AssayMax™

Human Factor IX 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 50 µl of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 10 minutes.

Step 5. Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key

Consult instructions for use.

Assay Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Human Factor IX ELISA Kit

Catalog No. EF1009-1

Sample insert for reference use only

Introduction

Factor IX (FIX) is a zymogen 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).

Principle of the Assay

The AssayMax Human Factor IX ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human factor IX in **plasma, serum, CSF, and cell culture samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures factor IX in less than 4 hours. A polyclonal antibody specific for factor IX has been pre-coated onto a 96-well microplate with removable strips. Factor IX in standards and samples is sandwiched by the immobilized antibody and the biotinylated polyclonal antibody specific for factor IX, which is recognized by a streptavidin-peroxidase 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 For Use In Diagnostic Procedures.
- Prepare all reagents (working diluent buffer, wash buffer, standard, 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 insert. 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.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human Factor IX Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against Factor IX.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Factor IX Standard:** Human Factor IX in a buffered protein base (220 ng, lyophilized).
- **Biotinylated Human Factor IX Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against FIX (120 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

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody 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.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- Store Standard 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 supernatants. Dilute samples 1:400 into EIA Diluent and assay. 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).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes, and remove serum. Dilute samples 1:400 into EIA Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Collect cell culture media and centrifuge at 3000 x *g* for 10 minutes at 4°C to remove debris. Collect supernatants and assay. Samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **CSF:** Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x *g* for 10 minutes and assay. Samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

Refer to Sample Dilution Guidelines below for further instruction.

Guidelines for Dilutions of 1:100 or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
1:100	**1:10000**
A) 4 ul sample: 396 µl buffer(100x) = 100 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000 fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1:1000	**1:100000**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000 fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	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 *Assuming the needed volume is less than or equal to 240 µl.*

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 Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.

- **Human Factor IX Standard:** Reconstitute the 220 ng (44 mU) of Human Factor IX Standard with 2.2 ml of EIA Diluent to generate a 100 ng/ml (20 mU/ml) standard stock solution. 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 stock solution (100 ng/ml) 1:2 with EIA Diluent to produce 50, 25, 12.5, 6.25, 3.125, and 1.563 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

Standard Point	Dilution	[FIX] (ng/ml)	[FIX] (mU/ml)
P1	1 part Standard	100.0	20.00
P2	1 part P1 + 1 part EIA Diluent	50.00	10.00
P3	1 part P2 + 1 part EIA Diluent	25.00	5.000
P4	1 part P3 + 1 part EIA Diluent	12.50	2.500
P5	1 part P4 + 1 part EIA Diluent	6.250	1.250
P6	1 part P5 + 1 part EIA Diluent	3.125	0.625
P7	1 part P6 + 1 part EIA Diluent	1.563	0.313
P8	EIA Diluent	0.000	0.000

- **Biotinylated Human Factor IX 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°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 EIA Diluent. Any remaining solution should be frozen 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 50 μl of Human Factor IX Standard or sample per well. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash five times with 200 μl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 μl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 μl of Biotinylated Human Factor IX 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 10 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 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 (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.

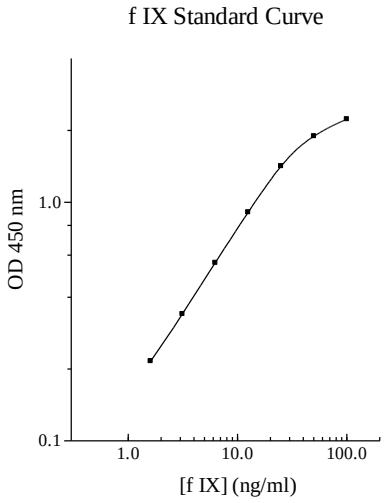
Typical Data

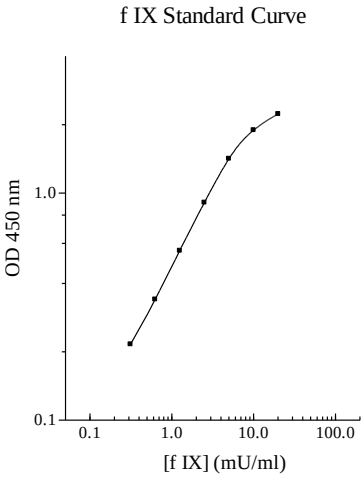
- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	ng/ml	OD	Average OD
P1	100.0	2.199 2.193	2.196
P2	50.00	1.850 1.856	1.853
P3	25.00	1.426 1.416	1.421
P4	12.50	0.940 0.942	0.941
P5	6.250	0.558 0.554	0.556
P6	3.125	0.345 0.343	0.344
P7	1.563	0.215 0.207	0.211
P8	0.000	0.092 0.090	0.091
Sample: Pool Normal, Sodium Citrate Plasma (400x)		1.067 1.071	1.069

Standard Curve

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





Reference Value

- Human plasma and serum samples from healthy adults were tested (n=40). On average, factor IX level was 4947 ng/ml.

Sample	n	Average Value (ng/ml)
Human Pool Normal Plasma	10	5218
Human Normal Plasma	20	5359
Human Pool Normal Serum	10	4265

Performance Characteristics

- The minimum detectable dose of factor IX as calculated by 2SD from the mean of a zero standard was established to be 0.4 ng/ml.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.
- **Kit standard has been calibrated against WHO International Standard.**

	Intra-Assay Precision			**Inter-Assay Precision**		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	3.6%	4.1%	3.3%	8.9%	8.6%	9.2%
Average CV (%)	3.7%			8.9%		

Spiking Recovery

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

Sample	Unspiked Sample (ng/ml)	Spike (ng/ml)	Expected	Observed	Recovery (%)
1	5.2	3.75	8.95	9.0	99%
		7.50	12.7	12.1	95%
		15.0	20.2	19.0	94%
2	10.3	3.75	14.05	15.7	112%
		7.50	17.8	18.4	103%
		15.0	25.3	23.1	91%
Average Recovery (%)					99%

Linearity

- Plasma and serum samples were serially-diluted to test for linearity.

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
1:200	92%	91%
1:400	98%	99%
1:800	103%	104%

Cross-Reactivity

Species	**Cross Reactivity (%)**
Beagle	None
Bovine	None
Monkey	<40%
Mouse	None
Rat	None
Swine	None
Rabbit	None
Proteins	**Cross Reactivity (%)**
Human Factor IX	100%
Human Factor IXa	100%

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

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of expired 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 dry 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.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	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) Wang X. *et al.* (2005) *J Thromb Haemost.* 3(4): 695-702
(2) Bogdanov VY *et al* (2003) *Nat Med.* 9(4): 458-62

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