Technical Bulletin

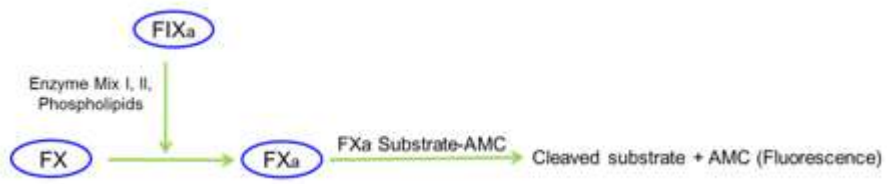
Factor IXa Activity Assay Kit (Fluorometric)

Catalog Number MAK240

Product Description

The coagulation factor IX (EC 3.4.21.22, Christmas factor) 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).

This 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.



Components

The kit is sufficient for 100 fluorometric assays in 96-well plates.

- FIXa Assay Buffer 15 mL
  Catalog Number MAK240A
- FXa Substrate-AMC 0.2 mL
  Catalog Number MAK240B
- Enzyme Mix I 1 vial
  Catalog Number MAK240C
- Enzyme Mix II 1 vial
  Catalog Number MAK240D
- Phospholipids 0.6 mL
  Catalog Number MAK240E
- FIXa Enzyme Standard (10 ng) 1 vial
  Catalog Number MAK240F

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Fluorescence multiwell plate reader
- Black flat-bottom 96-well plates. Cell culture or tissue culture treated plates are **not** recommended.

Precautions and Disclaimer

For R&D Use Only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C, protected from light.

Preparation Instructions

Briefly centrifuge small vials prior to opening.

FIXa Assay Buffer: Bring to room temperature before use.

Enzyme Mix I: Reconstitute in 220 µL of FIXa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot, and store at –20 °C. Avoid repeated freeze/thaw cycles.

Enzyme Mix II: Reconstitute in 220 µL of FIXa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot, and store at –80 °C. Avoid repeated freeze/thaw cycles.

Phospholipid Vesicles: Vortex for 10 seconds 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 cycles.

FIXa Enzyme Standard: Reconstitute in 20 µL of FIXa Assay Buffer to prepare a stock solution of 0.5 ng/µL. Mix well by pipetting up and down. Aliquot and store at –80 °C. Avoid repeated freeze/thaw cycles.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

1. Dilute serum and plasma samples 10× with FIXa Assay Buffer and add 2-10 µL/well into duplicate wells [Sample Well (S) and Background Control Well (Bck)] of a black 96-well plate.
2. For purified enzyme, add 2–10 µL (in the expected range of 10–500 pg) per well into desired well(s).
3. Adjust the volume of background control and sample wells to 10 µL/well with FIXa Assay Buffer.
4. For unknown samples, perform a pilot experiment and test several amounts of FIXa to ensure the readings are within the Standard Curve range.

Note: The Background Control Well is necessary to subtract basal factor Xa activity that might be present in the sample.

Standard Curve

Immediately prior to use, prepare FIXa Enzyme Working Solution (5 pg/µL) by adding 198 µL of FIXa Assay Buffer to 2 µL of FIXa Enzyme stock solution (0.5 ng/µL). prepare Standards according to Table 1. Mix well.

Table 1.
Preparation of Standards

Well	FIXa Enzyme Solution (5 pg/µL)	FIXa Assay Buffer	FIXa Enzyme (pg/ well)
1	0 µL	10 µL	0
2	2 µL	8 µL	10
3	4 µL	6 µL	20
4	6 µL	4 µL	30
5	8 µL	2 µL	40
6	10 µL	0 µL	50

Reaction Mixes

Mix enough reagents for the number of assays to be performed. For each well, prepare 10 µL of reaction mix according to Table 2. **Add reagents in the order shown.** Mix well.

Table 2.
Preparation of Reaction Mix

Reagent	**Volume**
Enzyme Mix I	2 µL
Phospholipids	6 µL
Enzyme Mix II	2 µL

Assay Procedure

1. Add 10 µL of Reaction Mix into each Standard and Sample (S) well.
2. Add 10 µL of FIXa Assay Buffer to all Background Control well(s).
3. Adjust the volume of each well to 98 µL/well with FIXa Assay Buffer.
4. Mix well by pipetting up and down.
5. Incubate for 15 minutes at 37 °C.
6. After incubation, add 2 µL of FXa substrate-AMC into standard, background control, and sample wells. Mix well.

Measurement

Measure fluorescence (RFU) in kinetic mode for 30–60 minutes at 37 °C (λ_{Ex} = 360 nm/ λ_{Em} = 450 nm).

Note: Incubation time depends on the FIXa activity in the samples. It is recommended to measure fluorescence in kinetic mode. For Samples (S) and Background Controls (Bck), choose two time points (T_1 and T_2) in the linear range to calculate the FIXa activity. For Standards, use the fluorescent readings (RFU) at the end of the incubation period for generation of the Standard Curve.

Results

1. Choose two time points (T_1 and T_2) 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}).
2. Subtract 0 Standard reading from all readings.
3. Subtract the Background Control signal (RFU_{Bck}) from the corresponding Sample signal (RFU_S) to obtain the corrected ΔRFU.
4. Plot the Factor IXa Standard Curve.
5. Apply the corrected ΔRFU for the Sample to the Factor IXa Standard Curve to obtain corresponding amount of Factor IXa (B, in pg). Calculate the activity of Factor IXa in the sample as:

FIXa Activity (pg/ mL or ng/ L) =

$$(B/V) \times DF$$

where

B = FIXa amount in the sample well from Standard Curve (pg)

V = Volume of sample added to the well (in mL)

DF = Dilution factor (if applicable; DF = 1 for undiluted samples)

Figure 1.
Typical Factor IXa Standard Curve

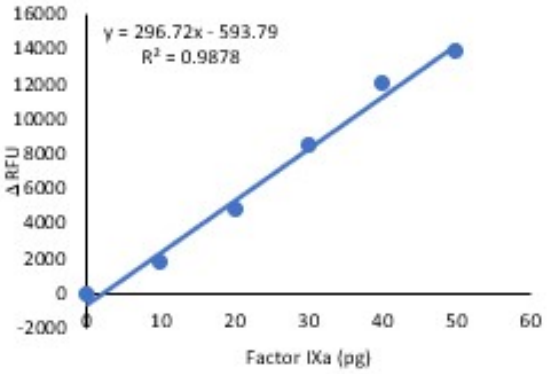
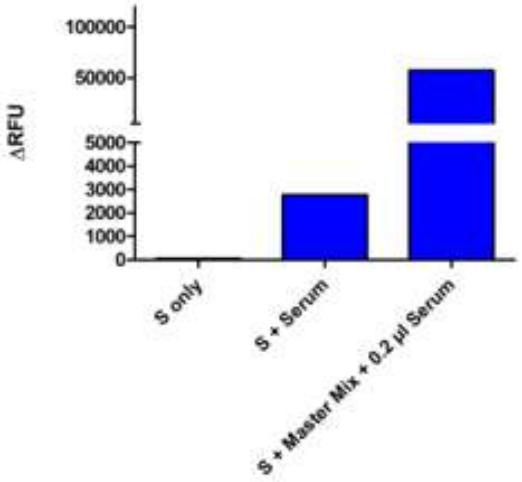


Figure 2.
Factor IXa activity was measured in serum samples in the presence and absence of the master mix. S = Substrate. Assays were performed following the kit protocol.



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