Factor VIII Chromogenic Assay

**Intended Use**
For photometric determination of Factor VIII (antihemophilic factor) activity in human plasma.

**Summary and Principle of the Method**
Hemophilia is a sex-linked hemorrhagic disease caused by circulating Factor VIII (F. VIII) or Factor IX (F.IX) deficiency. Hemophilia A is the term used for a deficiency of F. VIII, and is five to seven times more common than Hemophilia B, which is due to a deficiency in Factor IX\(^1\). Both diseases are transmitted as X-linked recessive traits and occur almost exclusively in males who receive the defective gene from the X-chromosome of a carrier mother. In Hemophilia A patients, the degree of F. VIII deficiency dictates the severity of the bleeding disorder. Approximately 10 % to 40 % of normal F. VIII activity is required for normal hemostasis; below this range, a tendency towards bleeding is apparent. Patients are generally classified by their F. VIII activity into three categories: mild, 25 % to 5 % of normal; moderate, 5 % to 1 % of normal; and severe, less than 1 % of normal. In this chromogenic assay, the Factor VIII in the sample is activated by thrombin\(^5\).
Activated Factor VIII (F. VIIIa) then accelerates the conversion of Factor X (F. X) into Factor Xa (F. Xa) in the presence of activated Factor IX (F. IXa), phospholipids (PL) and calcium ions. The F. Xa activity is assessed by hydrolosis of a p-nitroanilide substrate specific to F. Xa. The initial rate of release of p-nitroaniline (pNA) measured at 405 nm is proportional to the F. Xa activity, thus to the F. VIII activity in the sample. The chemistry of the Factor VIII Chromogenic Assay is illustrated by the following equations:

\[
\text{F. VIII} + \text{Thrombin} \rightarrow \text{F. VIIIa} \\
\text{F. X} \quad \text{F. VIIIa} \\
\quad \text{F. IXa, PL, Ca}^{2+} \rightarrow \text{F. Xa} \\
\text{CH}_3\text{OCO-D-CHG-Gly-Arg-pNA} \rightarrow \text{CH}_3\text{OCO-D-CHG-Gly-Arg-OH} + \text{pNA (yellow)}
\]

**Reagents**

**Note:** Factor VIII Chromogenic Assay can be used manually or on automated coagulation analyzers. Siemens Healthcare Diagnostics provides Reference Guides (Application Sheets) for several coagulation analyzers. The Reference Guides (Application Sheets) contain analyzer/assay-specific handling and performance information which may differ from that provided in these Instructions for Use. In such a case, the information contained in the Reference Guides (Application Sheets) supersedes the information in these Instructions for Use. In addition, please also consult the instruction manual of the instrument manufacturer.

**Materials provided**

- **Factor VIII Chromogenic**, REF B4238–40 with
- 2 x → 2 mL [REAGENT-FX], Factor X Reagent
- 2 x → 2 mL [REAGENT-FIX], Factor IXa Reagent
- 2 x → 1 mL [SUBSTRATE], Substrate Reagent
2 x 10 mL [SUBSTRATE BUFFER], Stopping Buffer

Composition

**Factor X Reagent:** lyophilized preparation containing approximately 2 nmol of bovine F.X, tris-(hydroxymethyl)-aminomethan (Tris) Buffer at pH 8 and stabilizers.

**Factor IXa Reagent:** lyophilized preparation containing approximately 0.6 nmol bovine F.IXa, approximately 0.6 nmol of bovine thrombin, approximately 0.06 mmol of calcium chloride, approximately 0.12 µmol of phospholipids, Tris buffer at pH 8, and stabilizers.

**Substrate Reagent:** lyophilized preparation containing approximately 3.4 µmol of CH$_3$OCO-D-CHG-Gly-Arg-pNA.AcOH, a F.Xa substrate, Nα-(2-Naphthylsulfonylglycyl)-D, L-amidino-phenylalanine piperidide (α-NAPAP)$^6$, a thrombin inhibitor, and stabilizers.

**Stopping Buffer:** solution containing Tris, ethylenediaminetetraacetic acid, sodium chloride and 0.02 % sodium azide.

Warnings and Precautions

For in-vitro diagnostic use only.

Contains sodium azide (< 1 g/L) as a preservative. Sodium azide can react with copper or lead pipes in drain lines to form explosive compounds. Dispose of properly in accordance with local regulations.

Preparations of the Reagents

**Note:** For analyzer-specific information on reagent preparation please refer to the respective Reference Guides (Application Sheet) provided by Siemens.

- **REAGENT FX** and **REAGENT FIX**:
  - Dissolve the contents of a vial with 2.0 mL of distilled or deionized water.

- **SUBSTRATE**:
  - Dissolve the contents of a vial with 1.0 mL of distilled or deionized water.

- **SUBSTRATE BUFFER**:
  - For use, mix together 1.0 mL of the [SUBSTRATE] with 10.0 mL of the [SUBSTRATE BUFFER] in the brown [SUBSTRATE] container.
  - Mix the reagents carefully once more before using.

Storage and Stability

The reagents may be used up to the expiry date indicated on the label if stored at 2 to 8 °C.

Stability after reconstitution ([REAGENT FX] and [REAGENT FIX], [SUBSTRATE], [SUBSTRATE BUFFER] mixture):

- at 37 °C: 2 hours
- at 15 to 25 °C: 8 hours
- at 2 to 8 °C: 3 days

Information about on-board stability is specified in the Reference Guides (Application Sheets) for the different coagulation analyzers.

**Signs of deterioration:** No evidence of vacuum in vial upon opening; difficulty in reconstituting reagents; extreme turbidity or presence of particulate matter in [SUBSTRATE BUFFER].

Specimens

Mix 9 parts of freshly collected patient blood with 1 part of 0.11 mol/L or 0.13 mol/L sodium citrate solution. Centrifuge the blood specimen at 1500 x g for no less than 15 minutes at room temperature. Immediately move plasma into a plastic tube and keep refrigerated until ready to test.
Patient plasma should be tested within 1 hour of blood collection, and can be stored up to 30 days at −20 °C. Due to the instability of Factor VIII, the plasma may be thawed only once and tested within 1 hour.

**Materials required, but not provided**

- **Sodium citrate 0.11 mol/L or 0.13 mol/L for blood collection.**
- Fresh plasma pool (FPP) or Standard Human Plasma ([REF ORKL](#)) for calibration.
- Coagulation Factor VIII Deficient Plasma (human), [REF OTXW](#).
- Distilled or deionized water.
- Spectrophotometer with the following capabilities:
  - Wavelength: 405 nm
  - Pathlength: 1 cm
  - Measuring temperatures: 25 °C, 30 °C or 37 °C.
- Sodium chloride solution, 0.9 %.
- For endpoint determination, 20 % acetic acid or 1 mol/L citric acid. (Mix 200 mL glacial acetic acid with 800 mL distilled or deionized water or dissolve 210 g citric acid monohydrate in distilled or deionized water and dilute to 1 liter.)
- Stopwatch.
- Pipettes for 0.1 mL, 0.5 mL and 3.0 mL.
- Plastic tubes and 1 cm semi-micro cuvettes,
- Control Plasma N, [REF ORKE](#)
- Control Plasma P, [REF OUPZ](#)
- Imidazole Buffer Solution, [REF OQAA](#) or
- Dade® Owren’s Veronal Buffer, [REF B4234](#) or
- Dade® CA System Buffer, [REF B4265](#)
- Coagulation analyzer

**Procedure**

**Manual:**

**Endpoint/Kinetic Assay Procedure**

The assay can be performed at 25 °C, 30 °C or 37 °C. Set the wavelength of the photometer at 405 nm and zero against 0.9 % sodium chloride solution.

**Sample/Calibration Plasma Dilution**

The assay is performed on a 1 : 31 dilution of sample or calibration plasma. This dilution is prepared as follows: Mix 0.1 mL of sample or calibration plasma with 3.0 mL of 0.9 % sodium chloride solution in a plastic tube.

**Preparation of the Calibration Curve:**

The assay can be performed using a single calibration plasma dilution, but a calibration curve should be calculated for greater accuracy. For this, use a calibration plasma with known level of F. VIII (e.g. 100 %) and dilute it with 0.9 % sodium chloride solution as follows:
### Dilution of calibration plasma

<table>
<thead>
<tr>
<th>Dilution of calibration plasma</th>
<th>1 : 21</th>
<th>1 : 31</th>
<th>1 : 62</th>
<th>1 : 124</th>
<th>zero standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9 % NaCl mL</td>
<td>2.0</td>
<td>3.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Calibrating plasma mL</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mix and transfer mL</td>
<td>1.0</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F. VIII* %</td>
<td>148</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

* When a commercial assayed reference plasma is used, the percent of factor assumed present in the 1 : 31 dilution will be the assay value published. The percent of factor assumed present for the other dilutions must be adjusted accordingly. The F. VIII concentration of the 1 : 21 dilution is calculated as follows: Concentration of F. VIII in 1 : 31 dilution x 1.48 = Concentration of F. VIII in 1 : 21 dilution, e.g. 100 % x 1.48 = 148 %.

### Procedure Outline

All reagents as well as the plastic tubes should be preincubated at the selected temperature. The reproducibility is optimal if the measuring temperature does not deviate by more than 0.2 °C from the selected temperature during the reaction.

#### Kinetic Assay

- **Diluted sample/calibration plasma 1 : 31**
  - Factor X Reagent: 0.1 mL
  - Factor IXa Reagent: 0.1 mL
- Mix well and incubate: 90 seconds at 37 °C
- Add substrate Reagent and Stopping Buffer: 0.5 mL
- Mix and measure the absorbance increase immediately (∆A/min): 60 s at 405 nm

#### Endpoint Assay

- **Diluted sample/calibration plasma 1 : 31**
  - Factor X Reagent: 0.1 mL
  - Factor IXa Reagent: 0.1 mL
- Mix well and incubate: 90 seconds at 37 °C
- Add substrate Reagent and Stopping Buffer: 0.5 mL
- Mix and measure the absorbance increase immediately (∆A/min): 60 s at 405 nm

**See chapter titled "Notes on the Assay Procedure"

*** 90 seconds at 37 °C; 120 seconds at 30 °C or 180 seconds at 25 °C.

For the endpoint assay a plasma bank must be prepared for the samples and each calibration plasma dilution.

- 20 % acetic acid: 0.1 mL
- Add Factor X Reagent. Mix.
- Add Factor IXa Reagent. Mix.
- Add diluted Sample. Mix.
- Add Substrate Reagent and Stopping Buffer. Mix.
- Mix and measure the absorbance against 0.9 % NaCl: 0.5 mL at 405 nm

### Internal Quality Control

**Normal range:** Control Plasma N

**Pathological range:** Control Plasma P

Two controls should be measured with each calibration and at least every 3 to 4 hours during each testing day (one in the normal range and one in the pathological range). The controls should be processed just like the samples. Each laboratory should determine its own quality control range, either by means of the target values and ranges provided by the manufacturer.
of the controls or by means of its own ranges established in the laboratory. This range is usually based on ± 2.0 to ± 2.5 standard deviations (SD) from the mean control value. If the measured control value lies outside the confidence level previously established, then the coagulation analyzer, the reagents and the calibration should be examined. Do not release patient results until the cause of deviation has been identified and corrected.

Calculation of the Results

a) Using a single calibration factor (F)

Since the calibration curve is linear between 0 to 100 %, the F. VIII activity in the sample can be calculated by using the known value of a calibration plasma (FPP, Standard Human Plasma).

I. For kinetic assays

\[
\text{Percent Factor VIII Activity of Calibration Plasma} = A \\
\Delta A/\text{min. of the Calibration Plasma} = C \\
\Delta A/\text{min. of the Zero Standard} = B \\
\Delta A/\text{min. of the Sample} = S \\
\text{Calibration factor} = F
\]

\[
\frac{A}{C - B} = F
\]

The factor VIII activity of the unknown sample in percent is calculated by the equation:

\[(S - B) \times F = \text{Factor VIII activity of the sample in \%}.
\]

II. For endpoint assays

\[
\text{Percent Factor VIII Activity of Calibration Plasma} = A \\
\text{Calibration Plasma Absorbance} = C \\
\text{Calibration Plasma Blank Absorbance} = C_b \\
\text{Sample Absorbance} = S \\
\text{Sample Blank Absorbance} = S_b \\
\text{Zero Standard Absorbance} = B \\
\text{Zero Standard Blank Absorbance} = B_b \\
\text{Calibration factor} = F
\]

\[
\frac{A}{(C - C_b) - (B - B_b)} = F
\]

The factor VIII activity of the unknown sample in percent is calculated by the equation:

\[
[(S - S_b) - (B - B_b)] \times F = \text{Percent Factor VIII activity of the sample.}
\]

b) Using a Calibration Curve

I. For kinetic assays

Plot the F. VIII activity in percent on the abscissa and the absorbance/minute measured at 405 nm, on the ordinate. Read the percent F. VIII activity of the unknown sample from the calibration curve by finding the point where the ∆A/minute intercepts the curve.

II. For endpoint assays

1. Subtract Calibration Plasma Blank Absorbance \((C_b)\) from the Calibration Plasma Absorbance \((C)\).
2. Subtract Sample Plasma Blank Absorbance \((S_b)\) from Sample Absorbance \((S)\).
3. Subtract the Zero Standard Blank Absorbance \((B_b)\) from the Zero Standard Absorbance \((B)\).

Plot the F. VIII activity in percent on the abscissa and \((C-C_b)\) on the ordinate using linear graph paper.

Read F. VIII activity of the unknown sample from the calibration curve by finding the point where \((S-S_b)\) intercepts the curve.
Limitations of the Procedure

Siemens has validated use of these reagents on various analyzers to optimize product performance and meet product specifications. User defined modifications are not supported by Siemens as they may affect performance of the system and assay results. It is the responsibility of the user to validate modifications to these instructions or use of the reagents on analyzers other than those included in Siemens Application Sheets or these Instructions for Use.

Results of this test should always be interpreted in conjunction with the patient's medical history, clinical presentation and other findings.

Expected Values

F. VIII levels of 60 to 168 % of normal were determined in 124 ostensibly healthy individuals using the Factor VIII Chromogenic Assay on BCS®/BCS® XP.

For other coagulation instruments the expected values are 70 to 150 % of normal.

It may be necessary to calculate your own reference range in the laboratory due to systematic differences in instrumentation.

Notes on the Assay Procedure

– The sensitivity of the assay in the low range of F. VIII (0 to 10 % of normal) may be improved if the Coagulation Factor VIII Deficient Plasma and FPP or Standard Human Plasma are used as standards. Prepare 4 standards by mixing both plasmas accordingly in order to obtain 100, 50, 25 and 0 % (Coagulation Factor VIII Deficient Plasma (human) alone). In this case, both standards and samples can be assayed at the 1 : 31 dilution.
– All reagents as well as the plastic tubes should be preincubated at the selected temperature. The reproducibility is optimal if the measuring temperature does not deviate by more than 0.2 °C from the selected temperature during the reaction.
– 1 mol/L citric acid can be used instead of 20 % acetic acid in the endpoint method.
– Disposable plastic materials are recommended for sampling and measuring.
– The reference curve must be re-determined for each change of device and for each new lot of Factor VIII Chromogenic Assay.

Specific Performance Characteristics

In a comparative performance study, 98 patient samples were assayed by both the Factor VIII Chromogenic Assay and the coagulometric assay using deficient plasma and Actin® Activated Cephaloplastin Reagent. The correlation coefficient obtained was 0.96 and the regression equation was \( y = 1.0 \times + 1.0 \% \).

The precision of the assay depends to some extent on the quality of the equipment and on the skill of the person performing the test. The following estimates of precision were made using the kinetic method at 37 °C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>N</th>
<th>Mean (%)</th>
<th>SD (%)</th>
<th>CV (%)</th>
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<tbody>
<tr>
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<td>118</td>
<td>5</td>
<td>4</td>
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<tr>
<td>Within-run precision</td>
<td>11</td>
<td>124</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Sample 2</td>
<td>N</td>
<td>Mean (%)</td>
<td>SD (%)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>Between-run precision</td>
<td>11</td>
<td>25</td>
<td>2.5</td>
<td>10</td>
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<tr>
<td>Within-run precision</td>
<td>11</td>
<td>28</td>
<td>2.6</td>
<td>9</td>
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</tbody>
</table>

Heparin concentrates of up to 10 U/mL do not interfere with the Factor VIII Chromogenic Assay.

The linearity of the Factor VIII Chromogenic Assay is from 0 to 100 % F. VIII°.
Bibliography


Definition of Symbols

<table>
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<th>Symbol</th>
<th>Description</th>
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<td>❌</td>
<td>Do not reuse</td>
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<tr>
<td>LOT</td>
<td>Batch Code</td>
</tr>
<tr>
<td>⚠️</td>
<td>Caution, consult accompanying documents</td>
</tr>
<tr>
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<tr>
<td>Reconstitution volume</td>
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<td>Keep away from sunlight and heat</td>
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