

# ELISpot

## Mouse CD4<sup>+</sup>/Granzyme B

Catalog Number EL6024

**For the quantitative determination of the frequency of CD4<sup>+</sup> 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).

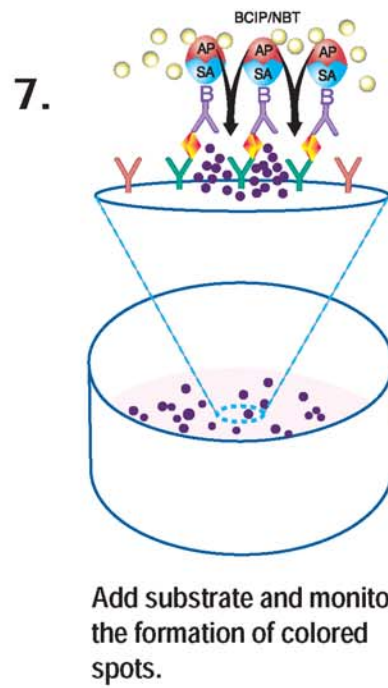
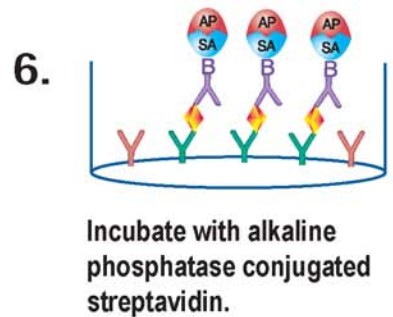
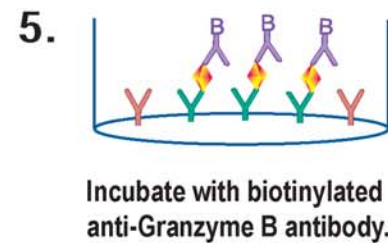
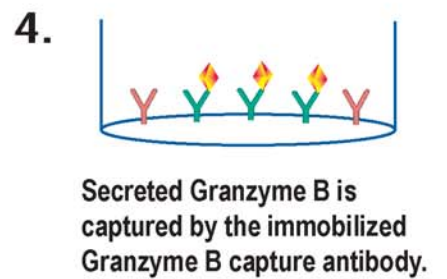
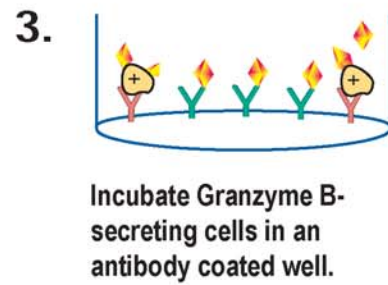
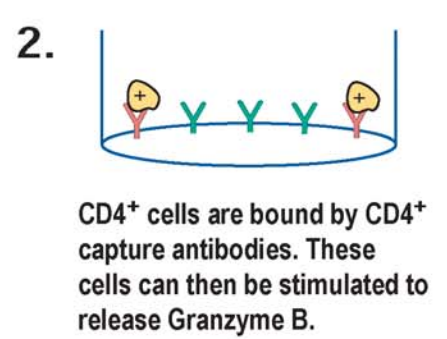
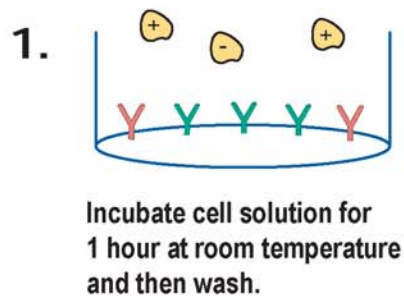
The Mouse CD4<sup>+</sup>/Granzyme B ELISpot assay is designed for the detection of Granzyme B secreted from CD4<sup>+</sup> cells at the individual single cell level and can be used to quantitate the frequency of mouse Granzyme B secreting cells. ELISpot assays are well suited for studying immune responses to various treatments and therapies and have been used for the quantitation of antigen-specific CD4<sup>+</sup> and/or CD8<sup>+</sup> 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<sup>+</sup> cells. After that, plates are washed to remove unbound non-CD4<sup>+</sup> cells. The ELISpot plate with CD4<sup>+</sup>-enriched cells is placed into a 37° C CO<sub>2</sub> incubator for a specified period of time. During the incubation, anti-mouse Granzyme B antibodies capture Granzyme B secreted primarily by CD4<sup>+</sup> 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 appear as 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 stereomicroscope.

# ELISpot SCHEMATIC

	CD4 <sup>+</sup> Cells
	CD4 <sup>-</sup> Cells
	Anti-CD4 Antibody
	Anti-Granzyme B Antibody
	Secreted Analyte
	Biotinylated Antibody
	Alkaline Phosphatase Conjugated Streptavidin
	Color Product



## 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 CD4<sup>+</sup>/Granzyme B Microplate** (Part 893007) - One 96-well PVDF-backed microplate coated with anti-mouse CD4<sup>+</sup> and anti-mouse Granzyme B monoclonal antibodies.

**Detection Antibody Concentrate** (Part 893008) - 150  $\mu$ L of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse Granzyme B with preservatives.

**Streptavidin-AP Concentrate A** (Part 895358) - 150  $\mu$ L 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 preservatives.

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

**Mouse CD4<sup>+</sup>/Granzyme B Positive Control** (Part 893009) - 1 vial (8 ng) of recombinant mouse Granzyme B; 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
- Dulbecco's phosphate-buffered saline (PBS)
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO<sub>2</sub> 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 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.

## 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 CD4<sup>+</sup>/Granzyme B Positive Control** - Reconstitute lyophilized mouse Granzyme B 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](http://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  $\mu$ 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  $\mu$ L of the appropriate cells to each well.
3. Incubate cells for 1 hour at room temperature on the benchtop.
4. Wash away cells which have not been captured by washing with PBS. Dump the contents into the sink and then fill with PBS and repeat two more times. **Do not aspirate cells.**
5. Add 100  $\mu$ L of culture media containing the appropriate stimulant concentration and 100  $\mu$ L of the appropriate controls (see Technical Hints for appropriate controls).
6. Incubate cells in a humidified 37° C CO<sub>2</sub> incubator. Optimal incubation time for each stimulus should be determined by the investigator. **Do not disturb the cells during the incubation period.**
7. 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  $\mu$ L) 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.**
8. Add 100  $\mu$ L of diluted Detection Antibody into each well and incubate at 2 - 8° C overnight.
9. Repeat step 7.
10. Add 100  $\mu$ L of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
11. Repeat step 47.
12. Add 100  $\mu$ L of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light.**
13. 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 ( $2.5 \times 10^6$  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  $\mu\text{g/mL}$  calcium ionomycin) were added to the culture media and incubated overnight at 37° C in a 5% CO<sub>2</sub> 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 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 - perhaps as a result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted 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>i.e.</i> , $1 \times 10^6$ , $5 \times 10^5$ , $1 \times 10^5$ , $5 \times 10^4$ , $1 \times 10^4$ 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.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	A	B	C	D	E	F	G	H