INTENDED USE

The IMUBIND® Factor VIII ELISA is an enzyme-linked immunoassay for the measurement of human factor VIII in purified concentrates and precipitates originating from cell culture media and plasma. The assay recognizes native and recombinant human factor VIII with equal efficiency. There is limited cross-reactivity with factor VIII from other species.

This assay is intended for research use only. It is not intended for diagnostic or therapeutic procedures.

EXPLANATION OF THE TEST

Factor VIII (FVIII) is a glycoprotein essential for the intrinsic pathway of blood coagulation because of its ability to accelerate the proteolytic activation of Factor X (FX) by the serine protease Factor IXa (FIXa). Synthesized mainly in hepatocytes, the mature form of FVIII is a single-chain, 2332 amino acid polypeptide, with a molecular ratio of approximately 265,000 Daltons. The molecule is comprised of two homologous groups separated by a third segment and organized with the domain structure of A1-A2-B-A3-C1-C2. Cleaved intracellularly into a two-chain heterodimer, a heavy-chain of domains A1-A2-B and a light-chain of domains A3-C1-C2, FVIII is secreted into the bloodstream and forms a stable, non-covalent complex with von Willebrand Factor (vWF). FVIII is activated by proteolytic cleavage and released from its vWF carrier protein by thrombin.

The activated protein, FVIIIa, consists of the domains A1-A2 and the A3-C1-C2 light chain, both of which are necessary for sustained activity. The B domain does not contribute to the active molecule and is lost after activation. FVIIIa is a cofactor for FIXa along with calcium and phospholipids. Binding to phospholipids and to platelets occurs via the light chain and has been determined to be associated with sequences within the C domain. The light chain is also responsible for the binding to vWF.

The role of FVIII in blood coagulation is demonstrated by the severe bleeding associated with hemophilia A, FVIII genetic deficiency. Along with hemophilia B, FIX deficiency, it occurs at a frequency of 1/10,000 of the whole population. In healthy normal individuals, FVIII is found circulating in plasma at a concentration of 100 - 200 ng/mL. The severity of hemophilia A is associated with the level of deficiency: mild being 5-40% of normal, moderate being 1-5% of normal, and severe being < 1% of normal. Hemophilia A is first diagnosed by clinical situation (family history) or the appearance of bleeding during the neonatal period. Severe hemophilia is usually diagnosed within the first year of life from a number of bleeding manifestations such as deep muscle and joint hemorrhaging and easy bleeding, or by post-trauma bleeding in later years. Mild hemophilia may not be diagnosed until year 10 or later.

The diagnosis is confirmed by a plasma assay for FVIII and successful treatment for moderate and severe deficiencies is accomplished by administration of FVIII concentrates.
PRINCIPLE OF THE METHOD

The IMUBIND Factor VIII ELISA is a “sandwich” ELISA using a monoclonal antibody against human factor VIII as the capture antibody. Samples incubate in microwells coated with an anti-human FVIII monoclonal antibody and a second monoclonal antibody, horseradish peroxidase (HRP) conjugated, is used to detect the bound FVIII antigen. The addition of a perborate/3,3',5,5' - tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP creates a blue colored solution. Sensitivity is enhanced by addition of a 0.5N sulfuric acid stop solution, yielding a yellow color. FVIII levels are determined by measuring solution absorbances at 450 nm and comparing the values to those of a standard curve.

REAGENTS

96 Anti-Factor VIII IgG coated microwells (6 x 16 well strips) with an acetate cover sheet
6 vials of Factor VIII standards, 0 - 200 mU/mL (lyophilized)
1 vial of Detection Antibody, HRP-conjugated Anti-Human Factor VIII (135 µL)
2 vials of Assay Diluent (lyophilized)
1 vial of Substrate, TMB (11 mL)
1 packet of Wash Buffer, PBS with 0.05% Tween 20, pH 7.4

WARNING

Source material for some of the reagents in this kit is of human origin. This material has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using FDA approved methods. As no known test method provides complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, reagents should be handled as recommended for any potentially infectious human specimen. Discard all waste associated with test specimens and human source reagents in a biohazard waste container.

Limited for research use only. Not for internal use in humans or animals. Do not use the kit components beyond the stated expiration date. Do not mix reagents from different kits. Avoid microbial contamination of the reagents. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled. Do not pipette reagents by mouth. Wear laboratory coat and disposable gloves throughout the test procedure and wash hands thoroughly afterwards. Avoid splashing or aerosol formation.

REAGENT PREPARATION AND STORAGE

1. Anti-Factor VIII IgG Coated Microwells: Once removed from the foil pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present and protected from moisture.

2. Factor VIII Standards: Note: Factor VIII standards should be prepared just prior to use in the assay. Do not prepare standards in advance.

Reconstitute each vial with 1.0 mL of filtered deionized water and gently mix. Do not vortex! Once reconstituted, standards may be stored at -20°C or colder for up to one month.

3. Detection Antibody: Supplied as a concentrate, dilute the Detection Antibody 1:100 with Assay Buffer just prior to use. For using all 96 microwells at one time, dilute 120 µL of Detection Antibody to 12 mL in Assay Buffer. If all 96 microwells are not to be used, dilute 20 µL of Detection Antibody to 2 mL in Assay Buffer for each 16 microwells that will be used. Working strength Detection Antibody is stable for 4 hours at 2°-8°C. Discard any unused working strength Detection Antibody.

4. Assay Diluent: Reconstitute each vial with 20 mL of filtered deionized water and mix well. Assay Diluent may be used for up to 4 weeks when stored at 2°-8°C.

5. Substrate, TMB: Supplied ready to use. Substrate may be used until the expiration date stated on the vial when stored in the dark at 2°-8°C.

6. Wash Buffer: Dissolve the contents of the Wash Buffer packet in 900 mL of filtered deionized water. Q.S. to a final volume of 1 Liter, mix well and confirm the pH is 7.4 (Adjust if necessary). Wash Buffer may be used for up to 4 weeks when stored at 2°-8°C.

SPECIMEN COLLECTION AND PREPARATION

Dilute Factor VIII concentrates at various ratios (1:100, 1:200, 1:500, etc.) with Assay Diluent until the sample produces an absorbance within the bounds of the standard curve.
PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided
- filtered deionized or distilled water
- 50-200 µL eight channel multi-pipette, 10-200 µL single pipette
- Microwell plate reader at 450 nm
- Microwell plate washer
- 0.5M H$_2$SO$_4$

Assay Procedure
1. Remove the necessary number of antibody coated microwells from the foil pouch. Return unused microwells to the pouch and reseal it with the desiccant inside and store at 2°-8°C.
2. Add 50 µL of FVIII Standard or diluted sample to the microwell. It is recommended to perform measurements in duplicate.
3. Add 100 µL of Assay Diluent to each microwell, cover with the acetate sheet and incubate for 90 minutes at room temperature (18°-25°C).
4. Empty the contents of the microwells and wash wells 4 times with Wash Buffer (250 µL per well). Washing may be performed using a microwell plate washer or manually. For manual washing, fill the wells with Wash Buffer with a pipette or squeeze bottle then empty and remove droplets by tapping the plate 4-5 times face down against absorbing material.
5. Add 100 µL of working strength Detection Antibody to each microwell, cover with the acetate sheet and incubate for 60 minutes at room temperature (18°-25°C).
6. Wash the microwells by repeating Step 4.
7. Add 100 µL of Substrate solution to each well, cover with the acetate sheet and incubate for 20 minutes at room temperature (18°-25°C), during which time the substrate will turn blue.
8. Stop the enzymatic reaction by adding 50 µL of 0.5M H$_2$SO$_4$. Add the acid with the same speed and order as you added the substrate. Shake the microwells to ensure even distribution of the H$_2$SO$_4$. The solution color will turn yellow. Read the absorbances using a microwell plate reader set to a wavelength of 450 nm within 10 minutes.

RESULTS

Representative Standard Curve

The standard curve is constructed by plotting the mean absorbance value for each Factor VIII standard versus the corresponding concentration of in mU/mL. Interpolate unknown values directly from the standard curve. For diluted samples multiply the value from the standard curve by the dilution factor to calculate the corrected sample value. A standard curve should be generated each time the assay is performed. The following curve is for demonstration purposes only.

IMUBIND® Factor VIII ELISA

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y = -2E^{-05}x^2 + 0.0113x + 0.2323
\]

\[R^2 = 0.9993\]

CALCULATIONS

Determine the amount of Factor VIII in the diluted sample by interpolating directly from the standard curve. Multiply the concentration determined from the standard curve by the dilution factor to obtain the Factor VIII concentration in the original sample.
REFERENCES


