ELISpot

Mouse/Rat IL-6

Catalog Number EL406

For the quantitative determination of the frequency of cells releasing mouse or rat IL-6.

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

Interleukin 6 (IL-6) is a multifunctional cytokine that plays important roles in host defense, acute phase reactions, bone remodeling, and hematopoiesis (1-8). The molecule is a 22-26 kDa secreted glycoprotein that is synthesized by many cell types (1). Although it is now known as an interleukin and serves as the prototype for a family of IL-6 type cytokines (2), IL-6 was originally isolated under a variety of names, including interferon β2, inducible 26 kD protein, interleukin-HP1, hepatocyte stimulating factor, and B cell stimulatory factor-2 (3). Mature mouse IL-6 is a 185 amino acid (aa) protein with four cysteine residues and two potential N-linked glycosylation sites (4, 9, 10). Although glycosylation has no effect on bioactivity (4), mouse IL-6 appears to be O-glycosylated (9, 11, 12). Mouse IL-6 shares 42% and 85% aa identity with human and rat IL-6, respectively (1, 13, 14). Whereas human IL-6 is active on both mouse and rat cells, mouse IL-6 is active on rat cells, but inactive on human cells (10). Mammalian cells known to express IL-6 include fibroblasts (15), bone marrow adipocytes (16), megakaryocytes (17), neurons (18), monocytes (19), keratinocytes (20), eosinophils and neutrophils (21), macrophages (22), renal tubular epithelial cells (23), osteoblasts (24), microglia and astrocytes (25), vascular smooth muscle cells (26), hepatocytes (27), adrenocortical cells (5), and B and T cells (28).

The functional IL-6 receptor complex is composed of an 80 kDa ligand-binding subunit (29), and a 130 kDa signal transducing subunit (30). The 80 kDa IL-6 receptor (IL-6R) is 449 aa in length, and is characterized by the presence of one Ig-like domain and two type III fibronectin domains in its extracellular region. IL-6 binds to IL-6R with high affinity (30). The signal transducing gp130 molecule is 896 aa in length, and extracellularly shows motifs characteristic of cytokine receptor superfamily molecules. On the cell surface, IL-6 is proposed to bind first to IL-6R, which then recruits gp130 into a trimer, which then associates with another trimer, forming a hexamer with gp130 homodimerization and subsequent signal transduction (31-34). Soluble forms of both IL-6R and gp130 have been identified in blood (35, 36). Soluble IL-6R has been shown to have IL-6 agonist activity, conferring IL-6 responsiveness to gp130 expressing cells that lack cell surface IL-6R (37).

The Mouse/Rat IL-6 ELISpot assay is designed for the detection of mouse and rat IL-6 secreting cells at the single cell level, and it can be used to quantify the frequency of mouse or rat IL-6 secreting cells. ELISpot assays are well suited for monitoring cellular responses to various stimuli, treatments and therapies, and they have been used specifically for the quantification of a multitude of physiologic responses. Other methods for the assessment of cytokine-secreting cells are tedious and require previous *in vitro* expansion of cytokine secreting cells for several days. These assays typically are not suitable for measuring infrequent cell responses that occur at less than 1 in 1,000. 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 IL-6 secreting cells, and thus they are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in areas as diverse as antigen recognition, vaccine development, and 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 (38, 39). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (40, 41). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique.

A polyclonal antibody specific for mouse/rat IL-6 has been pre-coated onto a polyvinylidene difluoride (PVDF)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37 °C CO_2 incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted IL-6. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse/rat IL-6 is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms and appears as spots at the sites of cytokine localization, with each individual spot representing an individual IL-6 secreting cell. The spots can be counted with an automated 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 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 for the rest of the experimental procedure and removed after the BCIP/NBT Substrate has been washed off.
- 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 BCIP/NBT Substrate.
- 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.
- 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 ELISpot 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 ELISpot experiment:

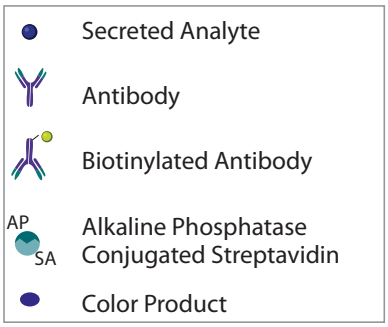
  Positive Control - Use recombinant mouse IL-6.

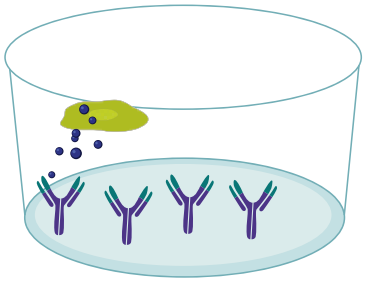
  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

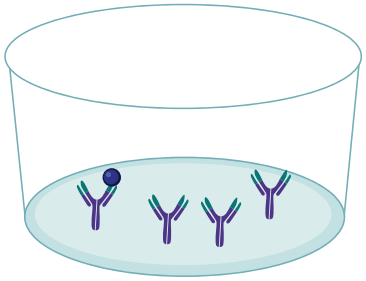
  Detection Antibody Control - Substitute phosphate buffered saline for detection antibody.

ELISPOT SCHEMATIC

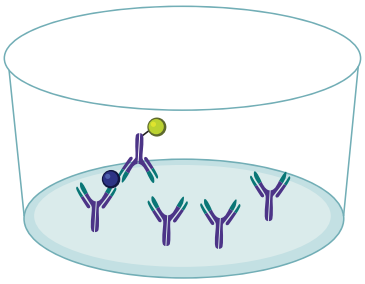




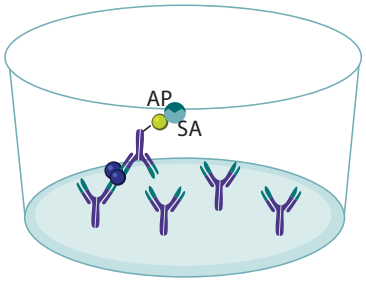
Incubate IL-6-secreting cells in an antibody-coated well.



Remove cells by washing. Secreted IL-6 is captured by the immobilized antibody.



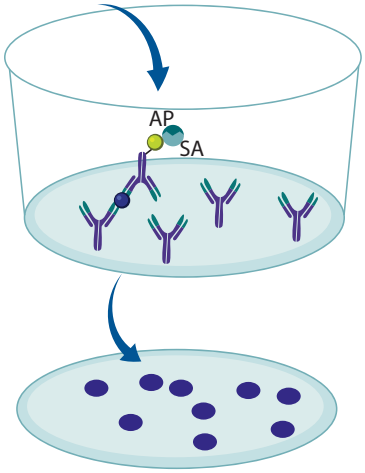
Incubate with biotinylated anti-IL-6 antibody.



Remove unbound biotinylated antibody by washing. Incubate with alkaline phosphatase conjugated streptavidin.

Wash to remove unbound enzyme.

Add BCIP/NBT Substrate



Add substrate and monitor the formation of colored spots. Analyze using either an ELISpot reader or dissection microscope.

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
Mouse/Rat IL-6 Microplate	891080	96-well PVDF-backed microplate coated with a polyclonal antibody specific for mouse/rat IL-6.
Mouse/Rat IL-6 Detection Antibody Concentrate	891081	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse/rat IL-6 with preservatives.
Streptavidin-AP Concentrate A	895358	150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.
Dilution Buffer 1	895307	12 mL of a buffer for diluting Mouse/Rat IL-6 Detection Antibody Concentrate 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.
BCIP/NBT Substrate	895867	12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).
Mouse/Rat IL-6 Positive Control	891082	2 ng of recombinant mouse IL-6 with preservatives; lyophilized.

OTHER SUPPLIES REQUIRED

- Dissection microscope or an ELISpot reader.
- Pipettes and pipette tips.
- Deionized 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.

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 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.

REAGENT PREPARATION

Bring all reagents to room temperature, except the Mouse/Rat IL-6 Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2-8 °C.

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.

Mouse/Rat IL-6 Positive Control - Reconstitute the lyophilized Mouse/Rat IL-6 Positive Control with 250 µL of culture medium that is used to incubate cells.

Detection Antibody Mixture - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Mouse/Rat IL-6 Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare the Detection Antibody mixture immediately before use.**

Streptavidin-AP Concentrate A - 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.**

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.

ASSAY PROCEDURE

Bring all reagents to room temperature, except the diluted Detection Antibody Mixture, which should remain at 2-8 °C. All samples and controls should be assayed at least in duplicate.

1. Fill all wells in the microplate with 200 µL 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 µL of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate 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.**
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 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 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 µL of the diluted Detection Antibody Mixture into each well, and incubate overnight at 2-8 °C. Alternatively, incubation with detection antibodies can be done for 2 hours at room temperature on a rocking platform.
6. Repeat the wash procedure described in step 4.
7. Add 100 µL of the diluted Streptavidin-AP Concentrate A into each well, and incubate for 2 hours at room temperature.
8. Repeat the wash procedure described in step 4.
9. Add 100 µL of the BCIP/NBT Substrate into each well, and incubate for 1 hour at room temperature. **Protect from light.**
10. Decant the the BCIP/NBT Substrate 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. 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 to the well.

REPRODUCIBILITY DATA

Splenocytes from a C57BL mouse (5 x 10^5 cells/mL) and a Sprague Dawley rat (5 x 10^5 cells/mL) were stimulated with 4.0 µg/mL of Concanavalin A overnight at 37 °C in a 5% CO_2 incubator. The samples were assayed in seven wells according to the procedure and analyzed with a dissection microscope.

Mouse Samples		Rat Samples	
Well	Number of Spots Counted	Well	Number of Spots Counted
1	285	1	441
2	274	2	447
3	278	3	484
4	272	4	442
5	259	5	437
6	259	6	475
7	256	7	456

TROUBLESHOOTING GUIDE

OBSERVATION	PROBLEM	CORRECTIVE ACTION
Following the incubation with the BCIP/NBT Substrate and rinsing the microplate with deionized water, the dark blue 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 and/or BCIP/NBT 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 blue-black.	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 were added to the wells.	Increase the number of cells added per well.
Following incubation with the BCIP/NBT Substrate and drying the microplate, the density of the spots makes them difficult to quantify.	Too many cells were added to the wells.	Make dilutions of cells (*i.e.* 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.

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