

american diagnostica inc.

500 West Avenue, P.O. Box 110215 Stamford, CT. 06911-0215

Tel. (203) 602-7777 • Fax. (203) 602-2221

IMUBIND® FVIII ELISA Kit

Product No. 884CON

for measuring human factor VIII
in purified concentrates

INTENDED USE

The IMUBIND® FVIII ELISA kit is an enzyme-linked immunoassay for the quantitation of human factor VIII purified concentrates originating from plasma or cell culture supernatants. 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 for research use only. It is not intended for diagnostic or therapeutic procedures. The assay's use with patient plasma is under investigation and remains undefined.

INTRODUCTION

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).¹ 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-C2.² 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 blood stream and forms a stable, non-covalent complex with von Willebrand Factor (vWF)^{3,4}. FVIII is activated by proteolytic cleavage and released from its vWF carrier protein by thrombin².

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.⁵ The B domain does not contribute to the active molecule and is lost after activation.^{6,7} 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.⁸ The light chain is also responsible for the binding to vWF⁹.

BIOLOGICAL SIGNIFICANCE

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/mL¹⁰.

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 later¹¹. 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 FVIII ELISA is a "sandwich" ELISA using a monoclonal antibody against human factor VIII as the capture antibody. Samples incubate in precoated micro-test wells 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 generated using calibrated antigen.

REAGENTS

6 x 16 well precoated micro-test strips with holder and lid
6 vials fVIII standards, 0 - 200 mU/mL (lyophilized)
1 vial Detection Antibody, HRP-conjugated anti-human fVIII (135 µL)
2 vials Assay Diluent (lyophilized)
1 vial Substrate, TMB (11 mL)
1 packet Wash Buffer, PBS with 0.05% Tween 20, pH 7.4

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED

0.22 µm filtered deionized H₂O
50-200 µL eight channel multi-pipette
10-200 µL single pipette
Micro-test plate reader at 450 nm
0.5N H₂SO₄

PREPARATION OF THE REAGENTS

A. fVIII Standards

Note: fVIII standards should be prepared just prior to use in the assay. Do not prepare standards in advance.

1. Add 1.0 mL filtered deionized H₂O to each of the **Standard** vials.
2. Agitate gently. Do not shake!

B. Assay Diluent

Add 20 mL of filtered deionized H₂O to each of the **Assay Diluent** vials and mix well.

C. Wash Buffer

1. Dissolve the contents of the **Wash Buffer** packet in 900 mL of filtered deionized H₂O.
2. Q.S. to a final volume of 1 Liter with distilled H₂O.
3. Mix well and confirm pH is 7.4 (adjust if necessary).

REAGENT STABILITY

Store unused micro-test strips and unreconstituted reagents at 2-8°C until the expiration date indicated on their labels. Aliquot and freeze reconstituted standards at -20°C. Store reconstituted reagents at 4°C for up to one month.

SAMPLE PREPARATION

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

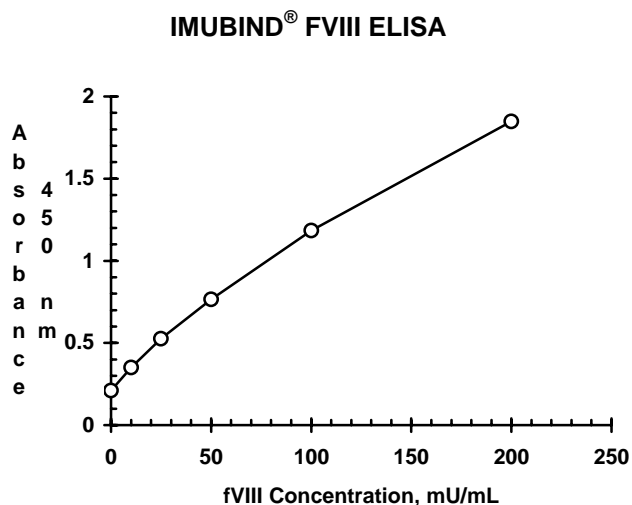
ASSAY PROCEDURE

1. Remove the necessary number of precoated micro-test strips from the foil pouch and place them in the plate holder. Tightly reseal the foil pouch with the desiccant inside and store at +2°C - +8°C.
2. Add 50 µL of **fVIII Standards** or diluted sample to the micro-test wells. Perform measurements in duplicate.
3. Add 100 µL of **Assay Diluent** to each well and incubate for 90 minutes at room temperature.
4. Wash wells 4 times with **Wash Buffer** (250 µL per well).
5. For each micro-test strip used, add 20 µL of HRP-conjugated **Detection Antibody** to 2 mL of **Assay Diluent**. Add 100 µL of diluted detection antibody to each well, cover with the lid and incubate for 60 minutes at room temperature.
6. Wash wells 4 times with **Wash Buffer** (250 µL per well).

7. Add 100 μL of **Substrate** solution to each well, cover with lid and incubate for 20 minutes at room temperature. A blue color will develop.
8. Stop the enzymatic reaction by adding 50 μL of 0.5N H_2SO_4 . Tap the sides of the strip-wells to ensure even distribution of the H_2SO_4 . The solution color will turn yellow. Read the absorbance of the solutions on a micro-test plate reader at a wavelength of 450 nm immediately. The background average of the "0" standard may be deducted from the absorbance of the remaining standards and the sample readings.

REPRESENTATIVE STANDARD CURVE

The standard curve is constructed by plotting the mean absorbance value calculated for each fVIII standard versus its corresponding concentration. A standard curve should be generated each time the assay is performed.



CALCULATION OF RESULTS

Use the mean absorbance value for each diluted sample to interpolate its fVIII concentration from the standard curve. Multiply the concentration determined from the standard curve by the dilution factor to obtain the fVIII concentration in the original sample.

REFERENCES

1. Mann, K., *et al.* Surface dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* 1990; **76**: 1-16.
2. Vehar G. A., *et al.* Structure of human factor VIII. *Nature* 1984; **312**: 337-342.
3. Kaufman, R. J., *et al.* Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *Journal of Biological Chemistry* 1988; **263**: 6352-6362.
4. Legaz, M., *et al.* Isolation and characterization of human factor VIII (anti-hemophilic factor). *Journal of Biological Chemistry* 1973; **248**: 3946-3955.
5. Fay, P. J., *et al.* Human factor VIIIa subunit structure. Reconstitution of factor VIIIa from isolated A1/A3-C1-C2 dimer and A2 subunit. *Journal of Biological Chemistry* 1991; **266**: 8957-8962.
6. Toole, J., *et al.* A large region (≈ 95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. *Proceedings of the National Academy of Sciences, USA* 1986; **83**: 5939-5942.
7. Fulcher, C., *et al.* Thrombin proteolysis of purified fVIII procoagulant protein: Correlation of activation with generation of specific polypeptides. *Blood* 1983; **60**: 807.
8. Foster, P. A., *et al.* Synthetic factor VIII peptides with amino sequences contained within the C2 domain of factor VIII inhibit factor VIII binding to phosphatidyl serine. *Blood* 1990; **75**: 1999-2004.
9. Saenko, E. L., *et al.* A role for the C2 domain of factor VIII in binding to von Willebrand factor. *Journal of Biological Chemistry* 1994; **269**: 11601.
10. Fulcher, C. and Zimmerman T. Characterization of the human fVIII procoagulant protein with a heterologous precipitating antibody. *Proceedings of the National Academy of Sciences, USA*, 1982; **79**: 1648.
11. Hathaway, W. E. and Goodnight Jr., S. H. *Disorders of Hemostasis and Thrombosis, A Clinical Guide* 1993, McGraw-Hill, 123-133.