Dual-Color FluoroSpot

Mouse IFN-γ/IL-17 Kit

Catalog Number ELD5007NL

For the quantitative determination of the frequency of cells releasing mouse Interferon gamma (IFN-γ) and/or mouse Interleukin 17 (IL-17).

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 Mouse IFN-γ/IL-17 FluoroSpot assay is designed for the simultaneous detection of mouse IFN-γ and mouse IL-17 secreting cells at the single cell level and can be used to simultaneously quantitate the frequency of mouse IFN-γ and IL-17 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 quantitive 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, the R&D Systems®' novel Dual-Color FluoroSpot assay utilizes NothernLights™ fluorescent probes for the detection of secreted cytokines.

A monoclonal antibody specific for mouse IFN-γ and a monoclonal antibody specific for mouse IL-17 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_2 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 IL-17. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IFN-γ is added to the wells. Following a wash to remove any unbound antibody, anti-IL-17 polyclonal antibody conjugated to a green fluorophore and streptavidin conjugated to a red fluorophore (for the detection of IFN-γ) 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 IL-17 secreting cells, and red fluorescent spots appear at the sites of IFN-γ 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 the 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 Fluorescence Enhancer has been aspirated.
- Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development. It may damage the PVDF membrane filter. The underdrain cover may be removed only after completing the incubation with the 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 mouse IFN-γ and recombinant mouse IL-17 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.

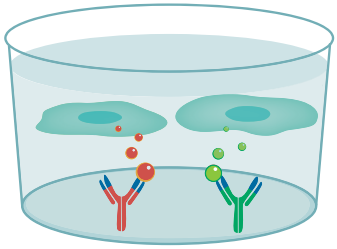
PRECAUTIONS

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.

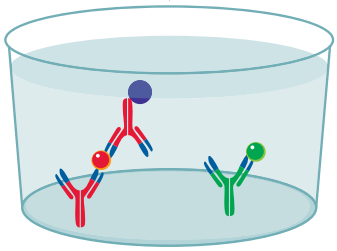
Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

ASSAY PRINCIPLE



Two different proteins secreted by cultured cells bind to the capture antibodies coated on the well.

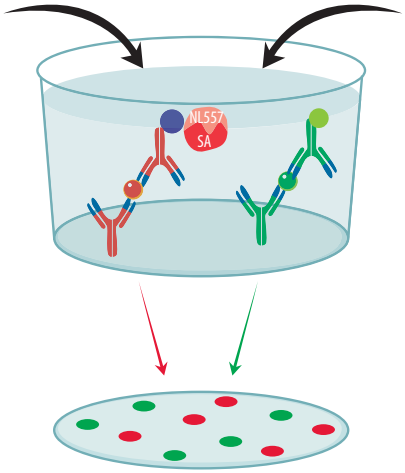


Remove cells by washing.
Add the biotinylated detection antibody which binds to the FIRST captured protein.

Remove the unbound biotinylated detection antibody by washing.

Add the Streptavidin conjugated to NL557 fluorescent tag (red).

Add the detection antibody conjugated to A488 fluorescent tag (green) which binds to the SECOND captured protein.



After incubation with the NorthernLights™ fluorescent tags, remove the unbound fluorescent tags by washing and add the fluorescent enhancer detection mixture. After incubation with this enhancer, aspirate the solution and allow microplate to dry. Analyze using either an ELISpot reader capable of detecting green and red fluoresent spots or a conventional epifluorescence microscope.

Red fluorescent spots represent the FIRST captured protein and green fluorescent spots represent the SECOND captured protein.

KEY

- FIRST secreted protein
- SECOND secreted protein
- Capture antibody against the FIRST secreted protein
- Capture antibody against the SECOND secreted protein
- Biotin-conjugated detection antibody against the FIRST protein
- A488-conjugated detection antibody against the SECOND protein
- Streptavidin conjugated to NL557 fluorescent tag

MATERIALS PROVIDED & STORAGE CONDITIONS

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

Note: *This kit is validated for single use only. 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.
Mouse IL-17 Capture Antibody Concentrate	894278	150 µL of a 120X concentrated solution of a monoclonal antibody specific for mouse IL-17.
Mouse IFN-γ Capture Antibody Concentrate	894279	150 µL of a 120X concentrated solution of a monoclonal antibody specific for mouse IFN-γ.
Mouse IFN-γ Detection Antibody Concentrate	894799	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IFN-γ with preservatives.
Mouse IL-17 Detection Antibody Concentrate A488	894280	250 µL of a 60X concentrated solution of fluorescent green conjugated polyclonal antibody specific for mouse IL-17 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 Mouse IFN-γ Detection Antibody Concentrate with preservatives.
Dilution Buffer 2	895354	12 mL of a buffer for diluting Mouse IL-17 Detection Antibody A488 and Streptavidin-NL557 with preservatives.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative.
Fluorescence Enhancer	894236	12 mL of a solution to enhance green NorthernLights™ fluorescence.
Mouse IL-17 Positive Control	892732	3 ng of recombinant mouse IL-17 with preservatives; lyophilized.
Mouse IFN-γ Positive Control	890896	8 ng of recombinant mouse IFN-γ with preservatives; lyophilized.

OTHER SUPPLIES REQUIRED

- ELISpot reader capable of detecting fluorescence or a conventional epifluorescence microscope.
- Pipettes and pipette tips.
- 35% Ethanol or Methanol.
- Deionized water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 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_2 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.

REAGENT PREPARATION

Bring all reagents to room temperature, except the detection antibody concentrates and Dilution Buffer 1, which should remain at 2-8 °C.

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 Mouse IL-17 Positive Control and Mouse IFN-γ Positive Control with 250 µL of culture medium that is used to incubate cells.

Capture Antibody Mixture (Mouse IL-17 + Mouse IFN-γ) - Tap or vortex each vial to release reagent collected in the cap. Transfer 100 µL of Mouse IL-17 Capture Antibody Concentrate and 100 µL of Mouse IFN-γ Capture Antibody Concentrate into 12 mL of PBS and mix well. **For optimal performance, prepare the Capture Antibody Mixture immediately before use.**

Mouse IFN-γ Detection Antibody - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Mouse IFN-γ Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare the Mouse IFN-γ Detection Antibody immediately before use.**

Mouse IL-17 Detection Antibody A488 + Streptavidin-NL557 Mixture - Tap or vortex each vial to release reagent collected in the cap. Transfer 200 µL of Mouse IL-17 Detection Antibody Concentrate A488 and 24 µL of Streptavidin-NL557 into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare the Mouse IL-17 Detection Antibody A488 + Streptavidin-NL557 Mixture immediately before use.**

ASSAY PROCEDURE

Bring all reagents to room temperature, except the Capture Antibody Mixture, Mouse IFN-γ Detection Antibody, and Mouse IL-17 Detection Antibody A488 + Streptavidin-NL557 Mixture, which should remain at 2-8 °C. All samples and controls should be assayed at least in duplicate. A plate layout is provided to record controls and samples assayed.

1. Prepare the membranes by adding 15 µL 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 µL) 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 µL of the 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 µL of 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 µL 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 µL of the appropriate cells or controls to each well. Refer to the Technical Hints section for appropriate controls.
8. Incubate the cells in a humidified 37 °C CO_2 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 µL of the diluted Mouse IFN-γ Detection Antibody to each well, and incubate overnight at 2-8 °C.
11. Repeat the wash procedure described in step 9.
12. Add 100 µL of the diluted Mouse IL-17 Detection Antibody A488 + Streptavidin-NL557 Mixture to each well, and incubate for 2 hours at room temperature. **Protect from light.**
13. Repeat the wash procedure described in step 9 **using 1X PBS instead of Wash Buffer.**
14. Add 100 µL of the Fluorescence Enhancer Solution to each well, and incubate for 15 minutes at room temperature. **Protect from light.**
15. Aspirate the Fluorescence Enhancer Solution. Remove the underdrain from the back of the microplate, and rinse with deionized 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

Mouse splenocytes (1 x 10^6 cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 µg/mL of calcium ionomycin overnight at 37 °C in a 5% CO_2 incubator. The sample was assayed in eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Green (IL-17) Spots Counted	Number of Red (IFN-γ) Spots Counted
1	45	334
2	38	347
3	36	349
4	50	379
5	48	363
6	46	339
7	47	357
8	44	359

TROUBLESHOOTING GUIDE

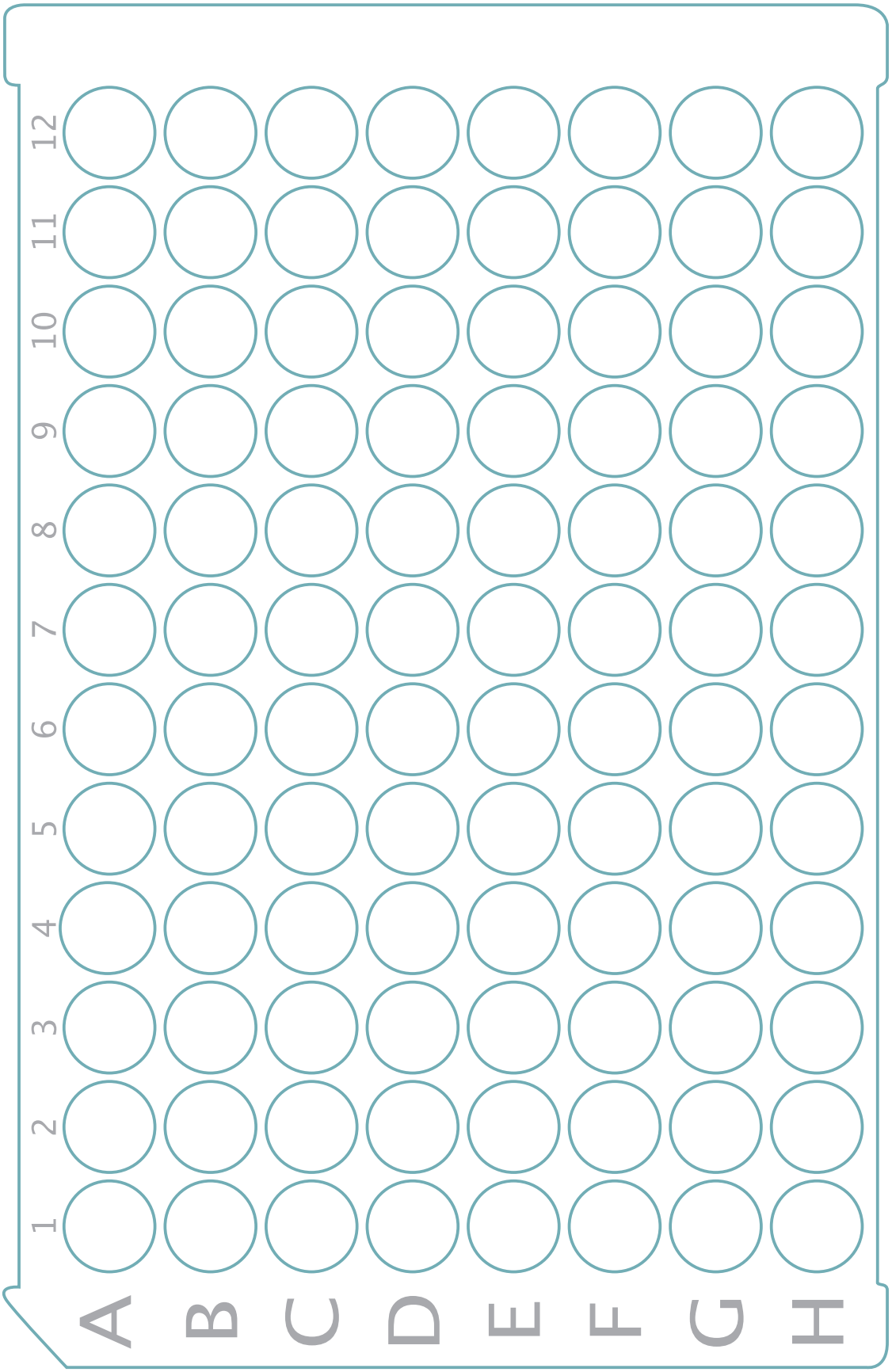
Observation	Problem	Corrective Action
Following the incubation with the Fluorescence Enhancer and rinsing the back of the microplate with deionized water, the green (or red) background color of the filter membrane attenuates visualization and quantification of spots.	The membrane is wet.	Microplates 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 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 the 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 x 10^6, 5 x 10^5, 1 x 10^5, 5 x 10^4, 1 x 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.



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