

**REAADS®****MONOCLONAL FREE PROTEIN S ANTIGEN TEST KIT**For *In Vitro* Diagnostic Use**This document is for informational use only; please refer to the package insert provided with each kit.****INTENDED USE**

An enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of 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.<sup>1,2</sup> 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.<sup>3</sup> Only Free Protein S is functionally active and able to bind to activated Protein C, while the complexed form of Protein S is not.<sup>4</sup>

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.<sup>5,6</sup> In young patients (<35 years) with a history of thrombosis, the prevalence may be as high as 15 to 18%.<sup>7</sup> 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.<sup>8</sup> 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).<sup>9,10</sup> 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.<sup>11,12</sup> This assay utilizes a monoclonal antibody specific for Free Protein S in an ELISA format to measure Free Protein S directly, without PEG precipitation.<sup>13,14</sup>

**PRINCIPLE OF THE TEST**

The Monoclonal Free Protein S Antigen assay is a sandwich ELISA. A capture monoclonal antibody specific for human Free 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 Free Protein S monoclonal antibody on the microwell surface. The plates are washed to remove unbound proteins or other plasma molecules. Bound Free Protein S is quantitated using horseradish peroxidase (HRP) conjugated polyclonal anti-human Protein S detection antibody. Following incubation, unbound conjugate 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 in optical density (O.D.) units with a spectrophotometer at 450 nm. Free Protein S relative percent concentrations in patient plasma are determined against a curve prepared from the 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**

Store at 2 - 8°C. Do Not Freeze.

Each REAADS Monoclonal Free Protein S Antigen (96-microwell) Test Kit contains the following reagents:

- 12 x 8 Monoclonal antibody to human Free Protein S coated microwells.
- 60 mL Sample Diluent (blue-green solution); contains sodium azide.
- 3 x 0.5 mL Lyophilized Reference Plasma, with assay sheet.
- 12 mL Anti-human Protein S HRP Conjugate (red solution).
- 13 mL Substrate (TMB and  $H_2O_2$ ).
- 15 mL Stopping Solution (0.36 N sulfuric acid).
- 30 mL 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.

**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. 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.
6. 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.
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. Avoid hemolysis. Remove the plasma and store at 2 - 8°C until testing can be performed. If not tested within eight hours of collection, the sample should be stored at -70°C and tested within one month.

**INSTRUCTIONS FOR USE****Materials Provided**

REAADS Monoclonal Free Protein S Antigen Test Kit; see "Reagents," for a complete listing.

**Materials Required but not Supplied**

- Free Protein S Control Plasma. Reconstitute Control Plasma selected for use following manufacturer's instructions, and store as recommended.
- Reagent grade water (1 L) 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 450 nm (with a 650 nm 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 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. 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 [33X phosphate buffered saline (PBS)/Tween 20]: Measure 30 mL Wash Solution and dilute to 1 liter with reagent grade water. The pH of the final solution should be 7.35 ± 0.1. Store unused Wash Solution at 2 - 8°C. Discard if solution shows signs of contamination.
2. Reconstitute Reference Plasma by adding 0.5 mL 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 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 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 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. Prepare six **reference plasma** dilutions as described in the table 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 is 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 working dilutions (reference plasmas x 6, controls, and patient samples) 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 wash solution (PBS/Tween 20). Each well should be filled with wash solution per wash. Wash solution in the empty well is intended to serve as a water blank and 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 Conjugate (red) to each well (except 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 wash solution (PBS/Tween 20) as in step 8. 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.
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. Blue color will develop in wells with positive samples.
13. Add 100 µL 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 200 µL 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 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.

#### Results

1. Calculate the mean O.D. 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.

- 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 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 Free Protein S Antigen value (as % of normal):  $40 \times 1.05 = 42\%$

- 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 0.100 when the spectrophotometer has been blanked against the water well. Readings greater than 0.100 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.
- Free Protein S Antigen values 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 VALUES<sup>15</sup>**

Normal Range: Free Protein S values are expressed in relative percent (%) as compared to pooled normal plasma. The normal range for Free Protein S for this assay is 50 - 150%. These ranges are consistent with normal ranges published in the literature and reported by other commercially available assays.<sup>6,10</sup> Samples with values above the range of the reference curve may need to be diluted and retested for accurate results.

**PERFORMANCE CHARACTERISTICS<sup>15</sup>**

Detection range:

The detection range for REAADS 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 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 Free 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 table below, 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 three 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 intra-assay CV for duplicates was 4.7% for Free Protein S.

Inter-assay precision:

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 (x16):	109-118%	2.2-6.1%
	59-65%	3.8-9.4%
	44-47%	3.9-10.4%
Overall mean CV:		6.0%
Duplicates: Overall mean CV:	entire range	4.7%
<hr/>		
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 REAADS Monoclonal Free Protein S Antigen Test Kit.

Accuracy:

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

**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 (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.<sup>5-10</sup>

The reactivity of the monoclonal capture antibody used in this assay has been evaluated and confirmed to be specific for human Free Protein S. The effect of hemolysis, icterus or lipemia on the performance of the test has not been fully investigated, but may interfere with assay results and should be considered in the interpretation of patient results.

Plasma samples can be inadvertently depleted or degraded of Free 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.

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