



## ZYMUTEST™ Free Protein S (II)

REF RK015B  
 96 tests

ELISA method for the quantitative determination of  
 Free Protein S

English, last revision: 11-2022

### INTENDED USE:

ZYMUTEST™ Free Protein S (II) is an ELISA method for the quantitative assay of Free Protein S (the Activated Protein C cofactor) in human plasma.

### SUMMARY AND EXPLANATION:

- Protein S concentration in normal human plasma is of about 25 µg/mL<sup>1</sup>. About 40% (i.e. 10 µg/mL) is in the Free form and 60% (i.e. 15 µg/mL) circulates in blood as a non-covalent complex with C4b-BP. Only the Free form has an anticoagulant activity as the cofactor of Activated Protein C.
- Protein S is synthesized in liver. It is a vitamin K dependent glycoprotein, with a molecular weight of 80,000 daltons. The balance between the free form and the C4b-BP bound form of Protein S plays an important role because only the Free Protein S is active. In the early stages of inflammatory diseases, Free Protein S concentration is decreased as a result of an elevation of C4b-BP. Protein S is decreased in dicoumarol or L-asparaginase therapy, and in hepatic diseases.

### PRINCIPLE:

The ZYMUTEST™ Free Protein S (II) assay is specific for the Free form of Protein S, and is designed with two monoclonal antibodies that don't react with Protein S complexed with C4b Binding Protein (C4b-BP). It measures specifically the free form of Protein S. First, the immunconjugate, a mouse monoclonal antibody specific for Free Protein S coupled to horse radish peroxidase (HRP), is introduced into the microwells coated with another mouse monoclonal antibody specific for Free Protein S. Then, the diluted tested plasma or biological fluid is immediately introduced, and the immunological reaction starts. When present, Free Protein S binds onto the monoclonal antibody coated solid phase through one epitope, and fixes the second monoclonal antibody coupled to HRP through another epitope. Only Free Protein S is bound while Protein S-C4b-BP complexes are not reactive in the assay. Following a washing step, the peroxidase substrate, 3,3',5,5' - Tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained that can be read at 450nm. The absorbance at 450nm is directly proportional to the concentration of human Free Protein S in the tested sample.

### REAGENTS:

- COAT ELISA Microplate**, 12x8 containing 12 strips of 8 wells, coated with a monoclonal antibody specific for the human Free Protein S, stabilised and packed in an aluminium pouch hermetically sealed in presence of a desiccant.
- SD ELISA Sample Diluent**: 2 vials of 50 mL, ready to use. Contains BSA.
- CAL PROTEIN S Plasma Calibrator**: 3 vials 2 mL, lyophilised. Each vial must be restored with 2 mL of Sample Diluent to obtain a plasma already diluted 1:50. This calibrator is related to the NIBSC international standard. Contains BSA.
- CI PROTEIN S Control I (High)**: 1 vial of 0.5 mL, lyophilised. Contains BSA.
- CII PROTEIN S Control II (Low)**: 1 vial of 0.5 mL, lyophilised. Contains BSA.
- IC Anti-(h)-Free PS (II)-HRP immunoconjugate**: 3 vials of 4mL, lyophilized. Monoclonal antibody coupled to HRP. Contains BSA.
- CD ELISA Conjugate Diluent**: 1 vial of 25 mL, ready to use. Contains BSA.
- WS ELISA Wash Solution**: 1 vial of 50 mL, 20x 20 fold concentrated. Contains Proclin.
- TMB 3,3', 5,5' - Tetramethylbenzidine**: 1 vial of 25 mL peroxidase substrate. Contains hydrogen peroxide. Ready to use.
- SA Sulfuric acid 0.45M**: 1 vial of 6 mL of stop solution, ready to use. Contains 0.45M sulfuric acid.

The calibrator and controls concentrations may vary slightly from one batch to the next. For the assay, see the exact values provided on the flyer provided with the kit used.

### WARNINGS AND PRECAUTIONS:

- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- The human plasma used has been tested by recorded methods and is certified free of HIV antibodies, Hbs Antigen and HCV antibodies.
- The bovine plasma used to prepare the BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- If the TMB substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- The disposal of waste materials must be carried out according to current local regulations
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay; they are optimized for each lot of kits.
- Reagents must be handled with care, in order to avoid any contamination during use. Take care to limit as much as possible any evaporation of the reagents during use, by limiting the liquid-air surface exchange.
- In order to preserve the stability of the reagents, close the vials with their original screw cap following each use.
- Stability studies show that the reagents can be shipped at room temperature for a short period without damage.
- For *in vitro* diagnostic use.

**CD ELISA** | **SD ELISA** | **WS ELISA** | H317: May cause an allergic skin reaction.

### REAGENT PREPARATION AND STABILITY:

Bring the kit at room temperature, at least 30 min before the assay. Store the unused reagents at 2-8°C. Remove carefully the stopper for lyophilized products, in order to avoid any loss of powder when opening the vials.

- COAT** Open the aluminium pouch and take off the required amounts of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 4 weeks in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided plastic microplate storage bag (minigrip).
- SD ELISA** Ready to use.  
 Reagent stability after opening, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.
- CAL PROTEIN S** Restored each vial with exactly 2 mL of Sample Diluent, shake thoroughly for complete dissolution. The reconstituted Plasma Protein S Calibrator contains a Free Protein S concentration "C" in %, already diluted in 1:50.  
 Reagent stability after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature 18-25°C.
- CI PROTEIN S** Restored each vial with exactly 0.5 mL of distilled water, shake thoroughly for complete dissolution.  
 Reagent stability after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature 18-25°C.
  - 2 months frozen at -20°C or less\*.
- CII PROTEIN S** Restored each vial with exactly 0.5 mL of distilled water, shake thoroughly for complete dissolution.  
 Reagent stability after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature 18-25°C.
  - 2 months frozen at -20°C or less\*.
 \*Thaw only once, as rapidly as possible at 37°C, adapting the incubation period to the volume of reagent. The stability of the thawed reagent should be checked under laboratory work conditions.
- IC** Restored each vial with exactly 4 mL of Conjugate Diluent, shake thoroughly for complete dissolution. Let the pellet to be completely dissolved before use, and shake the vial in order to homogenize the content.  
 Reagent stability after reconstitution, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.
  - 24 hours at room temperature 18-25°C.
- CD ELISA** Ready to use.  
 Reagent stability after opening, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.
- WS ELISA** Incubate, if necessary, the vial in a water bath, at 37°C, until complete dissolution of crystals. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL contained in the vial allow to prepare 1 liter of Wash Solution).  
 Stability of the wash solution after opening, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.
 Stability of the diluted wash solution, provided that any contamination or evaporation is avoided:
  - 7 days at 2-8°C, for the diluted solution.
- TMB** Ready to use. Reagent stability after opening, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.
- SA** Ready to use. Reagent stability after opening, provided that any contamination or evaporation is avoided, kept in its original vial, is of:
  - 4 weeks at 2-8°C.

### STORAGE CONDITIONS:

Unopened reagents must be stored at 2-8°C, in their original packaging box. They are then usable until the expiration date printed on the kit.

### REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

#### Reagents:

- Distilled water.

#### Materials:

- 8-channel pipettes allowing dispensing 50-300 µL.
- Pipettes at variable volumes from 0 to 20 µL, 20 to 200 µL and 200 to 1000 µL.
- Micro ELISA plate washing equipment and shaker (optional).
- Micro ELISA plate reader with a wavelength set up at 450 nm.

## SPECIMEN COLLECTION AND PREPARATION:

Preparation and storage of specimens must be performed according to the current local regulations (for the United States, see the CLSI GP44-A4<sup>1</sup> guidelines for general information on specimen collection, handling and storage).

### Specimens:

Human plasma poor in platelets obtained from trisodium citrate anticoagulated blood. EDTA collected human plasma may also be used. The storage conditions are the same with citrated plasma.

### Collection:

Blood (9 volume) must be collected on trisodium citrate anticoagulant (1 volume) (0.109M), with caution, through a net venipuncture. The first tube must be discarded.

### Centrifugation:

Within 2 hours, use a validated method in the laboratory to obtain a platelet-poor plasma, e.g., a minimum of 15 minutes at 2500 g at room temperature (18-25°C) and plasma must be decanted into a plastic tube.

### Storage of plasma:

- 8 hours at room temperature (18-25°C)
- 1 month at -20°C.
- 18 months at -70°C.

Frozen plasma specimens should be rapidly thawed at 37°C, then gently mixed and tested immediately. Resuspend any precipitation by thorough mixing immediately after thawing and before testing.

## PROCEDURE:

### Assay method:

1. Dilute the samples and controls using **sample diluent** as described in the table below:

Sample	Dilution
Plasma	1:50
Controls	1:50

In presence of sample with high amounts of Protein S, dilute at **1:100** or more. The obtained results should be multiplied by **2** or more (i.e. D:50). For low amounts of Protein S levels (<10%) the sample can be tested at a lower dilution. The obtained results should be divided by the dilution factor.

2. Using the **Calibrator (CAL PROTEIN S)**, with a Free Protein S concentration "C" (the **100% concentration of Free Protein S** corresponds to a normal pooled plasma **diluted 1:50**, which is the standard assay dilution) provided in the kit, prepare the following standard solutions:

Free Protein S concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Free Protein S calibrator	1 mL	0.5 mL	0.25 mL	0.1 mL	0.05 mL	0 mL
Vol. of Sample diluent	0 mL	0.5 mL	0.75 mL	0.9 mL	0.95 mL	1 mL

Mix for homogenization.

The standard dilutions are stable for **4 hours** at room temperature (18-25°C).

3. Remove the strips from the package and put in the frame provided. In the different wells of the micro ELISA plate, introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Conjugate anti (h)-Free PS (II)-HRP	100 µL	Introduce the <b>Immunoconjugate anti-(h)-Free Protein S (II)-HRP</b> into the plate wells.
Protein S Calibrator Or tested sample Or Sample Diluent (blank)	100 µL	Introduce <b>immediately</b> the : • <b>Standard solutions</b> or • <b>Tested samples</b> into the plate wells. (a).
<b>Mix gently then incubate for 1 hour at room temperature (18-25°C) (d)</b>		
Wash Solution (20 fold diluted in distilled water before use)	300 µL	Proceed to 5 successive washings (b).
TMB / H <sub>2</sub> O <sub>2</sub> Substrate	200 µL	Immediately after the washing, introduce the substrate into the wells (b). <b>Note:</b> The substrate distribution, row by row, must be accurate and at exact time intervals (c, d).
<b>Incubate for exactly 5 minutes at room temperature (18-25 °C) (d).</b>		
Stop solution 0.45 M Sulfuric Acid	50 µL	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the <b>0.45M sulfuric acid (c)</b> .
<b>Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 450 nm (A450). Subtract the blank value if necessary (e).</b>		

- (a) Distribute samples as rapidly as possible (≤10 minutes), in order to obtain a homogeneous immunological kinetics for antibodies binding. A too long delay between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results. **In case of the full plate is used, distribute the calibrator's dilutions on the center of the plate to reduce the kinetics effect.**
- (b) Never let the wells of ELISA plates empty between the addition of the reagents or following the washing step in order to preserve coated proteins. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates efficiently: a too drastic washing can lower plate reactivity, while an inefficient washing can increase blank value.
- (c) For addition of TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

- (d) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. An incubation temperature (18-25°C) must be respected. Results are affected by a too high (>25°C) or too low (<18°C) temperature, leading to abnormally high or low absorbance at 450nm. It has to be considered when analyzing the results. Absorbances at 450nm are significantly increased if a micro-ELISA plate shaker is used throughout the incubation steps.
- (e) For bichromatic readings, a reference wavelength between 620 nm or 690 nm can be used.

## QUALITY CONTROL:

Using quality controls allows validating the method compliance, as well as the inter-assays homogeneity for a same lot of reagents.

Quality control plasmas must be included in each series, as per good laboratory practice, in order to validate test results. A new calibration curve must be carried out for each test series.

Each laboratory can establish acceptance ranges and verify expected performances in its analytical system.

## RESULTS:

- For the manual method, plot the calibration curve, with the **OD 450 nm** along the Y-axis and the Free Protein S concentration, expressed as %, along the X-axis by choosing the "best fit" interpolation mode (refer to the flyer in the kit).
- Alternatively, an ELISA software (i.e., Dynex, Biolise, etc...) can be used for the calculation of concentrations.
- The concentrations of Free Protein S in the test specimen and in the controls I and II, when using the standard dilution (**D=50**), are directly deduced from the calibration curve, and expressed as % of Free Protein S.
- For higher or lower dilution factor, the concentration measured must be multiplied by the complementary dilution factor (as an example for **D=100**, results should be multiplied by 2).

## LIMITATIONS:

- In order to get the optimal assay performances and adhere to specifications, the procedural instructions validated by HYPHEN BioMed must be strictly respected. It is responsibility of the user laboratory to validate any modification to those instructions for use.
- Any reagent presenting an unusual aspect or signs of contamination must be rejected.
- If the washing step is not correctly performed, the negative control can produce a high absorbance value. In order to avoid non-specific coloration development, check that the washing step is performed efficiently.
- For the possible influence of interferences, no significant effect is observed for Heparin concentration up to 2 IU/mL, bilirubin concentration up to 0.6 mg/mL, hemoglobin concentration up to 10 mg/mL, intralipids concentration up to 10 mg/mL and DOACs concentration up to 400 ng/mL, by plasma overload tests.

## PATHOLOGICAL VARIATIONS:

- Free Protein S concentrations are decreased in type I and type III Protein S deficiencies.
- Free Protein S is decreased in VKA-based anticoagulant therapies, vitamin K deficiencies or in severe hepatic diseases.
- Transitory Free Protein S deficiencies are observed during the early stages of inflammatory diseases, as a result of increased C4b-BP concentrations, which form complexes with Protein S.
- Type I deficiency: Partial deficiency of total and Free Protein S antigen.
- Type II deficiency: Normal Total and Free Protein S antigen, reduced activity.
- Type III deficiency: Normal Total Protein S antigen, decreased activity and free antigen.<sup>6</sup>

## PERFORMANCES:

- The ZYMUTEST™ Free Protein S kit is specific for both forms of Free Protein S. The two monoclonal antibodies, are unreactive with Protein S-C4b-BP complexes. It measures specifically the functional, native, and active form of Protein S.
- Dynamic range: 5 to about 130%.
- Detection threshold ≤ 5%.
- Intra-assay CV: ≤ 6% (for samples of normal plasma), ≤ 8% (for samples of abnormal plasma).
- Inter-assay CV: ≤ 10%.
- Reference material: International Standard for Protein S (03/228).

## REFERENCES:

- CLSI Document GP44-A4: "Procedures for the handling and processing of blood specimens for common laboratory tests".
- Faioni E., Valsecchi C., Palla A., Taioli E., Razzari C., Mannucci P.: Free Protein S Deficiency is a Risk Factor for Venous Thrombosis: Thromb. Haemost., 1997, 78, 1343-46
- Henkens C.A.A., Bom V.S., Van der Schaaf W., Pelsma P.M., Smit Sibinga C.T, Kam P.S, Van der Meer J.: Plasma Levels of Protein S, Protein C, and Factor X : Effects of sex, Hormonal State and Age : Thromb. Haemost., 1995, 74, 1271-75
- Aiach M., Borgel D., Gaussem P., Emmerich J., Alhenc-gelas M., Gandrille S.: Protein C and Protein S deficiencies. Sem. in Hemat., 1997, 34, 205-17.
- Schwartz H.P., Fischer M., Hopmeier P., Batard M.A., and Griffin J.H.: Plasma Protein S Deficiency in Familial Thrombotic Disease; Blood, 1984, 64, 1297-1300.
- Martlar R. A., Gausman J. N. Protein S abnormalities a diagnostic nightmare. Am. J. Hematol, 2011, 86, 418-421.

## SYMBOLS:

Used symbols and signs listed in the ISO standard 15223-1, refer to the Definition of Symbols document.

Changes compared to the previous version.