Code No. KG612

Prolyl 4-Hydroxylated Human α-Fibrinogen ELISA Kit

INTRODUCTION

Fibrinogen is one of major component of plasma, and is a hexamer composed of pairs of three polypeptides (α-, β- and γ-chain). Prolyl hydroxylation is known to be essential for folding, secretion, and stability of collagen triple helix.

In the powerful study using 2-Dimensional Image Converted Analysis of Liquid chromatography and mass spectrometry (2DICAL), it is found that proline 565 and 530 residues in α-fibrinogen is hydroxylated by the Prolyl hydroxylase P4HA1. In addition, the plasma level of prolyl 4-hydroxylated α-fibrinogen (HP-FGA) was increased in individuals who suffer pancreatic cancer. The significant difference between the said group (n=43) and healthy subjects (n=43) was observed in the 2-DICAL analysis.

TEST PRINCIPLE

This assay kit employs the quantitative sandwich ELISA technique based on two mouse monoclonal antibodies against to different epitope of HP-FGA with hydroxylation on its proline 565 residue. One of antibody specifically binds the region which contains hydoxylated proline 565 residue on HP-FGA.

Standard solutions and samples (culture medium and clinical sample, e.g. plasma) are applied into the wells and incubated, HP-FGA is captured by the coated antibody. Following, the microplate is incubated with HRP-conjugated antibody. Finally, a chromogen (TMB) is applied to each test well. The strength of coloring is in proportion to the quantities of Human HP-FGA.

MEASUREMENT RANGE

2.73 - 175 ng/mL

For research use only, not for use in diagnostic procedures.

KIT COMPONENT

1	Precoated plate	: Anti-Human FGA Mouse IgG MoAb Affinity Purify	96Well x 1
2	Labeled antibody Conc.	: (30X) HRP conjugated Anti- Human HP-FGA Mouse IgG Fab' Affinity Purify	0.4mL x 1
3	Standard	: Human HP-FGA	0.5mL x 2
4	EIA buffer	: 1% BSA, 0.05% Tween20 in PBS	30mL x 1
5	Solution for Labeled antibody	: 1% BSA, 0.05% Tween20 in PBS	12mL x 1
6	Chromogen	: TMB solution	15mL x 1
7	Stop solution	: 1N H_2SO_4	12mL x 1
8	Wash buffer Conc.	: (40X) 0.05% Tween20 in phosphate buffer	50mL x 1

OPERATION MANUAL

1. Materials needed but not supplied

- Plate reader (450nm)
- Micropipette and tip
- Graduated cylinder and beaker
- Deionized water
- Refrigerator (as 4°C)
- Graph paper (log/log)
- Paper towel
- Tube for dilution of Standard
- Incubator (37°C ± 1°C)
- Washing bottle for precoated plate
- Disposable test tube for "2, Labeled antibody Conc." and "6, Chromogen"

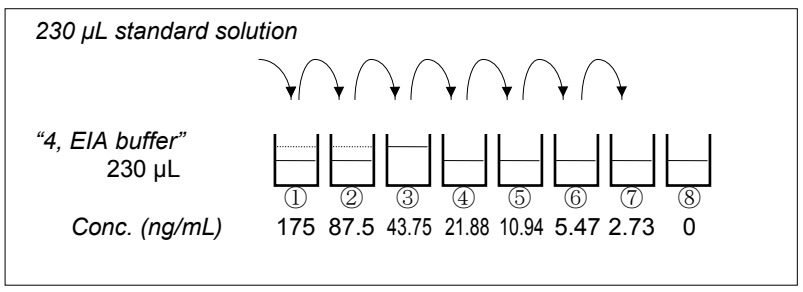
2. Preparation

1) Preparation of wash buffer
"8, Wash buffer Conc." is a concentrated (40X) buffer. Adjust the temperature of "8, Washing buffer Conc." to room temperature and then, mix it gently and completely before use. Dilute 50 mL of "8, Wash buffer Conc." with 1,950 mL of deionized water and mix it. This is the wash buffer for use. This prepared wash buffer shall be stored in refrigerator and used within 2 weeks after dilution.
2) Preparation of Labeled antibody
"2, Labeled antibody Conc." is a concentrated (30X). Dilute "2, Labeled antibody Conc." with "5, Solution for Labeled antibody" in 30 times according to required quantity into a disposable test tube. Use this resulting solution as Labeled antibody.
Example)
In case you use one strip (8 well), the required quantity of Labeled antibody is 800 µL. (Dilute 30 µL of "2, Labeled antibody Conc." with 870 µL of "5, Solution for Labeled antibody" and mix it. And use the resulting solution by 100 µL in each well.)
<u>This operation should be done just before applying labeled antibody.</u>
The remaining "2, Labeled antibody Conc." should be stored at 4°C in firmly sealed vial.
3) Preparation of Standard
Put just <u>0.5 mL</u> of deionized water into the vial of "3, Standard" and mix it gently and completely. This solution is 350 ng/mL Human HP-FGA standard.
4) Dilution of Standard
Prepare 8 tubes for dilution of "3, Standard". Put 230 µL each of "4, EIA buffer" into the tube.
Specify the following concentration of each tube."

Tube-1	175 ng/mL
Tube-2	87.5 ng/mL
Tube-3	43.75 ng/mL
Tube-4	21.88 ng/mL
Tube-5	10.94 ng/mL
Tube-6	5.47 ng/mL
Tube-7	2.73 ng/mL
Tube-8	0 ng/mL (Test Sample Blank)

Put 230 µL of Standard solution into tube-1 and mix it gently. Then, put 230 µL of tube-1 mixture into tube-2. Dilute two times standard solution in series to set up 7 points of diluted standard between 175 ng/mL and 2.73 ng/mL. Tube-8 is the test sample blank as 0 ng/mL.

See following picture.



5) Dilution of test sample
Test samples should be diluted with "4, EIA buffer" as necessary.
Example) EDTA-Plasma: x3,000

3. Measurement procedure

All reagents shall be brought to room temperature approximately 30 minutes before use. Then mix it gently and completely before use. Make sure of no change in quality of the reagents. Standard curve shall be prepared simultaneously with the measurement of test samples.

Reagents	Test Sample	Standard	Test Sample Blank	Reagent Blank
	Test sample 100 µL	Diluted standard (Tube 1-7) 100 µL	EIA buffer (Tube-8) 100 µL	EIA buffer 100 µL
Incubation for 60 minutes at 37°C with plate lid				
Washing 4 times				
Labeled Antibody	100 µL	100 µL	100 µL	-
Incubation for 30 minutes at 4°C with plate lid				
Washing 5 times				
Chromogen	100 µL	100 µL	100 µL	100 µL
Incubation for 30 minutes at room temperature (shielded)				
Stop solution	100 µL	100 µL	100 µL	100 µL
Read the plate at 450nm against a Reagent Blank within 30 minutes after addition of Stop solution.				

1) Determine wells for reagent blank. Put 100 µL each of "4, EIA buffer" into the wells.
2) Determine wells for test sample blank, test sample and diluted standard.
Then, put 100 µL each of test sample blank (tube-8), test sample and dilutions of standard (tube-1-7) into the appropriate wells.
3) Incubate the precoated plate for 60 minutes at 37°C after covering it with plate lid.
4) Wash each well of the precoated plate 4 times with wash buffer using a washing bottle or a plate washer in following way.
After shaking off (or aspiration of) the solution in wells, fill each well with wash buffer and shake off the wash buffer completely from the precoated plate. <u>This procedure must be repeated 4 times.</u> Then, drain the precoated plate completely on paper towel.
Please refer to 5) and 6) in SPECIAL ATTENION below, and be careful not to miss a well.
5) Pipette 100 µL of labeled antibody solution into the wells of test samples, diluted standard and test sample blank.
6) Incubate the precoated plate for 30 minutes at 4°C after covering it with plate lid.
7) Wash the precoated plate 5 times in the same manner as 4).
8) Take the required quantity of "6, Chromogen" into a disposable test tube. Then, pipette 100 µL from the test tube into every well. Please do not return the rest of used chromogen in the test tube into "6, Chromogen" bottle in order to avoid contamination.
9) Incubate the precoated plate for 30 minutes at room temperature in the dark. The solution of Chromogen will turn blue.
10) Add 100 µL of "7, Stop solution" to all wells. Mix the solution by tapping the side of precoated plate. The solution will turn yellow by addition of "7, Stop solution".
11) Remove any dirt or drop of water on the bottom of the precoated plate and confirm there is no bubble on the surface of the solution. Then, run the plate reader and conduct measurement at 450 nm against a reagent blank.
The measurement shall be done within 30 minutes after addition of "7, Stop solution".

SPECIAL ATTENTION

1) Test samples should be measured soon after collection. For the storage of test samples, store them frozen and do not repeat freeze/thaw cycles. Thaw the test samples at a low temperature and mix them completely before measurement.
2) Test samples should be diluted with "4, EIA buffer", as the need arises.
3) Duplicate measurement of test samples and standard is recommended.
4) Use test samples in neutral pH range. The contaminations of organic solvent may affect the measurement.
5) Use only wash buffer in this kit for washing the precoated plate.
Insufficient washing may lead to the failure in measurement.

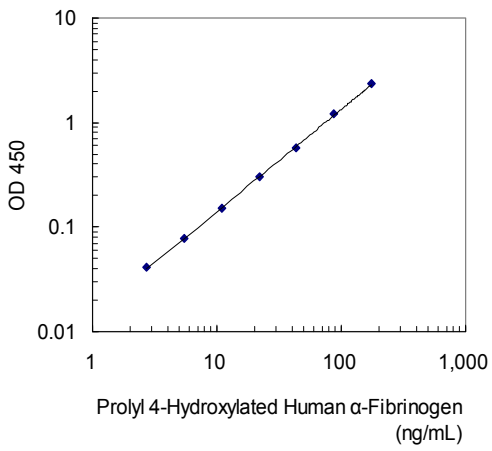
6) Remove the wash buffer completely by tapping the precoated plate on paper towel. Do not wipe wells with paper towel.
7) "6, Chromogen" should be stored in the dark due to its sensitivity against light. Avoid contact of Chromogen with metals.
8) Measurement should be done within 30 minutes after addition of "7, Stop solution".

HOW TO CALCULATE HP-FGA CONCENTRATION OF SAMPLES

Subtract the absorbance of test sample blank from all data, including standards and unknown samples before plotting. Plot the subtracted absorbance of the standards against the standard concentration on log-log graph paper. Draw the best smooth curve through these points to construct the standard curve. Read the concentration for unknown samples from the standard curve.

Example of standard curve

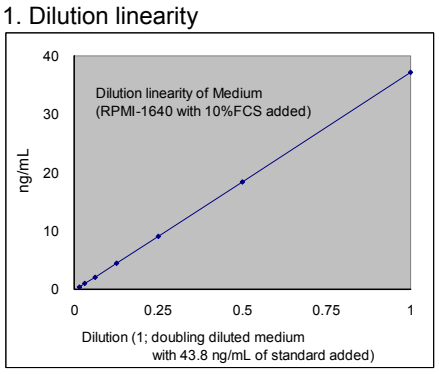
Conc. (ng/mL)	Absorbance (450nm)
175	2.355
87.5	1.207
43.75	0.578
21.88	0.308
10.94	0.159
5.47	0.086
2.73	0.049
0 (Test Sample Blank)	0.003

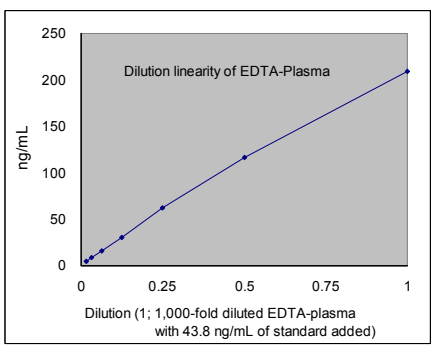


＊ The typical standard curve is shown above. This curve can not be used to derive test results. Please run a standard curve for each assay.

PERFORMANCE CHARACTERISTICS

1. Dilution linearity





2. Added Recovery Assay

Specimen	Theoretical Value (ng/mL)	Measured Value (ng/mL)	%
10%FCS added RPMI-1640 (x2)	43.75	40.59	92.8
	21.88	19.73	90.2
	112.66	111.32	98.8
Human Plasma (EDTA) (x3,000)	90.78	90.37	99.5
	43.75	40.59	92.8
	21.88	19.73	90.2

3. Intra - Assay

Mean Value (ng/mL)	SD (ng/mL)	CV (%)	n
6.76	0.53	7.8	16
19.67	1.87	9.5	16
79.03	6.63	8.4	16

4. Inter - Assay

Mean Value (ng/mL)	SD (ng/mL)	CV (%)	n
77.69	3.79	4.9	5
19.22	0.81	4.2	5
6.87	0.30	4.4	5

5. Specificity

Substance	Cross-Reactivity
Human HP-FGA	100 %
Human Serum Albumin	< 0.1 %
Human Transferrin	< 0.1 %
Human IgG	< 0.1 %

6. Sensitivity

0.43 ng/mL
The sensitivity for this kit was determined using the guidelines under the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols. (National Committee for Clinical Laboratory Standards Evaluation Protocols, SC1, (1989) Villanova, PA: NCCLS.)

PRECAUTION FOR INTENDED USE AND/OR HANDLING

1. All reagents should be stored at 2 - 8°C. All reagents shall be brought to room temperature approximately 30 minutes before use.
2. "3, Standard" is lyophilized products. Be careful to open this vial.
3. "7, Stop solution" is a strong acid substance. Therefore, be careful not to have your skin and clothes contact "7, Stop solution" and pay attention to the disposal of "7, Stop solution".
4. Dispose used materials after rinsing them with large quantity of water.
5. Precipitation may occur in "2, Labeled antibody Conc.", "4, EIA buffer" or "8, Wash buffer Conc.", however, there is no problem in the performance.
6. Wash hands after handling reagents.
7. Do not mix the reagents with the reagents from a different lot or kit.
8. Do not use expired reagents.
9. This kit is for research purpose only. Do not use for clinical diagnosis.

STORAGE AND THE TERM OF VALIDITY

Storage Condition : 2 - 8°C
The expiry date is specified on outer box.

REFERENCE

1. Ono M, Matsubara J, Honda K, Sakuma T, Hashiguchi T, Nose H, Nakamori S, Okusaka T, Kosuge T, Sata N, Nagai H, Ioka T, Tanaka S, Tsuchida A, Aoki T, Shimahara M, Yasunami Y, Itoi T, Moriyasu F, Negishi A, Kuwabara H, Shoji A, Hirohashi S, Yamada T. Prolyl 4-hydroxylation of alpha-fibrinogen: a novel protein modification revealed by plasma proteomics. J Biol Chem. 2009 Oct 16;284(42):29041-9.

Version 1.
Made in Japan.