

## IMUCLONE® Free Protein S ELISA

Product No. 842

**american diagnostica inc.**  
500 West Avenue • Stamford, CT 06902  
Tel: (203) 602-7777 Fax: (203) 602-2221

### INTENDED USE

The IMUCLONE® Free Protein S is an enzyme-linked immunosorbent assay for measuring human Free Protein S (the activated Protein C cofactor) in plasma or any biological fluid where Free Protein S may be present. The ELISA is limited to Research Use Only in the United States.

### EXPLANATION OF THE TEST

Protein S is an 80,000 D molecular ratio, vitamin K dependent glycoprotein synthesized in the liver. The concentration of Protein S in normal human plasma is approximately 25 µg/mL<sup>1</sup> and is found in two forms: Free Protein S comprises approximately 40% (10 µg/mL) of the total amount while approximately 60% (15 µg/mL) circulates in blood as a non-covalent complex with C4b Binding Protein (C4b-BP). Only the Free Protein S possesses anticoagulant activity as the cofactor of Activated Protein C. The balance between the Free and C4b-BP complexed forms of Protein S plays an important role as only the Free Protein S is active. In the early stages of inflammatory diseases, the Free Protein S concentration is decreased as a result of an elevation of C4b-BP. Protein S is also decreased by dicoumarol or L-asparaginase therapies and in hepatic diseases. Protein S deficiencies are defined as Type I Deficiency - Partial deficiency of Total and Free Protein S antigen, Type II Deficiency - Normal Total and Free Protein S antigen but reduced activity, and Type III Deficiency - Normal Total antigen with decreased activity and Free Protein S antigen.

### ASSAY PRINCIPLE

A calcium-dependent monoclonal antibody specific for Free Protein S coupled to horse radish peroxidase (HRP) is added to a microwell coated with another calcium-dependent monoclonal antibody specific for Free Protein S. Next, a diluted plasma sample or biological fluid is immediately added to the microwell and the immunological reaction begins. If present, Free Protein S binds onto the monoclonal antibody coated solid phase via one epitope and binds to the second monoclonal antibody coupled to HRP via a second epitope. Following a wash step, the peroxidase substrate, 3,3',5,5'-Tetramethylbenzidine (TMB), in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is added to the microwell and the subsequent enzymatic reaction yields a blue colored solution. The addition of sulphuric acid stops the reaction and turns the solution color to yellow. The amount of color is directly proportional to the concentration of human Free Protein S in the tested sample.

### REAGENTS

12 strips of 8 antibody coated microwells (total of 96 wells) in frame holder.  
2 vials of Protein S Sample Diluent (contains calcium), ready to use (50 mL).  
3 vials of Plasma Protein S Calibrator, 1:50 prediluted (lyophilized).  
1 vial of Protein S Control Plasma I, High (lyophilized).  
1 vial of Protein S Control Plasma II, Low (lyophilized).  
3 vials of Anti-Human Free Protein S-HRP Immunoconjugate (lyophilized).  
1 vial of Protein S Conjugate Diluent, ready to use (15 mL).  
1 vial of Wash Solution, 20 fold concentrate (50 mL).  
1 vial of TMB Substrate, ready to use (25 mL).  
1 vial of Stop Solution, 0.45M H<sub>2</sub>SO<sub>4</sub> (6 mL).

### WARNING

Source material for some of the reagents in this kit is of human origin. This material has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using FDA approved methods. As no known test method provides complete assurance that products derived from human blood will not transmit HBsAg, HCV, HIV-1, HIV-2 or other blood-borne pathogens, reagents should be handled as recommended for any potentially infectious human specimen. Discard all waste associated with test specimens and human source reagents in a biohazard waste container.

Limited for research use only in the United States. For *in vitro* use only. Not for internal use in humans or animals. Do not use the kit components beyond the stated expiration date. Do not mix reagents from different kits. Avoid microbial contamination of the reagents. Do not smoke, eat or drink in areas in which specimens or kit reagents are handled. Do not pipette reagents by mouth. Wear laboratory coat and disposable gloves throughout the test procedure and wash hands thoroughly afterwards. Avoid splashing or aerosol formation.

### REAGENT PREPARATION AND STORAGE

Unopened and lyophilized reagents are stable until the expiration date printed on the box when properly stored at 2°-8°C.

- 1. MAB against Human Free Protein S Coated Microwells:** Once removed from the aluminium pouch, the microwell strips must be used within 30 minutes. Unused strips may be stored at 2°-8°C for 4 weeks when sealed in the original pouch with the desiccant present, protected from any moisture, and stored in the provided storage bag.
- 2. Protein S Sample Diluent:** Supplied ready to use, once opened, the diluent may be used for up to 4 weeks when stored at 2°-8°C. The Diluent contains 0.05% Kathon CG.
- 3. Plasma Protein S Calibrator:** Reconstitute each vial with 2.0 mL of Protein S Sample Diluent. This calibrator is equivalent to normal plasma containing at a 1:50 dilution. Reconstituted calibrator is stable for at least 8 hours at room temperature (18°-25°C).
- 4. Protein S Control I:** Reconstitute this vial 0.5 mL of filtered deionized water. This control is a high plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at -20°C providing bacterial contamination is avoided.
- 5. Protein S Control II:** Reconstitute this vial 0.5 mL of filtered deionized water. This control is a low plasma control. See the enclosed data for the acceptable range. Reconstituted control is stable for 8 hours at room temperature (18°-25°C), 24 hours at 2°-8°C or 2 months at -20°C providing bacterial contamination is avoided.
- 6. Anti-Human Free Protein S-HRP Immunoconjugate:** Reconstitute each vial with 4.0 mL of Protein S Conjugate Diluent. Shake the vial gently to homogenize the content. Reconstituted immunoconjugate is stable for at least 24 hours at room temperature or for at least 4 weeks at 2°-8°C.
- 7. Protein S Conjugate Diluent:** Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C. This conjugate diluent contains 0.05% Kathon CG.
- 8. Protein S Wash Solution:** If solids are present, incubate the vial for 15-30 minutes in a 37°C water bath. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 mL is sufficient to prepare 1 Liter of Wash Solution). The Wash Solution may be used for up to 4 weeks after opening when stored at 2°-8°C in its original vial. Diluted Wash Solution may be used for up to 7 days when stored at 2°-8°C. This wash solution contains 0.05% Kathon CG and it contains calcium and must be used for this Free Protein S ELISA.
- 9. TMB Substrate:** Supplied ready to use. Once opened, it may be used for up to 4 weeks when stored at 2°-8°C.
- 10. Stop Solution, 0.45M H<sub>2</sub>SO<sub>4</sub>:** Supplied ready to use. **Caution:** Sulphuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

### SPECIMEN COLLECTION AND PREPARATION

Citrate collected platelet poor plasma or serum may be used for this assay. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 2,500 x g for 20 minutes.
3. Plasma should be stored at 2°-8°C and assayed within 4 hours. Alternatively, plasma may be stored at -20°C for up to 6 months.
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasmas should be stored at 2°-8°C and assayed within 4 hours.

Test samples and the Protein S Controls must be diluted 1:50 in the Protein S Sample Diluent. For expected Protein S levels >100%, samples must be assayed at higher dilutions, 1:100 or greater. For Protein S <10%, the sample may be assayed at a lower dilution, less than 1:50.

(...over)

## PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided

0.22 µm filtered deionized H<sub>2</sub>O

50-300 µL eight channel multi-pipette

0-200 µL, 200-1000 µL single pipettes

Microwell plate reader for reading absorbance at 450 nm

Microwell plate shaker, Microwell plate washer (optional)

### Preparation of the Standards

Free Protein S concentrations are expressed as a % of pooled normal plasma. The 100% concentration corresponds to a pooled normal human plasma diluted 1:50, the standard assay dilution. Using the Protein S Calibrator provided, with a Free Protein S concentration "C" as indicated on the flyer 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 Protein S Calibrator	1.0 mL	0.5 mL	0.25 mL	0.10 mL	0.05 mL	0 mL
Vol. of Protein S Sample Diluent	0 mL	0.5 mL	0.75 mL	0.90 mL	0.95 mL	1.0 mL

Mix gently for a complete homogenization. The standard dilutions are stable for at least 4 hours at room temperature (18°-25°C).

### Assay Procedure

Remove the required number of strips from the aluminium pouch sufficient for the number of assays to be performed. Place the strips in the frame provided. To the appropriate wells, add the reagents and perform the various assay steps as indicated on the following table:

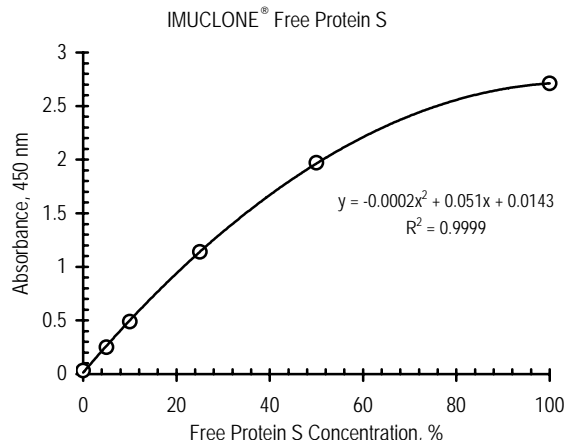
Reagent	Volume	Procedure
Anti-Human Free Protein S-HRP Immunoconjugate	100 µL	Add the Anti-Human Free Protein S-HRP Immunoconjugate to each microwell.
Protein S Standard, diluted sample or Protein S Sample Diluent (blank)	100 µL	Immediately add the Protein S standards or diluted sample to the appropriate microwell
Incubate for 1 hour at room temperature (18°-25°C) while gently shaking either manually or using an orbital microwell plate shaker		
Protein S Wash Solution	300 µL	Wash the wells 5 times
TMB Substrate	200 µL	Add the substrate to each microwell immediately after the wash step
Incubate for exactly 5 minutes at room temperature (18-25°C)		
0.45M H <sub>2</sub> SO <sub>4</sub>	50 µL	Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 0.45M H <sub>2</sub> SO <sub>4</sub>
Wait for 10 minutes in order to allow the color to stabilize and measure the absorbance at 450 nm. Subtract the blank value from the measurements.		

Notes:

1. Avoid letting the plate in the bright sunlight during incubations and particularly during color development.
2. Do not allow the microwells to dry out between the addition of reagents or following a washing step. Add the next reagent within 3 minutes in order to prevent the microwells from drying, which could damage the immobilized components. If necessary, fill the microwells with Wash Solution and empty it just before the introduction of the next reagent.
3. When adding the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

## RESULTS

Construct a standard curve by plotting the mean absorbance value for each Free Protein S standard (ordinate) versus its corresponding concentration in % (abscissa). A standard curve should be generated each time the assay is performed. The following standard curve is for demonstration purposes only.



### CALCULATIONS

From the standard curve generated, directly deduce the Free Protein S concentration in assayed samples at the standard 1:50 dilution. If higher dilutions are used, the Free Protein S concentration must be multiplied by the complementary dilution factor D (i.e. multiply the concentration by 2 for a 1:100 sample dilution or by 4 for a 1:200 sample dilution). If lower dilutions are used, the concentration obtained must be divided by 50:D. Alternatively, an ELISA software (i.e. Dynex, etc.) can be used for the calculation of concentrations.

### LIMITATIONS OF THE PROCEDURE

As the monoclonal antibodies used in the ELISA are calcium dependent, only the wash buffer supplied, which contains calcium, may be used in the assay. If the wash steps are not correctly performed, samples can produce a high absorbance value. In order to avoid non-specific color development, check that the wash steps are performed efficiently.

### EXPECTED VALUES

The Free Protein S concentration in normal human plasma is usually in the range 60–150%, therefore the abnormal range is <60%. As the concentration is higher in males than in females and tends to increase with age and blood lipid concentration, this cut off value must be analysed respectively to the patient context (age, gender, therapy, lipid metabolism, etc.) when diagnosing a Protein S deficiency.

Free Protein S concentrations are decreased in type I and type III Protein S deficiencies. It is decreased during pregnancy and during use of oral contraceptives. 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.

### PERFORMANCE CHARACTERISTICS

The IMUCLONE® Free Protein S ELISA is specific for the native and functional forms of Free Protein S. The ELISA is not reactive with Protein S-C4b-BP complexes.

### REFERENCES

1. Faioni, E., *et al.* Free Protein S Deficiency is a Risk Factor for Venous Thrombosis. *Thromb. Haemost.* 1997, **78**: 1343-46.
2. Henkens, C. A. A., *et al.* Plasma Levels of Protein S, Protein C, and Factor X: Effects of Sex, Hormonal State and Age. *Thromb. Haemost.* 1995, **74**: 1271-1275.
3. Aiach, M., *et al.* Protein C and Protein S deficiencies. *Sem. in Hemat.* 1997, **34**: 205-17.
4. Schwartz, H. P., *et al.* Plasma Protein S Deficiency in Familial Thrombotic Disease. *Blood* 1984, **64**: 1297-1300.
5. Hosaka, Y., *et al.* Thrombomodulin in human plasma contributes to inhibit fibrinolysis through acceleration of thrombin-dependent activation of plasma procarboxypeptidase B. *Thromb. Haemost.* 1998, **79**: 371-377.