

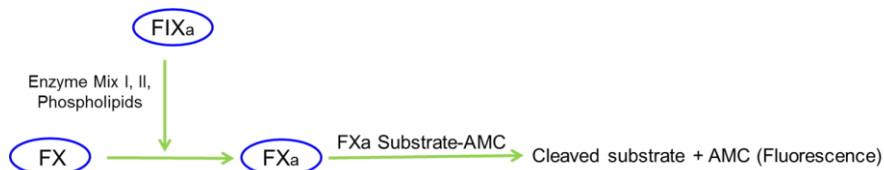
Factor IXa Activity Assay Kit (Fluorometric)

(Catalog # K364-100, 100 assays, Store kit at -20 °C)

rev 06/21

I. Introduction:

The coagulation Factor IX (or Christmas factor) (EC 3.4.21.22) is a vitamin K-dependent serine protease. Factor IX is produced as an inactive precursor and is activated via cleavage by either factor XIa (contact pathway) or factor VIIa (tissue factor pathway). In the presence of calcium ions and negatively charged membrane phospholipids, activated factor IX (FIXa) then binds to the activated Factor VIII (FVIIIa) and proteolytically activates factor X (FX) to factor Xa (FXa). **BioVision's Factor IXa Activity Assay kit** is based on the ability of FIXa to generate FXa. The generated FXa proteolytically cleaves a synthetic substrate and releases a fluorophore, AMC, which can be easily quantified by fluorescence microplate reader. The assay is simple, rapid and can detect activity as low as 10 pg of FIXa in a variety of samples.



II. Applications:

- Determine activity of pure FIXa
- Detect activity of FIXa in plasma, and serum

III. Sample Types:

- Purified enzyme
- Serum, plasma

IV. Kit Contents:

Components	K364-100	Cap Code	Part Number
FIXa Assay Buffer	15 ml	WM	K364-100-1
FXa Substrate-AMC	0.2 ml	Red	K364-100-2
Enzyme Mix I	1 vial	Blue	K364-100-3
Enzyme Mix II	1 vial	Purple	K364-100-4
Phospholipids	0.6 ml	Black	K364-100-5
FIXa Enzyme Standard (10 ng)	1 vial	Green	K364-100-6

V. User Supplied Reagents and Equipment:

- 96-well Black microplate with flat bottom
- Multi-well spectrophotometer.

VI. Storage Conditions and Reagent Preparation:

Store kit at -20 °C, protected from light. Briefly centrifuge small vials at low speed prior to opening. Read the entire protocol before performing the assay.

- **FIXa Assay Buffer:** Bring to room temperature (RT) before use.
- **Enzyme Mix I:** Reconstitute in 220 µl FIXa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot & store at -20 °C. Avoid repeated freeze/thaw.
- **Enzyme Mix II:** Reconstitute in 220 µl FIXa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot & store at -80 °C. Avoid repeated freeze/thaw.
- **Phospholipid Vesicles:** Vortex for 10 sec. before each use. Phospholipids can be stored at 4 °C for one month. For long term storage -20 °C is recommended. Avoid repeated freeze/thaw.
- **FIXa Enzyme Standard:** Reconstitute the vial in 20 µl FIXa Assay Buffer to prepare a stock solution of 0.5 ng/µl. Mix well by pipetting up and down. Divide into aliquots and store at -80 °C. Avoid repeated freeze/thaw cycles.

VII. Factor IXa Activity Assay Protocol:

- Sample Preparation:** Dilute serum and plasma samples 10 times with FIXa Assay Buffer and add 2-10 µl/well into a 96-well plate in two wells (Sample Well (**S**) and Background Control Well (**Bck**)). For purified enzyme, add 2-10 µl (in the expected range of 10 - 100 pg) per well into desired well(s). Adjust the volume of Background Control and sample wells to 10µl/well with FIXa Assay Buffer.

Notes:

- For unknown samples, we suggest doing pilot experiment and testing several amounts of FIXa to ensure the readings are within the Standard Curve range.
 - Background Control well is necessary to subtract basal Factor Xa activity that might be present in the sample.
- Standard Curve Preparation:** Prepare FIXa Enzyme Working Solution (5 pg/µl) just before use by adding 198 µl of FIXa Assay Buffer to 2 µl of FIXa Enzyme stock solution (0.5 ng/µl). Add 0, 2, 4, 6, 8, and 10 µl of FIXa Enzyme working solution (5 pg/µl) into a series of

wells in a 96-well plate to prepare 0, 10, 20, 30, 40, and 50 pg/well of FIXa Enzyme Standard. Adjust the volume of all Standard wells to 10µl with FIXa Assay Buffer. **Note:** Always make fresh FIXa Enzyme working solution.

3. **Reaction Mix:** Prepare the Reaction Mix of 10 µl/well by adding the components in the order shown:

Reaction Mix

Enzyme Mix I	2 µl
Phospholipids	6 µl
Enzyme Mix II	2 µl

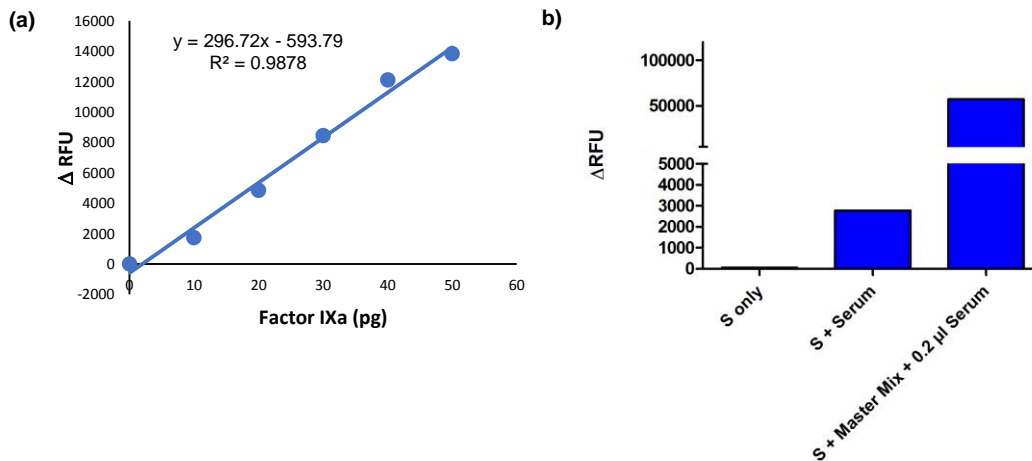
Mix and add 10 µl of the Reaction Mix into each Standard and Sample well. Add 10 µl of FIXa Assay Buffer to all Background Control well(s). Adjust the volume to 98 µl/well with FIXa Assay Buffer. Mix well by pipetting up and down. Incubate for 15 min at 37 °C. After incubation, add 2 µl of FXa substrate-AMC into Standard, Background Control and Sample wells. Mix well.

4. **Measurement:** Measure the fluorescence in kinetic mode for 30-60 min at 37 °C (Ex/Em = 360/450 nm). Choose any two time points (T₁ and T₂) in the linear range of the plot and obtain the corresponding values for the fluorescence (RFU_{S1} and RFU_{S2} and RFU_{bck1} and RFU_{bck2}). **Note:** Incubation time depends on the FIXa activity in the Samples. We recommend measuring fluorescence in kinetic mode, and choosing two time points (T₁ and T₂) in the linear range to calculate the FIXa activity of the Samples.

5. **Calculations:** Subtract 0 Standard reading from all readings. Subtract the Background Control signal from the Sample signal. Plot the Factor IXa Standard Curve. Apply corrected sample's ΔRFU to Factor IXa Standard Curve to obtain corresponding Factor IXa (B, in ng) and calculate the activity of Factor IXa in the sample as:

$$\text{Sample Factor IXa Activity} = \frac{B}{V} \times \text{Dilution Factor} = \frac{pg}{ml} = \frac{ng}{L}$$

Where, **B** is the FIXa amount in the Sample well from the Standard Curve (pg)
V is the volume of sample added into the reaction well (ml)



Figures: (a) Standard Curve of Factor IXa activity. (b) Factor IXa activity was measured in serum samples in the presence and absence of the master mix. S: Substrate. Assays were performed following the kit protocol.

VIII. Related Products:

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| Factor VIIIa Activity Fluorometric Assay Kit (K358) | Factor Xa Activity Fluorometric Assay Kit (K361) |
| Factor Xa Inhibitor Screening Kit (Fluorometric) (K362) | Factor Xa, Human Plasma (7689) |
| Thrombin Activity Fluorometric Assay Kit (K373) | Thrombin Inhibitor Screening Kit (Fluorometric) (K374) |
| Plasmin Activity Assay Kit (Fluorometric) (K381) | Plasmin Inhibitor Screening Kit (Fluorometric) (K382) |
| Plasmin Sepharose Beads (7926) | Urokinase Sepharose Beads (7927) |
| Urokinase, human recombinant (7696) | Urokinase Activity Fluorometric Assay Kit (K728) |

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