QUALITY CONTROL

1. The O.D. of the reagent blank should be less than 0.250 when the spectrophotometer has been blanked against the water well. Readings greater than 0.250 may indicate possible reagent contamination or inadequate plate washing.
2. O.D. for the duplicates of the controls or patient samples should be within 20% of the mean O.D. value for absorbance readings greater than 0.200.
3. Free Protein S Antigen values obtained for the controls should be within manufacturer's assigned ELISA ranges.
4. Each laboratory should establish their own reference range for this assay.

RESULTS

1. Calculate the mean O.D. values for the duplicates of the reference plasma dilutions, controls, and patient samples.
2. Plot the mean O.D. obtained for each dilution of the reference plasma (x axis) against the corresponding value of the reference level (y axis). A log-log graph is recommended, although a linear or point-to-point graph may also be used.
3. Using the mean O.D., determine the control and patient relative values from the graph, or, alternatively, calculate the linear regression for the reference curve.
4. To calculate Free Protein S Antigen levels in % of normal, multiply control and patient relative values obtained from the appropriate reference curve by the corresponding assigned value for the ELISA Reference Plasma.
   For example: Patient relative value (from the reference curve): 40
   Reference plasma assigned value: 105% of normal
   Actual patient Free Protein S Antigen value (as % of normal):
   40 x 1.05 = 42%
5. Ensure that all quality control parameters have been met (see Quality Control) before reporting test results.

REFERENCE RANGES[15]

Free Protein S values are expressed in relative percents as compared to pooled normal plasma. The normal range for Free Protein S for this assay is 50-150%. These Protein S ranges are consistent with those published in the literature and reported by commercially available assays.[6,10] Samples with values above the range of the reference curve may need to be diluted and retested for accurate results.

PERFORMANCE CHARACTERISTICS

Detection range:

The detection range for Monoclonal Free Protein S Antigen assay is 6-150%. However, the effective range of each run will depend on the assayed value of the reference plasma. For greatest accuracy, samples which generate absorbance readings outside the OD range of the reference curve should be retested at an appropriate dilution.

Precision:

Intra-assay: To determine variability within a plate, three plasma samples with known Free Protein S levels (one each high, medium and low) were tested in 16 wells by two operators on six plates from each of three lots. The data, presented in the following table, shows a mean intra-assay CV of 6.0% for Free Protein S across three lots. In addition, 30 commercially prepared plasma samples with Free Protein S levels spanning the entire detection range of the assay were tested in duplicate across 3 lots to demonstrate precision end users may expect when performing the assay according to package insert instructions. As shown in the table, the overall mean intra-assay CV for duplicates was 4.7% for Free Protein S.

Inter-assay: Eight (8) in-house control samples (prepared by mixing commercially immunodepleted citrated plasma and commercial healthy-donor citrated plasma samples) with values ranging from 29-56% were tested in duplicate on three lots to determine assay precision between lots. The mean inter-assay CV was 5.2% for Free Protein S, as seen in the table.

Intra-assay precision: (variability within a plate)	Free Protein S range (% of normal)	Free Protein S CV range (3 pilot lots)
Replicates (x 16):	109-118% 59-65% 44-47%	2.2- 6.1% 3.8- 9.4% 3.9-10.4%
Overall mean CV:		6.0%
Duplicates:	entire range	
Overall mean CV:		4.7%
Inter-assay precision (variability between lots) Duplicates:	30-58%	3.7- 6.4%
Overall mean CV:		5.2%

Linearity: Protein S reference plasma sample dilutions (prepared as directed in the package insert) demonstrate curves with a mean coefficient of determination (r-squared) of 0.994 when tested on three lots of Monoclonal Free Protein S Antigen Test Kit.

Accuracy: Accuracy was determined by testing mixtures of Protein S reference plasma with predetermined values on Monoclonal Free Protein S Antigen Assay and calculating the recovery of theoretical values. The overall mean percent recovery across 3 lots was 101.2% Free Protein S, with an average variation of 4.6% respectively.

LIMITATIONS OF THE TEST

The Free Protein S concentration values obtained from this assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings, and other diagnostic procedures. Patients with congenital homozygous deficiency of Protein S are rare and may show undetectable levels of Protein S, while those with heterozygous deficiency typically have levels below 50% of normal. Acquired Protein S deficiency may be seen in numerous clinical conditions: neonates (show 20-35% lower levels than adults), liver diseases, diabetes mellitus, pregnancy, oral contraceptives or oral anticoagulant therapy and disseminated intravascular coagulation (DIC). Increased levels of Protein S may be seen in patients with nephrotic syndrome.[5-10] Plasma samples could be inadvertently depleted or degraded of Protein S by improper collection or routine laboratory processing.

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15. Data on File.

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Monoclonal Free Protein S Elisa Kit

Instructions for use

REF: 5293

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HL-2-1964P 2009-07 (1)

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INTENDED USE
An enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of Free Protein S Antigen in citrated human plasma.

SUMMARY
Protein S is a vitamin K-dependent protein synthesized in the liver, vascular endothelium and megakaryocytes, which plays an important physiologic role in the Protein C Anticoagulant System.[1,2] This anticoagulant system is one of the major regulators of hemostasis by inhibiting clot formation and by promoting fibrinolysis. Protein S functions as a cofactor for activated Protein C on the vascular membrane to facilitate the degradation of clotting factors Va and VIIIa. In normal plasma approximately 40% of Protein S circulates as free molecule, while 60% is complexed with C4b, a plasma protein of the classical complement pathway.[3] Only Free Protein S is functionally active and able to bind to activated Protein C, while the complexed form of Protein S is not.[4] Protein S deficiency, either congenital or acquired, may lead to serious thrombotic events such as thrombophlebitis, deep vein thrombosis, or pulmonary embolism. The prevalence of Protein S deficiency has been estimated to be less than 1 case per 300 in the general population. Two-thirds of patients with a congenital deficiency of Protein S levels (less than 50% of normal) may present with venous thrombosis in young adulthood.[5,6] In young patients (< 35 years) with a history of thrombosis, the prevalence may be as high as 15 to 18%.7 Acquired Protein S deficiency may be seen during pregnancy, oral contraceptive use, liver disease, oral anticoagulant therapy, diabetes mellitus, postoperative complications, septicemia and various inflammatory syndromes.[8] A decreased Protein S activity in plasma may be the result of low concentrations or abnormal function of the Protein S molecule. The laboratory diagnosis of Protein S deficiency may require both quantitative and qualitative (functional) determinations. Quantitative determinations of Protein S Antigen are based on immunologic procedures such as radial immunodiffusion in gel, Laurell Rocket immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA).[9,10] ELISA procedures are less labor intensive and offer several advantages including more objective, accurate and reproducible results. In addition, the ELISA format allows automation with commonly available laboratory instrumentation. Measurement of plasma levels of both Total and Free Protein S are useful in determining the type of defect in patients with Protein S deficiency. Historically, ELISA procedures measuring Protein S used a polyclonal antibody specific to both the free and bound forms of Protein S. The addition of polyethylene glycol (PEG) to precipitate the bound protein S in the patient sample allowed determination of levels of free Protein S. While the PEG precipitation procedure allows the measurement of Free Protein S, it is non-specific, time consuming, and difficult to perform accurately.[11,12] This assay utilizes a monoclonal antibody specific for Free Protein S in an ELISA format to measure Free Protein S directly, without PEG precipitation.[13,14]

PRINCIPLE
The Monoclonal Free Protein S Antigen assay is a sandwich ELISA method. A capture antibody specific for human Protein S is coated to 96-microwell polystyrene plates. Diluted patient plasma is incubated in the wells allowing any available Free Protein S to bind to the anti-human Protein S monoclonal antibody on the microwell surface. The plates are washed to remove any unbound plasma molecules. Bound Free Protein S is quantitated using a horseradish peroxidase (HRP) conjugated polyclonal anti-human Protein S detection antibody. Following incubation, unbound conjugated anti-human Protein S is removed by washing. A chromogenic substrate of tetramethylbenzidine (TMB) and hydrogen peroxide (H_2O_2) is added to develop a colored reaction. The intensity of the color is measured spectrophotometrically at 450 nm in optical density (OD) units. Protein S relative percent concentrations in patient plasma are determined against a curve prepared from a reference plasma provided with the kit. The lyophilized assayed reference plasma is prepared from a frozen pool of citrated normal plasma and standardized against the Secondary Standard for Coagulation/International Society in Thrombosis and Haemostasis (SSC/ISTH) preparation, which is calibrated to World Health Organization (WHO) standards.

REAGENTS
WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY

1. **Free Protein S Antigen Microwells**
   Ingredients: 96 stabilized antibody coated microwells (12 strips of breakaway wells), with frame holder. Wells are coated with anti-human Protein S antibody.
   Preparation for Use: The microwells are ready for use as packaged.
   Storage and Stability: Store at 2-8°C. Do not freeze. Microwells are stable until the expiration date indicated on the package.
   Signs of Deterioration: Avoid microbial contamination.
2. **Sample Diluent**
   Ingredients: A blue-green solution containing buffers, salts, and sodium azide as a preservative
   WARNING: DO NOT INGEST. To prevent the formation of toxic vapors, sodium azide should not be mixed with acidic solutions. When discarding reagents containing sodium azide, always flush sink with copious quantities of water. This will prevent the formation of metallic azides which, when highly concentrated in metal plumbing, are potentially explosive. In addition to purging drain pipes with water, plumbing should occasionally be decontaminated with 10% NaOH.
   Preparation for Use: The diluent is ready for use as packaged.
   Storage and Stability: Store at 2-8°C. The diluent is stable until the expiration date indicated on the package.
   Signs of Deterioration: Discard if product shows signs of microbial growth.
3. **ELISA Reference Plasma**
   Ingredients: Contains human plasma.
   WARNING: DO NOT INGEST. Plasma has been tested and shown to be negative for Hepatitis B Antigen (HbsAg), HCV and HIV-1 antibody; however, the plasma should be handled as if capable of transmitting infection.
   Preparation for Use: Reconstitute Reference Plasma by adding 0.5 mL deionized water. Swirl gently to mix. Allow to stand for 10 minutes before use for complete dissolution.
   Storage and Stability: When stored at 2-8°C, the Reference Plasma is stable until the expiration date indicated on the package. Reconstituted solution is stable for 8 hours when stored at 2-8°C.
   Signs of Deterioration: Unreconstituted Reference Plasma should appear as a light yellow dry plug.
4. **Protein S Conjugate Solution**
   Ingredients: The red solution contains antibodies, specific for Protein S Factor which have been conjugated with horseradish peroxidase.
   WARNING: DO NOT INGEST.
   Preparation for Use: The conjugate solution is ready for use as packaged.
   Storage and Stability: When stored at 2-8°C, the solution is stable until the expiration date indicated on the package.
   Signs of Deterioration: Avoid microbial growth.
5. **Substrate**
   Ingredients: The Substrate contains 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide.
   WARNING: IRRITANT, DO NOT PIPETTE BY MOUTH. DO NOT INGEST –
   The substrate can cause irritation to the eyes and skin. Absorption through the skin is possible.
   Preparation for Use: The Substrate is ready for use as packaged.
   Storage and Stability: When stored at 2-8°C, the substrate is stable until the expiration date indicated on the package.
   Signs of Deterioration: Reagent should be clear and almost colorless.
6. **Stopping Solution**
   Ingredients: The solution is 0.36 N Sulfuric Acid.
   DANGER: DO NOT INGEST, CORROSIVE, DO NOT PIPETTE BY MOUTH.
   Avoid contact with skin or clothing.
   Preparation for Use: Solution is ready for use as packaged.
   Storage and Stability: The solution should be stored at 2-8°C and is stable until the expiration date indicated on the package.
7. **Phosphate Buffered Saline Concentrate (PBS)**
   Ingredients: 33X Phosphate Buffered Saline with 0.01% Tween 20.
   WARNING: DO NOT INGEST.
   Preparation for Use: Dilute 30 mL PBS Concentrate to 1 liter with deionized water. The pH of the final solution should be 7.35 ± 0.1.
   Storage and Stability: When stored at 2-8°C, the PBS is stable until the expiration date indicated on the package. The diluted PBS is stable for 1 year stored at 2-8°C.
   Signs of Deterioration: Discard if it shows signs of microbial or cross-contamination.

INSTRUMENTS
A spectrophotometer capable of reading microwell plates at 450 nm is required.

SPECIMEN COLLECTION AND PREPARATION
Specimen: The plasma collected by venipuncture with either 3.2% or 3.8% sodium citrate as an anticoagulant should be used as the sample matrix. Centrifuge sample immediately and remove the plasma.
Storage and Stability: Store at 2-8°C until testing can be performed. If not tested within 1 hour of collection, the sample must be stored at -70°C and tested within 1 month.

PROCEDURE
Materials Provided: The following materials needed for the procedure are contained in the kit.
Protein S:Ag Microwells (96)
ELISA Reference Plasma (3 x 0.5 mL)
Protein S Conjugate Solution (1 x 12 mL)
Sample Diluent (1 x 60 mL)
Substrate Solution (1 x 13 mL)
Stopping Solution (1 x 15 mL)
Phosphate Buffered Saline (1 x 30 mL)
Materials Required but not Supplied:
Specialty Assayed Control I (S.A.C. I) -5301
Specialty Assayed Control II (S.A.C. II) -5302
Deionized water
Graduated cylinders
Pipettors (delivering between 10 and 1000 µL)
Plate reading spectrophotometer capable of reading absorbance at 450 nm
Multichannel pipettors capable of delivering to 8 wells Centrifuge

Procedural Notes

1. Bring plasma samples and kit reagents to room temperature (18-26°C) and mix well before using; avoid foaming. Return all unused samples and reagents to refrigerated (2-8°C) storage as soon as possible.
2. All dilutions of reference plasma, control, and test plasma must be made just prior to use in the assay.
3. A single water blank well should be set up on each plate with each run. No sample or kit reagents are to be added to this well. Instead, add 200 µL of reagent grade water to the well immediately prior to reading the plate in the spectrophotometer. The plate reader should be programmed to "zero" or "blank" against this water well.
4. Good washing technique is critical for optimal performance of the assay. Adequate washing is best accomplished by directing a forceful stream of wash solution from a plastic squeeze bottle with a wide tip into the bottom of the microwells. An automated microtiter plate washing system can also be used.
5. **IMPORTANT:** Failure to adequately remove residual PBS can cause inconsistent color development of the substrate solution.
6. Use a multichannel pipettor capable of delivering to 8 wells simultaneously when possible. This speeds the process and allows for more uniform incubation and reaction times for all wells.
7. Carefully controlled timing of all steps is critical. All dilutions for curve points and samples must be added within a five minute period. Batch size of samples should not be larger than the amount that can be added within this time period.
8. For all incubations, the start of the incubation period begins with the completion of reagent or sample addition.
9. Addition of all samples and reagents should be performed at the same rate and in the same sequence.
10. Incubation temperatures above or below normal room temperature (18 to 26°C) may contribute to inaccurate results.
11. Avoid contamination of reagents when opening and removing aliquots from the primary vials.
12. Do not use kit components beyond expiration date.
13. **Do not use kit components from different kit lot numbers.**

STEP-BY-STEP METHOD

1. Remove any microwell strips that will not be used from the frame holder and store them in the bag provided.
2. Assay each reference plasma dilution in duplicate for Free Protein S. It is advised that duplicate determinations be made for all samples. One well should be run as a **reagent blank;** sample diluent without plasma is added to the well as explained in Step 6 of this section. This well will be treated the same as a control or patient sample in subsequent assay steps. A **water blank** well should be included with each plate; it is to remain empty until 200 µL of deionized water is added at the completion of the assay, immediately prior to reading the plate. The water blank well is to be used to zero the plate reader.
3. Using the Reference Plasma provided with the kit, prepare six reference dilutions as described below.

Volume Reference Plasma		Volume Sample Diluent		*Reference Level
30 µL	+	500 µL	=	150
20 µL	+	500 µL	=	100
15 µL	+	500 µL	=	75
10 µL	+	500 µL	=	50
10 µL	+	1000 µL	=	25
10 µL	+	2000 µL	=	12.5

*Reference level value to be used for constructing reference curve only

4. Prepare working dilutions of control and patient samples, as follows:
   Add 20 µL control or patient plasma to 500 µL Sample Diluent.
   (Note: These dilutions correspond to the 100% relative reference plasma dilution.)
5. Mix thoroughly and add 100 µL of the dilutions (reference plasmas x 6, patient samples and controls) to the appropriate microwells for Free Protein S determinations.
6. Add 100 µL of Sample Diluent to the reagent blank well. Leave the water blank well empty.
7. Incubate 40 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the sample fluid. Do not allow samples to contaminate other microwells.
8. Wash 4 times with working PBS solution. Each well should be filled with PBS solution per wash. PBS in the empty water blank well will not interfere with the procedure. Invert microwells between each wash to empty fluid. Use a snapping motion of the wrist to shake the liquid from the wells. The frame must be squeezed at the center on the top and bottom to retain microwell modules during washing. Blot on absorbent paper to remove residual wash fluid. Do not allow wells to dry out between steps.
9. Add 100 µL Protein S Conjugate Solution (red) to each well (except for the water blank well).
10. Incubate 10 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the Conjugate Solution.
11. Wash 4 times with working PBS solution as in Step 8. Use a snapping motion to drain the liquid, and blot on absorbent paper after the final wash. Do not allow the wells to dry out.
12. Add 100 µL Substrate to each well (except for the water blank well) and incubate for 10 minutes at room temperature. Add the substrate to the wells at a steady rate. The substrate will turn blue in wells with positive samples.
13. Add 100 µL of the Stopping Solution (0.36 N sulfuric acid) to each well (except for the water blank well) to stop the enzyme reaction. Be sure to add the acid to the wells in the same order and at the same rate as the working Substrate Solution was added to the wells. Blue Substrate will turn yellow and colorless substrate will remain colorless. Do not add Stopping Solution to the water blank well. Instead, add 200 µL of deionized water to the water blank well. Blank or zero the plate reader against the water blank well. Read the O.D. of each well at 450 nm against a 650 nm reference filter (if available). For best results, the O.D. values should be measured within 30 minutes after the addition of stopping solution.