ELISA Kit
Catalog #KHC2011

Human
GM-CSF

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INTRODUCTION

Human Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF; CSF-2; CSF-α) is a 127 amino acid protein with an apparent molecular weight ranging between 14 to 35 kDa due to variable glycosylation. This cytokine is produced by a large variety of cells including macrophages, endothelial cells and activated T cells. *In vitro* GM-CSF recruits different progenitors depending on its concentration; at its lowest, inducing macrophage colonies, increasing concentrations inducing granulocyte, eosinophil and finally megakaryocyte colonies. In addition to its growth promoting activity, GM-CSF also induces granulocyte and macrophage differentiation.

PURPOSE

The Invitrogen Human Granulocyte-Macrophage Colony Stimulating Factor (Hu GM-CSF) ELISA is to be used for the quantitative determination of GM-CSF in human serum, plasma, buffered solution, or cell culture medium in the range of 7.8 to 500 pg/mL. The assay will exclusively recognize both natural and recombinant GM-CSF.

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen GM-CSF kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for GM-CSF has been coated onto the wells of the microtiter strips provided. Samples, including standards of known GM-CSF content, control specimens, and unknowns, are pipetted into these wells after the addition of a biotinylated second monoclonal antibody.

During the first incubation, the GM-CSF antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.

After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of GM-CSF present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
Hu GM-CSF Standard, recombinant Hu GM-CSF. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer, *25* mL per bottle. Contains 0.05% Proclin® 300, 1.3 mM thymol and 1.5 mM sodium azide.	1 bottle
Hu GM-CSF Antibody-Coated Wells, 96 wells per plate.	1 plate
Hu GM-CSF Biotin Conjugate, (Biotin-labeled anti-GM-CSF), 15 mL per bottle. Contains human serum that tested negative for HIV and Hepatitis. Contains 9 mM sodium azide, 0.05% Proclin® 300 and 1.3 mM thymol.	1 bottle
Streptavidin-Peroxidase (HRP), (100x) concentrate. 0.125 mL per microtube. Contains 0.05% Proclin® 300.	1 vial
Streptavidin-Peroxidase (HRP) Diluent, 25 mL per bottle. Contains 0.05% Proclin® 300.	1 bottle
Wash Buffer Concentrate (25x). 100 mL per bottle.	1 bottle
Stabilized Chromogen, Tetramethylbenzidine (TMB). 25 mL per bottle.	1 bottle
Stop Solution. 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3

Disposal Note: This kit contains materials with small quantities of sodium azide and Proclin® 300. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Proclin® 300 is toxic. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.

3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. Samples that are >500 pg/mL should be further diluted with *Standard Diluent Buffer*.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots**.
11. Do not use reagents after the kit expiration date.
12. Read absorbances within 2 hours of assay completion.
13. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed. High pressure jets of some automated washers may adversely affect reproducibility.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Hu GM-CSF Standard

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute the standard to 6000 pg/mL with *Standard Diluent Buffer*. Refer to the standard vial label for instructions. Swirl gently and allow to sit for 10 minutes to ensure complete reconstitution.
2. Add 0.05 mL of the reconstituted standard to a tube containing 0.55 mL *Standard Diluent Buffer*. Label as 500 pg/mL GM-CSF. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 250, 125, 62.5, 31.2, 15.6, and 7.8 pg/mL GM-CSF.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of GM-CSF

Standard:	Add:	Into:
500 pg/mL	Prepare as described in Step 2.	
250 pg/mL	0.300 mL of the 250 pg/mL std.	0.300 mL of the Diluent Buffer
125 pg/mL	0.300 mL of the 125 pg/mL std.	0.300 mL of the Diluent Buffer
62.5 pg/mL	0.300 mL of the 62.5 pg/mL std.	0.300 mL of the Diluent Buffer
31.25 pg/mL	0.300 mL of the 31.2 pg/mL std.	0.300 mL of the Diluent Buffer
15.6 pg/mL	0.300 mL of the 15.6 pg/mL std.	0.300 mL of the Diluent Buffer
7.8 pg/mL	0.300 mL of the 7.8 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing assay. Return *Standard Diluent Buffer* to the refrigerator.

C. Storage and Final Dilution of Streptavidin-HRP

Please Note: The *Streptavidin-HRP* 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP* concentrate to reach room temperature. Gently mix. Pipette *Streptavidin-HRP* concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of the 100x concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP Concentrate	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

D. Dilution of Wash Buffer

Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x wash buffer concentrate with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 50 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for the chromogen blank should be left empty.
3. Add 50 µL of standards, samples or controls to each well.
4. Add 150 µL of biotinylated anti-GM-CSF (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Mix thoroughly on a plate shaker or by gently tapping on the side of the plate.
5. Cover plate with *plate cover* and incubate for **1 hour and 30 minutes at room temperature**.

6. Thoroughly aspirate or decant solution from wells and discard the liquid. Gently wash wells 4 times. See **DIRECTIONS FOR WASHING**.
7. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE,** Section C.)
8. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Gently wash wells 4 times. See **DIRECTIONS FOR WASHING**.
10. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
11. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The microtiter plate reader used often determines the incubation time for chromogen substrate. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
12. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

13. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.
14. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
15. Read the Hu GM-CSF concentrations for unknown samples and controls from the standard curve plotted in Step 14. Samples producing signals greater than that of the highest standard (500 pg/mL) should be diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 500 pg/mL Hu GM-CSF.

Standard Hu GM-CSF (pg/mL)	Optical Density (450 nm)
0	0.116
7.8	0.171
15.6	0.224
31.2	0.341
62.5	0.529
125	0.859
250	1.339
500	2.514

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 500 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples >500 pg/mL with *Standard Diluent Buffer*. Reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Hu GM-CSF in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies, binding molecules and soluble receptors. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Hu GM-CSF is <3 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 48 times.

PRECISION

1. Intra-Assay Precision

Samples of known GM-CSF concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	23.8	60.0	201
SD	1.38	2.38	14.7
%CV	5.8	4.0	7.3

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were measured 8 times in separate assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	23.7	68.0	215
SD	1.12	2.9	11.7
%CV	4.7	4.3	5.4

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Pools of human sera, EDTA plasma, heparinized plasma, urine or RPMI containing 10% FCS were spiked at 200 pg/mL with Hu GM-CSF and serially diluted in *Standard Diluent Buffer* over the range of the assay. All linear regression analyses of samples versus the expected concentrations yielded correlation coefficients of 0.99.

RECOVERY

The recovery of Hu GM-CSF added to tissue culture medium containing 10% fetal bovine serum averaged 105% (CV 5.1%). Recoveries of GM-CSF spiked into human serum, EDTA plasma, heparinized plasma and urine averaged respectively 94% (CV 4.9%), 99% (CV 7.8%), 98% (CV 5.3%) and 98% (CV 4.1%). Citrated plasma lead to poor recoveries (127%; CV 10%) and should be avoided.

SPECIFICITY

Buffered solutions of a panel of substances at 150 ng/mL were assayed with the Invitrogen Hu GM-CSF kit. The following substances were tested and found to have no cross-reactivity: human G-CSF, IFN-γ, TNF-α, IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, LIF, M-CSF, MCAF, MCP3, MIP-1α, MIP-1ß, Oncostatin-M, RANTES. Mouse GM-CSF does not react with this assay at levels up to 200 ng/mL.

HIGH DOSE HOOK EFFECT

A sample spiked with 150 ng/mL of GM-CSF gives a response higher than that obtained for the last standard point.

EXPECTED VALUES

Twenty-three sera, 25 EDTA plasma, and 19 heparin plasma samples from apparently healthy individuals were evaluated in this assay. All samples measured less than the lowest Hu GM-CSF standard (3.9 pg/mL), the highest value being one serum with an extrapolated value of 1.8 pg/mL.

Cell culture supernatants were evaluated in this assay. Fresh heparinized blood from 8 human donors was diluted five-fold in RPMI and cultured for 4, 24, 48 or 72 hrs. without stimulation, with a blend of LPS (25 µg/mL) and PHA (5 µg/mL), or with a blend of ionomycin (100 ng/mL) and PMA (100 ng/mL).

Release of Hu GM-CSF (pg/mL)

Culture Conditions	Time of Culture (h)			
	4	24	48	72
Unstimulated	<3.9	<3.9	<3.9	<3.9
LPS + PHA	<3.9	42	167	565
PMA + Ionomycin	<3.9	614	1298	2042

Highest and lowest Hu GM-CSF values for 72 hr. cell cultures were 5300 pg/mL - 80 pg/mL for the LPS and PHA stimulations and 4307 pg/mL - 1061 pg/mL for the ionomycin and PMA stimulations. Density gradient purified peripheral blood mononuclear cells (PBMCs) from two donors cultured at initial blood cell density in RPMI supplemented with 10% endotoxin-free FCS led to values similar to those observed with the whole blood cultures.

REFERENCES

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2. Costello, T. T. (1993) Acta Oncol. 32:403.
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Explanation of symbols

Symbol	Description	Symbol	Description
	Catalogue Number		Batch code
	Research Use Only		*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer		European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

NOTES

Human GM-CSF Assay Summary

Add 50 µL of standards, controls & samples

Add 150 µL of Biotin Conjugate
Incubate for 90 minutes at RT

aspirate and wash 4x

Incubate 100 µL of Streptavidin-HRP
Working Solution for 30 minutes at RT

aspirate and wash 4x

Incubate 100 µL of Stabilized Chromogen
for 30 minutes at RT

Add 100 µL of Stop Solution
and read at 450 nm

Total time: 2.5 hours