

VisuLize™ Factor IX Antigen Kit

96 Test Enzyme Immunoassay Kit for Factor IX antigen.

For *In Vitro* Diagnostic Use.

Product # FIX-AG



Store at 2–8°C. Do not freeze.

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INTENDED USE

The VisuLize™ FIX Antigen kit is an Enzyme Immunoassay for the quantitative determination of Factor IX antigen in human plasma and Factor IX concentrates using the double antibody enzyme linked immuno-sorbent assay (ELISA).

SUMMARY

Factor IX (Christmas Factor) is a vitamin K-dependent glycoprotein produced in the liver with a molecular weight of 56,000 daltons.¹ The plasma concentration of Factor IX is normally around 5 µg/ml (87 nM). Factor IX contains two EGF-like domains and an amino-terminal domain containing 12 γ-carboxy-glutamic acid (Gla) residues. These Gla residues allow Factor IX to bind divalent metal ions and participate in calcium-dependent binding interactions.¹ Factor IX can be activated through the intrinsic coagulation pathway by limited proteolysis in the presence of calcium by activated factor XI (FXI^a) and/or through the extrinsic coagulation pathway by a complex of VII^a/tissue factor/phospholipid and activated Factor X.^{1,2} The terminal activated product in either case is Factor IX^a, a two-chain enzyme consisting of a heavy chain (28,000 daltons), a light chain (18,000 daltons) and an activation peptide product of 11,000 daltons.¹ Activated Factor IX, along with Factor VIIIa, calcium ions and a phospholipid membrane, converts Factor X into Xa eventually leading to the formation of a fibrin clot.³

The biological importance of Factor IX is demonstrated in Haemophilia B (Christmas disease), an X-linked congenital bleeding disease resulting from a quantitative (low activity and low antigen) or qualitative (low activity and normal antigen) defect in Factor IX function.⁴ The congenital deficiency of Factor IX may be classified as severe (<1% Factor IX activity), moderate (between 1 and 5% Factor IX activity) or mild (between 5 and 40% Factor IX activity).⁵

The laboratory diagnosis of Factor IX deficiency typically involves quantitative determinations of procoagulant levels i.e. functional activity of Factor IX.⁵ An ELISA for Factor IX antigen may be used in conjunction with functional assays in the area of gene therapy, assessment of Factor IX concentrates, determination of carrier status as well as distinguishing those patients with cross-reactive material i.e. low functional activity but near normal antigen levels of Factor IX.

PRINCIPLE OF ENZYME IMMUNOASSAY

Strip wells are pre-coated with goat polyclonal antibody to human Factor IX. Plasma samples are diluted and applied to the wells. The Factor IX antigen present binds to the coated antibody. After washing away unbound material, peroxidase-labeled goat detecting antibody is applied and allowed to bind to the captured Factor IX. The wells are again washed and a solution of TMB (the peroxidase substrate tetramethylbenzidine) is applied and allowed to react for a fixed period of time. A blue color develops which changes to yellow upon quenching the reaction with acid. The color formed is measured spectrophotometrically in a microplate reader at 450 nm. The absorbance at 450 nm is directly proportional to the concentration of Factor IX. The assay is calibrated using the calibrator plasma provided in the kit.

REAGENTS

A. Description of Reagent Items

- Item 1:** Foil pouch containing 6 strips, each containing 16 wells coated with goat antibody to human Factor IX.
- Item 2:** 2 vials of Calibrator Plasma, each lyophilized from 1 mL plasma.
- Item 3:** 2 vials of Control Plasma A, each lyophilized from 1 mL plasma.
- Item 4:** 2 vials of Control Plasma B, each lyophilized from 1 mL plasma.
- Item 5:** 1 vial containing 50 mL of 20X Wash Buffer Concentrate.
- Item 6:** 3 vials, each containing 20 mL of 2X buffered Sample Diluent.
- Item 7:** 1 vial containing 12 mL peroxidase-labeled goat detecting antibody.
- Item 8:** 1 vial containing 12 mL of tetramethylbenzidine (TMB) substrate.
- Item 9:** 1 vial containing 12 mL Stop Solution (0.2 M Sulphuric acid).

B. Caution and Warning

This kit is intended for use by personnel trained in laboratory procedures and universal precautions for the use of chemicals and potentially biohazardous substances. Some items contain human source material. Each unit of source plasma used in the preparation of this product has been tested by FDA approved methods and found negative for HBsAg, syphilis and antibodies to HIV and HCV and non-reactive for HIV-1 rNA and HCV rNA. As no test can offer complete assurance that products derived from human blood will not transmit infectious diseases, this product should be handled as a potentially infectious material.

The substrate TMB (tetramethylbenzidine) has reduced toxicity, but precautions should still be taken to avoid direct contact. The use of gloves and safety glasses is recommended. The Stop Solution contains dilute sulphuric acid (0.2 M), which is corrosive. The use of gloves and safety glasses is recommended. The disposal of waste materials must be carried out according to current local regulations. For a Material Safety Data Sheet for this product contact Affinity Biologicals Inc.

C. Reagent Preparation

Item 1 (Antibody-coated strips with frame): Just prior to use open pouch and remove strips and frame. Unused strips should be replaced in the pouch and resealed. Strips may be used directly, see section C: Assay Procedure.

Item 2 (Calibrator plasma): Reconstitute one vial with 1.0 mL of reagent grade water. Allow contents to dissolve for 15 minutes at room temperature with occasional swirling. Stability after reconstitution is 4 hours at ambient (18-25°C), or 30 days at -20° C.

Items 3 and 4 (Control plasmas): Reconstitute one vial of each plasma with 1.0 mL of reagent grade water. Allow contents to dissolve for 15 minutes at room temperature with occasional swirling. Stability after reconstitution is 4 hours at ambient (18-25°C), or 30 days at -20° C.

Item 5 (20X Wash Buffer Concentrate): Allow vial to warm to room temperature before use. Ensure any crystals that may have formed are dissolved before proceeding. If necessary the vial can be warmed to 37°C until all crystals have dissolved. Dilute the concentrate 1/20 before use. For every 2 strips (32 wells), add 16 mL concentrate to 304 mL reagent grade water and mix. Stability after dilution is 1 week at 2–8°C.

Item 6 (2X Sample Diluent Concentrate): Allow vial to warm to room temperature before use. Ensure any crystals that may have formed are dissolved. Dilute the concentrate by adding concentrate to an equal volume of reagent grade water and mix. Stability after dilution is 1 week at 2–8°C.

Items 7-9 are supplied ready to use.

D. Storage and Stability

Intact kits and un-reconstituted reagents are stable until the expiration date stated on the box and individual reagent labels when stored at 2–8°C.

SPECIMEN COLLECTION

Blood is collected into 3.2% Buffered Citrate anticoagulant tubes at a ratio of 9 volumes blood to 1 volume anticoagulant and gently mixed by inversion. Centrifuge at a minimum of 1500 x g for 15 minutes (CLSI Guideline H21-A5⁶). Remove supernatant plasma and use within 4 hours or freeze below -20°C for up to 30 days.

(diluted Item 6), mix, then add 350 µL of this 1/100 dilution into 350 µL sample diluent to obtain the 1/200 dilution.

3. **Assay:**

PLATE PREPARATION	Place desired number of strips into frame.	
STEP	Pipette into each pre-coated well:	
Factor IX CAPTURE	Test Sample (run in duplicate)	100 µL
	Cover strips with the plate sealer and incubate 30 minutes at ambient temperature.	
Empty wells and wash with 300 µL diluted wash buffer 3 times.		
DETECTING ANTIBODY	Detecting Antibody Solution (Item 7)	100 µL
	Cover strips with the plate sealer and incubate 30 minutes at ambient temperature.	
Empty wells and wash with 300 µL diluted wash buffer 3 times.		
COLOR DEVELOPMENT	TMB Substrate (Item 8)	100 µL
	Allow color to develop for exactly 10 minutes at ambient temperature.	
	Stop Solution (Item 9)	100 µL (Add to each well in same order in which the TMB was added)
Read plate at a wavelength of 450 nm within 30 minutes of adding Stop Solution If necessary, keep plate frame for use with any unused wells. Discard used wells.		

PROCEDURE

A. Material Provided

Foil pouch containing 6 strips of antibody coated wells.
 Calibrator Plasma, lyophilized.
 Control Plasma A, lyophilized.
 Control Plasma B, lyophilized.
 20X Wash Buffer Concentrate.
 2X Sample Diluent Concentrate.
 Detecting antibody solution.
 TMB substrate.
 Stop Solution.
 Adhesive Plate Sealer.

B. Additional Material Required (but not provided)

Reagent grade water for reconstitution and for dilution
 Single-channel adjustable volume pipettes
 Multi-channel pipettes
 Pipette tips
 Laboratory timer
 Microplate strip-well washer device
 Microplate compatible spectrophotometer capable of 450 nm.

C. Assay Procedure

PROCEDURAL NOTES:

- Reconstitute reagents as described in REAGENTS, Section C, Reagent Preparation. Allow reagents to warm to room temperature before use.
- It is recommended that all calibrator, control and test sample dilutions be run in duplicate and that each run include a buffer blank (see Assay Calibration section).
- All dilutions must be made just prior to use in the assay.
- Do not allow the wells to become dry at any time. Keep plate covered during incubations.
- Plasma samples should not be applied at dilutions lower than 1/10.
- Do not use kit components from different lot numbers.
- Incubation temperatures above or below normal room temperature (18 -25°C) may contribute to inaccurate results.
- Do not use kit components beyond expiration date
- Used strips must be discarded and not re-used.

- Preparation of Calibrator Plasma Dilutions:** Dilute the Calibrator Plasma (reconstituted Item 2) into Sample diluent (diluted Item 6) as detailed in Table 1 below:

TABLE 1:

Dilution	Calibrator Plasma	Sample Diluent
100% **	10 µL	990 µL
50%	350 µL of 100%	350 µL
25%	350 µL of 50%	350 µL
12.5%	350 µL of 25%	350 µL
6.25%	350 µL of 12.5%	350 µL
3.13%	350 µL of 6.25%	350 µL

(NOTE: 100% = 1.0 IU/mL)

** Refer to Calibrator plasma vial (Item 2) for FIX antigen value to be used as the concentration of the initial dilution of the calibrator plasma. E.g. If the calibrator has an assigned value of 1.25 IU/ml, follow the dilution scheme above but call the first point of the calibration curve 1.25 IU/ml.

- Control Plasma A** (reconstituted Item 3) and normal test plasmas are diluted 1/200 and 1/400. Add 10 µL plasma into 1990 µL sample diluent (diluted Item 6), mix, then add 350 µL of this 1/200 dilution into 350 µL sample diluent to obtain the 1/400 dilution. **Control Plasma B** (reconstituted Item 4) and samples with expected Factor IX content of <10% (example: Haemophilia-B samples) should be run at lower dilutions of 1/10 and 1/20. Add 70 µL plasma into 630 µL sample diluent (diluted Item 6), mix, then add 350 µL of this 1/10 dilution into 350 µL sample diluent to obtain the 1/20 dilution. Test plasmas with expected Factor IX content of 10-30% should be diluted 1/100 and 1/200. Add 10 µL plasma into 990 µL sample diluent

CALIBRATION

A. Assay Calibration

The Factor IX antigen value stated on the Calibrator Plasma vial has been determined by comparison to a secondary standard that is traceable to the WHO international standard for Factor IX activity. This antigen value should be used as the concentration of the top point on the reference curve. It is recommended that the plate be blanked on wells that have received Sample Diluent alone instead of diluted sample (reagent blank wells).

B. Reference Curve and Calculation of Results

The reference curve is a log-log plot of the mean absorbance values (y axis) versus the Factor IX concentration (x axis). The Factor IX content of test samples and controls can be read from the reference curve and multiplied by the appropriate dilution factor. Under the conditions described here, a sample diluted 1/100 will have a dilution factor of 1, a dilution of 1/200 will have a dilution factor of 2, and a dilution of 1/400 has a dilution factor of 4. Samples applied at a lower dilution of 1/10 will have a dilution factor of 0.1, and a 1/20 dilution has a dilution factor of 0.2.

Example: Test plasma when diluted 1/200 gives an absorbance corresponding to 45% when read from the reference curve. This value would be multiplied by a dilution factor of 2 to obtain the corrected value of 90%.

QUALITY CONTROL

The supplied Control Plasmas (Item 3 and 4) should be assayed with every series of samples that are run. The Factor IX values obtained for test samples should be considered suspect if the values obtained for the control plasmas fall outside of the range stated on the Control Plasma labels.

LIMITATIONS AND INTERFERENCES

The Factor IX antigen values obtained using this assay should not be used in isolation to diagnose disease. Patient history, clinical presentation and findings from other diagnostic procedures should also be considered. Clinically significant states are known to exist in which plasma Factor IX antigen levels are normal or near-normal in the presence of a significant reduction in Factor IX activity.⁴

This kit has been developed for use with citrated plasma. The use of samples containing anticoagulants other than 3.2% sodium citrate is not recommended.

Assay interference due to the presence of drugs or due to the presence of heterophilic antibodies such as Lupus Anticoagulant (LA) and Rheumatoid factor (RF) has not been reported, however, the potential for interference by high levels of heterophilic antibodies cannot be excluded. The theoretical possibility of test samples containing antibodies to goat immunoglobulin may also interfere in the assay.

EXPECTED VALUES

The normal range for Factor IX as reported in the literature is 0.5-1.5 IU/mL.³ Each laboratory should determine a normal range independently but results from three lots measured in 101 healthy individuals indicate a normal reference interval of 0.69-1.28 IU/mL (mean = 0.987 IU/mL, SD = 0.148). Samples with values outside the range of the reference curve may need to be diluted and re-tested for accurate results.

PERFORMANCE CHARACTERISTICS

A. Specificity

This assay measures Factor IX antigen in human plasma, therapeutic Factor IX concentrates and recombinant Factor IX preparations.

B. Detection Limit

When assay is performed as indicated in Section C, Assay Procedure, the detection limit of this assay is 0.005 IU/mL (0.5%) Factor IX. For a given lot, the upper limit of the assay corresponds to two times the value of the Calibrator plasma (Item 2). Samples with values outside the range of the reference curve may need to be diluted and re-tested for accurate results.

C. Accuracy

The VisuLize™ Factor IX Antigen kit was compared to the Asserachrom IX:AG on 134 patient samples containing Factor IX levels ranging across the entire detection range. The correlation co-efficient (r) was 0.987 ($R^2 = 0.974$, $y=0.8628x+0.0474$).

D. Precision

Intra-assay Precision, Method 1: Normal and abnormal plasma samples were tested in 4 assays total in each of three lots with 40 replicates per sample per plate. The mean coefficient of variation (CV) from all results was 4.74%

Intra-assay Precision, Method 2: Three plasmas with different Factor IX concentrations were tested in replicates of 8 in 20 assay events using 3 lots of product. The intra-assay coefficient of variation (CV) was calculated according to NCCLS Guideline EP5-A⁷ and is indicated in the summary below for each Factor IX level. The mean CV from all results by this method was 4.70%.

	<u>Lot 1</u>	<u>Lot 2</u>	<u>Lot 3</u>
1.0-1.3 IU/mL sample	3.30%	3.24%	4.14%
0.5-0.7 IU/mL sample	3.72%	4.22%	8.78%
<0.05 IU/mL sample	4.76%	4.04%	6.14%


Inter-assay Precision: Three plasmas with different Factor IX concentrations were tested in replicates of 8 in 20 assay events using 3 lots of product. The inter-assay coefficient of variation (CV) was calculated according to NCCLS Guideline EP5-A⁷ and is indicated in the summary below for each Factor IX level. The mean CV from all results by this method was 4.88%

	<u>Lot 1</u>	<u>Lot 2</u>	<u>Lot 3</u>
1.0-1.3 IU/mL sample	6.84%	4.23%	3.49%
0.5-0.7 IU/mL sample	6.84%	4.59%	2.96%
<0.05 IU/mL sample	5.27%	6.20%	3.54%

E. Lot-to-Lot Variability

Ten control samples with Factor IX values ranging from 0.28 – 0.87 IU/mL were tested in duplicate on three lots to determine assay precision between lots. The mean lot-to-lot variability was 2.76%.

SYMBOL LEGEND ⁸

 For *in vitro* diagnostic use

 Manufacturer

 Batch code

 Authorized Representative



Expiry date



Catalogue Number



Temperature limitation



Consult instructions for use



Contains sufficient for <n> tests



Biological Risks

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