



AssayMax Human Factor IX (FIX) ELISA Kit

Catalog No. EF1009-1

Introduction

Factor IX (FIX) is zymogens of plasma serine proteases required for normal hemostasis (1). FIX and FX are activated by tissue factor (TF) and factor VIIa (FVIIa) complexes and initiates coagulation resulting in thrombin formation (2). Hemophilia B is an X-linked bleeding disorder that results from a deficiency in functional coagulation factor IX (hFIX) (3). On the other hand, increased plasma level of FIX was reported to be independent risk factor of venous thromboembolism (VTE) (4).

Principal of the Assay

The AssayMax Human Factor IX (FIX) ELISA kit is designed for detection of human factor IX in plasma, serum, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures FIX in less than 4 hours. A polyclonal antibody specific for FIX has been pre-coated onto a 96-well microplate with removable strips. FIX, in standards and samples, is sandwiched by the immobilized antibody and the biotinylated polyclonal antibody specific for FIX, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then 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

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, 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 protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **FIX Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against FIX.

- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **FIX Standard:** Human FIX in a buffered protein base (400 ng, lyophilized).
- **Biotinylated FIX Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against FIX (140 µl).
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate:** A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution:** A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8⁰C or -20⁰C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20⁰C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8⁰C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8⁰C.
- Store Standard at 2-8⁰C before reconstituting with Diluent and at -20⁰C 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 pipettes).
- 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 2000 x g for 10 minutes and collect supernatants. Dilute samples 1:400 into EIA Diluent and assay. The undiluted samples can be stored at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and dilute samples 1:400 into EIA Diluent. Store serum at -20⁰C or below. Avoid repeated freeze-thaw cycles
- **Cell Culture Supernatants:** Collect cell culture media and centrifuge at 2000 x g for 10 minutes at 4⁰C to remove debris. Collect supernatants and assay. The undiluted samples can be stored at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^oC.
- **FIX Standard:** Reconstitute the 400 ng of human FIX Standard with 4 ml of EIA Diluent to generate a stock standard solution of 100 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the Standard solution (100 ng/ml) 1:2 with EIA Diluent to produce 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/ml. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^oC.

Standard Point	Dilution	[FIX] (ng/ml)
P1	1 part Standard Stock (100 ng/ml)	100.00
P2	1 part P1 + 1 part EIA Diluent	50.00
P3	1 part P2 + 1 part EIA Diluent	25.00
P4	1 part P3 + 1 part EIA Diluent	12.50
P5	1 part P4 + 1 part EIA Diluent	6.25
P6	1 part P5 + 1 part EIA Diluent	3.13
P7	1 part P6 + 1 part EIA Diluent	1.56
P8	EIA Diluent	0.00

- **Biotinylated FIX Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with EIA Diluent. Any remaining solution should be frozen at -20^oC.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^oC.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^oC).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated FIX Antibody to each well and incubate for one hour.
- Wash the microplate as described above.

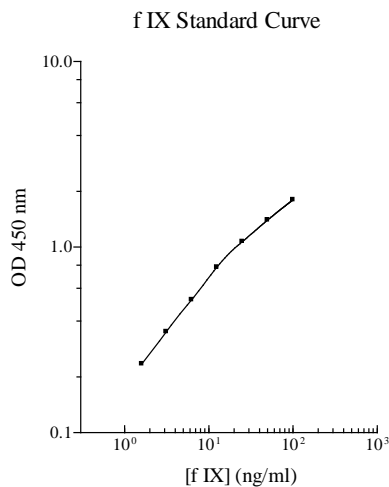
- Add 50 μ l of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μ l of Chromogen Substrate per well and incubate for about 15 minutes or till the optimal color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μ l of Stop Solution to each well. The color will change from blue to yellow.
- 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 high 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 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 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.

Standard Curve

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



Performance Characteristics

- The minimum detectable level of FIX was typically \sim 1.5 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 5.2 % and 7.1 % respectively.

Linearity

Sample Dilution	Average Percentage of Expected Value	
	Plasma	Serum
1:200	94%	87%
1:400	98%	97%
1:800	102%	104%

Recovery

Standard Added Value	5 – 50 ng/ml
Recovery %	92 - 110 %
Average Recovery %	97 %

Cross-Reactivity

Species	% Cross Reactivity
Beagle	None
Bovine	None
Monkey	< 10 (suggest dilution 1:4 for plasma)
Mouse	None
Rat	None
Swine	None
Rabbit	None
Human	100%
Human Factor IXa	100%

- 10% FBS in culture media will not affect the assay.

References

- (1) Wang X. *et al.* (2005) *J Thromb Haemost.* 3(4): 695-702
- (2) Bogdanov VY *et al* (2003) *Nat Med.* 9(4): 458-62
- (3) Pipe SW (2004) *Semin Thromb Hemost.* 30(2): 227-37
- (4) Bertina RM. (2003) *Pathophysiol Haemost Thromb.* 33(5-6): 395-400.

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