

ELISpot

Rat IL-2

Catalog Number EL502

For the quantitative determination of the frequency of cells releasing rat IL-2.

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

Rat Interleukin-2 (IL-2, also known as T Cell Growth Factor) is a 16 kDa, single chain, glycosylated polypeptide that has potent stimulatory activity for antigen-activated T cells (1-6). It is synthesized as a 155 amino acid (aa) precursor that contains a 20 aa signal peptide and a 135 aa mature segment (5). There is one intrachain disulfide bridge, and based on human IL-2 studies, rat IL-2 will likely be O-glycosylated on threonine at position #3 (3, 4). Rat IL-2 shares 80%, 68% and 54% aa identity with human, mouse and guinea pig IL-2, respectively (5, 10, 11). In apparent contrast to the rat, however, multiple forms of IL-2 exist in mouse that are subspecies specific. Relative to the rat IL-2 sequence, all mouse IL-2 forms show a serine-glutamine rich insert between rat aa's 7 and 8 that is anywhere from 11-23 aa in length (11). In terms of bioactivity, human IL-2 is active on mouse, rat, cat and guinea pig cells (12, 13). And while mouse and rat IL-2 are active on each others cells (12, 14, 15), mouse IL-2 is only marginally active on human cells (13). IL-2 is primarily expressed by CD4⁺ and CD8⁺ T cells (16), and has also been identified in visceral smooth muscle cells (17), eosinophils (18), $\gamma\delta$ T cells (19), B cells (20) and astrocytes (21).

The receptor complex for IL-2 consists of three distinct subunits in varying combinations (1, 22-24). Two of these are ligand-binding and are termed IL-2R α and IL-2R β . IL-2R α is 55 kDa and binds IL-2 with low affinity (22, 25). IL-2R β is 75 kDa and also binds IL-2 with a low affinity (22, 25). Signal transduction is performed by both IL-2R β and the 64 kDa common gamma chain (γ c) (22). The common gamma chain does not bind IL-2, but does heterodimerize with IL-2R β to form an intermediate affinity IL-2 receptor on macrophages and NK cells (22). It also heterotrimerizes with the α - and β -chains to form a high-affinity receptor on activated lymphocytes (22). There is also the potential for another receptor for IL-2. This is the delta (enkephalin)-opioid receptor, a 7-transmembrane G-protein coupled receptor. Although IL-2 lacks a classic opioid receptor-binding peptide motif (Y-x-x-F) in its primary structure, its three-dimensional structure creates a fold comprised of tyrosine and phenylalanine (26, 27).

Functionally, IL-2 is best known for its autocrine and paracrine activity on activated T cells. It is induced in T cells following the interaction of antigen with TCR. The TCR-Ag interaction drives resting T cells into active G1, inducing IL-2 and IL-2R α synthesis. Following cell surface receptor expression and IL-2 synthesis, IL-2-IL-2R binding forces the T cell into S phase which culminates in mitosis. Thus, IL-2 plays a critical role in T cell expansion (22, 23). IL-2 is also known to participate in NK cell activation, in part through its membrane upregulation of NKp44, a cytotoxicity-inducing molecule, and in part through a collaboration with IFN- γ (28-30). Finally, IL-2 may play a role in the induction of analgesia. This could be the result of direct IL-2 binding to neurons, or the consequence of IL-2-mediated release of leucine-enkephalin, which subsequently binds to opioid receptors (26, 31)

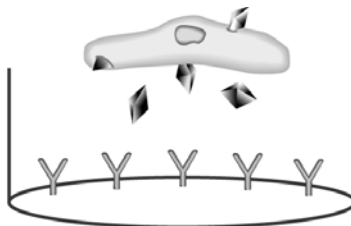
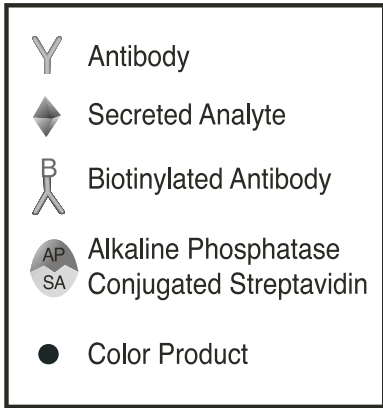
The Rat IL-2 ELISpot assay is designed for the detection of IL-2 secreting cells at the single cell level, and it can be used to quantitate the frequency of rat IL-2 secreting cells. ELISpot assays are well suited for monitoring cellular responses to various stimuli, treatments and therapies, and they have been used specifically for the 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 IL-2 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.

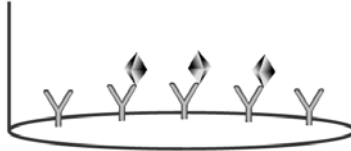
PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (32,33). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (34,35). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbant assay (ELISA) technique. A polyclonal antibody specific for rat IL-2 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 CO₂ 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-2. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for rat IL-2 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-2 secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

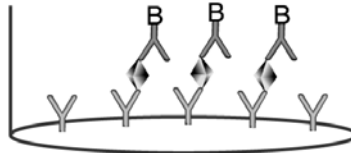
ELISpot SCHEMATIC



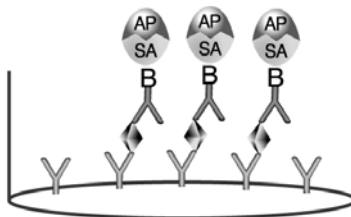
Incubate IL-2-secreting cells in an antibody-coated well.



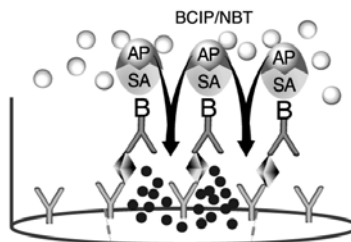
Remove cells by washing. Secreted IL-2 is captured by the immobilized antibody.



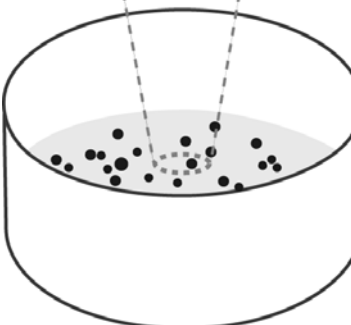
Incubate with biotinylated anti-IL-2 antibody.



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

Rat IL-2 Microplate (Part 890992) - One 96-well PVDF-backed microplate coated with polyclonal antibody specific for rat IL-2.

Detection Antibody Concentrate (Part 890993) - 150 μ L of a 120X concentrated solution of biotinylated polyclonal antibodies specific for rat IL-2 with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 μ L 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.

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

10X 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).

Rat IL-2 Positive Control (Part 890994) - 1 vial (2 ng/vial) of recombinant rat IL-2; 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
- Multi-channel pipette, squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Dissection microscope or an automated ELISpot reader
- 37° C CO₂ incubator

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 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 rat IL-2.

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, add 50 mL of 10X Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Rat IL-2 Positive Control - Reconstitute lyophilized rat IL-2 with 250 µL of culture medium that is used to incubate cells.

Detection Antibody - 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 - 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 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 μ L 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 μ L of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37° C CO₂ 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 μ L) 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 μ L of diluted Detection Antibody into each well and incubate at 2 - 8° C overnight.
6. Repeat step 4.
7. Add 100 μ L of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat step 4.
9. Add 100 μ L 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 it thoroughly with paper towels and allow it to 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

Rat splenocytes (1×10^5 cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μ g/mL of calcium ionomycin overnight at 37° C in a 5% CO₂ incubator. The sample was assayed in seven wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	327
2	270
3	264
4	262
5	297
6	283
7	288

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>i.e.</i> , 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.

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