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ab108831
Human Factor IX ELISA Kit

For the quantitative measurement of human Factor IX in plasma, serum CSF and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

Factor IX Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Factor IX concentrations in cell culture supernatants, serum and plasma.

A Factor IX specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently a Factor IX specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Conjugate is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Factor IX captured in plate.

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

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed

Add standard or sample to appropriate wells.

Incubate at room temperature.

Wash and add prepared biotin antibody to each well. Incubate at room temperature.

Wash and add prepared Streptavidin-Peroxidase Conjugate. Incubate at room temperature.

Add Chromogen Substrate to each well. Incubate at room temperature

Add Stop Solution to each well. Read immediately.

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage Condition
Factor IX Microplate (12 x 8 wells)	96 wells	4°C
Factor IX Standard	1 vial	4°C
10X Diluent M Concentrate	30 mL	4°C
50X Biotinylated Human Factor IX Antibody	1 vial	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	7 mL	4°C
Stop Solution	11 mL	4°C
20X Wash Buffer Concentrate	2 x 30 mL	4°C
Sealing Tapes	3	N/A

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 1 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.
- 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.

9.1 1X Diluent M

Dilute the 10X Diluent M Concentrate 1:10 with reagent grade water. Mix gently and thoroughly.

Δ Note Store for up to 1 month at 4°C.

9.2 1X Wash Buffer

Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

9.3 1X Biotinylated Factor IX Detector Antibody

9.3.1 The stock Biotinylated Factor IX Antibody must be diluted with 1X Diluent M according to the label concentration to prepare 1X Biotinylated Factor IX Antibody for use in the assay procedure. Observe the label for the "X" concentration on the vial of Biotinylated Factor IX Antibody.

9.3.2 Calculate the necessary amount of 1X Diluent M to dilute the Biotinylated Factor IX Antibody to prepare a 1X Biotinylated Factor IX Antibody solution for use in the assay procedure according to how many wells you wish to use and the following calculation:

Number of Wells Strips	Number of Wells	(V_T) Total Volume of 1X Biotinylated Antibody (µL)
4	32	1,760
6	48	2,640
8	64	3,520
10	80	4,400
12	96	5,280

Δ Note Any remaining solution should be frozen at -20°C.

Where:

C_S = Starting concentration (X) of stock Biotinylated Factor IX Antibody (variable)

C_F = Final concentration (always = 1X) of 1X Biotinylated Factor IX Antibody solution for the assay procedure

V_T = Total required volume of 1X Biotinylated Factor IX Antibody solution for the assay procedure

V_A = Total volume of (X) stock Biotinylated Factor IX Antibody

V_D = Total volume of 1X Diluent M required to dilute (X) stock Biotinylated Factor IX Antibody to prepare 1X Biotinylated Factor IX solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells:

$$(C_F / C_S) \times V_T = V_A$$

Calculate the final volume of 1X Diluent M required to prepare the 1X Biotinylated Factor IX Antibody:

$$V_T - V_A = V_D$$

Example:

NOTE: This example is for demonstration purposes only. Please remember to check your antibody vial for the actual concentration of antibody provided.

C_S = 50X Biotinylated Factor IX Antibody stock

C_F = 1X Biotinylated Factor IX Antibody solution for use in the assay procedure

V_T = 3,520 µL (8 well strips or 64 wells)

$$(1X/50X) \times 3{,}520\ \mu L = 70.4\ \mu L$$

$$3{,}520\ \mu L - 70.4\ \mu L = 3{,}449.6\ \mu L$$

V_A = 70.4 µL total volume of (X) stock Biotinylated Factor IX Antibody required

V_D = 3,449.6 µL total volume of 1X Diluent M required to dilute the 50X stock Biotinylated Antibody to prepare 1X Biotinylated Factor IX Antibody solution for assay procedures.

9.3.3 First spin the Biotinylated Factor IX Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount V_A of stock Biotinylated Factor IX Antibody to the calculated amount V_D of 1X Assay Diluent M. Mix gently and thoroughly.

9.4 1X SP Conjugate

Spin down the 100X Streptavidin-Peroxidase Conjugate (SP Conjugate) briefly and dilute the desired amount of the conjugate 1:100 with 1X Diluent M.

Δ Note Any remaining solution should be frozen at -20°C.

10. Standard Preparation

- Prepare serially diluted standards immediately prior to use.
- Always prepare a fresh set of standards for every use.
- Any remaining standard should be stored at -20°C after reconstitution and used within 30 days.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Reconstitute the Factor IX Stock to generate a 100 ng/mL **Standard #1**.

10.1.1 First consult the Factor IX Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent M to add when resuspending the Factor IX Standard vial to produce a 100 ng/mL Factor IX Standard stock by using the following equation:

C_S = Starting mass of Factor IX Standard (see vial label) (ng)

C_F = 100 ng/mL Factor IX **Standard #1** final required concentration

V_D = Required volume of 1X Diluent M for reconstitution (µL)

Calculate total required volume 1X Diluent M for resuspension:

$$(C_S / C_F) \times 1{,}000 = V_D$$

Example:

Δ Note This example is for demonstration purposes only. Please remember to check your standard vial for the actual amount of standard provided.

C_S = 240 ng of Factor IX Standard in vial

C_F = 100 ng/mL Factor IX **Standard #1** final concentration

V_D = Required volume of 1X Diluent M for reconstitution

(240 ng / 100 ng/mL) x 1,000 = 2,400 µL

10.1.3 First briefly centrifuge the Factor IX Standard Vial to collect the contents on the bottom of the tube.

10.1.4 Reconstitute the Factor IX Standard vial by adding the appropriate calculated amount V_D of 1X Diluent M to the vial to generate the 100 ng/mL Factor IX **Standard #1.** Mix gently and thoroughly.

10.2 Allow the reconstituted 100 ng/mL Factor IX **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions

10.3 Label seven tubes #2 – 8.

10.4 Add 120 µL of 1X Diluent M to tube #2 – 6.

10.5 To prepare **Standard #2**, add 120 µL of the **Standard #1** into tube #2 and mix gently.

10.6 To prepare **Standard #3**, add 120 µL of the **Standard #2** into tube #3 and mix gently.

10.7 Using the table below as a guide, prepare subsequent serial dilutions.

10.8 1X Diluent M serves as the zero standard (0 ng/mL).

Standard #	Volume to dilute (µL)	Volume Diluent M (µL)	Human Factor IX (ng/mL)
1	Step 10.1		100
2	120 µL Standard #1	120	50
3	120 µL Standard #2	120	25
4	120 µL Standard #3	120	12.5
5	120 µL Standard #4	120	6.25
6	120 µL Standard #5	120	3.125
7	120 µL Standard #6	120	1.563
8 (Blank)	N/A	120	0

11. Sample Preparation

11.1 Plasma:

Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3,000 x g for 10 minutes and collect supernatants. 1:400 sample dilution is suggested into 1X Diluent M; 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).

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3,000 x g for 10 minutes and remove serum. 1:400 sample dilution is suggested into 1X Diluent M; 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.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media and centrifuge at 3,000 x g for 10 minutes at 4°C to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 Cerebrospinal Fluid (CSF):

Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x g for 10 minutes. The sample is suggested for use at 1x or within the range of 2x – 10x into 1X Diluent M; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater *(for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
4 µl 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*
1000x	**100000x**
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 A + 216 µl buffer (10X) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl*

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.

13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).

13.2 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.

13.3 Add 50 µL of Factor IX Standard or sample per 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 two hours. Start the timer after the last sample addition.

13.4 Wash five times with 200 µL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine, wash six times with 300 µL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.

13.5 Add 50 µL of 1X Biotinylated Factor IX Antibody 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 one hour.

13.6 Wash microplate as described above.

13.7 Add 50 µL of 1X 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.

13.8 Wash microplate as described above.

13.9 Add 50 µL of Chromogen Substrate per well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate for 10 minutes in ambient light or until the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

13.10 Add 50 µL of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed.

13.11 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 12 minutes, which will reduce the readings.

13.12 Analyze the data as described below.

13.12.1 Calculate the mean value of the duplicate or triplicate readings for each standard and sample.

13.12.2 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.

13.12.3 Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

14. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.

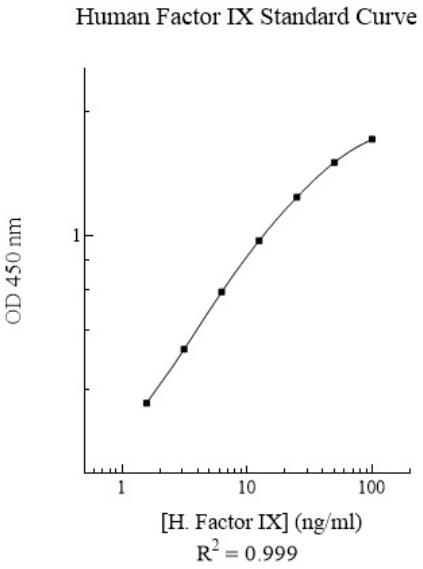

Figure 1. Example of Factor IX standard curve. The standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

15. Typical Sample Values

SENSITIVITY –

The minimum detectable dose (MDD) of Factor IX is typically ~0.4 ng/mL.

PRECISION –

	Intra-assay Precision	Inter-Assay Precision
CV (%)	5.1	10.2

RECOVERY –

Recovery (%)	91-112 %
Average Recovery (%)	99 %

LINEARITY OF DILUTION -

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

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

Average Percentage of Expected Value (%)		
Dilution Factor	Plasma	Serum
1:200	95	94
1:400	101	99
1:800	104	107

16. Assay Specificity

This kit recognizes Factor IX in plasma, serum, CSF and cell culture supernatants.

INTERFERENCES –

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

REFERENCE VALUES -

On average, normal Human factor IX plasma level is 4590 ng/mL.

CALIBRATION –

This immunoassay is calibrated against a highly purified Human Factor IX. The NIBSC/WHO unclassified purified Human Factor IX preparation 07/182 was evaluated in this kit.

The dose response curve of the unclassified standard 07/182 parallels the Factor IX standard curve. To convert sample values obtained with Factor IX Human ELISA Kit (ab46087) to approximate NIBSC Units/mL, use the equation below.

NIBSC (07/182) approximate value (Units/mL) = 9029.83 x Factor IX value (ng/mL).

17. Species Reactivity

Species	% Cross Reactivity
Canine	None
Bovine	None
Equine	None
Monkey	<40
Mouse	None
Rat	None
Swine	None
Rabbit	None
Protein	**% Cross Reactivity**
Human Factor IXa	100

18. Troubleshooting

Problem	Cause	Solution
Poor standard curve	Improper standard dilution	Confirm dilutions made correctly
	Standard improperly reconstituted (if applicable)	Briefly spin vial before opening; thoroughly resuspend powder (if applicable)
	Standard degraded	Store sample as recommended
	Curve doesn't fit scale	Try plotting using different scale
Low signal	Incubation time too short	Try overnight incubation at 4°C
	Target present below detection limits of assay	Decrease dilution factor; concentrate samples
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaws cycles)
High background/ Low sensitivity	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding STOP solution	Read plate immediately after adding STOP solution
	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)