INTENDED USE
This human coagulation Factor VIII antigen assay is intended for the quantitative determination of total Factor VIII antigen in human plasma. For research use only.

BACKGROUND
Factor VIII (aka Factor VIII:C or Antihemophilic Globulin) is a glycoprotein zymogen that circulates in a stabilized non-covalent complex with von Willebrand Factor (vWF) [1]. Following activation by thrombin or Factor Xa, Factor VIIIa dissociates from vWF and catalyzes the activation of Factor X by Factor IXa in the amplification phase of coagulation [2]. Factor VIIIa activity is quickly decreased by spontaneous dissociation and proteolytic degradation by activated Protein C, Factor Xa and Factor IXa [3]. Hemophilia A is caused by mutations in the Factor VIII gene; a majority of patients have decreased Factor VIII plasma levels while 5% of patients have normal levels of nonfunctioning protein [4].

ASSAY PRINCIPLE
Human Factor VIII will bind to the affinity purified capture antibody coated on the microtiter plate. After appropriate washing steps, biotin labeled anti-human Factor VIII primary antibody binds to the captured protein. Excess primary antibody is washed away and bound antibody is reacted with peroxidase conjugated streptavidin. Following an additional washing step, TMB substrate is used for color development at 450nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of human plasma. Color development is proportional to the concentration of Factor VIII in the samples.

STANDARD CALIBRATION
The Factor VIII level in the human plasma standard provided is calibrated against a secondary standard that is referenced to the WHO or ISTH International Standard.

Lot 317L: 0.77 IU/ml
PRECAUTIONS
• FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
• Do not mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
• Always pour peroxidase substrate out of the bottle into a clean test tube. Do not pipette out of the bottle as contamination could result.
• Keep plate covered except when adding reagents, washing, or reading.
• DO NOT pipette reagents by mouth and avoid contact of reagents and specimens with skin.
• DO NOT smoke, drink, or eat in areas where specimens or reagents are being handled.

PREPARATION OF REAGENTS
• TBS buffer: 0.1M Tris, 0.15M NaCl, pH 7.4
• Blocking buffer (BB): 3% BSA (w/v) in TBS
• 1X Wash buffer: Dilute 50ml of 10X wash buffer concentrate with 450ml of deionized water

SAMPLE COLLECTION
Collect plasma using EDTA or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles.

ASSAY PROCEDURE
Perform assay at room temperature. Vigorously shake plate (300rpm) at each step of the assay.

Preparation of Standard
Reconstitute standard by adding 1ml of deionized water directly to the vial and agitate gently to completely dissolve contents. This will result in a 0.77 IU/ml plasma standard.

Dilution table for preparation of human Factor VIII standard:

<table>
<thead>
<tr>
<th>Factor VIII concentration (IU/ml)</th>
<th>Dilutions</th>
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<tbody>
<tr>
<td>0.77</td>
<td>From vial</td>
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<tr>
<td>0.39</td>
<td>500µl (BB) + 500µl (0.77 IU/ml)</td>
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<tr>
<td>0.19</td>
<td>500µl (BB) + 500µl (0.39 IU/ml)</td>
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<tr>
<td>0.096</td>
<td>500µl (BB) + 500µl (0.19 IU/ml)</td>
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<tr>
<td>0.048</td>
<td>500µl (BB) + 500µl (0.096 IU/ml)</td>
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<td>0.024</td>
<td>500µl (BB) + 500µl (0.048 IU/ml)</td>
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<td>0.012</td>
<td>500µl (BB) + 500µl (0.024 IU/ml)</td>
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<tr>
<td>0.006</td>
<td>500µl (BB) + 500µl (0.012 IU/ml)</td>
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<tr>
<td>0.003</td>
<td>500µl (BB) + 500µl (0.006 IU/ml)</td>
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<tr>
<td>0.0015</td>
<td>500µl (BB) + 500µl (0.003 IU/ml)</td>
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<tr>
<td>0</td>
<td>500µl (BB) Zero point to determine background</td>
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NOTE: DILUTIONS FOR THE STANDARD CURVE AND ZERO STANDARD MUST BE MADE AND APPLIED TO THE PLATE IMMEDIATELY.

Standard and Unknown Addition
Remove microtiter plate from bag and add 100µl Factor VIII standards (in duplicate) and unknowns to wells. Carefully record position of standards and unknowns. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: The assay measures Factor VIII antigen in the 0-0.77 IU/ml range. 1:8 and 1:16 dilutions for normal plasma, or 1:4 and 1:8 dilutions for Haemophiliac plasma, are suggested for best results.

Primary Antibody Addition
Reconstitute primary antibody by adding 10ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. Add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.
**Streptavidin-HRP Addition**
Briefly centrifuge vial before opening. Dilute 2.5µl of HRP conjugated streptavidin into 2.5ml blocking buffer to generate a 1:1,000 dilution. Add 0.2ml of the 1:1,000 dilution to 9.8ml of blocking buffer to generate a 1:50,000 dilution. Add 100µl of the 1:50,000 dilution to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

**Substrate Incubation**
Add 100µl TMB substrate to all wells and shake plate for 2-10 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50µl of 1N H$_2$SO$_4$ or HCl stop solution to all wells when samples are visually in the same range as the standards. Add stop solution to wells in the same order as substrate upon which color will change from blue to yellow. Mix thoroughly by gently shaking the plate.

**Measurement**
Set the absorbance at 450nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A$_{450}$).

**Calculation of Results**
Plot A$_{450}$ against the amount of Factor VIII in the standards. Fit a straight line through the linear points of the standard curve using a linear fit procedure if unknowns appear on the linear portion of the standard curve. Alternatively, create a standard curve by analyzing the data using a software program capable of generating a four parameter logistic (4PL) curve fit. The amount of Factor VIII in the unknowns can be determined from this curve. If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

**EXPECTED VALUES**
The average normal plasma level of Factor VIII is defined as 1.0 IU/ml and the normal range is 0.4-1.8 IU/ml [5]. Hemaophilia A patients are classified by the following Factor VIII levels: 0.05-0.25 IU/ml = mild, 0.01-0.05 IU/ml = moderate, and <0.01 IU/ml = severe [6].

**PERFORMANCE CHARACTERISTICS**
**Sensitivity:** The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of eighteen zero standard replicates (range OD$_{450}$: 0.045-0.062) and calculating the corresponding concentration. The MDD was 0.00086 IU/ml.

**Intra-assay Precision:** These studies are currently in progress. Please contact us for more information.

**Inter-assay Precision:** These studies are currently in progress. Please contact us for more information.

**Recovery:** These studies are currently in progress. Please contact us for more information.

**Linearity:** These studies are currently in progress. Please contact us for more information.
**Specificity:** This assay recognizes total human Factor VIII. Pooled normal plasma from mouse, rat, rabbit, pig, horse, guinea pig, dog, and sheep was assayed and no significant cross-reactivity was observed. Pooled normal plasma from cyno monkey, rhesus monkey and baboon was assayed and significant cross-reactivity was observed.

**DISCLAIMER**
This information is believed to be correct but does not claim to be all-inclusive and shall be used only as a guide. The supplier of this kit shall not be held liable for any damage resulting from handling of or contact with the above product.

**REFERENCES**

Example of ELISA Plate Layout
96 Well Plate: 22 Standard wells, 74 Sample wells

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<td>A</td>
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