

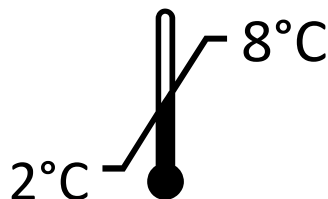


IMUCLONE™

Free Protein S ELISA

REF 842

For Research Use Only



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.

This assay is 'For Research Use Only' and is not intended for *in vitro* diagnostic use.

EXPLANATION OF THE TEST

Protein S is an 80,000 D molecular weight, vitamin K dependent glycoprotein synthesized in the liver. The concentration of Protein S in normal human plasma is approximately 25 µg/mL¹ 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.

PRINCIPLE OF THE METHOD

A calcium-dependent monoclonal antibody specific for Free Protein S coupled to horseradish 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 (H₂O₂), is added to the microwell and the subsequent enzymatic reaction yields a blue colored solution. The addition of sulfuric 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

- 96 Microtest Plate pre-coated with anti-Human Free Protein S; 12 strips of 8 wells in a frame holder plus storage bag with desiccant
- 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 PS-HRP ImmunoConjugate (lyophilized)
- 1 vial of Protein S Conjugate Diluent, ready to use (15 mL)
- 1 vial of Protein S Wash Solution, 20 fold concentrate (50 mL)
- 1 vial of TMB Substrate (Peroxidase Substrate), ready to use (25 mL)
- 1 vial of Stop Solution (0.45 M Sulfuric Acid), ready to use (6 mL)

WARNINGS AND PRECAUTIONS

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). 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.

For Research Use Only. Not for internal use in humans or animals. Do not use 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.

Conjugate Diluent	Warning 	CONT Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-2H-isothiazol-3-one (3:1) H317, P261, P272, P280, P302+P352, P333+P313, P363, P501
Sample Diluent	Warning 	CONT Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-2H-isothiazol-3-one (3:1) H317, P261, P272, P280, P302+P352, P333+P313, P363, P501
Wash Solution	Warning 	CONT Mixture of 5-Chloro-2-methyl-4-isothiazolin-3-one and 2-Methyl-2H-isothiazol-3-one (3:1) H317, P261, P272, P280, P302+P352, P333+P313, P363, P501
Stop Solution	Danger 	CONT Sulfuric acid H290, P234, P390

Hazard	H290 May be corrosive to metals.
Statements:	H317 May cause an allergic skin reaction.
Precautionary	P234 Keep only in original container.
Statements:	P261 Avoid breathing dust/fume/gas/mist/vapors/spray.
	P272 Contaminated work clothing should not be allowed out of the workplace.
	P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.
	P302 + P352 IF ON SKIN: Wash with plenty of soap water.
	P333 + P313 If skin irritation or rash occurs: Get medical advice/attention.
	P363 Wash contaminated clothing before reuse.
	P390 Absorb spillage to prevent material damage.
	P501 Dispose of contents/container in consultation with your regional waste disposer

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. Allow reagents to warm to room temperature for 30 minutes before use.

- 1. Microtest Strips pre-coated with anti-Human Free Protein S:** 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, 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 Plasma I:** Reconstitute this vial 0.5 mL of distilled 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 Plasma II:** Reconstitute this vial 0.5 mL of distilled 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 PS-HRP Immunoconjugate:** Reconstitute each vial with 4.0 mL of Protein S Conjugate Diluent. Shake vial gently to homogenize content. Reconstituted conjugate 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 calcium and must be used for this Free Protein S ELISA.
- 9. TMB Substrate (Peroxidase 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 (0.45 M H₂SO₄):** Supplied ready to use. Caution: Sulfuric acid is caustic. Handle with 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.

PROCEDURE

Materials Provided – See Reagents

Materials Required But Not Provided

Distilled water

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 washer (optional)

Preparation of the Free Protein S Concentration Levels

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 reconstituted Plasma Protein S Calibrator, with a Free Protein S concentration "C" as indicated on the flyer provided in the kit, prepare the following calibrator concentrations:

Free Protein S Concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma 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 mL

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

PROCEDURE

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

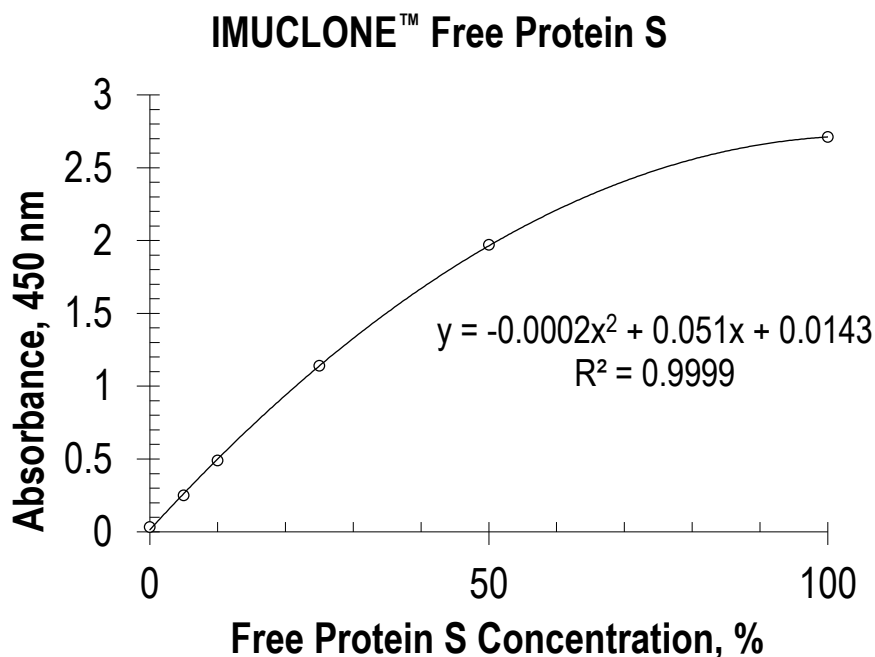
Reagent	Volume	Procedure
Anti-Human Free PS-HRP Immunoconjugate	100 µL	Add the immunoconjugate to each microwell.
Free Protein S concentrations, diluted Plasma Controls, diluted sample or Protein S Sample Diluent (blank)	100 µL	Immediately add the Free Protein S calibrator concentrations or diluted controls 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).		
Stop Solution (0.45 M H ₂ SO ₄)	50 µL	Following exactly the same time intervals used for adding the substrate, stop the reaction by adding 0.45 M H ₂ SO ₄
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 prepared 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 calibration curve by plotting the mean absorbance value for each Free Protein S concentration (ordinate) versus its corresponding concentration in % (abscissa). A calibration curve should be generated each time the assay is performed. The following calibration curve is for demonstration purposes only.



CALCULATIONS

From the calibration curve generated, directly deduce the Free Protein S concentration result in assayed samples at the standard 1:50 dilution. If higher dilutions are used, the Free Protein S concentration result 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 result obtained must be divided by 50:D (i.e. divide the concentration by 2 for a 1:25 sample dilution). Alternatively, an ELISA software (i.e. Dynex, etc.) can be used to calculate 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, ensure 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%. The concentration is higher in males than in females and tends to increase with age and blood lipid concentration.

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. 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.

DEFINITION OF SYMBOLS

	Consult instructions for use		Batch code / Lot number
	Temperature limitation: Store at 2°C to 8°C		Catalog number
	Manufactured by		Use By / Expiration Date
	Contains...		Contains sufficient for <n> tests