ELISpot

Human IL-13

Catalog Number EL213

For the quantitative determination of the frequency of cells releasing human IL-13.

The package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

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INTRODUCTION

Interleukin 13 (IL-13) is a pleiotropic cytokine that is critical in regulating inflammatory and immune responses (1-4). Human IL-13 is synthesized as a 132 amino acid (aa) precursor with a putative 20 aa signal peptide that is cleaved to generate a 112 aa mature protein containing four potential glycosylation sites (1, 5). Two alternatively spliced isoforms that differ by the presence or absence of the glutamine residue at position 98 have been reported (5). Genetic variants of IL-13 also exist, the variant with a glutamine substitution for arginine at residue 130 is a significant risk factor for asthma development (6). At the amino acid sequence level, human IL-13 shares approximately 58% and 63% identity with mouse and rat IL-13, respectively (5, 7, 8). IL-13 is primarily produced by Th2-like cells (3), but is also produced by Th1, Th0, and $CD8^+$ T cells (3- 5, 7, 9, 10), natural killer cells (11), mast cells and basophils (12, 13), alveolar macrophages (14), dendritic cells (15), and Reed-Sternberg cells (16).

IL-13 plays an important role in Th2-cell mediated immune responses elicited by allergic reactions and parasitic worm infections (3, 17, 22). On B cells, IL-13 induces IgE synthesis and upregulates the expression of CD23 (2, 4, 18). Also, IL-13 induces CD69 surface antigen expression and promotes cellular viability on eosinophils (19). VCAM-1 expression and eosinophil adhesion (20, 21) is promoted on endothelial cells whereas on monocytes/macrophages, IL-13 has anti-inflammatory functions and inhibits the production of pro-inflammatory cytokines, chemokines and other mediators, contributing to the downregulation of their effector functions (1, 22). On immature dendritic cells derived from GM-CSF and IL-4 treated monocytes, IL-13 induces functional maturation (23). IL-13 has also been shown to upregulate the expression of CXCR1 and CXCR2 in monocytes, macrophages and dendritic cells, thus reorienting the responsiveness of these mononuclear phagocytes to IL-8 and contributing to their accumulation in Th2-dominated responses (24, 25). *In vivo*, IL-13 plays a key role in the expulsion of gut-residing worms following helminth infection (4, 26).

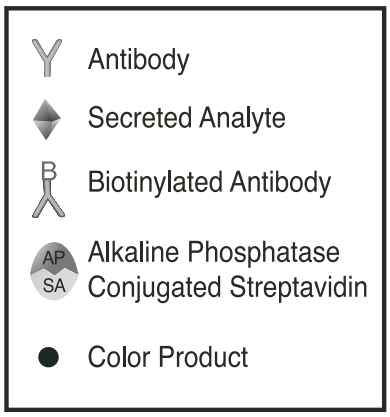
The functional high-affinity IL-13 receptor complex is composed of two type I transmembrane glycoproteins that are members of the hematopoietin receptor superfamily: the 140 kDa IL-4Rα chain, which does not bind IL-13 directly (27) and the 65 kDa IL-13Rα, which binds IL-13 with low-affinity (28-31). The presence of both the IL-4Rα and IL-13Rα1 is required to form the high-affinity IL-13 signaling receptor. A second IL-13Rα2 subunit that binds IL-13 with high-affinity and shares amino acid sequence homology with IL-13Rα1 has been identified (32-35). IL-13Rα2 does not signal IL-13 responses in the presence of IL-4Rα and is likely to be a decoy receptor for IL-13.

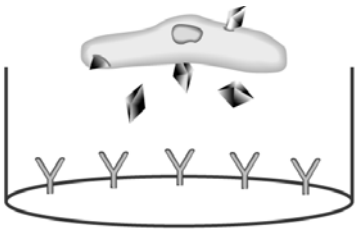
The Human IL-13 ELISpot assay is designed for the detection of IL-13 secreting cells at the single cell level, and it can be used to quantitate the frequency of human IL-13 secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and 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 the monitoring of various clinical trials.

PRINCIPLE OF THE ASSAY

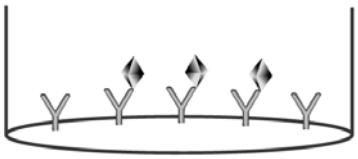
The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (36, 37). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (38, 39). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbant assay (ELISA) technique. A polyclonal antibody specific for human IL-13 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 bind secreted IL-13. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for human IL-13 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-13 secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

ELISpot SCHEMATIC

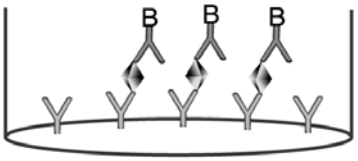




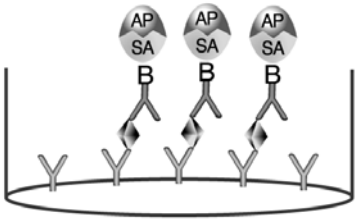
Incubate IL-13-secreting cells in an antibody-coated well.



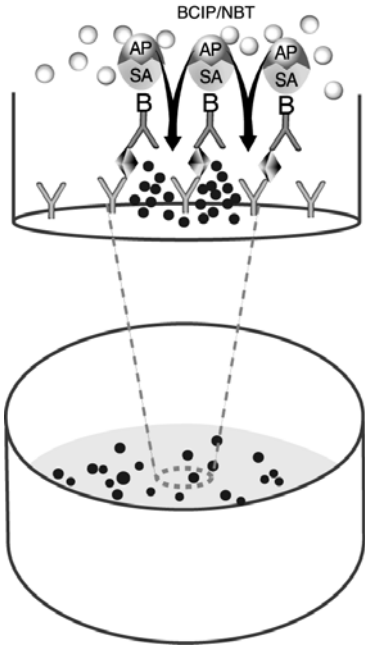
Remove cells by washing. Secreted IL-13 is captured by the immobilized antibody.



Incubate with biotinylated anti-IL-13 antibody.



Incubate with alkaline phosphatase conjugated streptavidin.



Add substrate and monitor the formation of colored spots.

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.

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.

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.

Although the toxicity of the chromogenic substrate BCIP/NBT is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used BCIP/NBT.

MATERIALS PROVIDED

Human IL-13 Microplate (Part 891001) - One 96-well PVDF-backed microplate coated with polyclonal antibody specific for human IL-13.

Detection Antibody Concentrate (Part 891002) - 150 µL of a 120X concentrated solution of biotinylated polyclonal antibodies specific for human IL-13 with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a diluent for diluting Detection Antibody Concentrate with preservatives.

10X Wash Buffer Concentrate (Part 895308) - 50 mL of a 10X concentrated solution of a buffered surfactant with preservative.

Dilution Buffer 2 (Part 895354) - 12 mL of a diluent for diluting Streptavidin-AP Concentrate A with preservatives.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3′ Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Human IL-13 Positive Control (Part 891003) - 1 vial (2 ng/vial) of recombinant human IL-13; lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer
- 1000 mL graduated cylinder
- Dissection microscope or an automated ELISpot reader

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of soft 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 color substrate 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 membrane.
- After completion of 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 human IL-13.

  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.

REAGENT PREPARATION

10X Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one microplate, dilute the 10X Wash Buffer Concentrate with deionized water. *i.e.,* add 50 mL of 10X Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Human IL-13 Positive Control - Reconstitute lyophilized human IL-13 with 250 μL of culture medium that is used to incubate cells.

Detection Antibody - Tap or vortex the 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 Detection Antibody 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 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. R&D Systems' cell selection products are suitable for the purification of effector and responder cells. For a complete product listing of human, mouse, and rat cell selection products, see the R&D Systems catalog or visit our website at www.RnDSystems.com.

ASSAY PROCEDURE

Bring all reagents as needed to room temperature. The Detection Antibody Concentrate and Dilution Buffer 1 should remain at 4° C. All samples and controls should be assayed at least in duplicates. An Assay Record Template 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, pipette, 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 Detection Antibody into each well and incubate at 4° C overnight.
6. Repeat step 4.
7. Add 100 µL of Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat 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 distilled water. Invert microplate and tap to remove excess distilled water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe microplate bottom thoroughly with paper towel 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

Peripheral blood mononuclear cells (5×10^5 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 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	149
2	164
3	153
4	156
5	136
6	144
7	139

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with distilled water, the dark-blue background color of filter membrane attenuates visualization and quantitation of spots	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry. Usually it takes from 15 to 30 minutes at ~37° C or about 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 as a result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted to room temperature	Adjust the temperature of the reagents to room temperature before adding to the wells
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue	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 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 it difficult to quantify them	Too many cells were added to the wells	Make dilutions of cells, *i.e.,* 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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ASSAY RECORD TEMPLATE

This template may be used as a record of samples and controls run in an assay.