Ab168546 Human Factor IX ELISA Kit (with plasma controls)

For the quantitative measurement of Human Factor IX concentrations in cell culture supernatants, plasma, CSF and serum. This product is for research use only and is not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab168546
(use abcam.cn/ab168546 for China, or abcam.co.jp/ab168546 for Japan)

Materials Supplied and Storage

Store kit at 4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C.

Item	Amount	Storage Condition (Before Preparation)
Factor IX Microplate (12 x 8 well strips)	96 Wells	4°C
Factor IX Standard	1 Vial	4°C
10X Diluent M Concentrate	30 mL	4°C
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
Positive control (Reference Plasma Control)	1 Vial	4°C
Low control (Depleted Human Plasma)	1 Vial	4°C
Sealing Tapes	3	N/A

Materials Required, Not Supplied:

- 1 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 tubes to prepare standard or sample dilutions.

1. Reagent Preparation

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare fresh reagents immediately prior to use. When diluting the concentrates, 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.

1.1 1X Diluent M: Dilute the 10X Diluent M Concentrate 1:10 with reagent grade water. Mix gently and thoroughly. *Store for up to 1 month at 4°C.*

1.2 1X Wash Buffer: Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

1.3 1X Biotinylated Factor IX Detector Antibody

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

1.3.2 Calculate the amount of 1X Diluent M to dilute the Biotinylated Factor IX Antibody to prepare a 1X Biotinylated Factor IX Detector 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 Detector Antibody (µL)
4	32	1,760
6	48	2,640
8	64	3,520
10	80	4,400
12	96	5,280

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 Detector Antibody solution for the assay procedure

V_T = Total required volume of 1X Biotinylated Factor IX Detector 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 Detector Antibody solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells: $\mathbf{V_A = (C_F / C_S) * V_T}$

Calculate the final volume of 1X Diluent M required to prepare the 1X Biotinylated Factor IX Detector Antibody: $\mathbf{V_D = V_T - V_A}$

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

1.3.4 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.

1.4 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. *Any remaining solution should be frozen at -20°C.*

1.5 Positive Control: Reconstitute 9 ng of the Positive Control with 1 mL of 1X Diluent M to generate a reference solution (9 ng/mL). Allow the reference plasma to sit for 10 minutes with gentle agitation prior to use. Prepare duplicate or triplicate control points. The useful range for this reference is: 6 ng/mL to 24 ng/mL. The data should be rejected if the values fall outside the range.

1.6 Low Control: Reconstitute the Low Control with 1 mL of 1X Diluent M. Allow the Low Control to sit for 10 minutes with gentle agitation prior to use. Prepare duplicate or triplicate control points. The useful range for this reference is: less than 2 ng/mL. The data should be rejected if the values fall outside the range.

2. 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. This procedure prepares sufficient standard dilutions for duplicate wells.

2.1 Reconstitution of the Factor IX Standard vial to prepare a 100 ng/mL Factor IX **Standard #1**.

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

2.1.2 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 #1** by using the 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: **$(C_S / C_F) \times 1{,}000 = V_D$**

2.1.3 Briefly spin the Factor IX Standard Vial to collect contents at the bottom of the tube.

2.1.4 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.

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

2.3 Label seven tubes #2 – 8.

2.4 Add 120 µL of 1X Diluent M to tubes #2 – 8.

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

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

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

2.8 1X Diluent M serves as the zero standard, 0 ng/mL (tube #8).

Standard #	Volume to Dilute (µL)	Volume Diluent M (µL)	Total Volume (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Step 2.1				100.0
2	120	120	240	100.0	50.00
3	120	120	240	50.00	25.00
4	120	120	240	25.00	12.50
5	120	120	240	12.50	6.25
6	120	120	240	6.25	3.13
7	120	120	240	3.13	1.56
8	-	120	120	-	0

3. Sample Preparation Avoid repeated freeze-thaw cycles of all samples.

3.1 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. A 400-fold sample dilution is suggested into 1X Diluent M; however, user should determine optimal dilution factor depending on application. Undiluted samples can be stored at-20°C or below for up to 3 months. EDTA/Heparin can be used as anticoagulant.

3.2 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. A 400-fold sample dilution is suggested into 1X Diluent M; however, user should determine optimal dilution factor depending on application. Undiluted samples can be stored at -20°C or below for up to 3 months.

3.3 Cell Culture Supernatants: Centrifuge cell culture media 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.

3.4 CSF: Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3,000 x g for 10 minutes and assay. 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.

4. Assay Procedure

Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use. Assay all standards, controls and samples in duplicate.

4.1 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).

4.2 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.

4.3 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 addition.

4.4 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.

4.5 Add 50 µL of 1X Biotinylated Factor IX Detector Antibody to each well. Gently tap plate to coat the wells. Break any bubbles that may have formed and incubate for one hour.

4.6 Wash microplate as described above.

4.7 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 and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

4.8 Wash microplate as described above.

4.9 Add 50 µL of Chromogen Substrate per well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed and incubate in ambient light for 10 minutes or till the optimal blue colour density develops.

4.10 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.

4.11 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 10 minutes, which will reduce the readings.

Calculations

Calculate the mean value of the 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.

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