ELISpot

Mouse IL-17

Catalog Number EL421

For the quantitative determination of the frequency of cells releasing mouse IL-17.

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

Mouse Interleukin 17 (IL-17; also known as IL-17A or CTLA-8) is a 30 - 35 kDa variably glycosylated homodimeric protein that belongs to a unique family of cysteine-knot related proteins (1 - 3). Its sequence was originally isolated from an activated hybridoma created from the fusion of a mouse cytotoxic and rat T cell lymphoma cell line (2 - 5). It is synthesized as a 158 amino acid (aa) precursor that contains a 25 aa signal sequence and a 15 kDa, 133 aa mature segment (5). Although there are two intrachain disulfide bonds that create a ring reminiscent of those found in cysteine-knot proteins, the actual closed knot structure does not appear to form (1, 2). IL-17 contains one potential N-linked glycosylation site. Mature mouse IL-17 shares 61% and 89% aa sequence identity to human and rat IL-17, respectively (4, 6). While rodent and human mature sequences show modest aa sequence identity, human IL-17 is active on both mouse and rat cells (5, 7). The cells principally known to produce IL-17 are the memory $CD4^+$ T cells. In addition, $CD8^+$ T cells as well as $\alpha\beta TCR^+$ $CD4^-CD8^-$ T cells, neutrophils (PMNs) and eosinophils have also been reported to express mRNA transcripts for IL-17 (3, 5, 8 - 10).

A high affinity receptor for mouse IL-17 (IL-17 R) has been reported and found to be ubiquitously expressed. It is synthesized as a 130 kDa, type I transmembrane glycoprotein that bears no resemblance to members of the cytokine, TNF or immunoglobulin receptor superfamily (2, 8, 11). It is synthesized as an 864 aa precursor that contains a 31 aa signal sequence, a 291 aa extracellular region, a 21 aa transmembrane segment, and a 521 aa cytoplasmic domain (11). The extracellular region has no distinguishing aa motifs or domains. IL-17 R signaling reportedly utilizes multiple classes of MAP kinases (1) and also activates the JAK/STAT family of transcription factors (8).

Functionally, IL-17 is best known for its participation in the recruitment and survival of neutrophils (3, 8, 12). Its induction is believed to be the result of antigen stimulation of dendritic cells, resulting in IL-23 secretion. In a TCR-independent event, IL-23 can induce T cell production of IL-17 (3). Once secreted, IL-17 in the bone marrow appears to induce stromal fibroblast expression of both G-CSF and stem cell factor (membrane form), an effect that increases PMN differentiation and production. IL-17 may complement this by directly blocking neutrophil apoptosis, promoting greater circulating PMN numbers (12). In the tissues, IL-17 appears to promote neutrophil extravasation, principally through its effects on macrophages and endothelial cells. On macrophages, IL-17 induces TNF-α, IL-1β and IL-6 production (13). TNF-α and IL-1β then act on local endothelial cells to induce G-CSF secretion, an effect that is potentiated by IL-17 (14). IL-17 further contributes to PMN influx by inducing endothelial cells to release CXC chemokines and nitric oxide production, which may increase vascular permeability (3, 7).

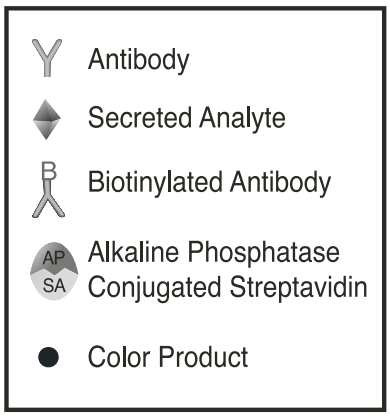
The Mouse IL-17 ELISpot assay is designed for the detection of IL-17 secreting cells at the single cell level, and it can be used to quantitate the frequency of mouse IL-17 secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and 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, cytokine secretion and the monitoring of various clinical trials.

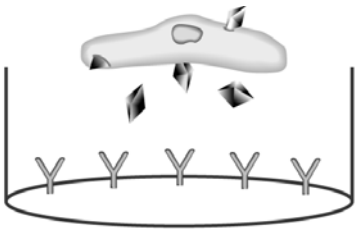
This kit detects mouse IL-17 and does not cross-react with mouse IL-17E or IL-17F. Cross-reactivity with other mouse IL-17 family members has not been determined.

PRINCIPLE OF THE ASSAY

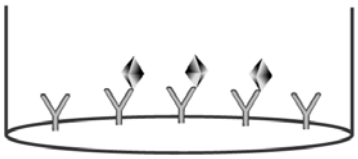
The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (15, 16). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (17, 18). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody specific for mouse IL-17 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-17. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IL-17 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 at the sites of cytokine localization and appears as spots, with each individual spot representing an individual IL-17 secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

ELISpot SCHEMATIC

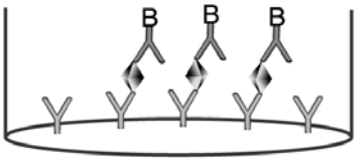




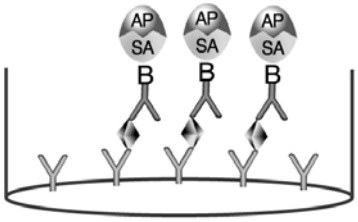
Incubate IL-17-secreting cells in an antibody-coated well.



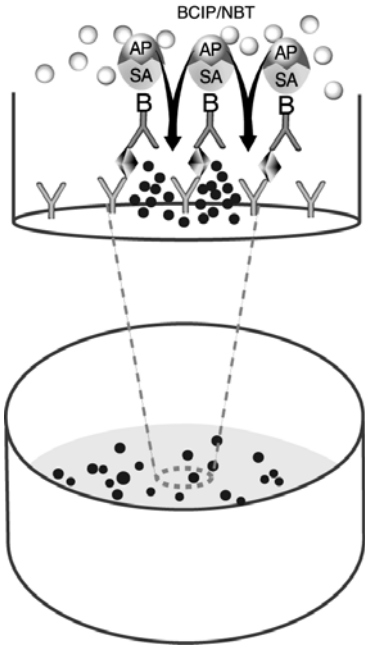
Remove cells by washing. Secreted IL-17 is captured by the immobilized antibody.



Incubate with biotinylated anti-IL-17 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.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in pipetting and washing techniques, incubation time or temperature, and kit age can cause variation in density of spots, intensity of specific staining and background level.

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 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

Mouse IL-17 Microplate (Part 892730) - One 96-well PVDF-backed microplate coated with monoclonal antibody specific for mouse IL-17.

Detection Antibody Concentrate (Part 892731) - 150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IL-17 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 buffer for diluting Detection Antibody Concentrate with preservatives.

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

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

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).

Mouse IL-17 Positive Control (Part 892732) - 1 vial (3 ng) of recombinant mouse IL-17; lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond the 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
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO_2 incubator
- Sterile culture media
- Dissection microscope or an automated ELISpot reader

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 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 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 the 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-17.

  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

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 IL-17 Positive Control - Reconstitute the lyophilized mouse IL-17 with 250 µL of the same 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' T-cell Separation/Enrichment Columns may be 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, 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. 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, 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 into each well and incubate at 2 - 8° C overnight.
6. Repeat step 4.
7. Add 100 µL of diluted 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 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 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 (1.0×10^6 cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μg/mL calcium ionomycin overnight at 37° C in a 5% CO_2 incubator. The sample was assayed in eight wells (100 μL/well) according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	104
2	103
3	116
4	107
5	110
6	109
7	113
8	98

SPECIFICITY

This kit detects mouse IL-17 and does not cross-react with mouse IL-17E or IL-17F. Cross-reactivity with other mouse IL-17 family members has not been determined.

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized 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 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 may result from using Streptavidin-AP and/or BCIP/NBT solutions that have not been brought to room temperature.	Bring 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.