

ZYMUTEST Factor IX

RK032A

(Complete ELISA kit for Factor IX)

In vitro research use only

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INTENDED USE:

The ZYMUTEST Factor IX kit is a one step immuno-assay for measuring human Factor IX (FIX) in plasma, or in any fluid where FIX can be present.

ASSAY PRINCIPLE:

First, the immunoconjugate, which is a polyclonal antibody specific for FIX coupled to horse radish peroxidase (HRP), is introduced into the microwells coated with a polyclonal antibody specific for FIX. Then, the diluted tested sample is immediately introduced, and the immunological reaction starts. When present, FIX 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 (H₂O₂), 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 FIX in the tested sample.

TEST SAMPLE:

- Trisodium Citrate or Na₂ EDTA anticoagulated human plasma.
- Any biological fluid where FIX must be measured.

REAGENTS:

1. **COAT: Micro ELISA plate**, containing 12 strips of 8 wells, coated with a rabbit polyclonal antibody specific for human FIX, 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 IX Calibrator (calibrator plasma)**, lyophilised. When restored with 2 ml of Sample Diluent, a plasma containing a concentration "C" (expressed in %) of human FIX 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 FIX Control I High** (human plasma).
5. **CL:** 1 vial containing 0.5 ml of lyophilised **Plasma FIX Control II Low** (human plasma).

Nota: The FIX 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)-FIX-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.

Nota: 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 IX Calibrator:** restore each vial with 2 ml of Sample Diluent in order to obtain a plasma containing a FIX 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 FIX Control I** (human plasma, high): restore with 0.5 ml distilled water.
5. **Plasma FIX Control II** (human plasma, low): restore with 0.5 ml distilled water.

Nota: 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)-FIX-HRP immunoconjugate:** each vial must be restored with 2 ml of **Conjugate Diluent**. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content. 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.

Nota: 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 FIX concentrations higher than "C" (in %), plasma or samples must be tested at a higher dilution, ie **1:100 (D=100), or more**. For low or very low FIX 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 FIX Calibrator**, with a FIX concentration "C" (in the range 130-170% according to the lot used), provided in the kit, prepare the following standard solutions.

Factor IX concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma FIX 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 homogenisation.

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)-FIX-HRP. (Restored with 2 ml of conjugate Diluent)	50 µl	Introduce the Anti-(h)-FIX- HRP immunoconjugate in the micro ELISA plate wells
FIX 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 ₂ O ₂ Substrate	200 µl	Immediately after the washing, introduce the substrate into the wells. Nota : 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).		

Nota:

Distribute calibrators, controls and tested specimen as rapidly as possible, in order to obtain an homogeneous immunological kinetics for FIX 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

- 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.
- 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.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.
- 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 FIX 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, **200µL** of the calibration solution (prepared as for the one step method) or 200µL of the diluted tested plasma are introduced. Following a **1 hour** incubation at room temperature (18-25°C) and a washing step, **200µl/well** of immunoconjugate (IC) are introduced. Following a new **1 hour** incubation at room temperature and a washing step, the colour development with TMB (**200µl/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 **FIX concentrations (%)** on abscissa and the corresponding absorbances (**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 FIX concentration for the tested sample. For obtaining the FIX concentration in a sample tested at a higher or lower dilution, this value must be multiplied by **D:50** (i.e. **x2 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.

EXPECTED RANGE:

By definition, the 100 % Factor IX concentration corresponds to the concentration in a normal human citrated plasma pool, obtained by pooling plasmas from healthy males or females aged from 18 to 55 years, and out of any medication or disease. The FIX concentration in adults is usually between 60 and 150%.

BIOCHEMISTRY:

Factor IX (FIX) is a vitamin K dependent glycoprotein, which participates in the middle phases of blood coagulation.

The normal Factor IX concentration in human plasma is of about 4 to 5 µg/ml.

When activated by factor XI, in the presence of calcium, FIX(a) forms an active complex with FVIII, in the presence of calcium and phospholipids, which converts FX into FXa.

PATHOLOGICAL VARIATIONS:

Factor IX (also called antihemophilic factor B) deficiency (<25%) results in haemophilia B (congenital bleeding disorder).

Low plasma levels are observed in patients under anti-vitamin K therapy, or in case of hepatic disease, cirrhosis.

High levels of FIX could be an indicator of an increased risk for venous thrombosis.

APPLICATIONS:

Assay of FIX:Ag in plasma samples.

ASSAY CHARACTERISTICS:

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

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