

Human GM-CSF Development Set for ELISpot

Pre-titered capture antibody, detection antibody and streptavidin-AP for the development of enzyme-linked immunospot (ELISpot) assays for the quantitation of single cells releasing human GM-CSF.

Catalogue Number: SL10020B

Designed for 5 x 96 tests

FOR LABORATORY RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.



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INTENDED USE

Pre-titered capture antibody, detection antibody and streptavidin-AP for the development of enzyme-linked immunospot (ELISpot) assays for the quantitation of single cells releasing human GM-CSF.

A recommended assay protocol is provided. The dilutions of capture antibody, detection antibody, and streptavidin-AP is determined according to this protocol. The researcher can optimize the dilutions if it is necessary.

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INTRODUCTION

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is a member of the hematopoietic cytokine family, which includes interleukin-3 (IL-3) and interleukin-5 (IL-5). It is a pleiotropic cytokine that was one of the first growth factors characterized and shown to be necessary for the proliferation, differentiation, activation, and survival of hematopoietic cells. Human GM-CSF precursor (144 a.a.) is cleaved at the amino-terminal end to form a mature polypeptide (23 kDa, 127 a.a.) that contains two intramolecular disulfide bonds, which are important for biological activity and two potential N-glycosylation sites. A single gene on chromosome 5 codes for the human GM-CSF protein. Human GM-CSF shows 56-60% amino acid (a.a) homology to murine GM-CSF but does not exhibit cross-species biological activity or receptor binding.^{1,2,3} Glycosylation does not appear to be essential for biological activity, since recombinant GM-CSF unlike native GM-CSF is non-glycosylated and it still retains high biologic activity. However, this glycoprotein does show a decrease in affinity for its receptor as a result of non-glycosylation.²

Human GM-CSF is different from other family members in that it can be produced and act upon a much wider range of cell types. T-lymphocytes, B-lymphocytes, monocytes/macrophages, endothelial cells, fibroblasts, stromal cells, mesothelial cells, keratinocytes, osteoblasts, uterine epithelial cells, synoviocytes, mast cells, and various solid tumours produce GM-CSF. Usually a cytokine, inflammatory agent, or antigen is needed to stimulate the above cells to synthesize GM-CSF.^{2,3} For human GM-CSF to exert its biologic effects it will bind to a single class of cell surface receptors on hematopoietic and non-hematopoietic cells.⁴ The GM-CSF receptor has been cloned³ and, the α and β chains (80 kDa and 130 kDa) were found to members of the hematopoietin receptor family.

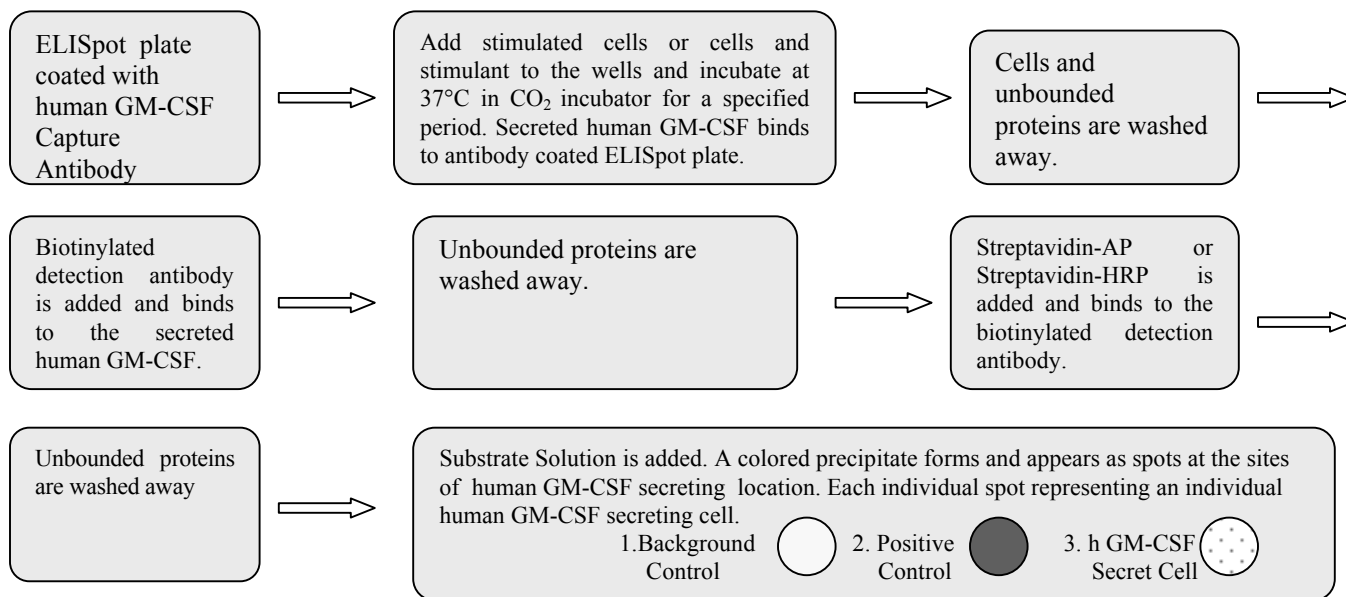
Numerous studies have shown diverse *in vitro* biological effects of GM-CSF on various cell types. GM-CSF can bind to pluripotent hematopoietic stem cells causing the proliferation and differentiation of various progenitor cells such as granulocyte and macrophage³, whereas eosinophil, erythroid and megakaryocyte colony formation is stimulated at much higher concentrations.^{2,3,5} GM-CSF is also required for growth and differentiation of typical dendritic cells from human bone marrow^{2,6}, causes activation and prolonged survival of mature hematopoietic cells^{2,3}, and activates mature neutrophils and eosinophils causing antibody dependent cellular cytotoxicity, phagocytosis, superoxide generation. Also, GM-CSF stimulates macrophage production of TNF, M-CSF, G-CSF, and IL-1, intensifies killing by granulocytes and macrophages³, and increases HIV-1 replication at the post-

transcriptional level.⁷ GM-CSF binds to non-hematopoietic cells causing the proliferation and/or migration of fibroblast, endothelial, and various tumour cell lines.^{8,9} The significance of GM-CSF receptor expression on these non-hematopoietic cell types is unknown. Very little is known about the *in vivo* biological effects of GM-CSF in various pathological states. However *in vivo* studies showed a significant eosinophilic response and macrophage granuloma formation accompanied with tissue damage when GM-CSF was overexpressed in the rat lung. Thus role GM-CSF may play a role in the development of fibrotic reactions.¹⁰ *In vivo*, GM-CSF induces the upregulation of CD11b on neutrophils, induces temporary neutrophil sequestration in the lung, followed by specific granule release, and enhanced *ex vivo* production of superoxide anion on neutrophils.¹¹

Various pathological conditions are associated with increased GM-CSF levels. These include: lung cancer,¹² acute myelogenous leukemia,¹³ tumour related thrombocytosis,¹⁴ myelodysplastic syndrome (MDS),¹⁵ thrombocytopenia,¹⁶ and psoriasis.¹⁷ GM-CSF expression is increased in bronchial asthma and lung inflammatory diseases;^{9,18} non-allergic respiratory diseases such as eosinophil pneumonia, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, sarcoidosis, cryptogenic organizing pneumonia, HIV infection,⁹ rheumatoid arthritis, and systemic lupus erythmatosus.¹⁹ GM-CSF shows therapeutic value by accelerating neutrophil recovery in disease induced myelosuppression such as bone marrow transplantation, chemotherapy, and infectious disease.^{2,3} It is suggested that a GM-CSF may be useful in autologous bone marrow transplantation to detect GM-CSF toxicity for the diagnosis of post-transplant liver disease²⁰ and in gestational trophoblastic disease (GTD) for the early identification of high risk choriocarcinoma cases.²¹

This ELISpot kit is developed to detect and visualize of single cells secreting human GM-CSF.

PRINCIPLES OF THE ASSAY



REAGENTS PROVIDED

All reagents provided are stored at -20°C. Refer to the expiration date on the label.

Name (Part No.)	Size	Description	Usage and Storage
1) Concentrated Human GM-CSF Capture Antibody (Part SL10020B-1)	1 Vial	Lyophilized mouse anti-human GM-CSF monoclonal antibody	<u>Stock Solution:</u> Reconstitute Concentrated Human GM-CSF Capture Antibody with 0.6 mL PBS. Aliquot if repeated use is expected. The stock solution can be stored frozen (-20°C to -70°C) for up to 6 months. Avoid freeze-thaw cycles. <u>Working Solution:</u> When PVDF -bottom Immunospot plates are used, the recommended dilution is <u>1: 100</u> . Calculate the volume of Capture Antibody Stock Solution needed and dilute to working solution in PBS. Use in 1 hour.
2) Concentrated Human GM-CSF Detection Antibody (Part SL 10020B-2)	1 Vial	Biotinylated mouse anti-human GM-CSF monoclonal antibody	<u>Stock Solution:</u> Reconstitute with 0.6 mL Reagent Diluent. Aliquot if repeated use is expected. The stock solution can be stored frozen (-20°C to -70°C) for up to 6 months. Avoid freeze-thaw cycles. <u>Working Solution:</u> When PVDF -bottom Immunospot plates are used, the recommended dilution is <u>1: 100</u> . Calculate the volume of Detection Antibody Stock Solution needed and dilute to working solution in Reagent Diluent. Use in 1 hour.
3) Concentrated Streptavidin - AP (Part SL 10020B-3)	1 Vial	120µL of Alkaline Phosphatase labeled Streptavidin.	When PVDF -bottom Immunospot plates are used, the recommended dilution is <u>1: 500</u> . Calculate the volume of Streptavidin - AP Concentrated needed and dilute to working solution in Reagent Diluent. Use in 1 hour.

MATERIALS REQUIRED BUT NOT SUPPLIED

- PBS**
137mM NaCl, 2.7mM KCl, 8.1mM Na₂HPO₄, 1.5mM KH₂PO₄, pH 7.2-7.4, 0.2µm filtered.
- Wash Buffer**
0.05% Tween-20 in PBS.
- Blocking Buffer**
1% BSA, 5% Sucrose in PBS, 0.2µm filtered.
- Reagent Diluent**
1% BSA in PBS.
- Positive Control (Recommended)**
Recombinant human GM-CSF (2ng/vial, Yes Biotech, Catalogue Number SL10020C) or equivalent.
- ELISpot Plates**

PVDF -bottom Immunospot plates or equivalent.

7. Substrate Solution

Yes Biotech BCIP/NBT Substrate Solution for ELISpot (10mL/bottle, Yes Biotech, Catalogue Number SS6006) or equivalent.

8. Pipettes with disposable tips, test tubes and racks, graduated cylinders, absorbent paper, and squirt bottle.

9. 37°C CO₂ incubator.

10. Deionized or distilled water.

11. Dissection microscope or ELISpot reader.

PRECAUTIONS

1. Allow kit reagents and materials to reach room temperature (20-25°C) before use.
2. Do not use kit components beyond their expiration date. Do not substitute reagents from one kit lot to another.
3. The toxicity of the Substrate Solution is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used Substrate Solution.
4. If 20 x Wash Buffer Concentrated is stored at lower temperature (2-8 °C), crystals may form which must be dissolved by warming prior to use.
5. When samples are added to the wells, don't let the pipette tips contact the membrane.
6. Don't let the plate dry during the assay.
7. In order to avoid edge effect don't stack plates during cell incubation.
8. Avoid move the plate during cells incubation period.
9. Don't dry the plate at a temperature higher than 37° C.
10. Spots can't be counted accurately until PVDF membranes were completely dry.

SAMPLE PREPARATION

Each researcher should optimize cell separation method, stimulant, stimulation mode and incubation time.

A recommended method to stimulate human GM-CSF secretion from peripheral blood mononuclear cells (PBMCs) is as following:

1. Add 10⁵ /mL PBMCs in 1ug / mL lipopolysaccharide (LPS).
2. Incubate for 12-24 hours at 37° C in CO₂ incubator.
3. Test according to this protocol.

ASSAY PROCEDURE

Aseptic Procedures: Steps 1 to 7 are aseptic procedures. Use sterile buffers and aseptic conditions, use laminar flow hood for procedures.

1. Prepare Human GM-CSF Capture Antibody Working Solution
As described in **REAGENT PROVIDED.**

2. Add 100 μ L of Human GM-CSF Capture Antibody Working Solution to each well of the plate. Cover the plate and incubate overnight at 2-8 °C.
3. Wash 3 times with PBS
Decant or aspirate contents of the plate into a waste container. Fill each well completely with PBS then decant or aspirate contents of the plate into a waste container. Repeat this procedure 2 more times for a total of 3 washes. After final wash, invert plate, and dry by tapping plate onto absorbent paper slightly.
4. Blocking
Immediately add 200 μ L of Blocking Buffer to each well of the plate. Cover the plate and incubate 2 hours at 37°C.
5. Prepare Positive Control
We recommend adding 2 wells positive control. If Yes Biotech GM-CSF Positive Control (2ng/vial, Catalogue Number SL10020C) was used, add 250 μ L Cell Culture Media to each vial. The final concentration is 8 ng/mL. Use within 1 hour of reconstituting. The reconstitution can be stored frozen (-20°C) for up to 30 days.
6. Wash 1 time with Cell Culture Media
Decant or aspirate contents of the plate into a waste container. Fill each well completely with Cell Culture Media. Don't discard until cells are ready to be plated.
7. Decant or aspirate contents of the plate into a waste container, invert plate, and dry by tapping plate onto absorbent paper slightly. Immediately add 100 μ L GM-CSF secreting cells with appropriate concentration to each well. We recommend adding 2 wells positive control, 2 wells negative control (unstimulated cells), and 2 wells background control (sterile cell culture media) in each plate, 100 μ L/well. Incubate at 37°C CO₂ incubator for 4-48 hours. Each researcher should determine the optimal incubation time based on the characteristics of the cell.

Non-aseptic Procedures: *The following steps are non-aseptic procedures.*

8. Prepare Human GM-CSF Detection Antibody Working Solution and Streptavidin - AP Working Solution
As described in **REAGENT PROVIDED**.
9. Wash the plate 5 times with Wash Buffer
Decant or aspirate contents of the plate into a waste container. Fill each well completely with Wash Buffer then decant or aspirate contents of the plate into a waste container. Repeat this procedure 4 more times for a total of 5 washes. After final wash, invert plate, and dry by tapping plate onto absorbent paper slightly.
10. Immediately add 100 μ L of Human GM-CSF Detection Antibody Working Solution to each well of the plate. Cover the plate and incubate 1hour at room temperature (20-25 °C).
11. Repeat wash procedure as described in step 9. Wash plate 5 times.
12. Immediately add 100 μ L of Streptavidin-AP Working Solution to each well of the plate. Cover the plate and incubate 1hour at room temperature (20-25 °C).
13. Repeat wash procedure as described in step 9. Wash plate 5 times.
14. Immediately add 100 μ L of Substrate Solution to each well of the plate. If Yes Biotech BCIP/NBT Substrate Solution for ELISpot (10mL/bottle Catalogue Number SS6006) is used, cover the plate and incubate 5-15 minutes at room temperature (20-25 °C) in dark. Each researcher may optimize the incubate time depending on the plate or substrate solution used.
15. Stop the assay

Rinse 5 times with deionized water/distilled water. After final wash, invert plate, and dry by tapping plate onto absorbent paper slightly.

16. Dry plate

Wet plates show higher background than completely dry plates. Remove the plastic underdrain of the plate. Allow the plate dry for 60-90 min at room temperature, or over night at room temperature, or 15-30 min at 37° C in dark. We recommend dry plate over night at room temperature.

17. Quantify spots using a dissection microscope or ELISpot reader.

18. Dried plate can be stored in sealed plastic bag in dark for 6 months.

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