Detection of fg/mL Levels of GM-CSF in Human Serum and Plasma

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❶ Abstract

Background: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a key cytokine in the adaptive and innate immune systems. The basal levels of GM-CSF in blood are in the fg/mL range, too low to be measurable with standard ELISAs.

Methods: To enable accurate measurements of GM-CSF, MSD developed an ultrasensitive S-PLEX℠ immunoassay and evaluated its performance with clinical samples. Monoclonal antibodies were screened and selected based on their sensitivity, specificity, and affinity for GM-CSF secreted by a differentiated THP-1 cell line. Development and characterization of the S-PLEX immunoassay were performed using a recombinant GM-CSF anchored to the WHO International Standard (NIBSC code: 88/646). Normal sera and plasma specimens and several cell models were screened to assess performance of the ultrasensitive assay.

Results: The S-PLEX GM-CSF assay provides a limit of detection (LOD) of < 1.0 fg/mL with a dynamic range of 4 orders of magnitude. The lower and upper limits of quantitation are 2.5 fg/mL and 8,000 fg/mL, respectively. The intra-plate coefficients of variation (CVs) were 4-16% and the inter-plate CVs were 7-14% (16 replicates on 4 plates). The average spike recovery of GM-CSF was 96% and the dilution linearity average recovery was 92% (n= 9 specimens). Analyte specificity was demonstrated by depleting 8 specimens (sera and plasma) with commercial anti-GM-CSF antibodies. Depletion results showed that the assay measures the true analyte, rather than some other non-specific interaction. GM-CSF was detectable in 100% of apparently healthy specimens (n=59)* with a median value of 44 fg/mL in serum (n=40) and 25 fg/mL in EDTA plasma (n=19). GM-CSF did not appear to be elevated in the autoimmune or sepsis specimens tested. An undifferentiated monocytic cell line, THP-1, was shown to secrete GM-CSF only after differentiation into a mature macrophage. GM-CSF secretion in a HaCaT cell line decreased substantially after differentiation into mature keratinocytes.

*After submission of abstract, additional samples were tested. In total, 40 serum and 19 plasma specimens have been tested.

❷ Methods
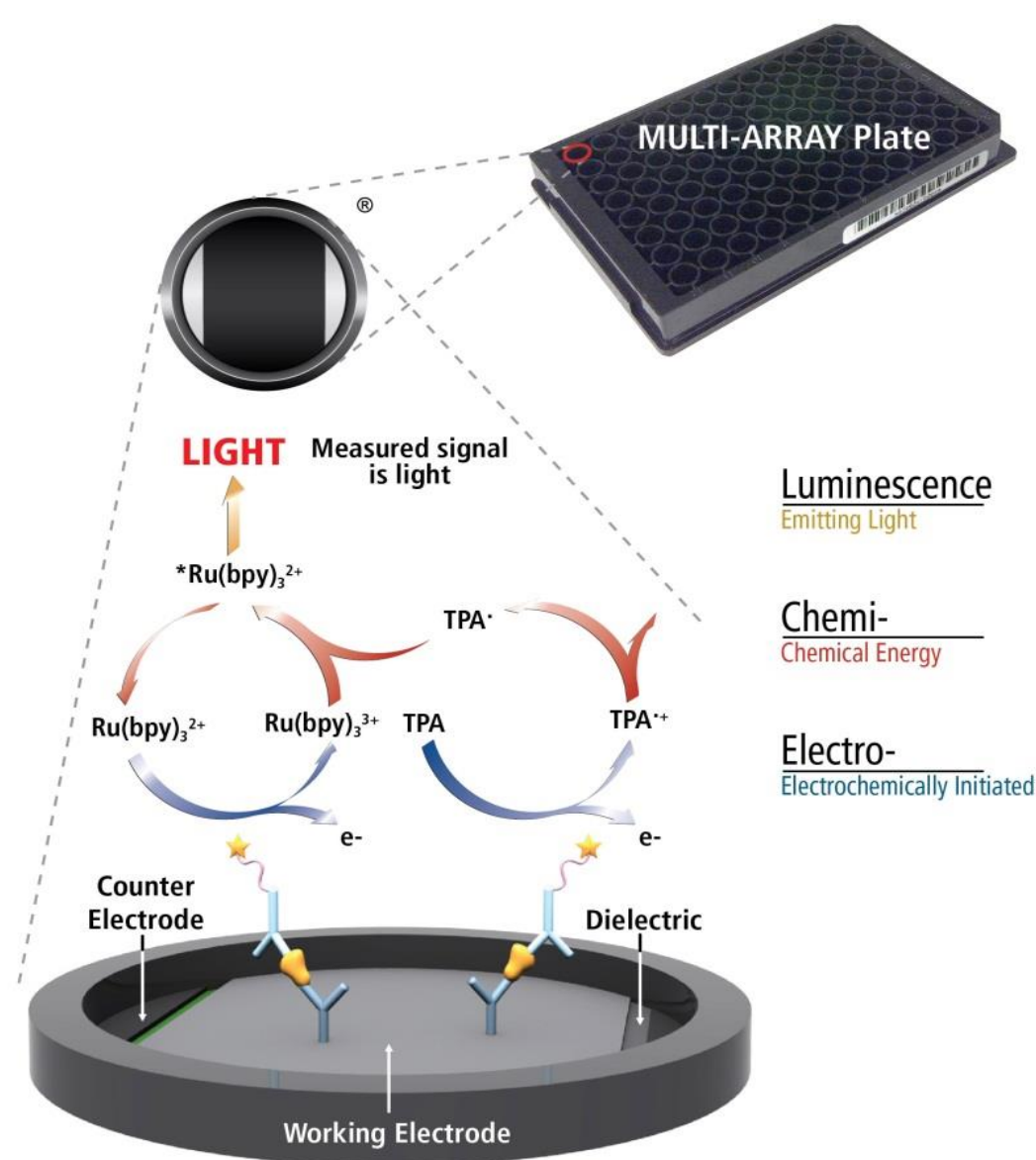
Electrochemiluminescence Technology

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates. We developed the S-PLEX assay platform, a next-generation MULTI-ARRAY technology with significantly higher sensitivity.

❸ Calibrator Curve
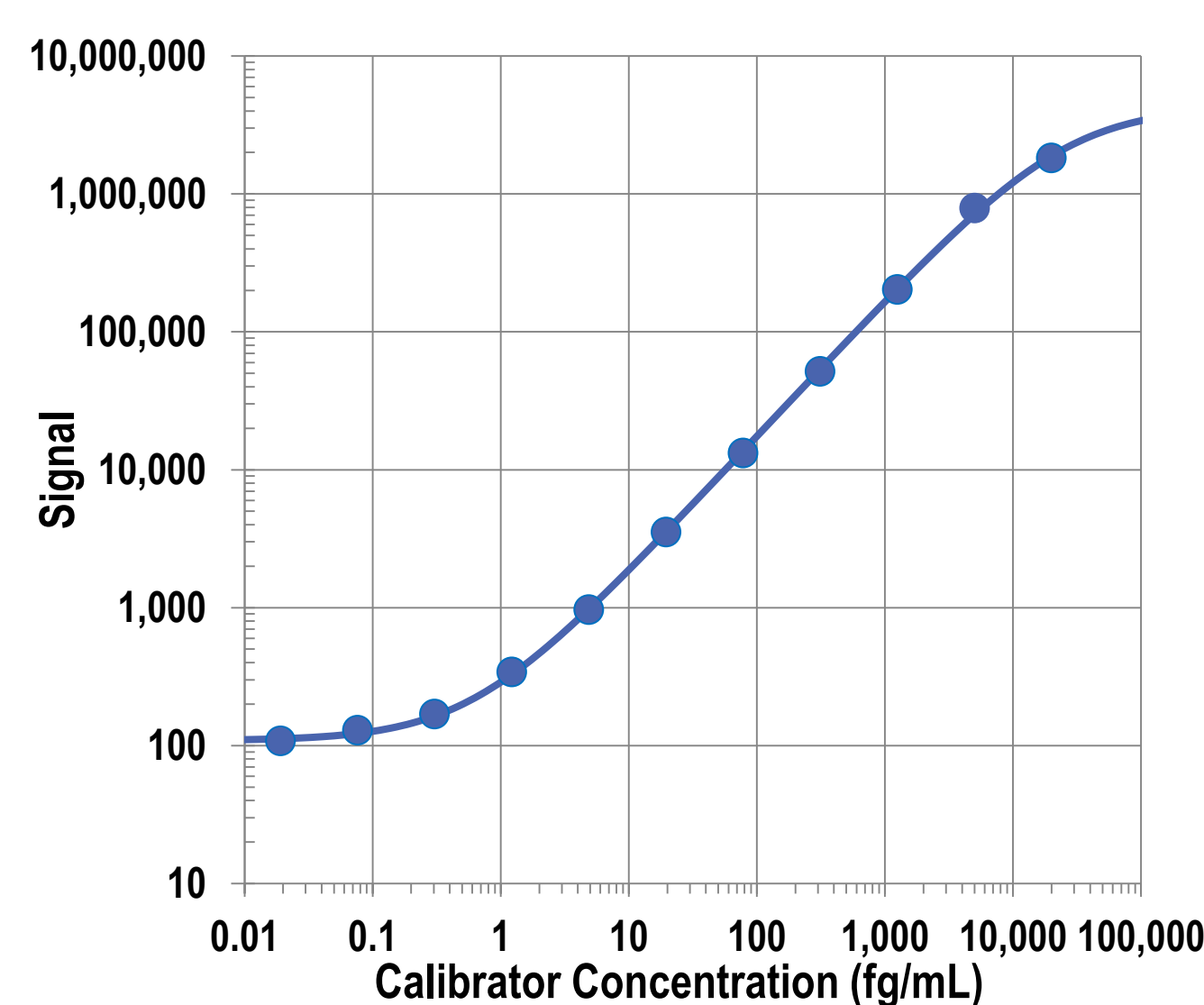
The extended calibration curve is representative. Limit of detection (LOD) was typically below 1.0 fg/mL.

❹ Lower Limit of Quantitation (LLOQ)

Lower limit of quantitation was determined using *E. coli* expressed recombinant human GM-CSF. Testing included four replicates per plate, four plates, and was performed in one day. Results are shown below. The LLOQ was determined to be 2.5 fg/mL; intra-plate and inter-plate concentration %CVs were < 20%.

Expected Concentration (fg/mL)	Average Measured Concentration (fg/mL) [n=16]	Accuracy (Average % Recovery)	Average Intra-plate Concentration (%CV) [n=4]	Average Inter-plate Concentration (%CV) [n=4]
8,000	7,829	98	4.5	2.4
5,600	5,223	93	4.8	1.3
204	187	92	4.8	3.5
10	9	94	9.0	2.5
5	5	93	10.3	1.2
2.5	2	91	9.8	11.6
1.3	1	85	19.8	7.4
Specification Range		70-130	< 20	< 20

Limit of Detection (fg/mL)	0.3
Lower Limit of Quantitation (fg/mL)	2.5

❺ Spike Recovery

Three serum, EDTA plasma, and heparin plasma specimens were spiked with three concentrations of recombinant GM-CSF. A diluent control was tested as well. All measurements were performed in quadruplicate and the average recovery was 96%.

Spike Level (fg/mL)	Serum		EDTA Plasma		Heparin Plasma	
	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
500	99	97-101	97	95-99	98	95-103
250	95	94-96	94	92-96	95	91-98
125	96	93-98	97	93-100	94	92-97

❻ Dilution Linearity

Three EDTA plasma, three heparin plasma, and two serum samples were prepared by spiking with supernatant from ConA stimulated peripheral blood mononuclear cells (PBMC). The GM-CSF level in one serum specimen was high enough to measure without spiking with analyte. Linearity was determined from 2-, 4- and 8-fold dilutions. All measurements were performed in quadruplicate and average recovery was 92%.

Fold Dilution	Serum		EDTA Plasma		Heparin Plasma	
	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range	Average % Recovery	% Recovery Range
2	88	83-91	89	86-92	90	89-90
4	84	74-91	89	86-92	88	79-93
8	87	78-94	89	84-93	92	87-98

## ❼ Depletion Study: Assay Specificity Verification
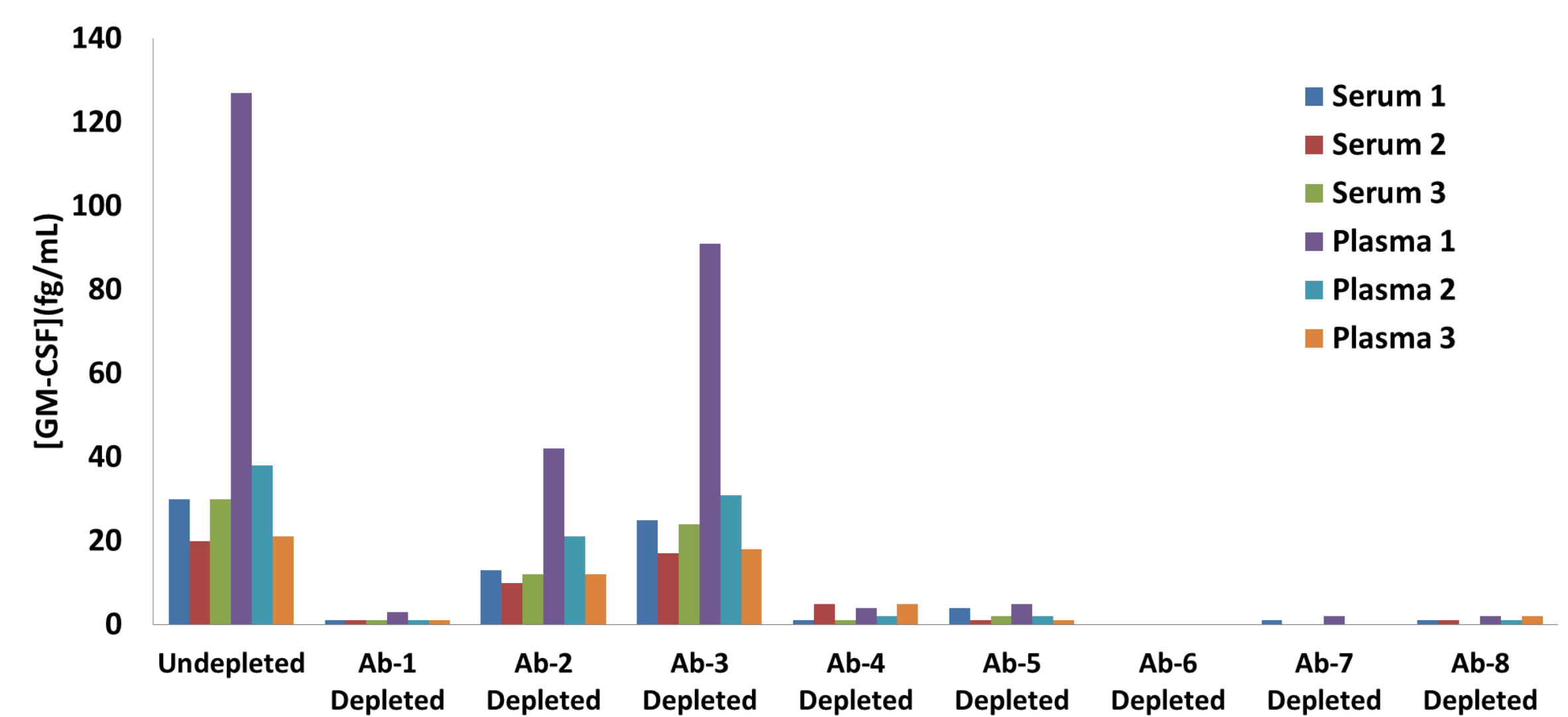
- Specificity of the GM-CSF assay was demonstrated with a depletion study. Eight anti-GM-CSF antibodies from four distinct commercial vendors and a control antibody (mouse IgG) were conjugated to magnetic beads, and used to deplete GM-CSF from three serum and three plasma samples.
- Pooled serum and diluent were spiked with supernatant from ConA stimulated PBMC. These controls were depleted similarly to apparently healthy specimens.
- With the exception of Antibody-2 and -3, all of the antibodies used were able to deplete GM-CSF efficiently. These results suggest that the analyte measured by the GM-CSF assay was in fact GM-CSF and not an artifact from the sample matrix.

❽ Sample Screening
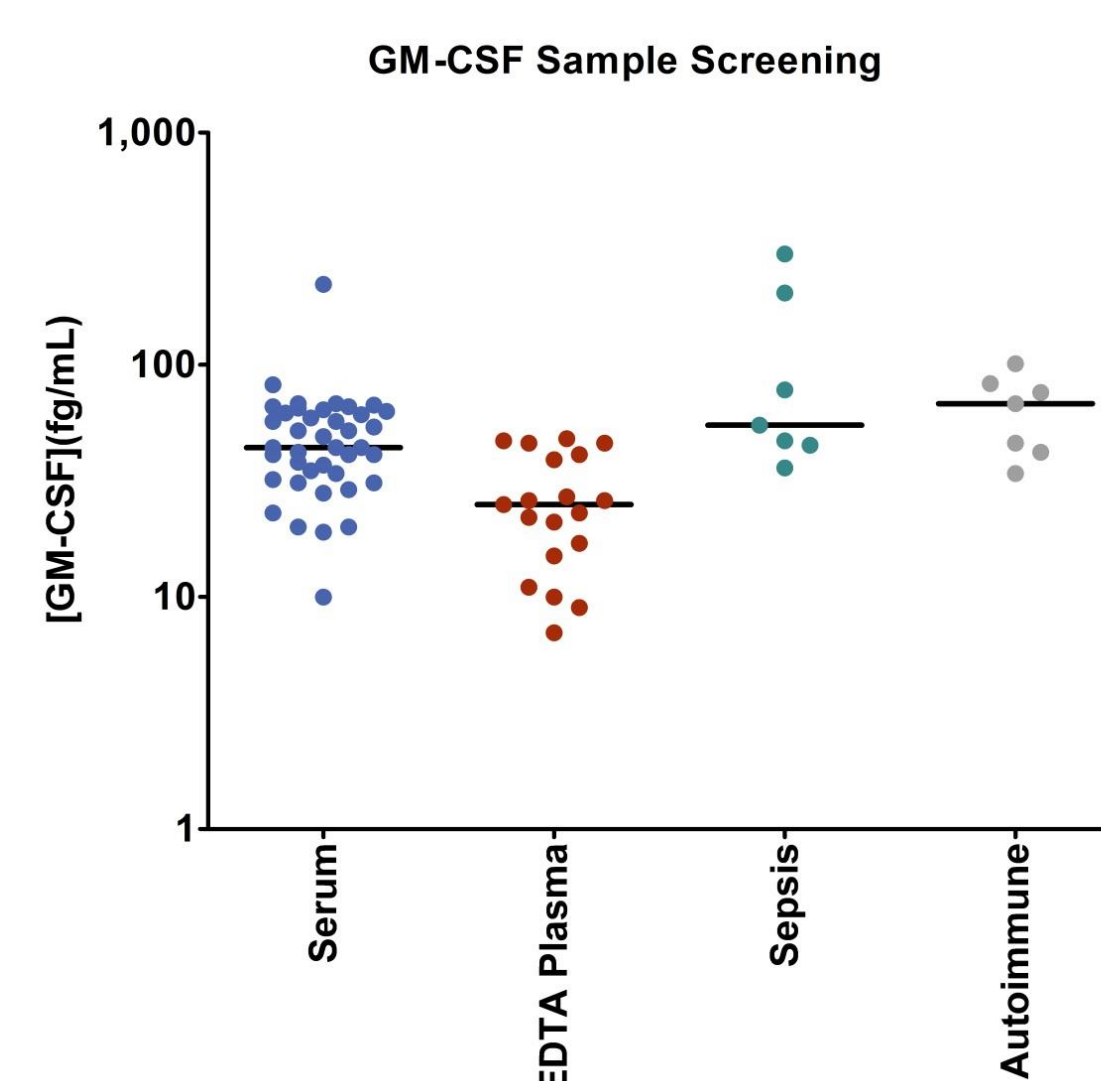
59 apparently healthy donors, 40 serum and 19 EDTA plasma samples, were tested. 100% of the samples had detectable levels of GM-CSF, with median levels of 44 fg/mL and 25 fg/mL for serum and EDTA plasma, respectively. A limited number of sepsis and autoimmune disease state samples (n=7) did not show elevated levels of GM-CSF relative to apparently healthy donors.

❾ Cell Model Verification
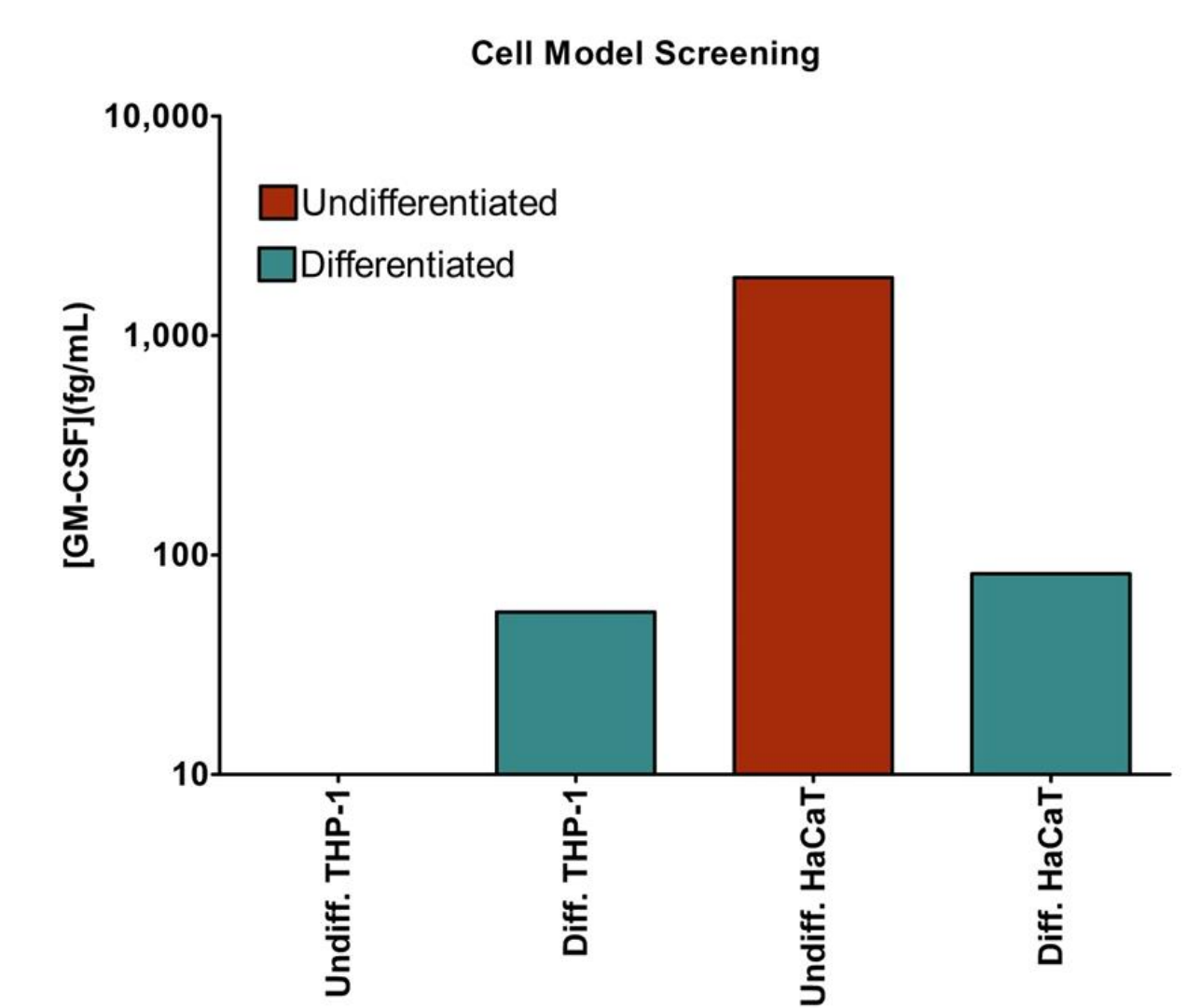
Cell models were used to verify the specificity of the GM-CSF assay. Differentiation of a monocytic cell line, THP-1 cells, into macrophages resulted in an increase in production of GM-CSF, from 0 to 55 fg/mL. Also, the basal cell phenotype of HaCaT cells is known to produce GM-CSF. Upon differentiation of the HaCaT cell line into mature keratinocytes, GM-CSF secretion in a HaCaT cell line decreased from 1,800 fg/mL to 80 fg/mL.

❿ Conclusion

This study demonstrated the performance of an ultrasensitive GM-CSF assay, with an LLOQ of 2.5 fg/mL (< 20%CV) and an LOD of <1.0 fg/mL. Analytic assay performance was characterized by an average spike recovery of 96% and linearity of 92%. The S-PLEX GM-CSF assay analyte specificity was suggested by depletion assays with serum and plasma samples. Additional assay specificity was verified via cell model screening, with the assay confirming expected levels of GM-CSF secretion. Furthermore, the S-PLEX assay format was able to quantify GM-CSF in 100% of the samples screened. The S-PLEX assay improves GM-CSF measurement and may further help in understanding the role of GM-CSF in inflammatory diseases.

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Summary of Assay Performance	
Limit of Detection	<1.0 fg/mL
Estimated Lower Limit of Quantitation	2.5 fg/mL
Median Normal Serum concentration (n=40)	44 fg/mL
Median Normal EDTA Plasma concentration (n=19)	25 fg/mL
Percentage of samples within assay range (n=73)	100%

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