ELISpot

Mouse IL-10 Kit

Catalog Number EL417

For the quantitative determination of the frequency of cells releasing mouse IL-10.

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 10, also known as cytokine synthesis inhibitory factor (CSIF), is the charter member of the IL-10 cytokine family. This family comprises IL-10, IL-19, IL-20, IL-22, IL-24 and AK155 (1-4). All IL-10 family members are secreted α-helical proteins. Mouse IL-10 is a secreted, glycosylated polypeptide that circulates as a nondisulfide-linked homodimer with a 38 kDa molecular weight (3, 5). Each mouse IL-10 molecule is synthesized as a 178 amino acid (aa) precursor with an 18 aa signal sequence and a 160 aa mature form. The mature segment has two potential N-linked glycosylation sites plus five cysteines, four of which form two intrachain disulfide bridges that are essential for activity (6, 7). Mature mouse IL-10 shows 85%, 73% and 73% aa identity to rat (4, 6, 8), human (4, 9) and porcine (4, 10) IL-10, respectively. Mouse IL-10 also shares 20%-23% aa identity with other known members of the mouse IL-10 family (11-13). Upon activation, mammalian cells known to secrete IL-10 include NK cells (14), cytotoxic $CD8^+$ T cells secreting Th2-like cytokines (15), $CD4^+CD45RA^-$ (memory) Th1 and Th2 cells (16), macrophages (17), monocytes (18), $CD5^+$ and $CD5^-$ B cells (19, 20), dendritic cells (21, 22), hepatic stellate (Ito) cells (23), keratinocytes (24), melanoma cells (25), bronchogenic carcinoma cells (26), placental cytotrophoblasts (27), and $CD14^+MHC\text{-}II^-B7^-$ monocytic cells (28).

The functional receptor for IL-10 (IL-10 R) is composed of two 110 kDa alpha (or IL-10 R1) and two 75 kDa beta (or IL-10 R2) chains (29-32). The α-chains bind IL-10 and transduce a signal in the presence of a β-chain complex (29, 30). Both receptors are members of the class II cytokine receptor family (CRF2) that is characterized by the presence of type III fibronectin domains and conserved tryptophans (30). This class does not possess the WSXWS motif characteristic of the class I CRF. There is no significant aa identity (17%) between mouse IL-10 R1 and IL-10 R2.

IL-10 has a myriad of effects on a variety of cell types. On activated B cells, IL-10 can induce plasma cell formation (33) and the secretion of either IgG (34, 35) or IgA (in the presence of TGF-β1 and/or IL-4) (35, 36). In the presence of IL-2, $CD56^+$ NK cells will respond to IL-10 with increased proliferation plus IFN-γ and TNF-α secretion (37). Conversely, on macrophages, IL-10 is known to downregulate IL-1, TNF-α and IL-6 production (38). On dendritic cells, IL-10 has been shown to interfere with APC function by downmodulating stimulatory and co-stimulatory molecules (39, 40). On monocytes, IL-10 is reported to direct monocyte differentiation into cytotoxic $CD16^+$ macrophages rather than antigen-presenting dendritic cells (41-43).

The Mouse IL-10 ELISpot assay is designed for the detection of IL-10 secreting cells at the single cell level, and it can be used to quantitate the frequency of mouse IL-10 secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and they have been used for the quantitation of antigen-specific CD4 and/or CD8 T cell responses. Other methods for the assessment of antigen-specific T cell responses, such as the chromium release assay 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 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 cytokine secretion.

PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (44, 45). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (46, 47). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody specific for mouse IL-10 has been pre-coated onto a PVDF (polyvinylidene difluoride)-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-10. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IL-10 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-10 secreting cell. The spots can be counted with an automated ELISpot reader system or manually 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.
- 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 4x6 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 has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membranes.
- After completion of the experiment, do not dry the microplate at a temperature higher than 37 °C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment:

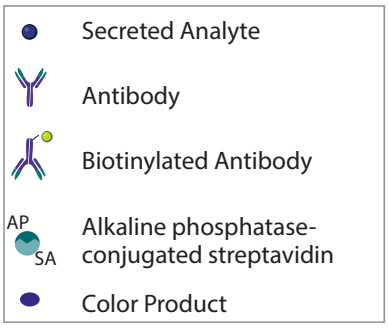
  Positive Control - Use recombinant mouse IL-10.

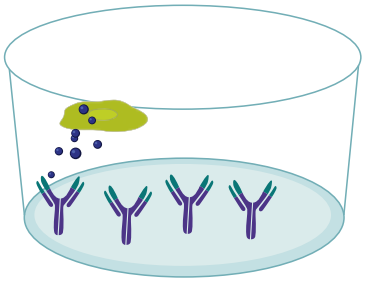
  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

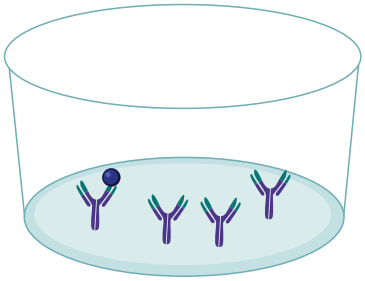
  Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

ELISpot SCHEMATIC

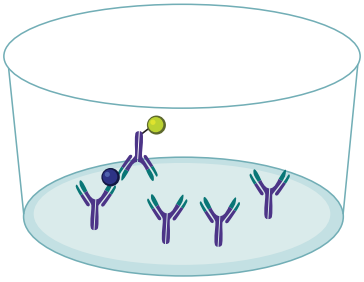




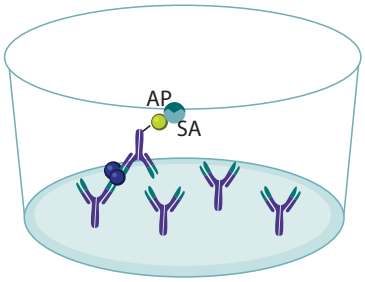
Incubate IL-10-secreting cells in an antibody-coated well.



Remove cells by washing. Secreted IL-10 is captured by the immobilized antibody.

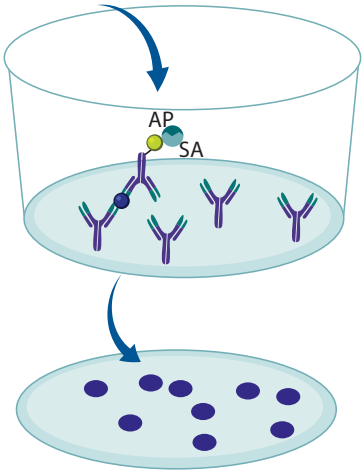


Incubate with biotinylated anti-IL-10 antibody.



Incubate with alkaline phosphatase conjugated streptavidin.

Add BCIP/NBT chromogen



Add substrate and monitor the formation of colored spots.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.
This kit is validated for single use only.

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

PART	PART #	DESCRIPTION
Mouse IL-10 Microplate	892199	96-well PVDF-backed microplate coated with monoclonal antibody specific for mouse IL-10.
Detection Antibody Concentrate	892200	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IL-10 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 with preservatives for diluting Detection Antibody Concentrate.
Dilution Buffer 2	895354	12 mL of a buffer with preservatives for diluting Streptavidin-AP Concentrate A.
Wash Buffer Concentrate	895308	50 mL of a 10X concentrated solution of a buffered surfactant with preservative.
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).
Mouse IL-10 Positive Control	892201	4 ng of recombinant mouse IL-10; lyophilized.

OTHER SUPPLIES REQUIRED

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

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 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.

Do not use reagents from this kit with components from other R&D Systems' ELISpot or ELISA kits and/or components manufactured by other vendors.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with BCIP/NBT chromogen.

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

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 50 mL of Wash Buffer Concentrate to 450 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Mouse IL-10 Positive Control - Reconstitute lyophilized mouse IL-10 Control with 250 µL of culture medium that is used to incubate cells. Mix well.

Detection Antibody - **Tap or vortex each vial to release reagent collected in the cap.** Transfer 100 µL of 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 - **Tap or vortex each 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 Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2-8 °C. All samples and controls should be assayed at least in duplicate. A plate layout is provided at the back of this insert to record controls and samples assayed.

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 stimuli 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 diluted Detection Antibody mixture into each well, and incubate overnight at 2-8 °C. Alternatively, incubation with Detection Antibody 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 diluted Streptavidin-AP 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 BCIP/NBT Chromogen into each well, and incubate for 1 hour at room temperature. **Protect from light.**
10. Discard the chromogen solution from the microplate and rinse the microplate with deionized or distilled 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 either manually using a dissection microscope or by using a specialized automated 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 into the well.

REPRODUCIBILITY DATA

Splenocytes from a C57BL mouse (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 seven wells according to the procedure and analyzed with a dissection microscope

Well	Number of Spots Counted
1	419
2	412
3	382
4	415
5	431
6	409
7	403

TROUBLESHOOTING GUIDE

OBSERVATION	PROBLEM	CORRECTIVE ACTION
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized or distilled 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 BCIP/NBT 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 (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.

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PLATE LAYOUT

Use this plate layout to record controls and samples assayed.