



AssayMax™
Human Fibrinogen ELISA Kit

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For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 25 μ l of Standard or Sample and 25 μ l of Biotinylated Protein per well.
Incubate 2 hours.

Step 2. Wash, then add 50 μ l of SP Conjugate per well.
Incubate 30 minutes.

Step 3. Wash, then add 50 μ l of Chromogen Substrate per well.
Incubate 12 minutes.

Step 4. Add 50 μ l of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key



Consult instructions for use.

Assay Template

[illegible]

AssayMax™ Human Fibrinogen (FBG) ELISA Kit

Catalog No. EF1040-27

Sample insert for reference use only

WHO Standard Calibrated Positive Control Included

Introduction

Fibrinogen (FBG) is a homodimer (340 kDa) that is made up of two sets of alpha, beta, and gamma polypeptide chains. FBG is synthesized in the parenchymal cell of the hepatocyte and in the megakaryocyte (1). FBG plays a major role in coagulation: Elevated and decreased levels have clinical significance. Upon cleavage by thrombin in the initial stages of coagulation activation, FBG self-assembles to yield a fibrin clot matrix that subsequently is cross-linked by factor XIIIa to form an insoluble network. FBG also binds to the platelet glycoprotein IIb/IIIa receptor to form bridges between platelets, thus facilitating aggregation (2). Elevated plasma FBG has been identified as an independent risk factor for coronary atherosclerosis and ischemic heart disease (3-4). Individuals with congenital absence of FBG, termed afibrinogenemia, have prolonged bleeding times.

Principle of the Assay

The AssayMax™ Human Fibrinogen ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of FBG in human **plasma samples**. This assay employs a quantitative **competitive enzyme immunoassay** technique that measures human FBG in approximately 3 hours. A murine antibody specific for human FBG has been pre-coated onto a 96-well microplate with removable strips. FBG in standards and samples is competed with a biotinylated human FBG protein sandwiched by the immobilized antibody and streptavidin-peroxidase (SP) conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated protein, and SP conjugate), as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.

- Spin down the SP conjugate vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human Fibrinogen Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a murine antibody against human FBG.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Fibrinogen Standard:** Human FBG in a buffered protein base, calibrated against WHO 3rd International Standard (40 µg, lyophilized).
- **Biotinylated Human Fibrinogen Protein (2x):** Lyophilized.
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml).
- **SP Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).
- **Positive Control:** 1 vial, lyophilized. See insert CEF104021.

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Store Standard and Biotinylated Protein at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation, and Storage

- Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x g for 10 minutes and collect plasma. A 250-fold sample dilution is suggested into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).

Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater <i>(for reference only; please follow the insert for specific dilution suggested)</i>	
100x	10000x
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution <i>Assuming the needed volume is less than or equal to 400 µl.</i>	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution <i>Assuming the needed volume is less than or equal to 400 µl.</i>
1000x	100000x
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution <i>Assuming the needed volume is less than or equal to 240 µl.</i>	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution <i>Assuming the needed volume is less than or equal to 240 µl.</i>

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- MIX Diluent Concentrate (10x):** Dilute the MIX Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.
- Human Fibrinogen Standard:** Reconstitute the Human Fibrinogen Standard (40 µg) with 0.5 ml of MIX Diluent to generate an 80 µg/ml standard stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate

standard points by serially diluting from the standard stock solution (80 µg/ml) 2-fold with equal volume of MIX Diluent to produce 40, 20, 10, and 5 µg/ml solutions. MIX Diluent serves as the zero standard (0 µg/ml). Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 5 days**.

Standard Point	Dilution	[FBG] (µg/ml)
P1	1 part Standard (80 µg/ml)	80
P2	1 part P1 + 1 part MIX Diluent	40
P3	1 part P2 + 1 part MIX Diluent	20
P4	1 part P3 + 1 part MIX Diluent	10
P5	1 part P4 + 1 part MIX Diluent	5.0
P6	MIX Diluent	0.0

- **Biotinylated Human Fibrinogen Protein (2x):** Reconstitute the Biotinylated Human Fibrinogen Protein with 3 ml of MIX Diluent to generate a stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to dilution. From the stock solution, dilute 2-fold with MIX Diluent to produce a 1x working solution. Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 5 days**.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with MIX Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 µl of Human Fibrinogen Standard or sample to each well, and immediately add 25 µl of Biotinylated Human Fibrinogen Protein to each well (on top of the standard or sample). Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed. Cover wells

with a sealing tape and incubate for 2 hours. Start the timer after the last addition.

- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 μ l of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer, wash six times with 300 μ l of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 μ l of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μ l of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate in ambient light for 12 minutes or until the optimal blue color density develops.
- Add 50 μ l of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at low concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance (OD) on the y-axis. The best fit line can be determined by regression analysis using log-log or four-parameter logistic curve fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Typical Data

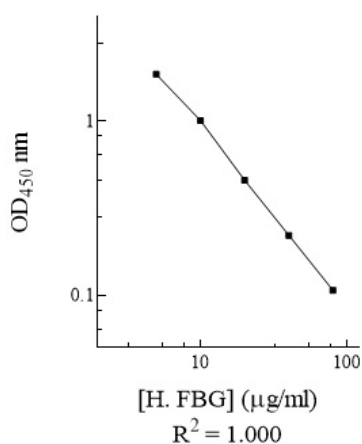
- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	µg/ml	OD	Average OD
P1	80	0.103 0.111	0.107
P2	40	0.214 0.228	0.221
P3	20	0.460 0.456	0.458
P4	10	1.009 1.007	1.008
P5	5.0	1.879 1.835	1.857
P6	0.0	2.478 2.250	2.364
Sample: Pooled Normal Sodium Citrate Plasma (250x)		1.032 1.044	1.038

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

Human FBG Standard Curve



Reference Value

- Normal human FBG plasma levels range from 1.5 – 4 mg/ml.
- Plasma samples from healthy adults were tested (n=10). On average, human FBG level was 2.9 mg/ml.

Performance Characteristics

- **Kit standard has been calibrated against WHO International Standard.**
- The minimum detectable dose of human FBG as calculated by 2SD from the mean of a zero standard was established to be 3.9 µg/ml.
- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	5.6%	3.9%	4.3%	11.0%	9.6%	10.4%
Average CV (%)	4.6%			10.3%		

Spiking Recovery

- Recovery was determined by spiking two plasma samples with different FBG concentrations.

Sample	Unspiked Sample (µg/ml)	Spiking Value (µg/ml)	Expected	Observed	Recovery (%)
1	6.599	7.871	14.470	13.172	91%
		3.631	10.230	10.629	104%
		1.867	8.466	7.626	90%
2	6.245	7.871	14.116	13.493	96%
		3.631	9.876	8.917	90%
		1.867	8.112	7.533	93%
Average Recovery (%)					94%

Linearity

- Plasma samples were serially diluted to test for linearity.

Average Percentage of Expected Value (%)	
Sample Dilution	Plasma
125x	90%
250x	98%
500x	110%

Cross-Reactivity

Species	Cross-Reactivity (%)
Canine	None
Bovine	None
Monkey	None
Mouse	None
Rat	None
Swine	None
Rabbit	None

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are empty after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.

Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	Each step of the procedure should be performed uninterrupted.
	Omission of step	Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	- Check pipette calibration. - Check pipette for proper performance.
	Wash step was skipped	Consult the provided procedure for all wash steps.
	Improper wash buffer	Check that the correct wash buffer is being used.
	Improper reagent preparation	Consult reagent preparation section for the correct dilutions of all reagents.
Deficient Standard Curve Fit	Insufficient or prolonged incubation periods	Consult the provided procedure for correct incubation time.
	Non-optimal sample dilution	- Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. - Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. - User should determine the optimal dilution factor for samples.
	Contamination of reagents	A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	- Pipette properly in a controlled and careful manner. - Check pipette calibration. - Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	- Thoroughly agitate the lyophilized components after reconstitution. - Thoroughly mix dilutions.

References

- (1) Doolittle RF. (1984) *Annu Rev Biochem.* 53:195.
- (2) Handley DA, Hughes TE. (1997) *Thromb Res.* 87:1.
- (3) Handa K *et al.* (1989) *Atherosclerosis.* 77:209.
- (4) Mannucci PM, Mari D. (1993) *Fibrinolysis.* 3:51.

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