

READS®**PROTEIN S ANTIGEN TEST KIT**For *In Vitro* Diagnostic Use

This package insert is for informational use only. When performing the test, please refer to the package insert provided with each kit.

INTENDED USE

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

SUMMARY AND EXPLANATION OF THE PROTEIN S TEST

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, down-regulating clot formation. In normal plasma approximately 40% of Protein S circulates as a 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 or oral anticoagulant therapy, liver disease, 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, ELISA allows automation with commonly available laboratory instrumentation. Measurement of plasma levels of both Total and Free Protein S are useful to determine the type of defect in patients with Protein S deficiency.

PRINCIPLE OF THE TEST

The Protein S Antigen assay is a sandwich ELISA. 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 Protein S to bind to the anti-human Protein S antibody on the microwell surface. The plates are washed to remove unbound proteins or other plasma molecules. Bound Protein S is quantitated using horseradish peroxidase (HRP) conjugated anti-human Protein S detection antibody. Following incubation, unbound conjugate is removed by washing. A chromogenic substrate of tetramethylbenzidine (TMB) and hydrogen peroxide (H₂O₂) is added to develop a colored reaction. The intensity of the color is measured in optical density (O.D.) units with a spectrophotometer at 450nm. Protein S relative percent concentrations in patient plasma are determined against a curve prepared from the reference plasma provided with the kit.

To measure Free Protein S, PEG is added to plasma samples prior to beginning the assay to precipitate the Protein S-C4b binding protein complex. The supernatant fraction containing Free Protein S may be tested along with the untreated plasma sample. Results obtained from diluted plasma samples not pretreated with polyethylene glycol (PEG) represent the Total Protein S concentration for that sample. Both Total (untreated) and Free (PEG-treated) Protein S concentrations are determined following the same assay procedure as described above using separate reference curves.

REAGENTS

Store at 2 - 8°C. Do Not Freeze.

Each READS Protein S Antigen 96-microwell Test Kit contains the following reagents:

- 12 x 8 anti-human Protein S antibody coated microwells
- 60mL Sample Diluent (blue-green solution); contains sodium azide.
- 3 vials x 0.5mL lyophilized Reference Plasma, with assay sheet.
- 12mL anti-human Protein S HRP Conjugate (red solution).
- 13mL Substrate (TMB and H₂O₂).
- 15mL Stopping Solution (0.36 N sulfuric acid).
- 30mL Wash Concentrate (33X PBS with 0.01% Tween 20). Note: turbidity may appear in wash concentrate which will not affect component performance and should disappear when working dilution is prepared.
- 2mL Free Protein S Reagent (PEG).

WARNINGS AND PRECAUTIONS**For *In Vitro* Diagnostic Use**

1. Human source material used to prepare the reference plasma included in this kit has been tested and shown to be negative for antibodies to HBsAg, HCV, and HIV-I & II by FDA required tests. However, all human blood derivatives, including patient samples, should be handled as potentially infectious material.
2. Do not pipette by mouth.
3. Do not smoke, eat, or drink in areas where specimens or kit reagents are handled.
4. Wear disposable gloves while handling kit reagents and wash hands thoroughly afterwards.
5. One component substrate can cause irritation to the eyes and skin. Absorption through the skin is possible. Use gloves when handling substrate and wash thoroughly after handling. Keep reagent away from ignition sources. Avoid contact with oxidizing agents.
6. The Sample Diluent contains sodium azide as a preservative. Sodium azide has been reported to form lead and copper azides when left in contact with these metals. These metal azides are explosive. Any solutions containing azide must be thoroughly flushed with copious amounts of water to prevent the build-up of explosive metal azides in the plumbing system.
7. Certain components are labeled with the following: Harmful if swallowed (R 22). Irritating to eyes and skin (R 36/38). Avoid contact with skin and eyes (S 24/25). In case of contact with eyes, flush affected areas with copious amounts of water and seek medical advice (S 26). Wear suitable protective clothing (S 36).

SPECIMEN COLLECTION AND PREPARATION

Plasma collected with either 3.2% or 3.8% sodium citrate as an anticoagulant should be used as the sample matrix. Blood should be collected by venipuncture, and the sample centrifuged immediately. Remove the plasma and store at 2 - 8°C until testing can be performed. If not tested within 8 hours of collection, the sample should be stored at -70°C and tested within 1 month.

INSTRUCTIONS FOR USE**Materials Provided**

READS Protein S Antigen Test Kit; see "Reagents," for a complete listing.

Materials Required but not Supplied

- Protein S Control Plasma (Total and/or Free). Reconstitute Control Plasma selected for use following manufacturer's instructions, and store as recommended.
- Reagent grade water (1L) to prepare PBS/Tween wash solution, to reconstitute Reference Plasma, and to zero or blank the plate reader during the final assay step.
- Graduated cylinders
- Precision pipettors capable of delivering between 5 and 1000 microliters, with appropriate tips

- Miscellaneous glassware appropriate for small volume handling
- Flask or bottle, 1 liter
- Wash bottles, preferably with the tip partially cut back to provide a wide stream, or an automated or semi-automated washing system
- Disposable gloves, powder-free recommended
- Plate reading spectrophotometer capable of reading absorbance at 450nm (with a 650nm reference if available)
- Multichannel pipettors capable of delivering to 8 wells simultaneously
- Microdilution tubes for patient sample preparation
- 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 storage (2 - 8°C) as soon as possible.
2. All dilutions of reference plasma, control plasma selected for use, and patient samples 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 200µL 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. Wash solution in the water blank well will not interfere with the procedure. An automated microtiter plate washing system can also be used.
5. **IMPORTANT:** Failure to adequately remove residual PBS/Tween 20 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 reference plasma dilutions, controls 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 - 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. Coated microwells, conjugate, and substrate are lot specific components that should not be used with different kit lots.

Reagent Preparation

1. Wash Solution - phosphate buffered saline (PBS)/Tween 20: Measure 30mL Wash Concentrate (33X PBS/Tween 20) and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1. Store unused PBS/Tween 20 solution at 2 - 8°C. Discard if solution shows signs of contamination.
2. Reconstitute Reference Plasma by adding 0.5mL reagent grade water. Swirl gently to mix. Allow to stand 10 minutes before use for complete dissolution. Stable for 8 hours when stored at 2 - 6°C. Reconstitute appropriate control plasma following manufacturer's instructions, and store as recommended.

Assay Procedure

1. Remove any microwell strips that will not be used from the frame and store them in the bag provided.
2. Assay each reference plasma dilution in duplicate for Total and Free Protein S. Duplicate determinations are also recommended for patient and control samples. One well should be run as a reagent blank; sample diluent without serum is added to the well as explained in step 8 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 200µL of reagent grade 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. **Pretreatment for Free Protein S determination:** all plasma samples to be tested for Free Protein S (Reference Plasma, controls, and patient samples) must be pretreated with polyethylene glycol (PEG) before setting up the Free Protein S assay. Do not dilute plasma samples before PEG pretreatment. Add 15µL of Free Protein S Reagent (PEG) to 85µL control or patient plasma. To yield sufficient supernatant to prepare the reference curve (95µL), add 45µL PEG to 255µL reference plasma. Vortex and place on ice for 30 minutes. Centrifuge for 10 minutes at 3,000 rpm. Use supernatant to prepare curve and sample dilutions as described in steps 5 and 6.
4. **Pre-dilute all plasmas for Total Protein S determination** (1:2 dilution in Sample Diluent) as follows:
Reference plasma: add 100µL reference plasma to 100µL Sample Diluent.
Control and patient samples: add 20µL plasma to 20µL Sample Diluent.
 Mix well. These pre-dilutions are utilized in preparing the working dilutions for Total Protein S in steps 5 and 6.
5. Prepare six reference dilutions as described in the table below. Separate reference curves are used for Total and Free Protein S assays. For **Total Protein S**, prepare one set of dilutions using pre-diluted reference plasma (step 4). For **Free Protein S**, prepare a second set of dilutions using the PEG-treated reference plasma (step 3).

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

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

6. Prepare working dilutions of control and patient samples, as follows:
Total Protein S: add 20µL pre-diluted control or patient plasma (1:2 dilution from step 4) to 500µL Sample Diluent.
Free Protein S: add 20µL PEG-treated control or patient plasma (from step 3) to 500µL Sample Diluent.
 (Note: these dilutions correspond to the 100% relative reference plasma dilution.)
7. Mix thoroughly, and add 100µL of the working dilutions (reference plasmas x 6, controls and patient samples) to the appropriate microwells for Total and Free Protein S determinations.
8. Add 100µL of Sample Diluent to the reagent blank well. Leave the water blank well empty.
9. 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.
10. Wash 4 times with working wash solution (PBS/Tween 20). Each well should be filled with wash solution per wash. Wash solution in the empty well intended to serve as a water blank 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.

- Add 100µL Conjugate (red) to each well (except the water blank well).
- Incubate 10 minutes at room temperature. After the incubation is complete, carefully invert the microwells and dump the conjugate solution.
- Wash 4 times with working wash solution (PBS/Tween 20) as in step 10. Wash solution in the water blank well does not interfere with the procedure. 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.
- Add 100µL 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. Blue color will develop in wells with positive samples.
- Add 100µL 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 Stopping Solution to the wells in the same order and at the same rate as the Substrate Solution was added. Blue Substrate will turn yellow and colorless substrate will remain colorless. Do not add Stopping Solution to the water blank well. Instead, add 200µL of reagent grade 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 450nm, against a 650nm reference filter (if available). For best results, the O.D. values should be measured within 30 minutes after the addition of Stopping Solution.

Results

- Calculate the mean O.D. for the duplicates of the reference plasma dilutions, controls, and patient samples.
- 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 semi-log or point-to-point graph is recommended, although a log-log may also be used. Prepare separate curves for Total and Free Protein S determinations.
- Using the mean O.D., determine the control and patient relative values from the graph, or, alternatively, use linear regression to calculate from the reference curve.
- To calculate Total or Free Protein S Antigen levels in % of normal, multiply the control and patient relative values obtained from the appropriate reference curve by the corresponding assigned value for the Reference Plasma (see vial label).

For example:*

Patient relative value (from the reference curve): 40
 Reference plasma assigned value (from vial label): 105% of normal
 Actual patient Protein S Antigen value (as % of normal): $40 \times 1.05 = 42\%$

* Example applies to both Total and Free Protein S calculations.

- Ensure that all quality control parameters have been met (see Quality Control) before reporting test results.

QUALITY CONTROL

- The mean O.D. of the reagent blank should be less than 0.1 when the spectrophotometer has been blanked against the water well. Readings greater than 0.1 may indicate possible reagent contamination or inadequate plate washing.
- Individual O.D.s for the duplicates of the controls or patient samples should be within 20% of the mean O.D. for absorbance readings greater than 0.200.
- Protein S Antigen values (Total and Free) obtained for the controls should be within manufacturer's assigned ELISA ranges. Occasional small deviations outside these ranges may be acceptable.
- Each laboratory should periodically determine their own reference range for this assay.

EXPECTED VALUES¹¹

Normal Range: Total and Free Protein S values are expressed in relative percent (%) as compared to pooled normal plasma. The normal range for Total Protein S for this assay is 60 - 150%. The normal range for Free Protein S for this assay is 50 - 130%. These ranges are consistent with normal ranges published in the literature and reported by other 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 CHARACTERISTICS¹¹

Detection range:

The detection range for REAADS Protein S Antigen assay (Total and Free) is 5 - 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 O.D. range of the reference curve should be retested at an appropriate dilution.

Precision:

Intra-assay precision:

To determine variability within a plate, three plasma samples with known Protein S levels (one high, one medium, and one 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 CV of 10.1% for Total Protein S, and 6.6% for Free Protein S across three lots. In addition, ninety-nine (99) patient samples with Total and Free Protein S levels spanning the entire detection range of the assay were tested in duplicate across 3 lots to demonstrate the precision end users may expect when performing the assay according to package insert instructions. As shown in the table, the overall mean CV for duplicates was 7.8% for Total Protein S and 4.7% for Free Protein S.

Inter-assay precision:

Six (6) commercially prepared, assayed plasma samples with values ranging from 57 - 159% were tested in duplicate on three lots to determine assay precision between lots. The mean inter-assay CV was 11% for Total Protein S, and 10.5% for Free Protein S, as seen in the table:

Intra-assay precision (variability within a plate)	Total Protein S range (% of normal)	Total Protein S CV range (3 pilot lots)	Free Protein S range (% of normal)	Free Protein S CV range (3 pilot lots)
Replicates (x16):	105 - 107%	10.3 - 11.1%	76 - 86%	4.4 - 7.9%
	53 - 83%	8.1 - 12.2%	38 - 80%	3.3 - 8.3%
	44 - 45%	7.1 - 10.4%	34 - 40%	3.4 - 10.4%
Overall mean CV:		10.1%		6.6%
Duplicates:	entire range	7.8%	entire range	4.7%
Overall mean CV:		7.8%		4.7%
Inter-assay precision (variability between lots)		CV range (2 lots)		CV range (2 lots)
Duplicates:	46 - 95%	2.4 - 19.5%	37 - 83%	1.3 - 22.6%
Overall mean CV:		11.0%		10.5%

Linearity:

Serial two-fold dilutions of Protein S reference plasma samples tested on 3 lots of REAADS Protein S Antigen assay demonstrated curves with a mean coefficient of determination (r-squared) of 0.985 for Total Protein S and 0.992 for Free Protein S.

Accuracy:

Accuracy was determined by testing mixtures of Protein S reference plasma with predetermined values on REAADS Protein S Antigen assay and calculating the recovery of theoretical values. The overall mean percent recovery across 3 lots was 101% for Total Protein S and 98% for Free Protein S, with an average variation of 7.1% and 5.0% respectively.

LIMITATIONS OF THE TEST

The 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 (levels 20-35% lower than adults), liver disease, diabetes mellitus, pregnancy, oral contraceptive 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 can be inadvertently depleted or degraded of Protein S by improper collection or laboratory processing.

As with any assay employing antibodies from an animal source (e.g. mouse, rabbit, goat, etc.) to capture a target molecule, the possibility exists for interference in the serum or plasma of patients who have been exposed to preparations containing animal antibodies for diagnosis or therapy. Falsely elevated or depressed values may be seen in these patients.

WARRANTY

This product is warranted to perform as described in this package insert. Corgenix, Inc. disclaims any implied warranty of merchantability or fitness for a particular use, and in no event shall Corgenix, Inc. be liable for consequential damage.

For Technical or Customer Service in the United States, phone 1-800-729-5661. Outside the United States, phone +303-457-4345, fax +303-457-4519, e-mail: technicalsupport@corgenix.com, or contact a Corgenix authorized distributor.