ab188393 – Human Factor IX SimpleStep ELISA® Kit

For the quantitative measurement of Factor IX in human serum and plasma.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab188393

Storage and Stability: Store kit at 2-8°C immediately upon receipt. Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Standard Preparation and Reagent preparation sections.

Materials Supplied

Item	Quantity	Storage Condition
Factor IX Capture Antibody (Lyophilized)	2 Vials	+4°C
Factor IX Detector Antibody (Lyophilized)	2 Vials	+4°C
Factor IX Human Lyophilized Native Protein	2 Vials	+4°C
Antibody Diluent 4BI	6 mL	+4°C
10X Wash Buffer PT	20 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+4°C
Plate Seal	1	+4°C

Materials Required, Not Supplied

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

Microplate reader capable of measuring absorbance at 450 or 600 nm.
Method for determining protein concentration (BCA assay recommended).
Deionized water.
PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
Multi- and single-channel pipettes.
Tubes for standard dilution.
Plate shaker for all incubation steps.
Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

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

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

10X Factor IX Capture Antibody: To reconstitute the lyophilized Factor IX Capture Antibody, centrifuge the vial at 10,000 x g for 2 minutes and then add 160 µL of nanopure water per vial; incubate at room temperature for 5 minutes and mix thoroughly. Add 160 µL 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.

10X Factor IX Detector Antibody: To reconstitute the lyophilized Factor IX Detector Antibody, centrifuge the vial at 10,000 x g for 2 minutes and then add 160 µL of nanopure water per vial; incubate at room temperature for 5 minutes and mix thoroughly. Add 160 µL 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.

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 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BI. Mix thoroughly and gently.

Standard Preparation

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use. The following section describes the preparation of a standard curve for duplicate measurements (recommended).

1. **IMPORTANT**: If the protein standard vial has a volume identified on the label, reconstitute the Factor IX standard 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 1,000 µL Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 100 ng/mL **Stock Standard Solution**. .
2. Label eight tubes, Standards 1– 8 and add 150 µL of Sample Diluent NS into each tube.
3. Use the Stock Standard to prepare the following dilution series:

Standard #	Dilution Sample	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	150	150	100	50
2	Standard#1	150	150	50	25
3	Standard#2	150	150	25	12.5
4	Standard#3	150	150	12.5	6.25
5	Standard#4	150	150	6.25	3.13
6	Standard#5	150	150	3.13	1.56
7	Standard#6	150	150	1.56	0.78
8	Blank Control	0	300	0	0

Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	**Range (% Dilution)**
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%

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.

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.

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 absorbance or "edge effects" have not been observed with this assay.

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

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
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.
3. Add 50 µL of all sample or standard to appropriate wells.
4. Add 50 µL of the Antibody Cocktail to each well.
5. Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
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. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.
7. Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.
   Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.
   Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
8. Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
   Alternative to 7 – 8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:

Mode	**Kinetic**
Wavelength	600 nm
Time	up to 20 min
Interval	20 sec - 1 min
Shake	Shake between readings

Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 µL Stop Solution to each well and recording the OD at 450 nm

Download our ELISA guide for technical hints, results, calculation, and troubleshooting tips:
www.abcam.com/protocols/the-complete-elisa-guide

For technical support contact information, visit: www.abcam.com/contactus

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ASSAY SPECIFICITY
This kit is designed for the quantification of Human Factor IX.

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

CALCULATION –
- Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- Create a standard curve by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.

  Δ Note: Most microplate 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.
- Determine the concentration of the target protein in the sample by interpolating the blank control subtracted absorbance 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.
- Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

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

Standard Curve Measurement			
Concentration (ng/mL)	O.D 450nm		Mean O.D
	1	2	
0	0.112	0.113	0.113
0.78	0.154	0.158	0.156
1.56	0.191	0.194	0.192
3.13	0.255	0.254	0.255
6.25	0.383	0.385	0.384
12.5	0.656	0.632	0.644
25	1.114	1.143	1.129
50	1.897	1.989	1.943

Figure 1. Example of Factor IX standard curve in Sample Diluent NS. The Factor IX 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.

TYPICAL SAMPLE VALUES

Sensitivity:
The calculated minimal detectable dose (MDD) is 0.230 ng/mL. The MDD was determined by calculating the mean of zero standard replicates (n=24) and adding 2 standard deviations then extrapolating the corresponding concentrations.

Recovery
Three concentrations of Factor IX 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

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

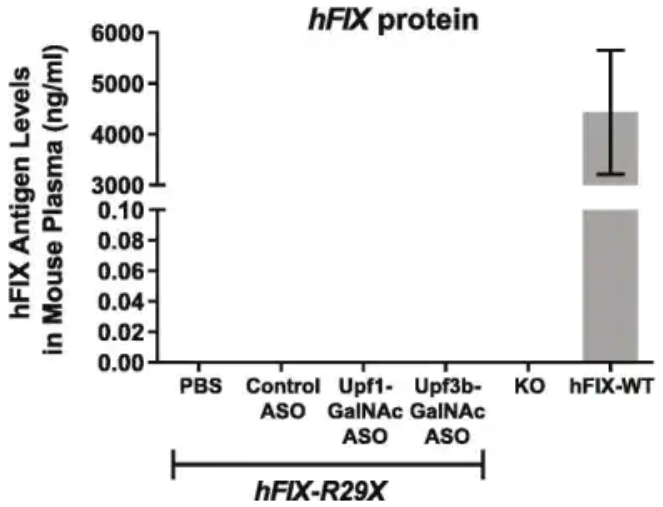
Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

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 from 3 concentrations of normal human serum within the working range of the assay.

	Intra-assay	Inter-assay
N=	9	3
CV (%)	4	6



Mouse plasma hFIX protein levels as measure by ELISA. Statistical significance was determined using a one-way ANOVA and Dunnett's multiple comparison test in Prism. All groups were compared to DPBS-treated *hFIX-R29X* group. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Image from Lulu Haung et al, Genome Biology, 19, 4, figure 5; doi: 10.1186/s13059-017-1386-9

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