PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Controls, Sample Diluent, and HRP Antibody. Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water.

MSDS for TMB, sulfuric acid, if not already on file, can be requested or obtained from the ADI website.

NOTES

Human G-CSF

ELISA Kit Cat. No. 0080

For Quantitative Determination of Human Granulocyte Colony-Stimulating Factor (G-CSF) in Biological Solutions

ALPHA DIAGNOSTIC INTERNATIONAL

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DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

INTENDED USE

The Human G-CSF ELISA Kit is an in vitro immunoassay for research use in the quantification of G-CSF in cultures of human cells and in appropriately qualified samples from serum, saliva, or other tissue fluids or extracts.

RESEARCH USE OF THE TEST

Human granulocyte colony-stimulating factor (hG-CSF) is a glycoprotein cytokine or growth factor, occurring in 2 forms (174- and 180-aa) of about 19,600MW, produced by a number of different tissues, including monocytes, macrophages, fibroblasts and epithelium, to stimulate the bone marrow to produce granulocytes and stem cells. In addition, G-CSF stimulates proliferation, differentiation, survival and release into the blood of precursors and mature neutrophils.

Recombinant hG-CSF has been used as a pharmaceutical product to treat myelo-suppresive chemotherapy for cancer patients, and has especially helped advance high-dose chemotherapy regimens, by stimulating white blood cell production and allowing for neutrophil recovery. hG-CSF is also used to increase hematopoietic sstem cells in the donor prior to collection for stem cell transplantation. Recently, hG-CSF has ben conjugated to polyethyleneglycol (called pegylation) to provide an effective drug that has essentially no renal clearance, and promises to increase the dose-effectiveness of hG-CSF in circulation. The behavior of pegylated hG-CSF in this ELISA has not been investigated.

PRINCIPLE OF THE TEST

The Human G-CSF ELISA kit is based on the binding of human G-CSF in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to biotin, which then binds to a streptavidin horseradish peroxidase (HRP) conjugate. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of hG-CSF present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of hG-CSF in samples is calculated from a standard curve of purified recombinant human G-CSF of designated concentration.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8^{o}C until the expiration date printed on the Kit label. Stabilities of the working solutions are indicated under Reagent Preparation.

PERFORMANCE CHARACTERISTICS

Range: 15.6pg/ml-1000pg/ml
Sensitivity: < 1pg/ml
Specificity: Natural and recombinant human GM-CSF
Cross-reactivity: No detectable cross-reactivity with other relevant proteins Culture Medium

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Sample Controls Each lab should also assay internal control samples, which represent the lab's expected sample population and that are maintained stabilized. A Diluent only Negative Control should also be run.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-continuously increasing or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. A Negative Diluent Control should be of lower signal than the lowest standard. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

CALCULATION OF RESULTS

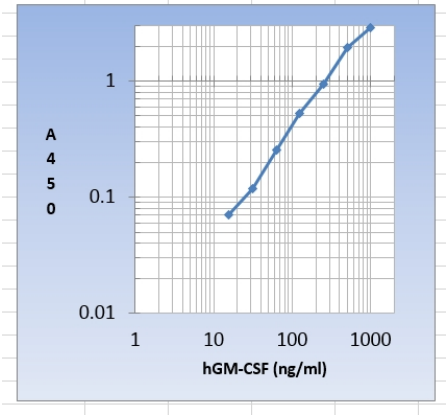
The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, hG-CSF concentrations may be determined as follows:

1. Calculate the mean OD of duplicate samples.
2. On graph paper plot the mean OD of the standards (y-axis) against the concentration (pg/ml) of hG-CSF (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
3. The hG-CSF concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
4. Multiply the values obtained for the samples by the dilution factor.
5. Samples producing signals higher than the 1000 pg/ml standard should be further diluted and re-assayed.

TYPICAL RESULTS

The following data are for illustration purposes only. A complete standard curve should be run in every assay to determine sample values.

Wells	Standards, Control & Samples	A450 nm	pg/ml
A1, A2	**Negative Diluent Control**	0.002	0
B1, B2	15.6 pg/ml **Standard**	0.070	15.6
C1, C2	31.25 pg/ml **Standard**	0.118	31.25
D1, D2	62.5 pg/ml **Standard**	0.253	62.5
E1, E2	125 pg/ml **Standard**	0.523	125
F1, F2	250 pg/ml **Standard**	0.948	250
G1, G2	500 pg/ml **Standard**	1.925	500
H1, H2	1000 pg/ml **Standard**	2.873	1000
A3, A4	**Sample** [Diluted 1:5]	1.27	585
	Calculated: 5-fold dilution x 350 pg/ml = **1.75** ng/ml in sample		



KIT CONTENTS

To Be Reconstituted: Store as indicated.

Component	Instructions for Use
Human G-CSF Standard 10 ng/vial Part No. 0082	Two (2) vials, each containing recombinant human G-CSF, lyophilized in buffer with protein Keep lyophilized vials refrigerated until used or kit lot expires. See preparation of Stds on page 3.
Sample Diluent Buffer Cat. No. 0085, 30ml	**High target protein concentration (10-100ng/ml).** The working dilution is 1:100. i.e. Add 1µl sample into 99 µl sample diluent buffer. **Medium target protein concentration (1-10ng/ml).** The working dilution is 1:10. i.e. Add 10µl sample into 90 µl sample diluent buffer. **Low target protein concentration (15.6-1000pg/ml).** The working dilution is 1:2. i.e. Add 50µl sample to 50 µl sample diluent buffer. **Very Low target protein concentration (≤15.6pg/ml).** No dilution necessary, or the working dilution is 1:2.
Wash Solution Concentrate (50x) Cat. No. WB-50, 10ml	Dilute the entire volume, 10ml, to 1L with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-Human GM-CSF Detection Antibody (100x) The solution should be prepared no more than 2 hours prior to the experiment. Part No. 0083,130 ul (dilution 1:100)	The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume) Biotinylated anti-human GM-CSF antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human GM-CSF antibody to 99µl antibody diluent buffer.).
Avidin-Biotin-Peroxidase Complex (ABC) working solution (100x) The solution should be prepared no more than 1 hour prior to the experiment. Part No. . 0084, 130 ul (dilution 1:100)	The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume) b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)
Antibody diluent buffer, 12 ml, Part No. 0080-ABD	Use for diluting detection antibody concentrate
ABC diluent buffer, 12 ml, Part No. 0080-ABC	Use for diluting ABC concentrate

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-Human G-CSF Microwell Strip Plate	0081	8-well strips (12)	Coated with purified anti-Human G-CSF antibodies. Return unused strips to the pouch with desiccant; re-seal and store refrigerated.
TMB Substrate	80-TMB	10 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80-SS	10 ml	1% sulfuric acid.

Preparation of Standards

Reconstitute 1 vial with 1.0 ml **Working Sample Diluent** to provide a 10,000 pg/ml Top Standard, sufficient for two entire curves. Prepare 2-fold dilutions, as follows.

Standard	+ Diluent	= Final Concn
Reconstituted Standard	None	10,000 pg/ml
100 ul of 10,000 pg/ml	900 ul	1000 pg/ml
300 ul of 1000 pg/ml	700 ul	500 pg/ml
300 ul of 500 pg/ml	700 ul	250 pg/ml
300 ul of 250 pg/ml	700 ul	125 pg/ml
300 ul of 125 pg/ml	700 ul	62.5 pg/ml
300 ul of 62.5 pg/ml	700 ul	31.25 pg/ml
300 ul of 31.25 pg/ml	700 ul	15.6 pg/ml

Use within 12 hrs of preparation, if stored @ 4° C; Or within 48 hrs, if stored frozen.

Materials Required But Not Provided:

- Microplate reader in standard size. Automated plate washer.
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
- Clean tubes and Eppendorf tubes

SPECIMEN COLLECTION AND HANDLING

Human serum may contain infectious material. Always wear gloves when handling serum-containing samples (standards and controls contain no human serum), and dispose of these samples and containers as biohazard waste.

Culture medium, serum and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference. For **serum**, collect blood by venipuncture, allow clotting, and separate the serum by centrifugation at room temperature. For other samples, including **tissue culture media**, clarify the sample by centrifugation and/or filtration prior to dilution in Working Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage. Avoid freeze-thaw cycles.

Samples, Standards and Controls

Dilute **Samples** in Working Sample Diluent according to expected G-CSF levels; for serum: dilute at least 5-fold (e.g., 50 ul sample + 200 ul Diluent) for reduced nonspecific signals.

Do not dilute the **Standards**. Include Working Sample Diluent as a Negative Control to determine proper assay performance (signal should be < 0.3 OD). Internal **Controls** that represent the lab's expected results should also be included in each assay run.

ASSAY PROCEDURE

Bring all reagents to 37° C temperature equilibration (at least 30 minutes).
ALL STEPS ARE PERFORMED AT 37° C TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. Set-up

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
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2. 1st Incubation [100ul – 90 min]

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for 90 minutes.
- Remove the cover, discard plate content, DO NOT WASH and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.

3. 2nd Incubation [100ul – 60 min; 3 washes]

- Add 100ul of Working Detection Antibody to each well.
- Incubate for 60 minutes.
- Wash wells 1X wash buffer 3 times and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
- Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.

4. 3rd Incubation [100ul – 30 min; 5 washes]

- Add 100ul of Working Streptavidin-HRP Conjugate to each well.
- Incubate for 30 minutes.
- Wash wells 5 times as in step 3.

5. Substrate Incubation [100ul – 15 min]

- Add 90 ul TMB Substrate to each well. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.
  Note: If your microplate reader does not register optical density (OD) above 2.0, incubate for less time, assuring the top standard does not surpass 2 OD.

6. Stop Step [Stop: 100ul]

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

7. Absorbance Reading

- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.