ZYMUTEST Factor V

 # RK009A

Human coagulation factor V antigen

(Complete ELISA kit for the assay of Factor V)

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTENDED USE:

The ZYMUTEST Factor V kit is a two-site immuno-assay for measuring human Factor V antigen in plasma, or in any fluid where Factor V can be present.

This kit is for research use only and should not be used for patient diagnosis or treatment.

ASSAY PRINCIPLE:

ZYMUTEST Factor V is a sandwich ELISA specific for human Factor V.

The diluted tested plasma or biological fluid is introduced into a microwell coated with a monoclonal antibody specific for human Factor V. When present, this protein is capted onto the solid phase. Following a washing step, the immunoconjugate, which is a horse polyclonal antibody coupled to horse-radish-peroxidase (HRP), is introduced, and binds to the free epitopes of immobilized Factor V. Following a new washing step, the peroxidase substrate, Tetramethylbenzidine (TMB) in presence of Hydrogen Peroxide (H_2O_2), is introduced and a blue colour develops. The colour turns yellow when the reaction is stopped with Sulfuric Acid. The amount of colour developed is directly proportional to the concentration of human Factor V in the tested sample.

TEST SAMPLE:

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

REAGENTS:

1. **COAT: Micro ELISA plate,** containing 12 strips of 8 wells, coated with a mouse monoclonal antibody specific for human Factor V, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 mL of **Factor V Sample Diluent**, ready to use.
3. **CAL**: 3 vials of **Plasma Factor V calibrator**, (normal human plasma calibrated with a reference plasma pool), lyophilised, prediluted.

Each vial, when restored with **2 mL** of Factor V Sample Diluent, allows obtaining the calibrator plasma, **already diluted 1:50**. The exact Factor V concentration is indicated on the flyer provided in the kit.

4. **CI :** 1 vial of **0.5 mL** of **Plasma Factor V Control I (high)**, human plasma lyophilised.
5. **CII :** 1 vial of **0.5 mL** of **Plasma Factor V Control II (low)**, human plasma lyophilised.

   The exact Factor V concentration and the confidence range for the controls are indicated on the flyer provided in the kit.
6. **IC:** 3 vials of **Anti-(h)-Factor V-HRP immunoconjugate**, a horse polyclonal antibody coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 mL of **Factor V Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 mL of 20 fold concentrated **Wash Solution**.
9. **TMB:** One vial of 25 mL peroxidase substrate: 3,3',5,5' **– Tetramethylbenzidine** containing hydrogen peroxide, ready to use.
10. **SA:** One vial of 6 mL of **0.45 M Sulfuric Acid (Stop Solution)**, ready to use.

Note:

Use only components from a same kit lot number. 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 amount 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. **Factor V 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. It contains 0.05% Kathon CG.
3. **Plasma Factor V calibrator:** restore each vial with **2 mL of** Factor V Sample Diluent in order to obtain the calibrator plasma, **already diluted 50 fold**. This solution is stable for at least **8 hours** at room temperature.
4. **Plasma Factor V Control I (high) :** restore with **0.5 mL** distilled water.
5. **Plasma Factor V Control II (low) :** restore with **0.5 mL** distilled water.

   Note:

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

Warning:

Plasma Factor V calibrator (3) and controls (4&5) 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)-Factor V-HRP immunoconjugate**: each vial must be restored with **7.5 mL of Factor V Conjugate Diluent**. Leave the pellet to be completely dissolved before use, and shake the vial gently in order to homogeneize 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. **Factor V 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. It 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**. It 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 :** 0.45M Sulfuric acid, 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 below 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:

The sample must be tested diluted **fifty fold (1:50)** in the Factor V Sample Diluent. For expected Factor V concentrations > 100 %, plasma or samples can be tested at a higher dilution, 1:100, or 1:200, or more. If the dilution factor is **D**, concentrations obtained must then be multiplied by the complementary dilution factor which is **D:50** (i.e. x2 for 1/100, x4 for 1/200 etc…).
Controls I and II must be diluted **1:50** in the Factor V Sample Diluent.

Calibration:

Factor V concentrations are expressed as % of a normal pooled plasma (which concentration is assigned to 100%). For the Factor V assay the 100% concentration corresponds to a normal human plasma pool diluted **1:50**, which is the standard assay dilution.

Using the Plasma Factor V calibrator provided in the kit (2 mL of calibrator, already prediluted 1:50, and with a Factor V concentration "**C**" indicated on the flyer provided in the kit, for each lot of reagents), prepare the following standard solutions:

Factor V concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma FV calibrator	1 mL	0.5 mL	0.25 mL	0.1 mL	0.05 mL	0 mL
Vol. of FV Sample Diluent	0 mL	0.5 mL	0.75 mL	0.9 mL	0.95 mL	1 mL

Mix gently for a complete homogeneisation.
The standard dilutions are stable for at least **4 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
Plasma Factor V calibrator or tested sample or controls or sample diluent (blank)	**200 µl**	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well **(1).**
Incubate for 2 hours at 37°C (2, 3)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument **(1)**.
Conjugate (anti Factor V polyclonal antibody coupled with peroxidase. Restored with 7.5 mL of Conjugate Diluent)	**200 µl**	Introduce the Anti-(h)-Factor V- HRP immunoconjugate in the micro ELISA plate wells **(1)**.
Incubate for 2 hours at 37°C (2, 3)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument.
TMB / H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells **(1, 4)**. **Note**: The substrate distribution, row by row, must be accurate and at exact time intervals.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (2)		
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.
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **450 nm (A450)**. Substract blank values. **(5)**		

Note:

1. 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 immobilized 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 avoid a too drastic emptying, which could lower plate reactivity.
2. Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro ELISA plate shaker can be used.
3. In order to allow the complete antigen/antibody reaction, incubating the plates at 37°C is necessary for the Factor V ELISA.
4. For addition of the TMB/H_2O_2 substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.
5. For biochromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RESULTS:

Users must construct their own calibration curve obtained using their standard dilutions. The calibration curve provided on the flyer is an example only.
- On a linear graph paper plot the **Factor V concentrations** on abscissae and the corresponding absorbances on ordinates.
- From the curve obtained, deduce directly the Factor V concentration in samples tested at the standard **1:50** dilution. When higher dilutions are used (i.e **D**), the Factor V concentration must be multiplied by the complementary dilution factor (i.e. **D:50**) in order to get the factor V concentration in the tested sample (ex: x2 fo D=1:100).

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 V circulates in the plasma as a single chain glycoprotein with a molecular weight of 330,000. It is activated by thrombin to factor Va, a two-chain glycoprotein (a heavy and a light chain linked by a Ca2+ ion). Normal factor Va is degraded and inactivated by activated protein C, while factor Va with a mutation on amino acid 306 (R306Q) is resistant to this degradation. The plasma level of factor V is of 10 µg/mL in humans. It is synthesized by hepatocytes and megacaryocytes.

ASSAY CHARACTERISTICS:

- Assay range: 2 to 200 ng/mL (corresponding to a normal human plasma diluted from 1:50 to 1:5000).
- Detection threshold: 2 ng/mL.

LIMITATIONS OF THE METHOD:

- A whole mouse monoclonal antibody is used for coating. In some cases a possible interference of rheumatoid factor cannot be excluded.
- Factor V is very sensitive to activation. Factor Va is underestimated in the assay as part of the molecule is cleaved away, and less epitopes are available for binding the anti-factor V-HRP immunoconjugate.