Human GM-CSF ELISpot Kit

For detection and enumeration of cells secreting human granulocyte-macrophage colony-stimulating factor

Catalog #200-1001 1 Kit

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Product Description

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) ELISpot Kit is designed for the quantitative detection and enumeration of cells secreting human GM-CSF in a suspension of peripheral blood mononuclear cells (PBMCs). ELISpot is commonly used for evaluating specific immune responses, such as determining the frequency of activated T cell subsets in response to antigenic stimulation. GM-CSF is secreted by many cell types including T helper (Th) subsets Th1, Th2, Th17, and ThGM cells as well as CD1a-reactive T cells involved in inflammatory and allergic diseases. GM-CSF can also be produced by monocytes and dendritic cells in response to pathogen recognition. ELISpot is highly sensitive and can detect secreted analytes at a single-cell level. This assay is used in research of infectious diseases, cancers, allergies, and autoimmune diseases.

The assay is based on a modified version of the sandwich ELISA method, in which cells are added to ELISpot plates pre-coated with capture antibodies specific for the analyte. The analyte is then captured throughout the culture period and is detected by the addition of a biotinylated detection antibody, followed by streptavidin-alkaline phosphatase (SA-ALP), which binds to the biotinylated antibody. Addition of the chromogenic enzyme substrate, 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP)/nitroblue tetrazolium (NBT), results in the formation of colored spots. The number, size, and intensity of these spots are directly proportional to the frequency of analyte-secreting cells in the sample and the amount of analyte secreted.

Product Information

All components listed below are stable until expiry date (EXP) on label. Components may be shipped at room temperature (15 - 25°C) but should be stored as indicated upon receipt.

COMPONENT NAME	SIZE	STORAGE	DESCRIPTION
Anti-human GM-CSF mAb (23B6), biotin	40 µL	Store at 2 - 8°C.	Biotinylated anti-human GM-CSF antibody (1 mg/mL); detection antibody
ELISpot substrate: BCIP/NBT-plus for ALP*	25 mL	Store at 2 - 8°C.	BCIP/NBT solution
Human GM-CSF pre-coated ELISpot white plate	2 Plates	Store at 2 - 8°C or room temperature.	96-well plate coated with anti-human GM-CSF antibody, mAb clone 21C11
Streptavidin-ALP for ELISpot	40 µL	Store at 2 - 8°C.	SA-ALP conjugate

* This product is hazardous. Please refer to the Safety Data Sheet (SDS).

Materials Required but Not Included

- Absorbent paper
- Aluminum foil (optional)
- Biohazard safety cabinet certified for Level II handling of biological materials
- ELISpot reader (e.g. Mabtech IRIS™ 2/ASTOR™ 2) or dissection microscope
- Incubator, 37°C
- Multi-channel pipettor (e.g. Catalog #38110) with appropriate pipette tips or automatic plate washer adapted for ELISpot plates for non-sterile steps of washing
- Phosphate-buffered saline (PBS) containing 0.5% fetal bovine serum (FBS; PBS + 0.5% FBS)
- PBS, sterile and filtered (0.2 µm), OR Dulbecco's phosphate-buffered saline (D-PBS; Without Ca++ and Mg++), pH 7.4 (Catalog #37350)
- Pipettor (e.g. Catalog #38059/38060) with appropriate pipette tips (e.g. Catalog #38032/38033)
- Reagent reservoir (e.g. Catalog #38080)
- Syringe and syringe filter (0.45 µm and 0.2 µm, e.g. Catalog #200-0583)
- Timer

- Tubes for preparing dilutions (e.g. Catalog #100-0092/200-0521)

Preparation of Reagents and Materials

A. Samples and Cell Culture Medium

Fresh or cryopreserved PBMCs may be used in the assay. If using cryopreserved cells, it is recommended to incubate cells in culture medium at 37°C for ≥ 1 hour to return the cells to their resting state before beginning the experiment. For optimal results, remove any cell debris by allowing it to settle and carefully transfer the cell suspension to a new tube or filter the cell suspension through a cell strainer (e.g. Catalog #27215).

NOTE: The suggested seeding density for detecting antigen-specific responses is 2.5 x 10^5 cells/well. Cell density may be reduced to prevent confluent spot formation when using polyclonal activators. Duplicate or triplicate wells for all samples are recommended.

CELL CULTURE MEDIUM

Complete medium (cell culture medium + serum) should be screened for low background staining. FBS is recommended over human serum which may contain heterophilic antibodies or secreted analytes that can interfere with the assay. Alternatively, serum-free medium such as ImmunoCult™-XF T Cell Expansion Medium (Catalog #10981) may also be used.

B. Assay Controls

A polyclonal activator is often used as a positive control for the assay to confirm cell viability and functionality.

Example: Use 1 - 10 µg/mL of phytohemagglutinin (PHA). Alternatively, for monocyte and dendritic cell activation, use 1 - 10 µg/mL of lipopolysaccharide (LPS; e.g. Catalog #100-1270).

It is recommended to also include negative controls (e.g. unstimulated cells) in your assay to determine the number of cells spontaneously producing the analyte of interest. Wells with medium only may also be used to determine the presence of any background staining.

C. Detection Antibody

Dilute Anti-human GM-CSF mAb (23B6), biotin 1 in 1000 in PBS + 0.5% FBS (final concentration of 1 µg/mL). Diluted detection antibody may be filtered using a 0.2 µm syringe filter to reduce the risk of non-specific background.

Example: For one plate, add 10 µL Anti-human GM-CSF mAb (23B6), biotin to 10 mL PBS + 0.5% FBS.

D. SA-ALP

Dilute Streptavidin-ALP for ELISpot 1 in 1000 in PBS + 0.5% FBS.

Example: For one plate, add 10 µL Streptavidin-ALP for ELISpot to 10 mL PBS + 0.5% FBS.

E. BCIP/NBT Substrate Solution

Bring ELISpot substrate: BCIP/NBT-plus for ALP to room temperature (15 - 25°C) prior to use and filter using a 0.45 µm syringe filter.

Directions for Use

The following instructions are for detection of antigen-specific immune responses using PBMCs. If using other cell types (e.g. isolated cells), adjust accordingly.

Please read the entire protocol before proceeding. Sterile technique should be used for steps 1 - 5.

NOTE: In all washing steps, each well must be washed thoroughly and blotted dry by tapping the plate upside down on absorbent paper to remove any remaining liquid before adding the next reagent. Once the PVDF membrane is wetted, ensure it does not dry out during the assay.

NOTE: Cross-contamination of reagents may invalidate assay results. Permanently labeled, dedicated, multi-channel pipettor reservoirs for reagents are recommended.

1. Prepare samples as described in Preparation of Reagents and Materials.
2. Wash each well of Human GM-CSF pre-coated white plate four times with 200 µL PBS. Remove PBS completely.
3. Add 200 µL/well of cell culture medium used in Preparation of Reagents and Materials, section A to condition the plate. Incubate at room temperature (15 - 25°C) for at least 30 minutes.
4. Remove cell culture medium and add stimulus (e.g. antigen or polyclonal activator; 50 µL/well) followed by the cell suspension (e.g. 50 µL/well). Alternatively, cells and stimulus can be pre-mixed prior to addition to the plate.
5. Put the plate in a 37°C humidified incubator with 5% CO_2 and incubate for 18 - 48 hours. Cover the plate (e.g. by wrapping the plate in aluminum foil) to avoid evaporation.

   NOTE: Do not stack or move plate during incubation.
6. Remove cells and wash each well five times with 200 µL PBS and blot dry.

7. Add 100 µL/well of diluted Detection Antibody (see Preparation of Reagents and Materials, section C) and incubate at room temperature for 2 hours.
8. Wash each well five times with 200 µL PBS and blot dry.
9. Add 100 µL/well of diluted SA-ALP (see Preparation of Reagents and Materials, section D) and incubate at room temperature for 1 hour.
10. Wash each well five times with 200 µL PBS and blot dry.
11. Add 100 µL/well of BCIP/NBT Substrate Solution (see Preparation of Reagents and Materials, section E). Develop until distinct spots emerge (approximately 5 - 30 minutes).

    NOTE: Darkening of the membrane may occur during development, but it disappears after drying.
12. Wash the plate thoroughly with tap water to stop color development. If needed, remove the underdrain (i.e. soft plastic under the plate) and rinse the bottom of the membrane.
13. Leave the plate to dry overnight at room temperature.
14. Using an ELISpot reader (recommended) or dissection microscope, inspect and count spots. Each spot represents the footprint of an individual cell.

    NOTE: The number of cells responding to a stimulus should be compared to the number of cells spontaneously secreting the analyte in your unstimulated cells (i.e. negative control).
15. Store the plate at room temperature in the dark.

Troubleshooting

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
Blank wells	ELISA substrate or incorrect ELISpot substrate used	Ensure BCIP/NBT ELISpot substrate is used.
	Reagents not added in correct sequence or missed from some wells	Follow the sequence of incubation and wash steps as outlined in Directions for Use. Wells may be able to be recovered by repeating Step 6 onward in the Directions for Use.
Too few spots	Low seeding density	Increase seeding density. Follow the suggested seeding density outlined in Preparation of Reagents and Materials, section A.
	Low/no cell viability	Ensure cell viability is ≥ 90% before beginning the assay. Include a positive control to confirm cells are viable and functional.
Too many spots	High seeding density	Reduce seeding density. Follow the suggested seeding density outlined in Preparation of Reagents and Materials, section A.
	Stimulation time too long	Incubation time may need to be optimized depending on the stimulus used. Follow recommended incubation time outlined in Directions for Use.
High spot count in negative control wells	Cells not rested	Ensure cryopreserved cells are rested for ≥ 1 hour before use.
	Non-specific activation	Ensure cell culture medium does not contain any reagents that may stimulate the cells non-specifically.
	Contamination	Start assay with new cells and ensure sterile technique is used.
Spots very faint	Incorrect storage conditions or expired reagents	Ensure reagents are stored at the recommended storage conditions and have not passed their expiry dates.
	Incubation time too short	Follow the directions for the multiple incubation steps outlined in Directions for Use. Incubation time may need to be optimized depending on the stimulus used.
	Incorrect dilution of Detection Antibody or SA-ALP	Follow the directions for dilution of Detection Antibody and SA-ALP outlined in Preparation of Reagents and Materials.
	Spots underdeveloped	Ensure BCIP/NBT Substrate Solution is brought to room temperature before use. The substrate should be left on until distinct spots are visible by eye in the positive control wells (approximately 5 - 30 minutes).
Spots unevenly distributed or smeary	Cells unevenly distributed	It is recommended to first add the stimulus or medium to the wells, then the cell suspension. Ensure the plate is kept in a horizontal position during incubation.

PROBLEM	POSSIBLE CAUSE	RECOMMENDED ACTION
	Plate moved during incubation	Ensure the plate is not disturbed during the incubation period.
	Presence of cell clumps	A cell strainer may be used to remove aggregates from the cell suspension before use.
	PVDF membrane dried out	Work efficiently to ensure that PVDF membrane does not dry out between steps.
	Uneven dispersion of reagents	Gently tap the plate upside down on an absorbent tissue to remove any leftover wash buffer. After the addition of diluted antibody, ensure the solution covers the bottom of the well. Avoid bubble formation.
Poor replicates	Unequal volumes in wells	Ensure that pipettes function properly.
	Unequal cell numbers between wells	Ensure cell suspensions are thoroughly mixed before adding to each well.
	Carry-over between wells	Ensure pipette tips are changed between wells.
Dark membrane	Inappropriate serum selection	Use FBS or serum-free medium. FBS should be screened to ensure low background staining. Avoid using human serum as this may contain heterophilic antibodies or secreted analytes that can interfere with the assay.
	Presence of detergents	Avoid including Tween or other detergents in washing and incubation buffers.
	Presence of dimethyl sulfoxide (DMSO)	Ensure final DMSO concentration (if used) is < 0.5%.
	Presence of previously secreted analyte	Wash cells thoroughly and resuspend in fresh culture medium just before plating.
	Insufficient washing	Ensure that each well is washed five times with 200 µL PBS. Ensure that multi-channel pipettor fills and empties reproducibly without touching the reagents on the plate. Blot dry wells before proceeding to the next step. Increase cycles of washes and soaking time between washes.
	Overdevelopment	BCIP/NBT Substrate Solution should be left on until distinct spots are visible by eye in the positive control wells (approximately 5 - 30 minutes). Monitor the wells closely during this time.
	PVDF membrane still wet	Ensure the membrane is completely dry before imaging.
Assay artifacts	Presence of cell debris	Ensure cryopreserved cells are rested before use, debris is removed, and cell viability is ≥ 90%.
	Precipitate present in assay reagents or buffers	Ensure BCIP/NBT Substrate Solution is filtered (0.45 µm) before use. PBS and diluted Detection Antibody may also be filtered (0.2 µm) for optimal results.
	Membrane damaged by pipette tips	Ensure pipette tips do not touch the bottom of the wells.
	False positives	Check for false positives by plating wells with medium only (no cells).

Related Products

For a complete list of ELISpot kits, ELISA kits, and related products from STEMCELL Technologies, visit www.stemcell.com, or contact us at techsupport@stemcell.com.