

Version 3 Last updated 2 November 2021

ab229439 Human Factor IX CatchPoint® SimpleStep ELISA® Kit

For the quantitative measurement of Factor IX in human serum and plasma.

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

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1. Overview

Factor IX *in vitro* CatchPoint SimpleStep ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Factor IX protein in human serum and plasma.

The CatchPoint SimpleStep ELISA employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. CatchPoint HRP Development Solution containing the Stoplight Red Substrate is added. During incubation, the substrate is catalyzed by HRP generating a fluorescent product. Signal is generated proportionally to the amount of bound analyte and the intensity is measured in a fluorescence plate reader at 530/570/590 nm Excitation/Cutoff/Emission.

Factor IX, also known as Christmas Factor, is a 52 kDa zymogen of plasma serine proteases that initiates coagulation through activation by Tissue Factor (TF) and Factor VIIa. The activated form of Factor IX, known as Factor IXa, further activates an intrinsic activation complex that consists of factor X and factor VIIIa in the presence of Ca (2+) ions, and phospholipids. Mutations that cause a deficiency in Factor IX lead to x-linked hemophilia B, whereas overproduction of Factor IX has been shown to be a risk factor in venous thromboembolism (VTE). In 2013 the Food and Drug Administration approved the use of recombinant factor IX as a prophylactic treatment of patients with hemophilia B.

2. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50 μ L standard or sample to appropriate wells



Add 50 μ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350 μ L 1X Wash Buffer
PT



Add 100 μ L of prepared CatchPoint HRP Development Solution to
each well and incubate for 10 minutes



Read fluorescence at Ex/Cutoff/Em 530/570/590 nm

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. 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.
- All data, except Typical Standard Curve and Sensitivity were collected using the colorimetric version of this kit (ab188393).

6. Materials Supplied

Item	Quantity	Storage Condition
Human Factor IX Lyophilized Capture Antibody	600 µL	+4°C
Human Factor IX Lyophilized Detector Antibody	600 µL	+4°C
Human Factor IX Lyophilized Native Protein	2 Vials	+4°C
Antibody Diluent 4BI	6 mL	+4°C
Wash Buffer PT 10X	20 mL	+4°C
Stoplight Red Substrate Buffer	12 mL	+4°C
100X Stoplight Red Substrate	120 µL	+4°C
500X Hydrogen Peroxide (H ₂ O ₂ , 3%)	50 µL	+4°C
Sample Diluent NS*	50 mL	+4°C
SimpleStep Pre-Coated Black 96-Well Microplate	96 Wells	+4°C
Plate Seal	1	+4°C

*Sample Diluent NS is provided but not necessary for this product.

7. Materials Required, Not Supplied

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

- Fluorescence microplate reader Ex/Cutoff/Em 530/570/590 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- 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.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The incubation times provided in this protocol were optimized for fastest results with good signal. It is possible to increase the signal with longer incubation times, further optimization might be necessary.
- Keep in mind any RFU values shown are relative, NOT absolute. RFU from one plate reader are not comparable to another, especially if different make or model.
- To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.
- 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.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.2 10X Factor IX Capture Antibody:

To reconstitute the lyophilized Factor IX Capture Antibody, centrifuge the vial at 10,000 $\times g$ for 2 minutes and then add 160 μ L of nanopure water per vial; incubate at room temperature for 5 minutes and mix thoroughly. Add 160 μ L of Sample Diluent NS and incubate at room temperature for 5 minutes with rotation. For long term storage replace Sample Diluent NS for glycerol and store at -20°C.

9.3 10X Factor IX Detector Antibody:

To reconstitute the lyophilized Factor IX Detector Antibody, centrifuge the vial at 10,000 $\times g$ for 2 minutes and then add 160 μ L of nanopure water per vial; incubate at room temperature for 5 minutes and mix thoroughly. Add 160 μ L Sample Diluent NS and incubate at room temperature for 5 minutes with rotation. For long term storage replace Sample Diluent NS for glycerol and store at -20°C.

9.4 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BI. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent 4BI. Mix thoroughly and gently.

9.5 CatchPoint HRP Development Solution

Just prior to use prepare CatchPoint HRP Development Solution by diluting the 100X Stoplight Red Substrate and the 500X Hydrogen Peroxide in Stoplight Red Substrate Buffer.

For example, to make 6 mL of the CatchPoint HRP Development Solution combine 60 μ L 100X Stoplight Red Substrate and 12 μ L of 500X Hydrogen Peroxide with 5.928 mL Stoplight Red Substrate Buffer. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 IMPORTANT: If the protein standard vial has a volume identified on the label, reconstitute the Factor IX by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the Factor IX standard by adding 1 mL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 100 ng/mL **Stock Standard** Solution.

10.2 Label 11 tubes, Standards 2– 11. The **Stock Standard** solution will be standard #1.

10.3 Add 150 µL of Sample Diluent NS into numbers 2-11.

10.4 Use the Stock Standard to prepare the following dilution series. Standard #11 contains no protein and is the Blank control.

Standards will be added to the plate in step 13.3. If desired all 11 standards can be used for a full standard curve.

Alternatively, to commit fewer wells to the standard curve, select a subset of at least 7 standards plus the blank control. If 7 standards are desired, we recommend standards #2-8.

Standar d #	Sample to Dilute	Volume to Dilute (μ L)	Volume of Diluent (μ L)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock				100
2	Standard#1	150	150	100	50
3	Standard#2	150	150	50	25
4	Standard#3	150	150	25	12.5
5	Standard#4	150	150	12.5	6.25
6	Standard#5	150	150	6.25	3.13
7	Standard#6	150	150	3.13	1.56
8	Standard#7	150	150	1.56	0.78
9	Standard#8	150	150	0.78	0.39
10	Standard#9	150	150	0.39	0.20
11	None	0	150	0	0

11. Sample Preparation

Typical Sample Dynamic Range	
Sample Type	Range
Normal Human Serum	0.031 – 1%
Normal Human Plasma - EDTA	0.016 – 0.5%
Normal Human Plasma - Heparin	0.016 – 0.5%
Normal Human Plasma - Citrate	0.016 – 0.5%

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

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 plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well fluorescence or "edge effects" have not been observed with this assay.
- Ensure plate and all materials are equilibrated to room temperature during use.
- Cover the plate with a plate seal during incubation steps.

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.
 - Prepare all reagents, working standards, and samples as directed in the previous sections.
- 13.1** Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 13.2** Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
 - 13.3** Add 50 µL of all sample or standard to appropriate wells.
 - 13.4** Add 50 µL of the Antibody Cocktail to each well.
 - 13.5** Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
 - 13.6** Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
 - 13.7** Add 100 µL of prepared CatchPoint HRP Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
 - 13.8** Record the fluorescence at Ex/Cutoff/Em 530/570/590 nm. If using a Molecular Devices' plate reader supported by SoftMax® Pro software, a preconfigured protocol for these CatchPoint SimpleStep ELISA Kits is available with all the protocol and analysis settings at www.softmaxpro.org

Mode:	Fluorescence
Instrument settings:	Endpoint
Excitation:	530 nm
Cutoff:	570 nm
Emission:	590 nm
Sensitivity:	6 flashes/read or 200ms
PMT:	Auto
Auto calibrate:	On
Read:	Top

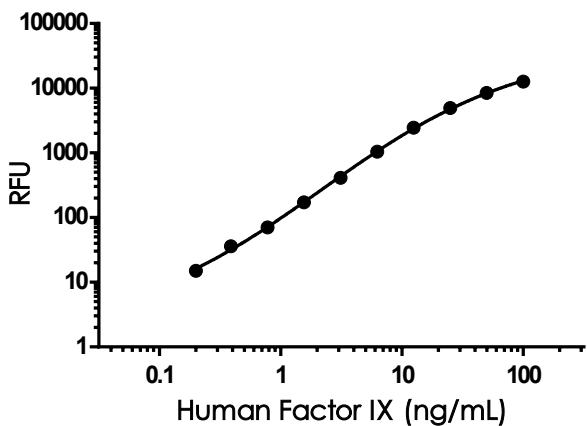
13.9 Analyze the data as described below.

14. Calculations

- 14.1 Preconfigured protocols are available when using SoftMax Pro software from Molecular Devices
 - 14.2 Calculate the average fluorescence value for the blank control (zero) standards. Subtract the average blank control standard fluorescence value from all other fluorescence values.
 - 14.3 **Create a standard curve** by plotting the average blank control subtracted fluorescence value for each standard concentration (yaxis) against the target protein concentration (xaxis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
- Δ **Note:** Most fluorescence reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- 14.4 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted fluorescence **values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
 - 14.5 Samples generating fluorescence values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at fluorescence values less than that of the lowest standard should be retested in a less dilute form.

15. Typical Data

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



Standard Curve Measurements			
Concentration (ng/mL)	RFU		Mean RFU
	1	2	
0	95	101	98
0.20	112	114	113
0.39	140	129	134
0.78	166	171	169
1.56	273	267	270
3.13	507	511	509
6.25	1,152	1,124	1,138
12.5	2,548	2,542	2,545
25	5,097	4,948	5,022
50	8,614	8,491	8,552
100	12,622	12,822	12,722

Figure 1. Example of human Factor IX standard curve in Sample Diluent NS. The Factor IX standard curve was prepared as described in Section 10. Raw data generated on SpectraMax M4 Multi-Mode Microplate Reader is shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 0.17 ng/mL. The MDD was determined by calculating the mean of zero standard replicates (n=17) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of Factor IX native protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
Normal Human Serum	108	86-130
Normal Human Plasma - EDTA	98	78-107
Normal Human Plasma - Heparin	103	85-119
Normal Human Plasma - Citrate	104	94-121

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.

Native Factor IX was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	1% NHS	0.5% Human Plasma (EDTA)	0.5% Human Plasma (Heparin)	0.5% Human Plasma (Citrate)
Undiluted	ng/mL	13.2	14.9	15.7	13.9
	% Expected value	100	100	100	100
2	ng/mL	5.94	6.62	7.05	6.04
	% Expected value	90	89	90	87
4	ng/mL	2.84	3.12	3.15	2.88
	% Expected value	86	83	80	83
8	ng/mL	1.59	1.58	1.72	1.52
	% Expected value	97	84	88	87
16	ng/mL	0.738	0.859	1.03	0.893
	% Expected value	90	92	105	103

PRECISION –

Mean coefficient of variations of interpolated values of Factor IX from three concentrations of human serum within the working range of the assay.

	Intra- Assay	Inter- Assay
n =	9	3
CV(%)	4	6

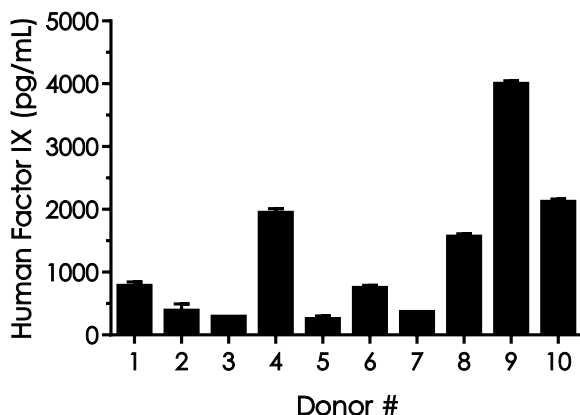


Figure 2. Ten individual healthy donors were evaluated for the presence of Factor IX in serum using this assay. Results were interpolated from the standard curve in Sample Diluent NS and corrected by sample dilution (400 fold). The mean level of Factor IX is 1,240 ng/mL with a range of 246 – 3,994 ng/mL and a standard deviation of 1,193 ng/mL.

17. Assay Specificity

This kit recognizes native human Factor IX protein in serum and plasma samples only.

18. Species Reactivity

This kit recognizes human Factor IX protein.

Other species reactivity was determined by measuring 1% serum samples of various species, interpolating the protein concentrations from the human standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in human serum assayed at the same dilution.

Reactivity < 3% was determined for the following species:

- Mouse
- Rat
- Hamster
- Guinea Pig
- Rabbit
- Dog
- Goat
- Pig
- Cow

Please contact our Technical Support team for more information.

19. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with CatchPoint HRP Development Solution too brief	Read plate again after longer incubation time
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions.
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store your reconstituted standards at -80°C, all other assay components 4°C. Keep Stoplight Red Substrate protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

20. Notes

Technical Support

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