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ZYMUTEST Factor X

 # RK033A

(Complete ELISA kit for Factor X)

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTENDED USE:

The ZYMUTEST Factor X kit is a one step immuno-assay for measuring human Factor X (FX) in plasma, or in any fluid where FX can be present.
This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

First, the immunoconjugate, which is a polyclonal antibody specific for FX coupled to horse radish peroxidase (HRP), is introduced into the microwells coated with a polyclonal antibody specific for FX. Then, the diluted tested sample is immediately introduced, and the immunological reaction starts. When present, FX binds onto the polyclonal antibody coated solid phase through one epitope, and fixes the polyclonal antibody coupled to HRP through free epitopes. Following a washing step, the peroxidase substrate, 3,3',5,5' – Tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H_2O_2), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained. The amount of colour developed is directly proportional to the concentration of human FX in the tested sample.

TEST SAMPLE:

- Trisodium Citrate or Na_2 EDTA anticoagulated human plasma.
- Any biological fluid where FX must be measured.

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with a rabbit polyclonal antibody specific for human FX, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50ml of **Sample Diluent**, ready to use.
3. **Cal:** 3 vials of **Factor X Calibrator (calibrator plasma)**, lyophilised. When restored with **2 ml** of Sample Diluent, a plasma containing a concentration "**C"** (expressed in %) of human FX is obtained. This concentration (in the range 130-170% according to the lot), established by reference to the NIBSC international standard and a normal plasma pool, is accurately determined for each lot.
4. **CI:** 1 vial containing **0.5 ml** of lyophilised **Plasma FX Control I High** (human plasma).
5. **CII**: 1 vial containing **0.5 ml** of lyophilised **Plasma FX Control II Low** (human plasma).

Note: The FX concentrations and acceptancy ranges for controls can vary from lot to lot, and are indicated on the flyer provided in the kit.

6. **IC:** 3 vials of **Anti-(h)-FX-HRP immunoconjugate**, a polyclonal antibody coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
9. **TMB:** 1 vial of 25 ml peroxidase substrate: **3,3',5,5' – Tetramethylbenzidine** containing hydrogen peroxide. Ready to use.
10. **SA:** 1 vial of 6 ml of **0.45M Sulfuric acid** (Stop solution). Ready to use.

***Note*:** Use only components from a same kit lot. Do not mix components from different lots, when running the assay.

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 50-300 µl.
- **1-channel pipettes** at variable volumes from 0 to 20 µl, 20 to 200 µl and 200 to 1000 µl.
- **Micro ELISA plate** washing equipment and shaker.
- Micro ELISA plate **reader** with a wavelength set up at 450 nm.
- Distilled water.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at **2-8°C** for **4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
2. **Sample Diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
3. **Factor X Calibrator:** restore each vial with **2 ml** of Sample Diluent in order to obtain a plasma containing a FX concentration **"C",** already diluted 1:50 (fifty fold). This solution is stable for at least **24 hours at room temperature** or **72 hours at 2-8°C.**
4. **Plasma FX Control I** (human plasma, high): restore with **0.5 ml** distilled water.
5. **Plasma FX Control II** (human plasma, low): restore with **0.5 ml** distilled water.

Note: when restored, PAI-1 controls are stable for **24 hours** at room temperature, **72 hours at 2-8°C** or **2 months** frozen **at –20°C** or below.

Warning: Plasma controls I and II (4&5) and calibrator (3) are prepared with normal human plasma. This latter was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.

6. **Anti-(h)-FX-HRP immunoconjugate**: each vial must be restored with **2 ml of Conjugate Diluent**. Let the pellet completely dissolve before use, and shake the vial gently in order to homogenize the contents. The restored conjugate is stable for at least **24 hours** at room temperature or for at least **4 weeks at 2-8°C**.
7. **Conjugate Diluent**: It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
8. **Wash Solution**: Incubate the vial for 15-30 minutes in a water bath **at 37°C** until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow preparing 1 liter of Wash Solution). The Wash Solution must be stored at **2-8°C** in its original vial and used within **4 weeks** following opening. The diluted Wash Solution must be used within **7 days**, when protected from any contamination and stored at **2-8°C**. This reagent contains 0.05% Kathon CG.
9. **TMB substrate:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use.
10. **Stop solution:** It is ready to use.

Cautions: Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

***Note*:** Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.
The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

PROCEDURE:

Specimen collection:
Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within **8 hours** or stored frozen at –20°C or colder for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within **4 hours**.
EDTA collected human plasma may also be used. Conditions of storage are the same than those for citrated plasma.

Tested plasma or sample or controls:

The sample must be tested diluted **fifty fold (1:50)** in the Sample Diluent. For expected FX concentrations higher than "**C**" (in %), plasma or samples must be tested at a higher dilution, i.e., **1:100 (D=100), or more.** For low or very low FX concentrations lower dilutions can be used.
Plasma Controls I and II must be tested diluted **fifty fold (1:50)**, with Sample Diluent.

Calibration:
Using the **Plasma FX Calibrator,** with a FX concentration "**C**" (in the range 130-170% according to the lot used), provided in the kit, prepare the following standard solutions.

Factor X concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma FX calibrator at C	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05ml	0 ml
Vol. of Sample diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95ml	1 ml

Mix gently for a complete homogenization.
The standard dilutions are stable for at least **8 hours** at room temperature.

Assay procedure:
Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Conjugate anti (h)-FX-HRP. (Restored with 2 ml of conjugate Diluent)	**50 µl**	Introduce the Anti-(h)-FX- HRP immunoconjugate in the micro ELISA plate wells
FX calibrator or tested sample or sample diluent (blank)	**200 µl**	Introduce **immediately** the standard solutions or the tested samples in the corresponding micro ELISA plate well
Mix gently on a plate shaker or manually and incubate for 1 hour at room temperature (18-25°C)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument. **(a)**
TMB/H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. ***Note:*** The substrate distribution, row by row, must be accurate and at exact time intervals **(b, c)**.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (c)		
0.45 M Sulfuric Acid (5)	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid **(b).**
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **450 nm (A450)**. Subtract the blank value **(d)**.		

Note:

Distribute calibrators, controls and tested specimen as rapidly as possible, in order to obtain homogeneous immunological kinetics for FX binding. A too long delay (>15 min) between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results

a. Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilised components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity.
b. For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
c. Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.
d. For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

TWO STEP METHOD:

- The assay can also be performed with a two step method. The calibration curve is from **0 to C%** (as for the one step method), the FX calibrator being reconstituted with **2 ml** of Sample Diluent (**SD**).
- The immunoconjugate (**IC**) must be reconstituted with **7.5 ml** of Conjugate Diluent (**CD**).
- Tested plasma must be assayed at a fifty fold (**1:50**) dilution or at higher dilutions in Sample Diluent (**SD**), if required.
- In each microwell, **200 µL** of the calibration solution (prepared as for the one step method) or 200 µL of the diluted tested plasma are introduced. Following a **1 hour** incubation at room temperature (18-25°C) and a washing step, **200 µl**/well of immunoconjugate (**IC**) are introduced. Following a new **1 hour** incubation at room temperature and a washing step, the colour development with TMB (**200 µl**/well) is allowed to develop for **5 min**, and is then stopped with **50 µl** of 0.45M sulfuric acid (**SA**). A450 is then measured. Washing and operating cautions, as well as results interpretation, are the same as recommended for the one step method.

EXPRESSION OF RESULTS:

- On a linear graph paper plot the **FX concentration (%)** on abscissa and the corresponding absorbance (**A450**) on ordinates.
- Users must construct their own calibration curve, obtained using their calibrator dilutions (See model on the flyer). From the curve obtained, deduce directly the FX concentration for the tested sample. For obtaining the FX concentration in a sample tested at a higher or lower dilution, this value must be multiplied by **D:50** (i.e., x**2 for D=100**, or **x 0.40 for D=20**...).
- For **controls I and II**, the concentrations are directly deduced from the calibration curve.
- Alternatively, an ELISA software (i.e., Dynex, Biolise, etc.) can be used for the calculation of concentrations.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

BIOCHEMISTRY:

Factor X (FX) is a vitamin-K dependent serine-protease, glycoprotein, which can be activated by both intrinsic and extrinsic blood coagulation pathways. In the presence of calcium and phospholipids, activated FX(a) forms a complex with FV(a), which activated prothrombin into thrombin. The normal Factor X concentration in human plasma is of about 10µg/ml.

ASSAY CHARACTERISTICS:

- Detection threshold ≤ 5%.
- Intra-assay: 3-8%.
- Inter-assay: 5-10%.
- No significant heparin interference up to 2 IU/ml.

REFERENCES:

Ahmad SS, London FS, Walsh PN, "The assembly of the factor X-activating complex on activated human platelets", J Thromb Haemost, 1(1):48-59, 2003.