

# Dual-Color B Cell ELISpot

## Mouse IgG/IgM Kit

Catalog Number ELDB8080

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 ELISpot 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 ELISpot assay provides a method for accurate simultaneous enumeration of B cells which secrete two immunoglobulin molecules: IgG and IgM (3,4). This assay uses enzyme conjugates and chromogenic substrates 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<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 IgG and IgM. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IgM and a horseradish peroxidase-conjugated polyclonal antibody specific for IgG 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 deionized water, an AEC chromogen solution is added to the wells. A blue-black colored precipitate forms and appears as spots at the sites of antigen localization, with each individual spot representing an individual IgM secreting cell. A red precipitate also forms and appears as spots, with each red spot representing an individual IgG 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.
- 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 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.
- 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** PVDF-backed microplate with antigen provided by user.

**Add** the stimulated cells into the wells.

**Incubate** in a humidified 37 °C CO<sub>2</sub> incubator.

**Wash** away any cells and unbound substances.

**Add** a biotinylated polyclonal antibody specific for the first immunoglobulin and an HRP-conjugated polyclonal antibody specific for the second immunoglobulin to the wells.

**Wash** away any unbound biotinylated antibodies.

**Add** alkaline phosphatase-conjugated streptavidin.

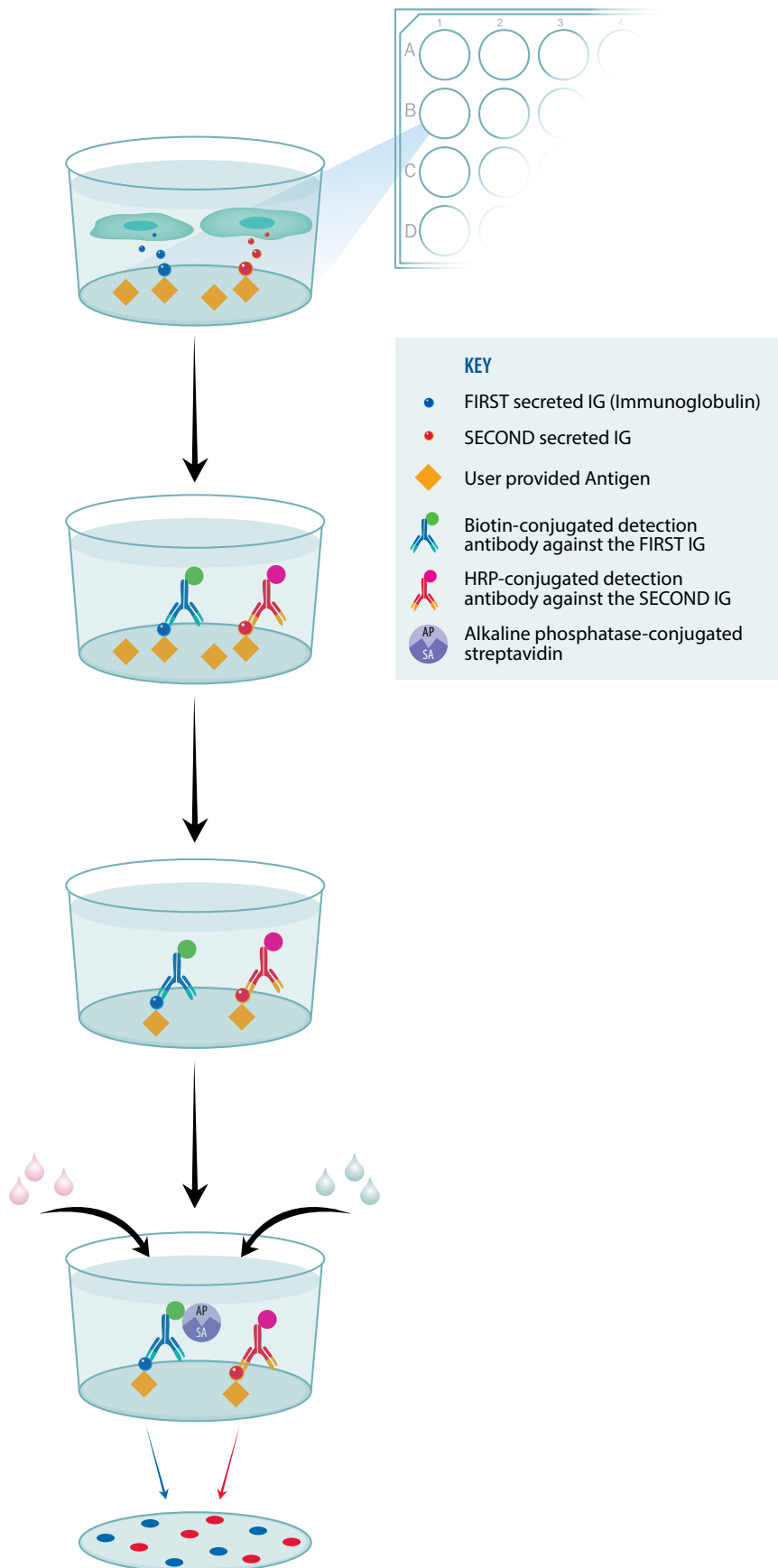
**Wash** away any unbound enzyme.

**Add** the BCIP/NBT chromogen.

**Incubate** until a blue-black precipitate forms.

**Add** the AEC chromogen.

**Identify** and count the spots with an automated ELISpot reader or manually with a stereomicroscope.



## SANDWICH ASSAY PRINCIPLE

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

**Add** the stimulated cells into the wells.

**Incubate** in a humidified 37 °C CO<sub>2</sub> incubator.

**Wash** away any cells and unbound substances.

**Add** a biotinylated polyclonal detection antibody specific for the first immunoglobulin and an HRP-conjugated polyclonal antibody specific for the second immunoglobulin to the wells.

**Wash** away any unbound biotinylated antibodies.

**Add** alkaline phosphatase-conjugated streptavidin.

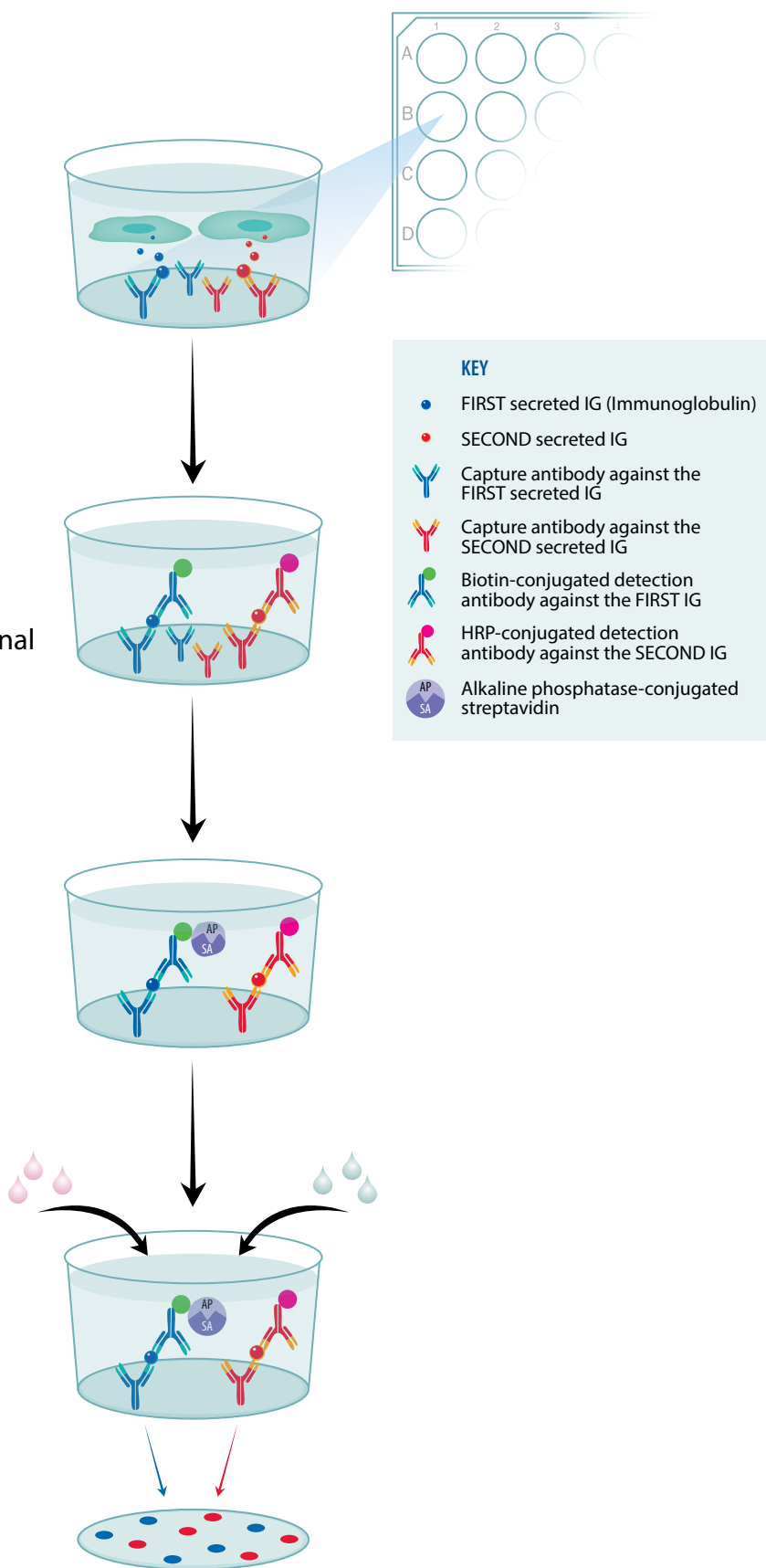
**Wash** away any unbound enzyme.

**Add** the BCIP/NBT chromogen.

**Incubate** until a blue-black precipitate forms.

**Add** the AEC chromogen.

**Identify** and count the spots with an automated ELISpot reader or manually with a stereomicroscope.



## 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	607856	96-well PVDF-backed microplate to be coated with provided capture antibodies or customer provided antigen of interest.
Mouse IgM Detection Antibody Concentrate A	894707	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IgM with preservatives.
Mouse IgG Detection Antibody Concentrate B	894706	250 µL of a 60X concentrated solution of horseradish peroxidase-conjugated polyclonal antibody specific for mouse IgG with preservatives.
Streptavidin-AP Concentrate A	895358	150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.
Mouse IgM Capture Antibody Concentrate A	894705	150 µL of a 120X concentrated solution of polyclonal antibody specific for mouse IgM.
Mouse IgG Capture Antibody Concentrate B	894704	150 µL of a 120X concentrated solution of polyclonal antibody specific for mouse IgG.
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-AP Concentrate A with preservatives.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative. <i>May turn yellow over time.</i>
BCIP/NBT Chromogen	895867	12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).
AEC Chromogen	895922	300 µL of 3-Amino-9-Ethyl-Carbazole (AEC) in stabilizing buffer.
AEC Chromogen Buffer	895923	12 mL of a buffer for diluting AEC Chromogen.

## OTHER SUPPLIES REQUIRED

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

## 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.

AEC Chromogen may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

BCIP/NBT is toxic if swallowed, in contact with skin, or if inhaled. It is a highly flammable liquid and vapor may cause serious irritation and damage to organs. Do not eat, drink, or smoke when using this product. Do not breathe fumes. Use only in a well-ventilated area. Keep away from heat, sparks, open flames, and hot surfaces. Keep the container tightly closed.

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

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



## 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.**

**1. For Detection of antigen-specific IgG/IgM producing B cells (Antigen-Down Assay Principle):**

Dilute the antigen (5-15 µg/mL) in 1X 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 the diluted Capture Antibody Mixture (A + B) into each well and incubate at 2-8 °C overnight.

2. Aspirate each well and wash, repeating the process once for a total of two washes. Wash by filling each well with PBS (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 PBS 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.*

3. Fill all wells in the microplate with 200 µL of Block Buffer and incubate for 2 hours at room temperature.

4. Aspirate each well. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for 20 minutes at room temperature.

5. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells to each well (see Technical Hints for appropriate controls).

6. Incubate 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.**

7. Aspirate each well and wash as in step 2, using 1X Wash Buffer, repeating the process three times for a total of four washes.

8. Add 100 µL of diluted Detection Antibody Mixture (A + B) into each well and incubate at 2-8 °C overnight.

9. Aspirate/wash as in step 7.

10. Add 100 µL of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.

11. Aspirate/wash as in step 7.

12. Add 100 µL of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light.**

13. Discard the BCIP/NBT Chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water.

14. Add 100 µL of the prepared AEC Chromogen solution into each well and incubate for 20 minutes at room temperature. **Protect from light.**

15. 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 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. 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 ( $5 \times 10^4$  cells/mL) were stimulated with 1  $\mu$ g/mL of Lipopolysaccharide (LPS) and 1  $\mu$ g/mL Pokeweed mitogen and incubated overnight at 37 °C in a 5% CO<sub>2</sub> 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 Blue-Black (IgG) Spots Counted	Number of Red (IgM) Spots Counted
1	23	7
2	26	5
3	25	7
4	25	8
5	24	5
6	25	7
7	25	9
8	30	12

## TROUBLESHOOTING GUIDE

OBSERVATION	PROBLEM	CORRECTIVE ACTION
Following the incubation with BCIP/NBT (or AEC) chromogen and rinsing the back of the microplate with deionized or distilled water, the dark blue (or red) background color of the filter membrane attenuates visualization and quantitation 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-AP, BCIP/NBT, and/or AEC 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 black-blue (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 AEC 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

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**PLATE LAYOUT**

Use this plate layout to record controls and samples assayed.

