



ZYMUTEST vWF:CBA

RK038A

(Complete ELISA kit for measuring von Willebrand Factor Collagen Binding Activity)

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INTENDED USE:

The ZYMUTEST vWF:CBA kit is an enzyme-immuno-assay for measuring human von Willebrand Factor (vWF) Collagen Binding Activity (CBA) in plasma, or in any fluid where vWF:CBA can be present.

ASSAY PRINCIPLE:

In a first step, the diluted tested plasma or biological fluid is introduced into a microwell coated with fibrillar collagen. When present, vWF is captured onto the solid phase through its collagen binding activity. Following a washing step, the immunoconjugate, which is a polyclonal antibody coupled to horse radish peroxidase (HRP), is introduced, and binds to free epitopes of immobilized vWF. 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 vWF:CBA in the tested sample.

TEST SAMPLE:

- Trisodium Citrate anticoagulated human plasma.
- Any biological fluid where vWF:CBA must be measured.

REAGENTS:

- COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with equine collagen (types I and III), then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
- SD:** 2 vials containing 50 ml of Sample Diluent, ready to use.
- Cal:** 3 vials of vWF Calibrator, lyophilised. When restored with 2 ml of Sample Diluent, a plasma containing a concentration "C" (expressed in %) of human vWF is obtained. This concentration (in the range 120-160% according to the lot), established by reference to the NIBSC international standard is accurately determined for each lot.
- CI:** 1 vial containing 0.5 ml of lyophilised vWF Plasma Control I High (human plasma).
- CII:** 1 vial containing 0.5 ml of lyophilised vWF Plasma Control II Low (human plasma).

Note: The vWF concentrations and acceptance ranges for control plasma I and II and calibrator can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.

- IC:** 3 vials of Anti-(h)-vWF-HRP immunoconjugate, a polyclonal rabbit antibody specific for human vWF, coupled to Horse-Radish-Peroxidase (HRP), lyophilised.
- CD:** 1 vial of 25 ml of Conjugate Diluent, ready to use.
- WS:** 1 vial of 50 ml of 20 fold concentrated Wash Solution.
- TMB:** 1 vial of 25 ml peroxidase substrate: 3,3',5,5' - Tetramethylbenzidine containing hydrogen peroxide. Ready to use.
- SA:** 1 vial of 6 ml of 0.45M Sulfuric acid (Stop solution). Ready to use.

Note: Use only components from a same lot of kits. Do not mix components from different lots of kits, 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.

- Micro ELISA plate (COAT):** open the plastic pouch and take off the required amount of 8 well strips for the test series to be performed. 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).
- Sample Diluent (SD):** 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.
- vWF Calibrator (Cal):** Restore each vial with 2 ml of Sample Diluent (at least 30 minutes before use) in order to obtain a plasma containing the vWF concentration "C", already diluted 1:50 (fifty fold). This solution is stable for at least 8 hours at room temperature.
- vWF Plasma Control I (High) (CI):** Restore with 0.5 ml distilled water (at least 30 minutes before use).
- vWF Plasma Control II (Low) (CII):** Restore with 0.5 ml distilled water (at least 30 minutes before use).

Note: When restored, vWF control plasma I and II are stable for 8 hours at room temperature (18-25°C), or 24 hours at 2-8°C, or 1 month frozen at -20°C or below.

Warning: Plasma vWF calibrator (3) and controls I and II (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. 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.

- Anti-(h)-vWF-HRP immunoconjugate (IC):** Each vial must be restored with 7.5 ml of Conjugate Diluent. Let 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.
- Conjugate Diluent (CD):** 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.
- Wash Solution (WS):** 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.
- TMB substrate (TBM):** 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.
- Stop solution (SA):** 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 performed at 30°C show that the reagents keep their performances and can be shipped at room temperature without any damage.

PROCEDURE:

Specimen collection:

Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.) by a clean venipuncture; plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma must 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.

Tested plasma or sample or controls:

The sample must be tested diluted fifty fold (1:50) in the sample diluent. For expected vWF concentrations above "C"%, plasma or samples must be tested at a higher dilution, 1:100 (D=100) or more.

Controls I and II must be tested diluted fifty fold (1:50), with sample diluent.

Calibration:

Using the vWF Calibrator with a vWF concentration "C" (in the range 120-160%), provided in the kit, prepare the following standard solutions.

vWF concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of vWF Calibrator	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Vol. of Sample Diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently for a complete homogenisation (slightly vortex).

The standard dilutions are stable for at least **6 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
vWF standards (1:1) or tested sample or controls diluted 1:50 or Sample Diluent (blank)	200 µl	Introduce the standard solutions or the tested samples in the corresponding micro ELISA plate well (a).
Incubate for 2 hours at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 10 successive washings using the washing instrument (c).
Anti (h)-vWF-HRP conjugate (restored with 7.5 ml of Conjugate Diluent)	200 µl	Introduce the Anti-(h)-vWF-HRP immunoconjugate in the micro ELISA plate wells (c).
Incubate for 1 hour at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	300 µl	Proceed to 10 successive washings using the washing instrument. (c)
TMB/H ₂ O ₂ Substrate	200 µl	Immediately after the washing, introduce the substrate into the wells. <i>Nota:</i> The substrate distribution, row by row, must be accurate and at exact time intervals (c, d).
Incubate for exactly 15 minutes at room temperature (18-25°C) (b)		
0.45 M Sulfuric Acid	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 (d).
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450) (e). Subtract the blank values.		

Note:

- Distribute callibrators, controls and tested specimen as rapidly as possible (within 10 minutes), in order to obtain an homogeneous immunological kinetics for vWF binding. A too long delay between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results.
- Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.
- 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. **Each washing step (10 successive washings) must be strictly adhered to for optimal performance of the kit.**
- 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.
- For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used

EXPRESSION OF RESULTS:

- On a linear graph paper plot the **vWF concentrations in %** on abscissa and the corresponding absorbances (**A450**) on ordinates in order to establish the calibration curve.
- Users must construct their own calibration curve, obtained using their standard dilutions. From the curve obtained, deduce directly the vWF concentration for the tested sample. For obtaining the vWF concentration in a sample tested at a higher dilution, this value must be multiplied by D:50 (e.g. x2 for D=100 ...) (See model on the flyer).
- 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:

- The vWF concentration in normal human plasma is of about 10 µg/ml. vWF:CBA presents a large distribution (from 50 to 160%) in the general population.
- Plasma vWF level is highly influenced by the ABO histo group (decreased of about 25% in type O subjects), the gender (vWF is higher in women than in men), and the ethnic origin (vWF is lower in Caucasians). vWF is positively associated with diabetes and it increases with age.

BIOCHEMISTRY:

- Plasma von Willebrand Factor (vWF) is a high molecular weight (MW), multimeric glycoprotein (MW range from 1,000 to 20,000 KD), composed of identical disulfide-linked subunits (of about 280 KD), synthesized by endothelial cells and megakaryocytes. After a complex processing, the molecule is released in blood and is present both in plasma and in platelets, as well as in endothelial cells and subendothelial matrix of the vessel wall.
- Especially the higher molecular forms mediate platelet adhesion to subendothelial connective tissue following vascular injury and support platelet aggregation to a lesser extent. vWF is also a carrier protein in plasma for FVIII:C, stabilizing its coagulant activity and therefore playing a key role in the coagulation process.

PATHOLOGICAL VARIATIONS:

- Von Willebrand disease (vWD) is the most commonly inherited (autosomally) bleeding disorder, present at about 1% in the general population. It may affect males and females. The disease presents very heterogeneous manifestations, usually classified in 3 major subtypes:
 - Type I vWD (about 70 to 80% of cases): quantitative and moderate deficiency of vWF (15 to 50% of normal); asymptomatic or mild bleeding symptoms (mucocutaneous bleeding).
 - Type II vWD (about 15 to 20% cases): qualitative abnormalities of vWF (2A, 2B, 2M), mostly associated with high MW multimers deficiency. Patients present mild to severe bleeding manifestations.
 - Type III vWD (about 1 to 5% of cases): severe hemorrhagic disorders, in which plasma vWF is severely reduced or absent from plasma and platelets.

Considering the heterogeneity of vWF concentration in the normal population and in clinical manifestations, a complete biological study is necessary for vWD diagnosis, combining the assay of at least 3 parameters: FVIII:C activity, vWF activity and vWF antigen assay, and with regards to the ABO group and the familial context.

vWF:CBA assay, based on the ability of vWF (High Molecular Weight forms) to bind collagen, is thus useful to reflect the "true" physiological activity of vWF, and better characterize the vWD type.

APPLICATIONS:

- Diagnosis of von Willebrand disease (vWD).
- Assay of vWF:CBA in clinical samples, as a disease marker or as indicator for cardiovascular diseases.

ASSAY CHARACTERISTICS:

- Dynamic range: 0 to about 150%.
- Detection threshold ≤ 5%.
- Intra-assay CV: <10%.
- Inter-assay CV: <10%.

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