BioVendor Group

ENG

Instructions for Use:

HUMAN FIBRINOGEN-LIKE PROTEIN 1 ELISA

Catalogue number:

RAG027R

For research use only!

BioVendor R&D

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CONTENT

HISTORY OF CHANGES

Previous version	Current version
	ENG.001.A
New edition	

1. INTENDED USE

The FGL1 (human) ELISA Kit is to be used for the in vitro quantitative determination of human FGL1 in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. STORAGE, EXPIRATION

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

3. INTRODUCTION

FGL-1 (Fibrinogen-like protein 1, also called Hepatocyte-derived fibrinogen-related protein 1, HFREP-1 or Hepassocin) was initially identified as an overexpressed transcript in hepatocyte carcinoma (1) and as a transcript enriched in regenerating liver (2). Under physiological conditions, FGL-1 is a liver secreted factor and contributes to its mitogenic and metabolic functions (3), but it has also been shown to be expressed at lower levels in brown adipose tissue in the setting of liver injury (4). Structurally, FGL-1 is a 34 kDa protein similar to Angiopoietin-like factors 2, 3, 4 and 6, regulating also lipid metabolism and energy utilization. Thus, it was proposed to be a member of an emerging group of proteins with key roles in metabolism and liver regeneration (4).
Recently, FGL-1 has also been shown to be upregulated in human cancers, especially non-small cell lung carcinomas (NSCLC), acting as a functional inhibitory ligand of lymphocyte-activation gene 3 (LAG-3) (5). FGL-1/LAG-3 interaction occurs in a MHC-II-independent manner and involves the FGL-1 fibrinogen-like domain (FD) and the LAG-3 D1-D2 domains (5). FGL-1 forms two disulfide- linked homodimers (2) and higher molecular weight oligomers that bind to LAG-3 much better than dimeric forms (5). Thus, it acts as an immune suppressive molecule inhibiting antigen-specific T-cell activation and being responsible for LAG-3 T-cell inhibitory function (5). Because FGL-1/LAG-3 interaction is independent from the B7-H1-PD-1 pathway, elevated FGL-1 in the plasma of cancer patients is associated with poor prognosis and resistance to anti-PD therapy (5).

4. TEST PRINCIPLE

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human FGL1 in cell culture supernatants, serum and plasma. A monoclonal antibody specific for human FGL1 has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, FGL1 (h) is recognized by the addition of a biotinylated monoclonal antibody specific for human FGL1 (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'- tetramethylbenzidine (TMB). The intensity of the

color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of FGL1 in the samples.

5. TECHNICAL HINTS

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

6. REAGENT SUPPLIED

1 vial human FGL1 Standard (lyophilized)	(100 ng)
1 vial Detection Antibody	(20 µl)
1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)
2 bottles Wash Buffer 10X	(2 x 30 ml)
1 bottle ELISA Buffer 10X	(1 x 30 ml)
1 bottle TMB Substrate Solution	(12 ml)
1 bottle Stop Solution	(12 ml)
1 plate coated with FGL1 Antibody	(6 x 16-well strips)
2 plate Covers (plastic film)	
2 silica Gel Minibags	

7. MATERIAL REQUIRED BUT NOT SUPPLIED

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. PREPARATION AND STORAGE OF REAGENTS

NOTE: Prepare just the appropriate amount of the buffers necessary for the assay.

8.1 Wash Buffer 10X

has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.

8.2 ELISA Buffer 10X

has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.

8.3 Detection Antibody (DET)

has to be diluted to 1:1000 in ELISA Buffer 1X (10 µl DET + 10 ml ELISA Buffer 1X).
NOTE: The diluted Detection Antibody is not stable and cannot be stored!

8.4 HRP Labeled Streptavidin (STREP-HRP)

has to be reconstituted with 100 µl of ELISA Buffer 1X.

– After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
– Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

NOTE: The diluted STREP-HRP is not stable and cannot be stored!

8.5 Human FGL1 Standard (STD)

has to be reconstituted with 100 µl of ELISA Buffer 1X.

– This reconstitution produces a stock solution of 1 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

NOTE: The reconstituted standard is aliquoted and stored at -20°C!

– Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven- point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

– Suggested standard points are:
500, 250, 125, 62.5., 31.125, 15.5625, 7.78125 and 0 pg/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of FGL1 (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X
1 ng/ml	100µl of FGL1 (10 ng/ml)	900 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
500 pg/ml	300 µl of FGL1 (1 ng/ml)	300 µl of ELISA Buffer 1X
250 pg/ml	300 µl of FGL1 (500 pg/ml)	300 µl of ELISA Buffer 1X
125 pg/ml	300 µl of FGL1(250 pg/ml)	300 µl of ELISA Buffer 1X
62.5 pg/ml	300 µl of FGL1 (125 pg/ml)	300 µl of ELISA Buffer 1X
31.25 pg/ml	300 µl of FGL1 (62.5 pg/ml)	300 µl of ELISA Buffer 1X
15.625 pg/ml	300 µl of FGL1 (31.25 pg/ml)	300 µl of ELISA Buffer 1X
7.781 pg/ml	300 µl of FGL1 (15.625 pg/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

9. PREPARATION OF SAMPLES

9.1 Serum

Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

9.2 Plasma

Collect plasma using heparin, citrate or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -80°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma and Cell Culture Supernatant have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: As a starting point, 1/1000 dilution of serum or of plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required

10. CALCULATIONS

- Average the duplicate readings for each standard and sample and subtract the average blank value (obtained with the 0 pg/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding FGL1 concentration (pg/ml) on the vertical axis (see TYPICAL DATA).
- Calculate the FGL1 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human FGL1 in the sample.

11. TYPICAL DATA

The following data are obtained using the different concentrations of standard as described in this protocol:

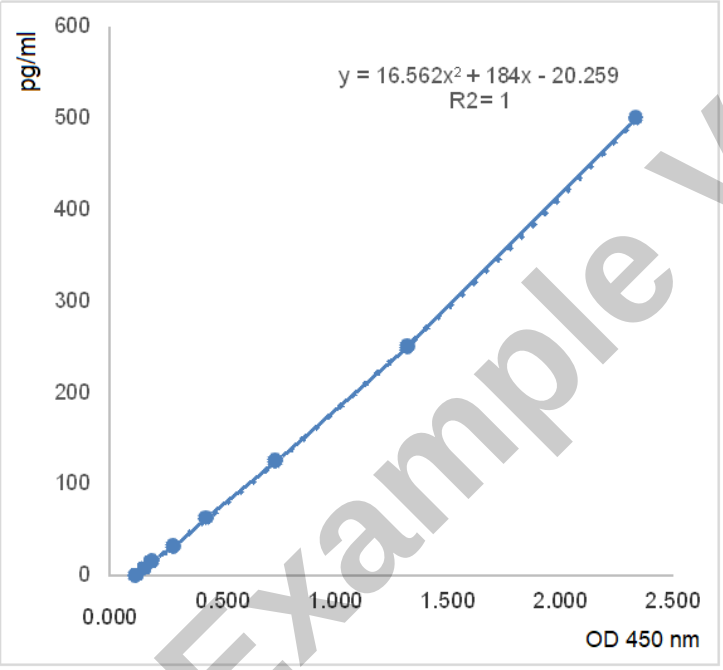


Figure: Standard curve

Standard FGL1 (pg/ml)	Optical Density (mean)
500	2.335
250	1.318
125	0.736
62.5	0.424
31.25	0.283
15.625	0.185
7.781	0.153
0	0.113

12. PERFORMANCE CHARACTERISTICS

12.1 Sensitivity (Limit of detection):

The lowest level of human FGL1 that can be detected by this assay is **1.8 pg/ml**.

NOTE: The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.

12.2 Assay range:

7.78 pg/ml – 500 pg/ml

12.3 Specificity:

This ELISA is specific for the measurement of natural and recombinant human FGL1.

12.4 Intra-assay precision:

Four samples of known concentrations of human FGL1 were assayed in replicates 4 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	99.92	3.52	3.52	4
A2	123.93	5.65	4.56	4
A3	39.66	1.23	3.11	4
A4	63.96	1.2	3.77	4

12.5 Inter-assay precision:

Four samples of known concentrations of human FGL1 were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	62.50	2.11	3.37	5
B2	30.95	2.049	6.61	5
B3	83.94	2.38	2.84	5
B4	110.00	6.99	6.35	5

12.6 Recovery:

When samples are spiked with known concentrations of human FGL1, the recovery averages range from 92% to 107%.

12.7 Linearity:

Different samples containing human FGL1 were diluted several fold (1/1000 to 1/2000 for sera and plasmas) and the measured recoveries ranged from 90% to 108%.

12.8 Expected values:

Human FGL1 protein levels range in serum from **30ng/ml to >500ng/ml** and in plasma from **200ng/ml to >1000ng/ml.**

Note: FGL1 protein levels are lower in serum due to a loss of the protein in the serum after blood coagulation.

13. TROUBLESHOOTING

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double- check calculations.

14. REFERENCES

14.1 General references

1. Molecular cloning and initial characterization of a novel fibrinogen-related gene, HFREP-1: T. Yamamoto, et al.; BBRC 193, 681 (1993)
2. Isolation and characterization of a novel liver-specific gene, hepassocin, upregulated during liver regeneration: H. Hara, et al.; Biochim. Biophys. Acta 1492, 31 (2000)
3. Recombinant human hepassocin stimulates proliferation of hepatocytes in vivo and improves survival in rats with fulminant hepatic failure: C. Li, et al.; Gut 59, 817 (2010)
4. Targeted deletion of fibrinogen like protein 1 reveals a novel role in energy substrate utilization:V. Demchev, et al.; PloS One 8, e58084 (2013)
5. Fibrinogen-like Protein 1 Is a Major Immune Inhibitory Ligand of LAG-3: J. Wang, et al.; Cell 176, 334 (2019)

15. ASSAY PROCEDURE - SUMMARY

1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C.
NOTE: Remaining 16-well strips coated with FGL1 antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.

2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma, serum or cell culture supernatant samples in duplicate to the wells **(see Preparation and Storage of Reagents and Preparation of Samples).**

3. Cover the plate with plastic film and incubate for **2 hours at Room Temperature.**

4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see Preparation and Storage of Reagents)**.

6. Cover the plate with plastic film and incubate for **1 hour at Room Temperature.**

7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see Preparation and Storage of Reagents)**.

9. Cover the plate with plastic film and incubate for **30 min at Room Temperature**.

10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.

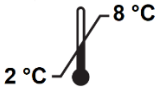
11. Add 100 µl to each well of TMB substrate solution **(TMB)**.

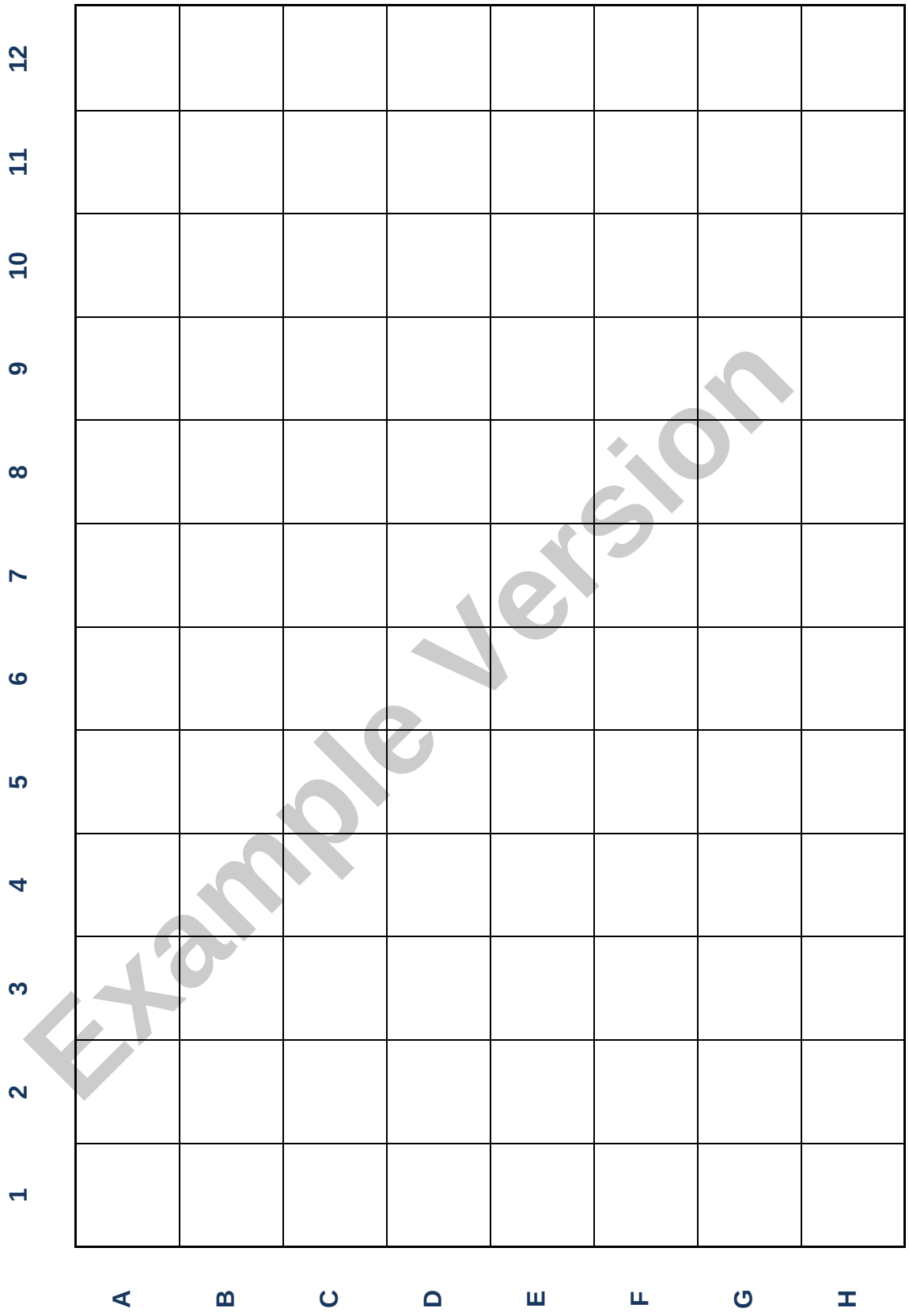
12. Allow the color reaction to develop **at Room Temperature in the dark for 20 minutes**. Do not cover the plate.

13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
! CAUTION: CORROSIVE SOLUTION !

14. Measure the OD at 450 nm in an ELISA reader.

16. EXPLANATION OF SYMBOLS

	Catalogue number
	Batch code
	Caution
	Use by date
	Temperature limit
	Manufacturer
	Read electronic instructions for use - eIFU
	The content is sufficient for 96 tests
	Biological risks



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