirated.
- Do not remove the flexible plastic under drain on the bottom of the microplate before or during incubation and development. It may damage the PVDF membrane filters. The under drain cover may be removed only after completing the incubation with NorthernLights Fluorescence Enhancer.
- To avoid damage to the membrane, do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents.
- Upon completing the experiment, do not dry the microplate at a temperature above 37°C. It may cause the PVDF membrane filters to crack.
- The 96-well microplate provided in this kit is not sterile. Due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been shown to be an issue with this FluoroSpot procedure.
- This kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the provided microplate and reagents.
- The controls listed are recommended for each FluoroSpot experiment:
  - Positive Control - Use recombinant human IFN- $\gamma$  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.

## PRECAUTION

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.

## MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8°C. Do not use past kit expiration date.

**Note:** Results obtained using previously opened or reconstituted reagents may not be reliable.

| PART  | PART # | DESCRIPTION  |
|---|--------|--|
| Microplate                                      | 607760 | 96-well PVDF-backed microplate used for fluorescent applications.  |
| Human Granzyme B Capture Antibody Concentrate   | 894264 | 150 µL of a 120X concentrated solution of polyclonal antibody specific for human Granzyme B.   |
| Human IFN-γ Capture Antibody Concentrate        | 894237 | 150 µL of a 120X concentrated solution of monoclonal antibody specific for human IFN-γ.  |
| Human Granzyme B Detection Antibody Concentrate | 894802 | 150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for human Granzyme B with preservatives.           |
| Human IFN-γ Detection Antibody Concentrate A488 | 894238 | 250 µL of a 60X concentrated solution of fluorescent green conjugated polyclonal antibody specific for human IFN-γ with preservatives. |
| Streptavidin-NL557                              | 967367 | 50 µL of Streptavidin conjugated to NorthernLights™ 557 with preservatives.  |
| Dilution Buffer 1                               | 895307 | 12 mL of a buffer for diluting Human Granzyme B Detection Antibody Concentrate with preservatives.                                     |
| Dilution Buffer 2                               | 895354 | 12 mL of a buffer for diluting Human IFN-γ Detection Antibody NL493 and Streptavidin-NL557 with preservatives.                         |
| Wash Buffer Concentrate                         | 895308 | 50 mL of a 10X concentrated solution of a buffered surfactant with preservative.   |
| NorthernLights™ Fluorescence Enhancer           | 894236 | 12 mL of a solution to enhance green NorthernLights™ fluorescence.   |
| Human Granzyme B Positive Control               | 893202 | 3 ng of recombinant human Granzyme B; lyophilized with preservatives.  |
| Human IFN-γ Positive Control                    | 890893 | 4 ng of recombinant human IFN-γ; lyophilized with preservatives.   |
| Dual-Color FluoroSpot Schematic                 | 749061 | A full-color diagram of the assay principle.   |

## OTHER SUPPLIES REQUIRED

- ELISpot reader capable of detecting fluorescence or a conventional epifluorescence microscope.
- Pipettes and pipette tips.
- 35% ethanol or methanol.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2-7.4, 0.2 µm filtered).
- Block Buffer (1% BSA and 5% sucrose in PBS).
- 500 mL graduated cylinder.
- 37°C CO<sub>2</sub> incubator.
- Sterile culture media.

## 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, visit [www.RnDSystems.com/go/CellSelection](http://www.RnDSystems.com/go/CellSelection).

## REAGENT PREPARATION

**Bring all reagents to room temperature before use.**

**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 or distilled water and mix well.

**Positive Controls** - Reconstitute the lyophilized human Granzyme B and human IFN- $\gamma$  with 250  $\mu$ L of culture medium that is used to incubate cells.

**Capture Antibody Mixture (Human Granzyme B + Human IFN- $\gamma$ )** - Tap or vortex each vial to release reagent collected in the cap. Transfer 100  $\mu$ L of Human Granzyme B Capture Antibody Concentrate and 100  $\mu$ L of Human IFN- $\gamma$  Capture Antibody Concentrate into 12 mL of PBS and mix well. **For optimal performance, prepare the Capture Antibody Mixture immediately before use.**

**Human Granzyme B Detection Antibody** - Tap or vortex the vial to release reagent collected in the cap. Transfer 100  $\mu$ L of Human Granzyme B Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare the Human Granzyme B Detection Antibody immediately before use.**

**Human IFN- $\gamma$  Detection Antibody A488 + Streptavidin-NL557 Mixture** - Tap or vortex each vial to release reagent collected in the cap. Transfer 200  $\mu$ L of Human IFN- $\gamma$  Detection Antibody Concentrate A488 and 24  $\mu$ L of Streptavidin-NL557 into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare the Human IFN- $\gamma$  Detection Antibody A488 + Streptavidin-NL557 Mixture immediately before use.**

## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature, except the Antibody Concentrates and Dilution Buffer 1, which should remain at 2-8°C. All samples and controls should be assayed at least in duplicate.**

1. Prepare the membranes by adding 15  $\mu\text{L}$  of 35% alcohol to each well. Incubate for 1 minute.
2. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with deionized or distilled water (250-300  $\mu\text{L}$ ) 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 liquid by aspirating or decanting. Invert the plate and blot it against clean paper towels. **Note:** *Adjust the height of the manifold dispenser or autowasher to prevent damage to the membranes.*
3. Add 100  $\mu\text{L}$  of diluted Capture Antibody Mixture into each well, and incubate overnight at 2-8°C.
4. Aspirate each well and wash **with 1X PBS** (as in step 2), repeating the process three times for a total of four washes.
5. Fill each well of the microplate with 200  $\mu\text{L}$  Block Buffer, and incubate the microplate for approximately 90 minutes at room temperature.
6. Aspirate the Block Buffer, and fill each well of the microplate with 200  $\mu\text{L}$  of sterile culture media. Incubate for approximately 20 minutes at room temperature.
7. When the cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100  $\mu\text{L}$  of the appropriate cells or controls to each well. Refer to the Technical Hints section for appropriate controls. A plate layout is provided for a record of controls and samples assayed.
8. Incubate the cells in a humidified 37°C CO<sub>2</sub> incubator. Optimal incubation time for each stimulus should be determined by the investigator. **Do not disturb the cells during the incubation period.**
9. Aspirate each well and wash **with Wash Buffer** (as in step 2), repeating the process three times for a total of four washes.
10. Add 100  $\mu\text{L}$  of diluted Human Granzyme B Detection Antibody to each well, and incubate overnight at 2-8°C.
11. Repeat the wash described in step 9.
12. Add 100  $\mu\text{L}$  of diluted Human IFN- $\gamma$  Detection Antibody A488 + Streptavidin-NL557 Mixture to each well, and incubate for 2 hours at room temperature. **Protect from light.**
13. Repeat the wash described in step 9 **using 1X PBS instead of Wash Buffer.**
14. Add 100  $\mu\text{L}$  of NorthernLights Fluorescence Enhancer Solution to each well, and incubate for 15 minutes at room temperature. **Protect from light.**
15. Aspirate the NorthernLights Fluorescence Enhancer Solution. Remove the under drain from the back of the microplate, and rinse with deionized or distilled water. **Do not rinse the wells.** Let the microplate dry completely before analyzing. **Protect from light until analysis.**

## CALCULATION OF RESULTS

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

## REPRODUCIBILITY DATA

Peripheral blood mononuclear cells ( $5 \times 10^5$  cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5  $\mu$ g/mL of calcium ionomycin overnight at 37°C in a 5% CO<sub>2</sub> incubator. The sample was assayed in five wells according to the procedure and analyzed with a dissection microscope.

| Well | Number of Green (IFN- $\gamma$ ) Spots Counted | Number of Red (Granzyme B) Spots Counted |
|------|--|--|
| 1    | 88   | 288                                      |
| 2    | 116  | 272                                      |
| 3    | 88   | 266                                      |
| 4    | 84   | 264                                      |
| 5    | 92   | 264                                      |



## TROUBLESHOOTING GUIDE

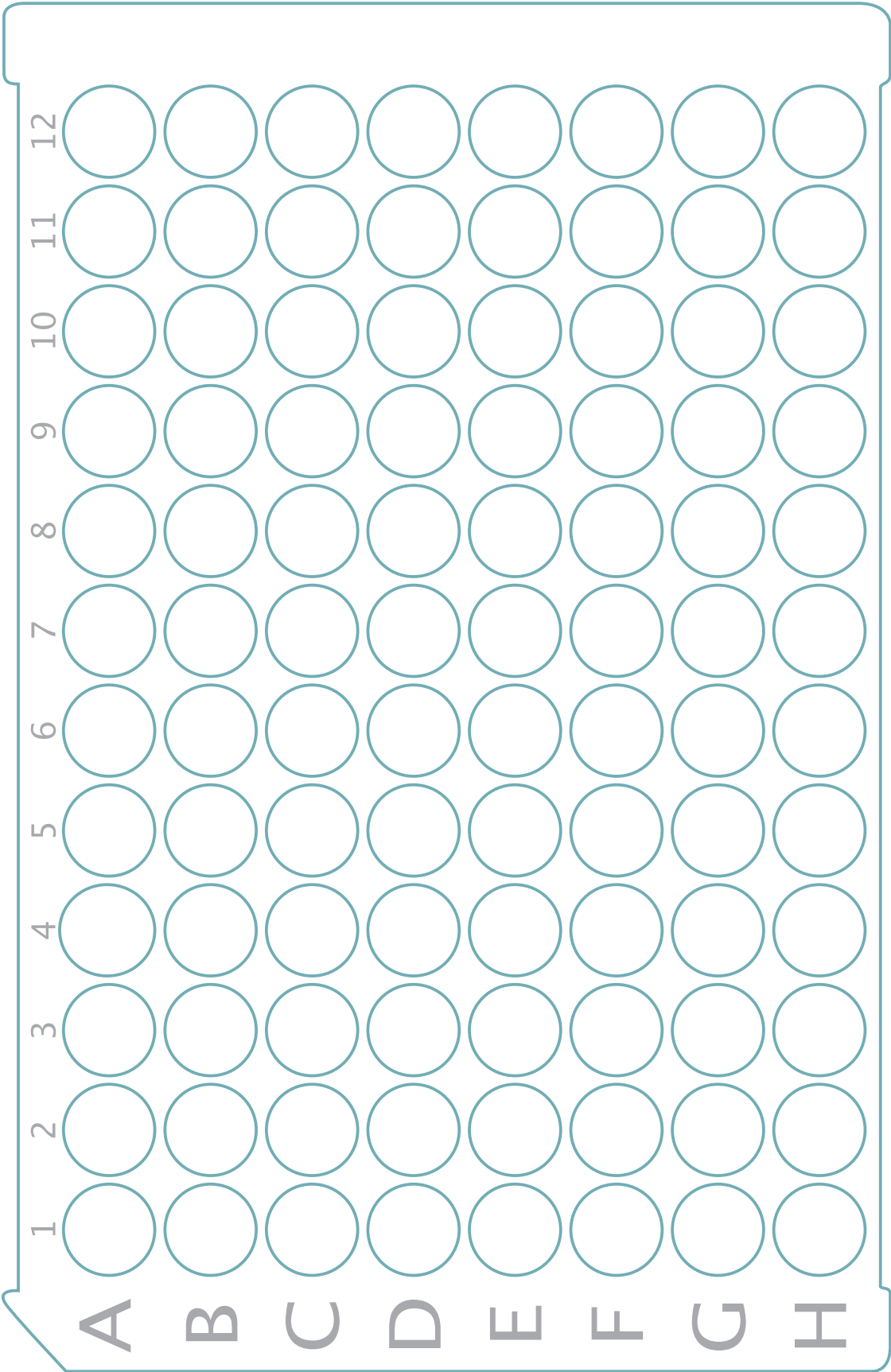
| Observation  | Problem   | Corrective Action   |
|--|---|---|
| Following the incubation with NorthernLights™ Fluorescence Enhancer and rinsing the back of the microplate with deionized or distilled water, the green (or red) background color of the filter membrane attenuates visualization and quantification of spots. | The membrane is wet.  | Microplates cannot be analyzed accurately until the PVDF filter membranes are completely dry. Wait until the membrane becomes dry (typically 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-NL557 and/or NorthernLights™ Fluorescence Enhancer solutions that have not been brought to room temperature. | Warm the appropriate reagents to room temperature before adding them to the wells.  |
| The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned green (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 immunohistochemistry on fixed cells after stimulation.                        |
|  | Too few cells were added to the wells.  | Increase the number of cells added per well.  |
| Following incubation with NorthernLights™ Fluorescence Enhancer and drying the microplate, the density of the spots makes it difficult to quantify.  | Too many cells were added to the wells.   | Make dilutions of cells ( $1 \times 10^6$ , $5 \times 10^5$ , $1 \times 10^5$ , $5 \times 10^4$ , $1 \times 10^4$ 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.

**PLATE LAYOUT**

Use this plate layout to record controls and samples assayed.



**NOTES**

**NOTES**