



AssayMax Human Factor X (FX) ELISA Kit

Catalog No. EF1010-1

Introduction

Factor X (FX) is a plasma serine protease zymogen involved in the blood coagulation cascade. FX is purified from plasma as a two-chain protein consisting of a 45 kDa heavy chain and a 17 kDa light chain. The FX heavy chain is cleaved during coagulation by several different proteases including the intrinsic Xase complex, the FX-activating enzyme from Russell's viper venom (RVV) and trypsin, and also by extrinsic (tissue factor/factor VIIa) pathway to give an active enzyme FXa. FXa as the activator of prothrombin occupies a central position linking the two blood coagulation pathways (1 - 4).

Principal of the Assay

The AssayMax Human Factor X (FX) ELISA kit is designed for detection of human factor X in plasma, serum, milk, urine, saliva, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures FX in less than 4 hours. A monoclonal antibody specific for FX has been pre-coated onto a 96-well microplate with removable strips. FX in standards and samples is sandwiched by the immobilized antibody and the peroxidase conjugated polyclonal antibody specific for FX. 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

- **FX Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against human FX.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **FX Standard:** Plasma human FX in a buffered protein base (400 ng, lyophilized).

- **Biotinylated FX Antibody (50x):** A 50-fold biotinylated polyclonal antibody against H. FX (140 µl).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **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, 2 bottles).
- **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)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using 3.8% sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes. Dilute plasma 1:800 into MIX Diluent. 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 assay. Dilute samples 1:800 into MIX Diluent. The undiluted samples can be stored at -20⁰C or below for up to 3 months. 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. Store samples at < -20⁰C. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 600 x g for 10 minutes. Milk dilution is suggested at 1:2 in MIX Diluent; however, the user should determine the optimal dilution factor. Store samples at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample tube. Centrifuge samples at 600 x g for 10 minutes and assay. Store samples at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 600 x g for 10 minutes and assay. Store samples 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.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8°C.
- **Standard Curve:** Reconstitute the 400 ng of human FX Standard with 2 ml of MIX Diluent to generate a Stock solution of 200 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by diluting the Stock solution (200 ng/ml) 1:2 with MIX Diluent to generate solution of 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C.

Standard Point	Dilution	[FX] (ng/ml)
P1	1 part Stock (200 ng/ml) + 1 part MIX Diluent	100.000
P2	1 part P1 + 1 part MIX Diluent	50.000
P3	1 part P2 + 1 part MIX Diluent	25.000
P4	1 part P3 + 1 part MIX Diluent	12.500
P5	1 part P4 + 1 part MIX Diluent	6.250
P6	1 part P5 + 1 part MIX Diluent	3.125
P7	1 part P6 + 1 part MIX Diluent	1.563
P8	MIX Diluent	0.000

- **Biotinylated FX Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- **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 MIX Diluent. Any remaining solution should be frozen at -20°C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed.
- 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 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 FX Antibody to each well and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 µl 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.

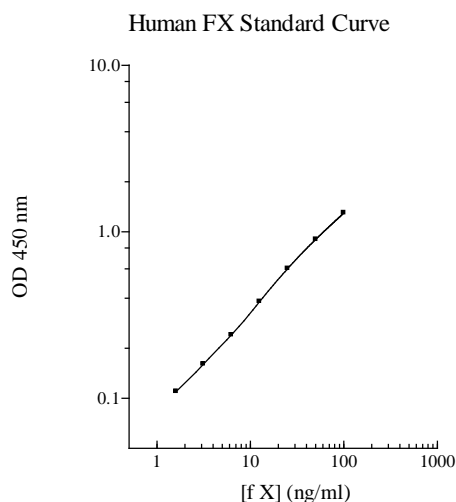
- Add 50 µl of Chromogen Substrate per well and incubate for approximately 20 minutes or till the optimal blue color density develop. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from 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 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 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 used for illustration only. A standard curve should be generated each time the assay is performed.



Precision, Sensitivity and Specificity

- The minimum detectable dose of human FX is typically ~1.5 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.1% respectively.

Linearity

Sample Dilution	Average Percentage of Expected Value	
	Plasma	Serum
1:400	96%	94%
1:800	99%	98%
1:1600	101%	97%

Sample Dilution	Average Percentage of Expected Value		
	Saliva	Urine	Milk
No dilution	89%	95%	92%
1:2	99%	96%	99%
1:4	97%	94%	98%

Recovery

Standard Added Value	5 – 50 ng/ml
Recovery %	84-117 %
Average Recovery %	96%

Cross-Reactivities

Species	% Cross Reactivity
Canine	0.5%
Bovine	0.1%
Monkey	2%
Mouse	0.5%
Rat	0.5%
Swine	0.1%
Rabbit	0.1%
Human	100%
Human Factor Xa	100%

References

- (1) Ruf, W. and Edgington, T.S. (1994) *FASEB J.* 8:385
- (2) Neuenschwander, P.F. *et al.* (1993) *Thrombosis and Haemostasis* 70:970
- (3) Messier, T.L. *et al.* (1991) *Gene* 99:291
- (4) Di Scipio, R.G. *et al.* (1977) *Biochemistry* 16:5253

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