***REPRESENTATIVE DATA SHEET ***

Matched-Pair Antibody Set
for ELISA of human
Factor VIII antigen (F.VIII:C)

Sufficient reagent for **4 x 96 well** plates

Product #:	**F8C-EIA**
Lot #:	SAMPLE
Expiry Date:	SAMPLE

Store at 2-8°C

For Research Use Only
Not for use in diagnostic procedures.

Description of Factor VIII (F.VIII)

Factor VIII (formerly referred to as antihemophilic globulin and Factor VIII:C) is a large glycoprotein (320 kDa) that circulates in plasma at approximately 200 ng/ml. Synthesized in the liver, the majority of Factor VIII is cleaved during expression, resulting in a heterogeneous mixture of partially cleaved forms of F.VIII ranging in size from 200-280 kDa. The F.VIII is stabilized by association with von Willebrand Factor to form a F.VIII-vWF complex required for the normal survival of F.VIII *in vivo* ($t_{1/2}$ of 8-12 hours).
F.VIII is a pro-cofactor that is activated through limited proteolysis by thrombin. In this process F.VIIIa dissociates from vWF to combine with activated Factor IX, calcium and a phospholipid surface where it is an essential cofactor in the assembly of the Factor X activator complex. Once dissociated from vWF, F.VIIIa is susceptible to inactivation by activated Protein C and by non-enzymatic decay.
Hemophilia A is a congenital bleeding disorder resulting from an X-chromosome-linked deficiency of F.VIII. The severity of the deficiency generally correlates with the severity of the disease. Some Hemophiliacs (~10%) produce a F.VIII protein that is partially or totally inactive. The production of neutralizing antibodies to F.VIII also occurs in 5-20% of Hemophiliacs [1-3].

Principle of Sandwich-style ELISA

Affinity-purified antibody to F.VIII is coated onto the wells of a microtitre plate. The plate is washed and plasma or other fluids containing F.VIII are applied. The coated antibody will capture the F.VIII in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to F.VIII is added to the plate to bind to the captured F.VIII. After washing the plate to remove unbound conjugated antibody, the peroxidase activity is expressed by incubation with o-phenylenediamine (OPD). After a fixed development time the reaction is quenched with the addition of H_2SO_4 and the colour produced is quantified using a microplate reader. The colour generated is proportional to the concentration of F.VIII present in the sample.

Supplied Materials:

1. **Capture Antibody (F8C-EIA-C):** One yellow-capped vial containing 0.4 ml of polyclonal affinity purified anti-FVIII antibody for coating plates.
2. **Detecting Antibody (F8C-EIA-D):** Four neutral-capped tubes each containing 10 ml of pre-diluted peroxidase conjugated polyclonal anti-FVIII antibody for detection of captured F.VIII.
3. **Sample Diluent (F8C-EIA-SD):** 100 ml bottle containing a green-coloured diluent optimised for dilution of samples.

Store reagents at 2-8°C

Materials Required but not Provided:

1. **Coating Buffer:** 50 mM Carbonate
1.59g of Na_2CO_3 and 2.93g of $NaHCO_3$ up to 1 litre. Adjust pH to 9.6. Store at 2-8°C up to 1 month.

2. **PBS:** (base for wash buffer)
8.0g NaCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4 and 0.2g KCl, up to 1 litre. Adjust pH to 7.4, if necessary. Store at 2-8°C up to 1 month, discard if there is evidence of microbial growth.

3. **Wash Buffer:** PBS-Tween (0.1%,v/v)
To 1 litre of PBS add 1.0 ml of Tween-20. Check that the pH is 7.4. Store at 2-8°C up to 1 week.

4. **Substrate Buffer:** Citrate-Phosphate buffer pH 5.0
2.6g Citric acid and 6.9g Na_2HPO_4 up to a final volume of 500 ml with purified H_2O. Store at 2-8°C up to 1 month.

5. **OPD Substrate:** (o-Phenylenediamine.2HCl) TOXIC!
(5mg tablets: Sigma # P-6912). Make up immediately before use. Dissolve 5mg OPD in 12 ml substrate buffer then add 12 µl 30% H_2O_2. Do not store.

6. **Stopping Solution:** 2.5 M H_2SO_4
Caution: VERY CORROSIVE! GENERATES HEAT ON DILUTION! Where stock sulphuric acid is 18 Molar, add 13.9 ml to 86 ml H_2O. Store at room temperature.

7. **Other:**

Microplates, 96-well Immulon 4-HBX (http://www.labsystems.fi)

Microplate washer (optional)

Microplate reader.

Assay Procedure:

1. Coating of plates:
Dilute the capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 µl to every well in the plate.
Incubate 2 hrs @ 22°C.

2. **Blocking:**
Blocking is not required under the conditions described. Washing the plate with PBS-Tween is sufficient to block non-specific interactions.
Wash plate X 3 with wash buffer.

3. **Samples:**
Reference plasma is diluted 1/2 (100%) then serial 1/2's down to 1/64 (3.13%). Sample plasmas are diluted 1/4, 1/8 & 1/16. All dilutions are made in the provided green sample diluent. Apply 100 µl/well and incubate plate @ 22°C for 120 minutes. Wash plate X 3 with wash buffer.

4. Detecting Antibody:
Apply the pre-diluted detecting antibody, 100 µl to each well. Incubate plate @ 22°C for 60 minutes.
Wash plate X 3 with wash buffer.

5. **OPD Substrate:**
Apply 100 µl of freshly prepared OPD substrate to every well. Allow colour to develop for **10-15 minutes** then stop colour reaction with the addition of 50 µl/well of 2.5 M H_2SO_4. The plate can be read at wavelength of 490 nm.

Calculation of Results:

The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit [4,5]. In general, the simplest model that defines the concentration-response relationship should be used [6].
The "back-fit" test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits[6]. The coefficient of determination (R^2) is a valuable indicator of the overall fit, but should not be used by itself in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone [5,6]

In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed using serial dilutions of normal pooled plasma, will produce a correlation coefficient (R^2) of at least 0.980 using a semi-log fit, and an R^2 of at least 0.990 using a 4-parameter logistic curve fit algorithm. However, the performance characteristics of in-house assays developed using this product in other laboratories may vary slightly from ours. Different curve fitting methods may be employed but we recommend that the back-fit test be applied as evidence that the fitting method is appropriate.

Technical Notes:

- This paired antibody product is intended to facilitate the end user in establishing an in-house immunoassay for research purposes only. It must not be used for diagnostic applications. Assay validation is the responsibility of the end user and should be done according to user-defined protocols[6].
- Reference calibrators should be of the same matrix and anticoagulant as the samples to be tested (example serum or plasma, citrate or EDTA).
- The optimal colour development time should be determined empirically as the time required to obtain an absorbance of at least 1.000 at 490 nm for the 100% reference point, not to exceed 20 minutes.
- Do not use samples diluted less than 1/2, as falsely high readings may result.
- Rheumatoid factor in samples may interfere in ELISA by binding to the capture and/or detecting antibodies.
- The wells should not be allowed to become dry. Keep plate covered or in a humid chamber during incubations.
- The capture antibody is supplied in a microcentrifuge tube and can be centrifuged to gather residual reagent from the cap and walls of the tube.

References:

1. Lollar P, Fay PJ, Fass DN; Factor VIII and Factor VIIIa. Methods in Enzymology, 222, pg 122, 1993.
2. Hoyer, LW, Wyshock EG, Colman RW, in Hemostasis and Thrombosis, 3rd Edition, eds. RW Colman, J Hirsh, VJ Marder and EW Salzman, pp. 109-133, J.B. Lippincott Co., Philadelphia, 1994.
3. Pittman DD, Kaufman RJ. Structure-Function Relationships of Factor VIII Elucidated through Recombinant DNA Technology. Thromb. Haemostas. 61:161-165, 1989.
4. Nix,B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.
5. NCCLS. Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guideline – Second Edition. NCCLS Document EP6-P2 (ISBN 1-56238-446-5, NCCLS, Wayne, Pennsylvania, 2001.
6. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available online: www.fda.gov/cder/guidance/index.htm .