Dual-Color B Cell FluoroSpot

Mouse IgG/IgM Kit

Catalog Number ELDB8080NL

For the quantitative determination of the frequency of cells releasing mouse Immunoglobulin G (IgG) and/or Immunoglobulin M (IgM).

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 B Cell Mouse IgG/IgM FluoroSpot assay is designed for the simultaneous detection of antigen-specific IgG and IgM secreting cells at the single cell level and can be used to simultaneously quantitate the frequency of human IgG and IgM 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 B cell responses. Other methods for assessment of antigen-specific B cell responses may require previous *in vitro* expansion and subcloning of B cells. These assays can be tedious and time consuming and typically are not suitable for measuring infrequent B 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 B cells and are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research antigen recognition in vaccine development and for the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay is a sandwich immunoassay that was originally developed for the detection of individual B cells secreting antigen-specific antibodies (1, 2). The B Cell Dual-Color FluoroSpot assay provides a method for accurate simultaneous enumeration of B cells which secrete two immunoglobulin molecules: IgG and IgM (3,4). Instead of using enzyme conjugates and chromogenic substrates the novel B Cell Dual-Color FluoroSpot assay utilizes NorthernLights™ fluorescent probes for the detection of secreted antibodies. It can be used for the simultaneous detection of IgG and IgM from B cells secreting either antigen-specific antibodies or total IgG/IgM proteins (5).

Antigen Specific antibodies (Antigen-Down Assay Principle): Antigen of interest is coated onto a polyvinylidene difluoride (PVDF)-backed microplate.

Total IgG/IgM (Sandwich Assay Principle): A polyclonal antibody specific for mouse IgG and a polyclonal antibody specific for mouse IgM 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 IgG and IgM. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IgM is added to the wells. Following a wash to remove any unbound antibody, anti-IgG polyclonal antibody conjugated to a green fluorophore and streptavidin conjugated to a red fluorophore (for the detection of IgM) 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 IgG secreting cells, and red fluorescent spots appear at the sites of IgM 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 for the rest of the experimental procedure and removed after the final wash.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- Upon completing the experiment, do not dry the microplate at a temperature above 37 °C. It may cause the PVDF membrane filters to crack.
- 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 ELISpot experiment:

  Positive Control - Use capture and detection antibodies provided to get "total" IgG and IgM 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.

ANTIGEN-DOWN ASSAY PRINCIPLE

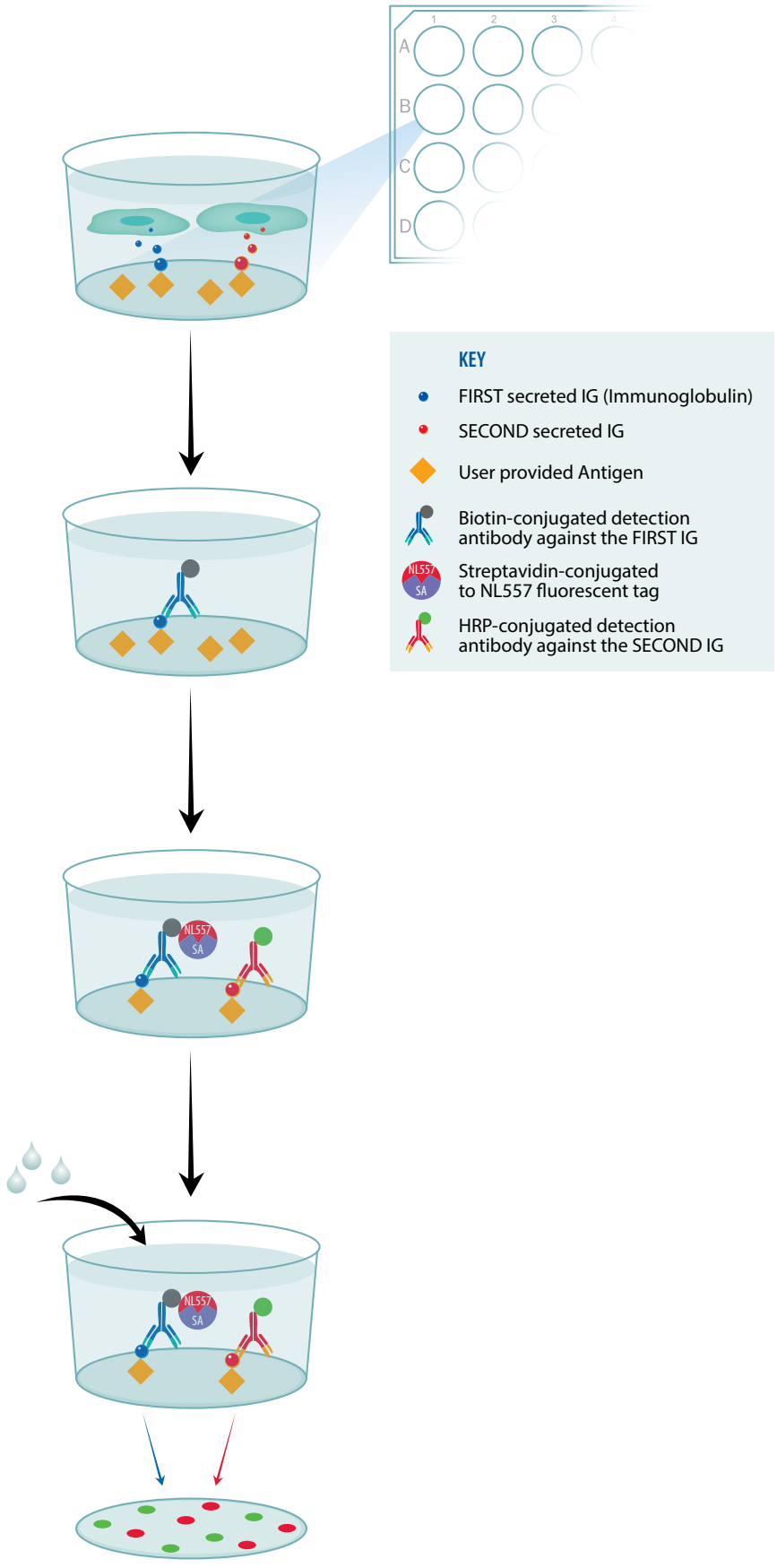
Coat a PVDF-backed microplate with antigen provided by user.

Add the stimulated cells into the wells.
Incubate the plate at 37 °C and 5% CO_2.

Wash away any cells and unbound substances.
Add a biotinylated detection antibody specific for the first immunoglobulin.

Wash the plate.
Add NL557-conjugated streptavidin and the NL493-conjugated detection antibody specific for the second immunoglobulin.

Wash the plate.
Add NorthernLights Fluorescence Enhancer.
Aspirate the enhancer solution and dry the plate.
Analyze the spots using a conventional epifluorescence microscope or an automated ELISpot reader.



SANDWICH ASSAY PRINCIPLE

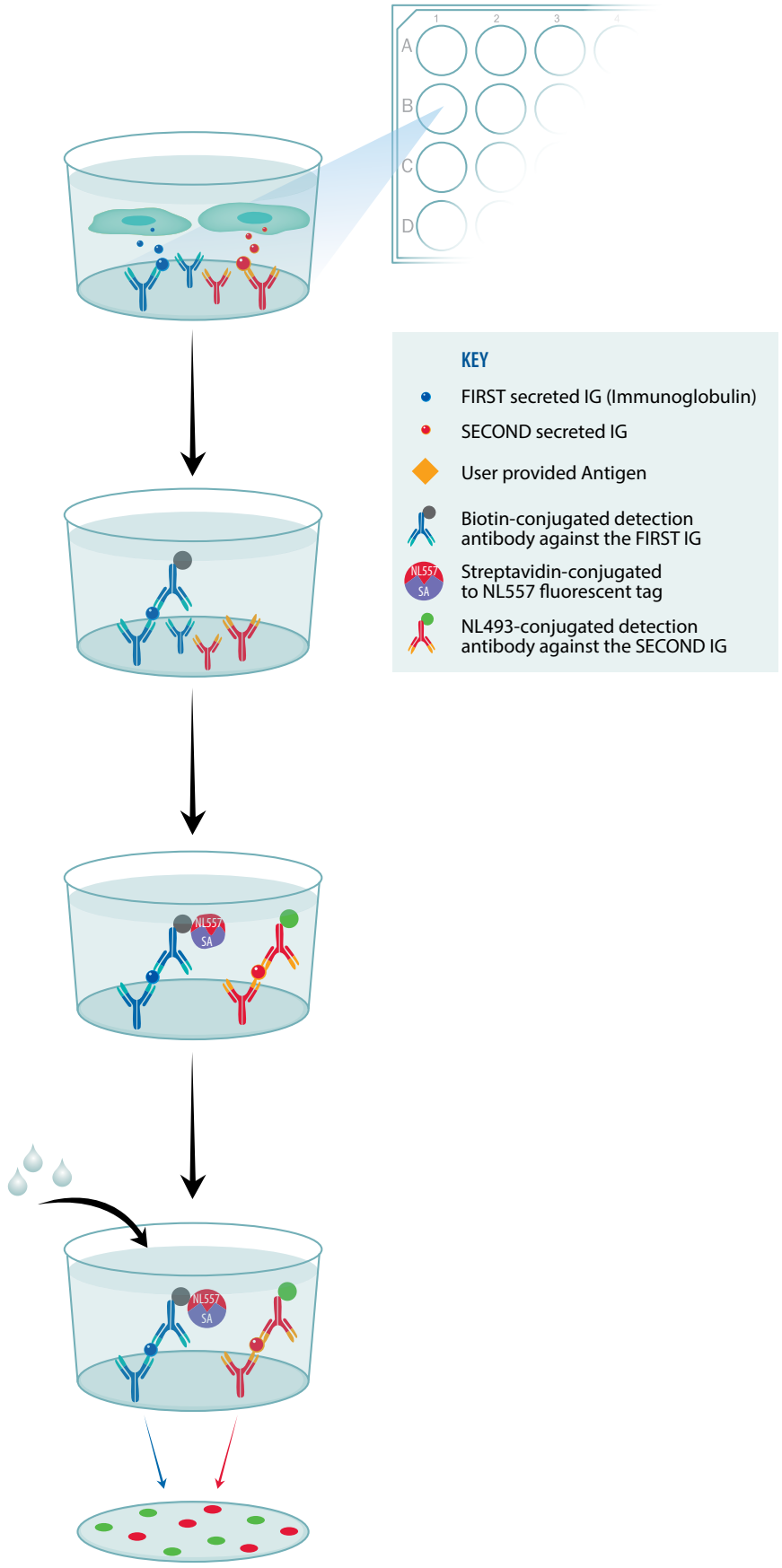
Coat a PVDF-backed microplate with antibodies specific for the chosen immunoglobulins.

Add the stimulated cells into the wells.
Incubate the plate at 37 °C and 5% CO_2.

Wash away any cells and unbound substances.
Add a biotinylated polyclonal detection antibody specific for the first immunoglobulin.

Wash the plate.
Add NL557-conjugated streptavidin and the NL493-conjugated detection antibody specific for the second immunoglobulin.

Wash the plate.
Add NorthernLights Fluorescence Enhancer.
Aspirate the enhancer solution and dry the plate.
Analyze the spots using a conventional epifluorescence microscope or an automated ELISpot reader.



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.
Mouse IgG Capture Antibody Concentrate	894062	150 µL of a 120X concentrated solution of monoclonal antibody specific for mouse IgG.
Mouse IgM Capture Antibody Concentrate	894063	150 µL of a 120X concentrated solution of polyclonal antibody specific for mouse IgM.
Mouse IgG Detection Antibody Concentrate NL493	894708	250 µL of a 60X concentrated solution of fluorescent green conjugated polyclonal antibody specific for mouse IgG with preservatives.
Mouse IgM Detection Antibody Concentrate	894064	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IgM with preservatives.
Streptavidin-NL557 Concentrate	967367	50 µL of Streptavidin conjugated to NorthernLights 557 with preservatives.
Dilution Buffer 1	895307	12 mL of a buffer for diluting detection antibody concentrates with preservatives.
Dilution Buffer 2	895354	12 mL of a buffer for diluting Streptavidin-NL557 Concentrate with preservatives.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative. *May turn yellow over time.*
NorthernLights Fluorescence Enhancer	894236	12 mL of a solution to enhance green NorthernLights fluorescence.

OTHER SUPPLIES REQUIRED

- ELISpot reader capable of detecting fluorescence or a conventional epifluorescence microscope.
- PBS (137 mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.2-7.4, 0.2 µm filtered)
- Block Buffer (1% BSA, 5% Sucrose in PBS)
- Pipettes and pipette tips.
- 35% ethanol or methanol
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- 37 °C CO_2 incubator.
- Sterile culture media.

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 ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to 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.

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 1X Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized or distilled water and mix well.

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

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

Mouse IgG Detection Antibody NL493 + Streptavidin-NL557 Mixture - Tap or vortex each vial to release reagent collected in the cap. Transfer 200 µL of Mouse IgG Detection Antibody Concentrate NL493 and 24 µL of Streptavidin-NL557 into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare the Mouse IgG Detection Antibody NL493 + 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 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. **For Detection of antigen-specific IgG/IgM producing B cells (Antigen-Down Assay Principle):** Dilute antigen (5-15 µg/mL) in PBS and add 100 µL to each well. Incubate at 2-8 °C overnight. **For Detection of total IgG/IgM producing B cells (Sandwich Assay Principle):** Add 100 µL of Capture Antibody Mixture (A + B) into each well and incubate at 2-8 °C overnight.
4. Aspirate each well and wash as in step 2 using **PBS**, repeating the process three times for a total of four washes.
5. Fill each well of the microplate with 200 µL Block Buffer, and incubate the microplate for 2 hours at room temperature.
6. Aspirate the Block Buffer, and fill each well of the microplate with 200 µL of sterile culture media. Incubate for 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 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_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 as in step 2 using **1X Wash Buffer**, repeating the process three times for a total of four washes.
10. Add 100 µL of diluted Mouse IgM Detection Antibody to each well, and incubate overnight at 2-8 °C.
11. Repeat the wash described in step 9.
12. Add 100 µL of Mouse IgG Detection Antibody NL493 + 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 PBS instead of 1X Wash Buffer.**
14. Add 100 µL of NorthernLights Fluorescence Enhancer to each well, and incubate for 15 minutes at room temperature. Protect from light.
15. Aspirate the NorthernLights Fluorescence Enhancer. 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

Mouse Splenocytes (2 x 10^5 cells/mL) were stimulated with 1 µg/mL Lipopolysaccharide (LPS) and 1 µg/mL of Pokeweed mitogen 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. The numbers below reflect detection of total IgG/IgM producing B cells.

Well	Number of Green (IgG) Spots Counted	Number of Red (IgM) Spots Counted
1	36	32
2	33	36
3	29	26
4	32	31
5	43	34
6	47	42
7	34	23
8	42	43

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 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 immunoglobulin release from the cells retained their biological activity. One way to check is to perform immunocytochemistry 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 (1×10^6, 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

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3. Jahnmatz, M. *et al.* (2013) J. Immunol. Methods **391**:50.
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5. Walsh PN. *et al.* (2013) J. Immunol. Methods **394**:84.

PLATE LAYOUT

Use this plate layout to record controls and samples assayed.