

KRIBIOLISA® Anti-Filgrastim (GCSF) ELISA

REF : KBBA13

Ver 1.0

RUO

Enzyme Immunoassay for the quantitative estimation of Anti-Filgrastim (GCSF) ELISA in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

RUO	For Research Use Only	REF	Catalog Number
	Store At	LOT	Batch Code
	Manufactured By		Biological Risk
	Expiry Date		Consult Operating Instructions

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REF KBBA13 **96 tests****Krishgen Biosystems Private Limited**

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Introduction:

Filgrastim is a recombinant form of human G-CSF that stimulates the proliferation, differentiation, and activation of neutrophil precursors, thereby promoting neutrophil recovery in patients undergoing chemotherapy, bone marrow transplantation, or treatment for severe neutropenia. Monitoring the presence of Anti-Filgrastim antibodies is therefore important during immunogenicity assessment studies, clinical research, and therapeutic monitoring programs. The KRIBIOLISA® Anti-Filgrastim (GCSF) ELISA provides a reliable, sensitive, and reproducible method for the detection of Anti-Filgrastim antibodies, making it suitable for immunogenicity testing, preclinical and clinical studies, biopharmaceutical development, and research applications.

Intended Use:

The KRIBIOLISA® Anti-Filgrastim (GCSF) ELISA is designed for the quantitative detection of Anti-Filgrastim antibodies in biological samples. This assay can be used for the screening and evaluation of immune responses against Filgrastim (recombinant human Granulocyte Colony-Stimulating Factor, G-CSF) human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

Principle:

This ELISA is a sandwich immunoassay. Microplate wells are pre-coated with Filgrastim (GCSF) antigen. During the assay, Anti-Filgrastim antibodies present in standards, controls, and samples bind specifically to the immobilized Filgrastim on the microplate surface. Following incubation, unbound components are removed by washing. Filgrastim Conjugate is then added to the wells. The conjugate binds to the captured Anti-Filgrastim antibodies, forming an antigen-antibody-conjugate complex. After a washing step to remove excess conjugate, 3, 3', 5, 5' Tetra Methyl Benzidine (TMB) substrate is added. The HRP enzyme catalyzes the conversion of the substrate to a blue-colored product. The enzymatic reaction is stopped by the addition of Stop Solution, resulting in a color change from blue to yellow. The absorbance is measured at 450 nm using a microplate reader. The intensity of the color developed is directly proportional to the amount of Anti-Filgrastim antibodies present in the sample.

Materials Provided:

Part	Description	Qty
Filgrastim Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Filgrastim.	1 x 96 wells
Anti-Filgrastim Standard	Anti-Filgrastim standard in a buffered protein base with preservatives 0.02% methylisothiazolinone and 0.02% Bromonitrodioxane – (lyophilized, 1 ug/ml)	2 vials
Filgrastim:HRP Conjugate Concentrated	Filgrastim conjugated to HRP concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffered protein base with preservatives 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane.	12 ml
(1X) Standard Diluent	Buffered protein base with 1:1000 dilution human serum and preservatives 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane.	10 ml
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% Bromonitrodioxane	2 x 50 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal <0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen.	12 ml
Stop Solution	0.73M Phosphoric Acid.	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul.
3. Deionized (DI) water.

4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and store the Filgrastim:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Filgrastim:HRP Conjugate concentrated after running your assay.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25°C). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

**Sample Preparation and Storage:**

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. Extract as soon as possible after specimen collection as per relevant procedure. The samples should be tested as soon as possible after the extraction. Alternately the extracted samples can be kept in -20°C. Avoid repeated freeze-thaw cycles.
2. **Serum-** Coagulate at room temperature for 10-20 minutes; centrifuge for 20-min at 2000-3000 rpm. Remove the supernatant. If precipitation appears, recentrifuge.
3. **Plasma-** Use EDTA or citrate plasma as an anticoagulant, mix for 10-20 minutes; centrifuge for 15-min at 2000-3000 rpm. Remove the supernatant carefully. If precipitation appears, recentrifuge.
4. **Cell Culture Supernatant-** Collect sample in a sterile container. Centrifuge for 20-mins at 2000-3000 rpm. Remove the supernatant carefully. When examining the components within the cell, dilute cell suspension with PBS (pH 7.2-7.4), if cell concentration is greater than 1 million/ml. Damage the cells by repeated freeze-thaw cycles to release intracellular components. Centrifuge for 20-min at 2000-3000 rpm. If precipitation appears, centrifuge again.
5. **Tissue Samples-** Rinse tissues in PBS (pH 7.4) to remove excess blood thoroughly and weigh before homogenization. Mince tissues and homogenize them in PBS (pH7.4) with a glass homogenizer on ice. Thaw at 2-8°C or freeze at -20°C. Centrifuge at 2000-3000 RPM for approximately 20 minutes and collect the supernatant carefully.

Note: Grossly hemolyzed samples are not suitable for use in this assay.

Preparation Before Use:

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul Sample Diluent) prior to assay. The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room Temperature before use.
3. To make Wash Buffer (1X); dilute 25 ml of 20X Wash Buffer in 475 ml of DI water.

4. **Standards Preparation:** Reconstitute the Lyophilized Anti-Filgrastim Standard with the 1 ml of Standard Diluent (1X) specified on the vial label to obtain a stock concentration of 1 ug/ml (1000 ng/ml). Keep the vial for 15 mins with gentle agitation before making further dilutions. Prepare **Standards** as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1000 ng/ml	Reconstituted Standard	Reconstitute with 1 ml Standard Diluent (1X)
640 ng/ml	Standard No.8	640 ul reconstituted standard + 360 ul Standard Diluent (1X)
320 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent (1X)
160 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
80 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
40 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
20 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
10 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.1	500 ul Standard Diluent (1X) only

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

5. **Preparation of Working Filgrastim:HRP Conjugate - Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

Procedural Notes:

- In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
- High Dose Hook Effect may be observed in samples with very high concentrations of Anti-Filgrastim. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Filgrastim present in the sample.
- Anti-Filgrastim concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
- Avoid assay of Samples containing sodium azide (NaN₃), as it could destroy the HRP activity resulting in under-estimation of the amount of Anti-Filgrastim.
- It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
- Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
- If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromise of the sensitivity of the assay.
- The plates should be read within 30 minutes after adding the Stop Solution.
- Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

- It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.
- Add **100 µL of prepared Standards, Controls, and Samples** to the respective.
- Cover the plate and incubate for incubate at 37°C for 1 hour.
- Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
- Pipette **100 ul of Filgrastim:HRP Conjugate** to all wells.
- Cover the plate and incubate for incubate at 37°C for 1 hour.
- Aspirate and wash plate 4 times with **Wash Buffer (1X)** as mentioned in step 4.

8. Pipette **100 µl TMB Substrate** in all the wells.
9. Incubate the plate at **37°C** for **30 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
10. Pipette **100 µl of Stop Solution** to all wells. The wells should turn from blue to yellow in color.
11. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph Paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Integrin $\alpha 4 \beta 7$ Pathway Inhibitor concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Anti-Filgrastim Concentration.

If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4-PL is best recommended for automated results

Note:

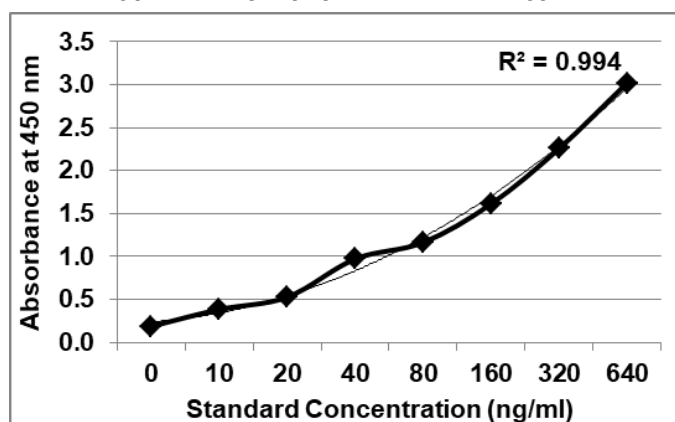
It is recommended to repeat the assay at a different dilution factor in the following cases:

- If the sample absorbance value is below the first standard.

Typical Data (representative only)

Standard Concentration (ng/ml)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.188	--	--
10	0.385	7.0	70.0
20	0.529	15.2	76.0
40	0.973	55.0	137.4
80	1.164	78.9	98.6
160	1.613	153.5	95.9
320	2.258	317.8	99.3
640	3.021	644.4	100.7

Typical Graph (representative only)



Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Standard Range:

10– 640 ng/ml.

Sensitivity:**Limit Of Quantification:**

It is defined as the lowest detectable concentration that can be determined with an acceptable repeatability and the LOQ was found to be 10 ng/ml.

Specificity:

This assay has high sensitivity and excellent specificity for detection of Anti-Filgrastim. No significant cross-reactivity or interference between Anti-Filgrastim and analogues were observed.

Precision:

Intra-Assay: CV<10%

Inter-Assay: CV<12%

Safety Precautions:

- **This kit is For Research Use only.** Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents
- All reagents should be kept in the original shipping container.
- However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Source materials maybe derived from **human body fluids** or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed.
 - Do not smoke, eat or drink while handling kit material.
 - Always use protective gloves.
 - Never pipette material by mouth.
 - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.



Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Anti-Filgrastim equivalent
1A	Standard No.1			
2A	Standard No.1			
1B	Standard No.2			
2B	Standard No.2			
1C	Standard No.3			
2C	Standard No.3			
1D	Standard No.4			
2D	Standard No.4			
1E	Standard No.5			
2E	Standard No.5			
1F	Standard No.6			
2F	Standard No.6			
1G	Standard No.7			
2G	Standard No.7			
1H	Standard No.8			
2H	Standard No.8			
3A	Sample			
4A	Sample			
3B	Sample			
4B	Sample			

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SYMBOLS KEY

MTP	Filgrastim Coated Microtiter Plate (12 x 8 wells)
STD	Anti-Filgrastim standard, Lyophilized
HRP CONJ	Filgrastim:HRP Conjugate, Concentrated
DETN DIL	Detection Diluent
1X STD DIL	(1X) Standard Diluent
1X SAMP DIL	(1X) Sample Diluent
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature