ELISpot

Mouse CD4$^+$/Granzyme B

Catalog Number EL6024

For the quantitative determination of the frequency of CD4$^+$ cells releasing mouse Granzyme B

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

Granzyme B is a member of the granzyme family of the serine proteases found specifically in the cytotoxic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells (1, 2). As the two major populations of cytotoxic lymphocytes (CL), CTL and NK cells are key components of the innate and adaptive cellular immune responses against intracellular pathogens and transformed cells (3). Released through exocytosis of CL secretory granule contents and aided by perforin, Granzyme B is able to access the target cell cytosol, where it processes key substrates to trigger cell death (3). In addition to playing an essential role in granule-mediated apoptosis, Granzyme B may have roles in rheumatoid arthritis and in bacterial and viral infection (4). The substrates that have been identified for Granzyme B include caspases, BH3 Interacting Domain Death Agonist (BID), lamins, poly(ADP-ribose) polymerase (PARP), neuronal glutamate receptor, and cartilage proteoglycan (3).

As one of the eleven granzymes (A, B, C, D, E, F, G, K, M, N, and O) found in the mouse genome, Granzyme B is synthesized as a precursor of 247 amino acid residues (5-7). It consists of a signal sequence (residues 1-18), a pro peptide (residues 19-20) and a mature chain (residues 21-247). The amino acid sequence of mouse Granzyme B is 69% and 80% identical to its human and rat counterparts, respectively (5). Once inside granules, Granzyme B is fully processed into the mature chain and becomes an active protease (1). The protease activity of Granzyme B can be inhibited by serpin B9/proteinase inhibitor 9 (8).

Mouse CD4 is a 55 kDa type I transmembrane palmitoylated glycoprotein that contains four extracellular Ig-like domains (9, 10). It binds to MHC-II molecules, and acts as a co-receptor for the T cell receptor (TCR). This facilitates TCR signaling, and either expands the repertoire of antigens that the TCR recognizes or increases the avidity of TCR for antigen (11, 12). It also binds IL-16, inducing T cell migration (13). In the extracellular region, CD4 shows 72%, 53% and 46% aa sequence identity to rat, human, and canine CD4, respectively (9).

The Mouse $CD4^+$/Granzyme B ELISpot assay is designed for the detection of Granzyme B secreted from $CD4^+$ cells at the individual single cell level and can be used to quantitate the frequency of mouse Granzyme B secreting cells. ELISpot assays are well suited for studying immune responses to various treatments and therapies and have been used for the quantitation of antigen-specific $CD4^+$ and/or $CD8^+$ T cell responses. Other methods for assessment of antigen-specific T cell responses, such as 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 are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in vaccine development and for 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 (14, 15). This method has since been adapted for the detection of a selected cell type secreting specific cytokines or other antigens (16, 17). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique.

PVDF (polyvinylidene difluoride)-backed microplates are coated with both anti-mouse CD4 and anti-mouse Granzyme B monoclonal antibodies. Cells are added to the plate and incubated for a short period of time to capture $CD4^+$ cells. After that, plates are washed to remove unbound non-$CD4^+$ cells. The ELISpot plate with $CD4^+$ -enriched cells is placed into a 37° C CO_2 incubator for a specified period of time. During the incubation, anti-mouse Granzyme B antibodies capture Granzyme B secreted primarily by $CD4^+$ cells. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse Granzyme B 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 appearas spots, with each individual spot representing an individual Granzyme B secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a dissection scope.

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 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 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:

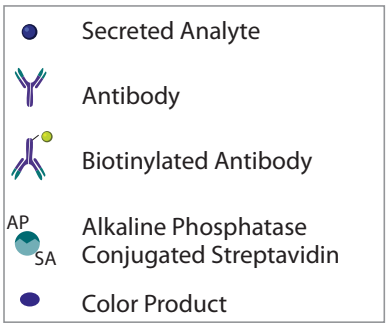
  Positive Control - Use recombinant mouse Granzyme B.

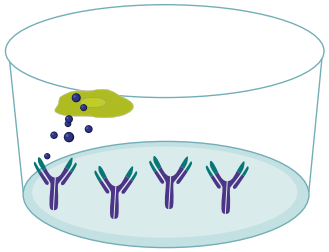
  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

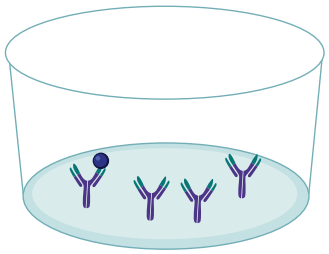
  Detection Antibody Control - Substitute phosphate buffered saline for detection antibody.

ELISPOT SCHEMATIC

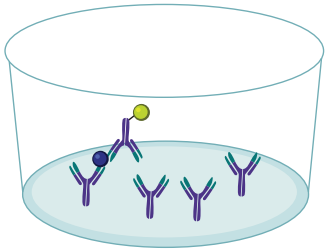




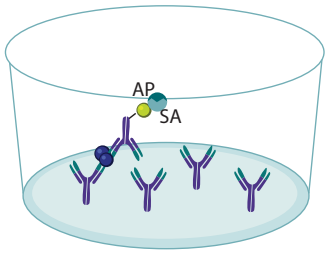
Incubate Granzyme B-secreting cells in an antibody-coated well.



Remove cells by washing. Secreted Granzyme B is captured by the immobilized antibody.

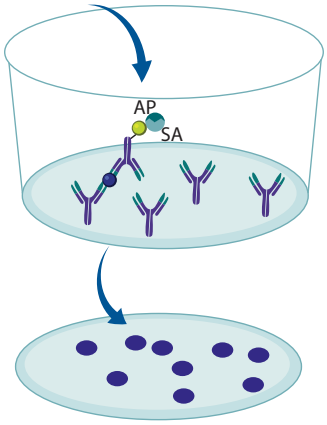


Incubate with biotinylated anti-Granzyme B antibody.



Add BCIP/NBT Substrate

Incubate with alkaline phosphatase conjugated streptavidin.



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.

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 CD4$^+$/Granzyme B Microplate	893007	96-well PVDF-backed microplate coated with a monoclonal antibody specific for mouse Granzyme B.
Detection Antibody Concentrate	893008	150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse Granzyme B 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 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 CD4$^+$/Granzyme B Positive Control	893009	8 ng of recombinant mouse Granzyme B with preservatives; lyophilized.

OTHER SUPPLIES REQUIRED

- 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
- Dissection microscope or an ELISpot reader

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.

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 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 $CD4^+$/Granzyme B Positive Control - Reconstitute the lyophilized Mouse Granzyme B Control 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 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 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 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 BCIP/NBT Substrate into each well, and incubate for 1 hour at room temperature. **Protect from light.**
10. Decant 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 (2.5 x 10^6 cells/mL) were incubated for 1 hour at room temperature. Following three washes with PBS, stimulants (50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 µg/mL calcium ionomycin) were added to the culture media and incubated overnight at 37 °C in a 5% CO_2 incubator. The sample was assayed in eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	325
2	322
3	310
4	323
5	322
6	297
7	326
8	297

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 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 (*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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