

INTENDED USE

COALIZA® Protein S - Free is intended for the quantitative determination of free protein S antigen in human citrated plasma by enzyme immunoassay.

SUMMARY AND PRINCIPLE

Protein S is a vitamin K-dependent, potent natural anticoagulant plasma glycoprotein. Protein S has a molecular weight of approximately 70 kDa and circulates in plasma at a concentration of 25 µg/mL with a half-life of approximately two days.^{1,4} About 40% of Protein S in plasma is in a free form, whereas 60% is complexed with C4b-binding protein (C4BP). Only the free Protein S (FPS) functions as a cofactor to APC in the APC-dependent degradation of FVa and FVIIIa.^{5,6} Protein S deficiency is associated with an increased risk of thrombosis.⁷⁻¹¹ The Coaliza Protein S - Free kit method is based on a procedure described by Dahlbäck and colleagues.¹² The microplate wells are pre-coated with C4BP, which has a very high affinity for binding free Protein S (FPS) antigen in plasma. A monoclonal antibody (HPS 54) conjugated with the enzyme horseradish peroxidase (HRP) is added together with the plasma sample. After the sample and conjugate incubation, unbound material is washed away and bound protein S, in complex with C4BP is detected with the addition of a substrate-chromogen. The amount of color in the wells, when expressed in a double logarithmic scale, is directly proportional to the amount of free Protein S antigen in the plasma sample.

KIT COMPONENTS

- 1. Microtitre Plate** 12 x 8
Wells coated with human purified C4BP.
- 2. Conjugate Concentrate** 1 x 0.3 mL
A monoclonal antibody anti-Protein S (HPS 54) conjugated with peroxidase, containing preservatives and protein stabilisers.
- 3. Diluent (Conjugate and Sample)** 2 x 60 mL
Tris buffer containing additives and preservatives.
- 4. Wash Buffer** 2 x 50 mL
Phosphate buffer (10x concentrate) containing Tween 20 (1%) and 2-Chloroacetamide.
- 5. Substrate Buffer** 2 x 14 mL
Citrate-acetate buffer containing hydrogen peroxide.
- 6. Chromogen TMB** 1 x 1.5 mL
3,3',5,5'-Tetramethylbenzidine (TMB)
- 7. Calibration Plasma** 2 x 1 mL (lyophilised)
Human plasma containing a known concentration (IU/mL) of Free Protein S as specified in the enclosed data sheet.
The concentration of the Free Protein S in the calibration plasma has been referenced against the 1st International Standard 93/950.¹³
- 8. Normal Control Plasma** 1 x 1 mL (lyophilised)
Human plasma containing a known concentration range (IU/mL) of Free Protein S as specified in the enclosed data sheet.
- 9. Stopping Solution** 1 x 12 mL
Sulfuric acid (1N H₂SO₄)
- 10. Adhesive Plate Covers** x 6
Adhesive seals to cover the microplate strips during incubations.
- 11. Resealable Bag** x 1
For storage of unused microplate strips.

CAUTION: Each donor unit used in the preparation of the control, calibrator and the human material contained in the microwell has been tested for the presence of Hepatitis B surface antigen and antibodies to HIV 1&2 and Hepatitis C and found to be negative. Since no test can completely rule out of the presence of blood borne diseases, the handling and disposal of human source reagents from this product should be made with care.¹⁴ Avoid contact with skin and eyes (S24/25). In case of contact with eyes, rinse immediately with plenty of water and seek medical advice (S26). Do not empty into drains (S29). Wear suitable protective clothing (S36). In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible) (S45).

This product is for *in vitro* diagnostic use.

REAGENT STORAGE AND STABILITY

All kit reagents are stable until the expiration dates shown on the labels when stored at 2-8°C.

- Microtitre Plate: To avoid condensation in the wells, the bag containing the microtitre plate should be brought to room temperature before it is opened. Store unused strips at 2-8°C in the resealable bag with the silica gel sachet provided in the kit.
- Wash Buffer: diluted wash buffer is stable for 14 days when stored at 2-8°C.
- Conjugate: diluted conjugate is stable for 14 days when stored at 2-8°C.
- Chromogen: store the chromogen protected from light. As the Substrate-Chromogen TMB solution is not stable once prepared, instructions for use should be closely followed.
- Calibrator and Normal Control plasmas: after reconstitution, aliquots can be stored for 4 months at -20°C. Do not refreeze. Thoroughly mix all dilutions of the plasma.

MATERIAL REQUIRED BUT NOT PROVIDED

- Sterile distilled or deionized water or NCCLS Type II water¹⁵
- Multichannel pipettes capable of delivering 100 µL and 200 µL
- Micropipettes for pipetting volumes of 10 µL, 500 µL and 1000 µL
- Glass or plastic graduated cylinders for preparation of wash buffer
- Manual, semi-automated or automated wash system
- Microplate reader at 450 nm (preferably with a 620 nm / 630 nm reference filter)
- Microtubes/containers for preparation of the calibrators, controls, sample dilutions, conjugate and substrate working solutions
- Vortex mixer

SPECIMEN COLLECTION

Nine parts of freshly drawn venous blood is collected into one part trisodium citrate. Immediately after collection, centrifuge at 2000 g for at least 10 minutes. Refer to NCCLS Document H21-A3 for further instructions on specimen collection, handling and storage.¹⁶

Specimens containing visible particulate matter should be clarified by centrifugation before testing. DO NOT USE HEAT INACTIVATED PLASMA SAMPLES.

QUALITY CONTROL

The normal control contained within the kit is intended to ensure that all the steps and elements of the assay are working. The free protein S antigen values obtained for the control should be within the limits specified in the enclosed data sheet.

If a second point for quality control is necessary, dilute 1:2 (1+1) the normal control using the procedure described under "Reagent Preparation". The free protein S antigen values obtained should be within half of the limits specified in the enclosed data sheet of the normal control.

Abnormal and normal levels of free Protein S controls are recommended for a complete quality control program.¹⁷ Each laboratory should establish its own mean and standard deviation and should establish a quality control program to monitor laboratory testing. Controls should be analyzed in each plate. Refer to Westgard et al for identification and resolution of out-control situations.¹⁸

TRACEABILITY OF CALIBRATORS AND CONTROL MATERIALS

The Calibration plasma and Control values are traceable to the International Standard for Protein S supplied by the NIBSC (National Institute for Biological Standards and Controls) according to the WHO recommendations. This International standard is used during the assignment of reference values to a House Standard Calibration plasma which is then used to assign values to all the lots of Calibration plasma and Control.

PROCEDURE (See SUMMARY ASSAY PROCEDURE on the last page)

Important Notes

- Before running the assay, allow all reagents to reach room temperature (18-25°C).
- Chromogen TMB contains DMSO, which has a melting point of 18°C. Bring it to a temperature of 20-25°C until completely liquefied. Mix thoroughly before use.

NOTE: A yellowish color for the Chromogen TMB is normal.

- Gently mix all liquids before use.
- All dilutions of calibration plasma, normal control plasma and samples must be made just prior to use in the assay.
- To perform the test as efficiently as possible, use a microtube for the dilutions of calibrator, control and samples.
- Use a multichannel pipette capable of delivering to 8 wells simultaneously to obtain uniform incubation and reaction time for all wells.
- Add calibrators and samples within 5 minutes.
- Commence incubation immediately after the first addition of calibrator/control(s)/sample or reagent.
- Do not use kit components beyond the expiration date.
- Do not use kit components from different kit lot numbers.

Reagent Preparation

1. Wash Buffer Dilution (stable for 14 days at 2-8°C)
Dilute the concentrated Wash Buffer 1:10 (1+9) with sterile distilled or deionised water or NCCLS Type II water¹⁵. If the entire plate is used, mix 50 mL of concentrated Wash Buffer with 450 mL of water. If the plate is only partially used, prepare a proportional volume of solution.
2. Conjugate Dilution (stable for 14 days at 2-8°C)
Dilute the Conjugate Concentrate 1:51 (1+50) with the Diluent according to Table 1 below. Mix thoroughly.

Table 1: Conjugate Dilution

Number of Strips Used	1	2	4	6	8	10	12
Diluent-Conjugate and Sample (mL)	1	2	4	6	8	10	12
Conjugate Concentrate (µL)	20	40	80	120	160	200	240

3. Calibration and Normal Control Plasmas Reconstitution (stable for 4 months at -20°C)
Reconstitute each vial of Calibration and Normal Control Plasmas with 1 mL of sterile distilled or deionised water or NCCLS Type II water¹⁵. Let the plasma stand for 30 minutes. Swirl gently to mix.
4. Calibration Plasma Dilutions (use immediately; stable 30 minutes at 18-25°C)
Prepare four proportional Calibration Plasma Dilutions according to Table 2 below starting from the reconstituted Calibration Plasma. The Cal 1 (highest calibrator) will correspond to the value stated on the enclosed data sheet (approximately 1.0 IU/mL).

Table 2: Calibration Plasma Dilutions

Cal1	1.0 IU/mL free PS	10 µL of Cal Plasma	1000 µL of Diluent
Cal2	0.5 IU/mL free PS	500 µL of Cal 1	500 µL of Diluent
Cal3	0.25 IU/mL free PS	500 µL of Cal 2	500 µL of Diluent
Cal4	0.125 IU/mL free PS	500 µL of Cal 3	500 µL of Diluent

NOTE: The exact value for each calibrator dilution (Cal1, Cal2, Cal3, Cal4) is derived by dividing the value stated on the enclosed data sheet by 1, 2, 4 and 8 respectively.

5. Normal Control Dilutions (use immediately; stable 30 minutes at 18-25°C)
Dilute the Normal Control Plasma 1:101 by adding 10 µL of Control Plasma to 1 mL of Diluent. Mix thoroughly. To obtain a second point for quality control make a dilution 1:2 (1+1) dilution using 500 µL of diluted Normal Control plus 500 µL of Diluent. Swirl gently to mix.
6. Substrate-Chromogen TMB Dilution (prepare during last 5-10 minutes of conjugate/sample incubation)

- **NOTE:** The final solution should be colorless. Discard if it turns blue. Prepare the Substrate-Chromogen TMB Dilution just prior to its use as follows:
- If the entire plate is used combine the two 14 mL bottles of substrate buffer and add 560 µL of Chromogen TMB. Mix well.
- If the plate is only partially used, follow the instructions as described in Table 3 below.

Table 3: Substrate-Chromogen TMB Dilution

Number of Strips Used	1	2	4	6	8	10	12
Substrate (mL)	2	4	8	12	16	20	24
Chromogen TMB (µL)	40	80	160	240	320	400	480

Assay Procedure

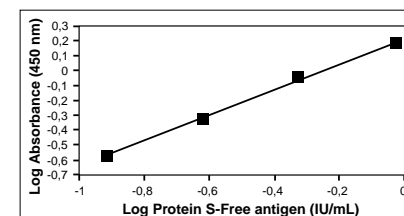
1. Dilute the samples 1:101 (1+100) by adding 10 µL of plasma to 1 mL of Diluent. Mix thoroughly.
2. Use only the number of strips required for the test. Remove the remaining strips from the frame holder and store them in the plastic bag with the silica gel sachet.
3. Reserve 6 wells for the blank (A1), 4 levels of Calibration Plasma Dilutions and Normal Control Plasma.

- NOTE:** If a second point for quality control is used, reserve 7 wells.
4. Add 100 µL of the Conjugate Dilution to all wells except the blank (A1).
 5. Leaving the blank well (A1) empty, transfer 100 µL of the different freshly prepared Calibrator Plasma Dilutions, the control(s) and samples to the corresponding wells.
 6. Cover the plate with an adhesive seal, mix with a few gentle taps and incubate for 45 (± 5) minutes at room temperature (18-25°C).
 7. During the last 5-10 minutes of the conjugate and sample incubation, prepare the Substrate-Chromogen TMB Dilution as described under the Reagent Preparation section above.
 8. Following the incubation, remove and discard the adhesive plate cover.
 9. Aspirate the content of the wells and then fill them completely (approximately 300 µL) with the diluted Washing Buffer.

10. Repeat the process of aspiration and wash 3 more times.
11. After the last wash, blot the microplate on absorbent paper to remove any excess liquid from (in and around) the wells.
12. Add 200 µL of freshly prepared Substrate-Chromogen TMB Dilution to each well, including the blank wells.
13. Incubate the uncovered plate at room temperature (18-25°C) for 10 minutes.
14. Stop the reaction by adding 100 µL of Stopping Solution in the same sequence and time intervals as the Substrate-Chromogen TMB Dilution additions.
15. Using a microplate reader, read the plate at 450 nm. If the reader is capable of dual wavelength reading, the reference wavelength should be 620 nm / 630 nm. Read the absorbance of each well.

Results

- Plot the calibrator values of free protein S antigen on the abscissa (x-axis) using Log-Log scale and the corresponding absorbance on the ordinate (y-axis). Draw a line of best fit.
- Determine the concentration of free protein S antigen (IU/mL) of samples and control from the graph or, if a computer system or manual calculator is used, calculate them through Log-Log regression.
- An example of a typical standard curve at 23°C is shown below.



Important Notes:

- Results > 1.0 IU/mL or < 0.125 IU/mL can be extrapolated but not to exceed the linearity claims of the assay.
- If required, samples with results above the linear range can be assayed again at a higher dilution. However, the value must be multiplied by the dilution factor to obtain the final result.
- 1.0 IU/mL of FPS is equivalent to 100% FPS.

Expected Values

The normal range for Coaliza Protein S-Free was established from analysis of 205 samples from healthy individuals (103 males/102 females).

Men: from 0.68 to 1.29 IU/mL 68 to 129% FPS
 Women: from 0.54 to 1.08 IU/mL 54 to 108% FPS

Due to the many variables, which may affect results, each laboratory should establish its own normal range.

During pregnancy and oral anticoagulant therapy (OAT), the free protein S antigen levels drop significantly.^{19,20}

Performance Characteristics

Limitations

Optimal assay performance requires strict adherence to the procedures described. Deviation from the procedure may lead to aberrant results. The results obtained with Coaliza Protein S-Free must be evaluated by a physician in light of the clinical symptoms shown by the patient.

Precision

The intra-assay and inter-assay variability of the kit was evaluated by testing 2 samples (Normal and Abnormal Controls) run in replicates of six over five days using two different operators (n=60 per control).

	Intra-assay %CV	Inter-assay %CV
Normal Control		
Mean 0.89 IU/mL (89% FPS)	2.1%	3.7%
Abnormal Control		
Mean 0.29 IU/mL (29% FPS)	2.7%	3.7%

Correlation

Correlation studies were performed on three different lots of Coaliza Protein S-Free versus commercially available free protein S ELISA using a total of 106 plasma samples with free protein S antigen level ranging from 0.097 to 1.48 IU/mL (9.7 to 148 % FPS). The obtained slopes and correlation coefficients are shown below:

Coaliza Protein S Free vs. ELISA Slope	Lot 1	Lot 2	Lot 3
r	0.965	0.957	0.887
r	0.972	0.970	0.967

In addition, Coaliza Protein S-Free was compared with the same commercially available free Protein S ELISA in a clinical study on samples from 97 patients (52 undergoing OAT, 32 on no OAT and 15 with protein S deficiency). The correlation coefficient (r) was 0.961. The precision and correlation results were obtained using specific lots of reagents and controls.

Linearity

Linearity studies were performed using a series of plasma dilutions with known concentrations of free protein S. The recovery range for free protein S with a deviation of < 10% from the expected value is 0.06 to 1.20 IU/mL (6 to 120%FPS).

Detection Limit

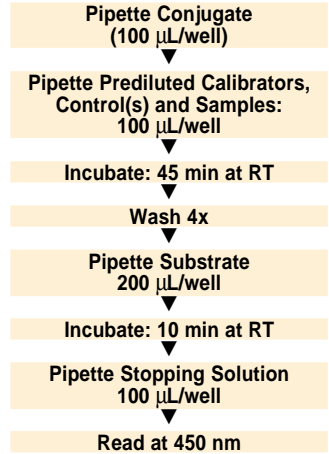
The detection limit of this assay is 0.025 IU/mL (2.5% FPS) when performed in accordance with insert instructions.

Interferences

Free protein S results are not affected by:

- Rheumatoid Factor at concentrations of ≤ 400 IU/mL
- Fibrinogen at concentrations of ≤ 10 mg/mL
- Protein C at concentrations of ≤ 8 µg/mL
- Triglycerides at concentrations of ≤ 10 mg/mL
- Bilirubin at concentrations of ≤ 0.2 mg/mL
- Unfractionated Heparin at concentrations of ≤ 1 IU/mL
- LMW Heparin at concentrations of ≤ 1 IU/mL
- Hemoglobin at concentrations of ≤ 2 mg/mL

Summary Assay Procedure



Troubleshooting Guide

Problem	Possible Causes	Solution	
1. Controls out of range	1a. Incorrect temperature, timing or pipetting.	Check procedure. Repeat assay.	
	1b. Improper preparation of reagents, error of dilution, reagents not well mixed.	Check procedure. Repeat assay.	
	1c. Incorrect reading filter.	Check that the wavelength of the filter used is 450 nm. If no reference wavelength is used, absorbances increase by approximately 0.050.	
	1d. Interference in the optical pathway.	Check the reader. Clean or dry the bottom of microplate. Check for air bubbles. Repeat reading.	
	1e. Used components from different lots.	Do not use components from different lots as they are adjusted for each batch released.	
	1f. Expired reagents.	Check the kit expiry date. Use current kit.	
	2. No color or only a light color in microplate wells	2a. One or more reagents not added or added in wrong sequence.	Check procedure. Repeat assay.
		2b. Inactive conjugate: improper storage.	Check for contamination. Recheck procedure. Repeat assay.
		2c. Inactive microplate: Improper storage.	Always use components from the resealable plastic bag, very well closed, with the desiccant bag inside. Repeat assay.
		2d. Inactive substrate: Improper storage or dilution, cross-contamination with the stop solution.	Always use freshly prepared mixture of substrate buffer and TMB. Recheck procedure. Repeat assay.
		3. Too much color in microplate wells	3a. Contaminated, oxidised or improperly prepared substrate.
3b. Contaminated or improperly prepared reagents.			Check for contamination: Turbid aspect. Check dilutions. Repeat assay.
3c. Contaminated wash buffer (1x).	Check the quality of distilled or deionised water used for dilution. Repeat assay.		
4. Poor reproducibility	3d. Insufficient washing or washing not consistent: Filling volume and/or aspiration insufficient or not uniform. Insufficient number of wash cycles, contaminated device.	Check the washing device. Fill wells with washing solution close to the top, aspirate completely. Increase the number of wash cycles. After washing, blot the inverted microplate on tissue paper.	
	3e. Improper dilution of samples.	Check procedure. Repeat assay.	
	4a. Washing problems.	See 3c, 3d, 3e.	
	4b. Uncalibrated pipettes or tips not well fitted. Improper pipetting.	Use only calibrated pipettes, with well fitting tips and pipette carefully, without bubbles and splashing. Repeat assay.	
	4c. Reagents and plasma not at room temperature or not well mixed before using.	Equilibrate reagents to room temperature and mix thoroughly before using.	
	4d. Air currents over the microplate during incubations.	Keep the microplate protected from air currents.	
	4e. Too long time for addition of samples and/or reagents. Inconsistency in time intervals. Air bubbles.	Develop consistent and uniform technique.	
	4f. Interference in the optical pathway.	See 1d.	

**Bibliography / Literatur / Bibliografía / Bibliographie /
Bibliografia / Bibliografia / Litteratur / Litteraturförteckning /
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**Symbols used / Verwendete Symbole / Símbolos utilizados /
Symboles utilisés / Simboli impiegati / Simbolos utilizados /
Anvendte symboler / Använda Symboler /
Χρησιμοποιηθέντα σύμβολα**

IVD

In Vitro Diagnostic Medical Device
In Vitro Diagnostikum
Dispositif médical de diagnostic in vitro
Producto sanitario para diagnóstico in vitro
Dispositivo médico-diagnóstico in vitro
Dispositivo médico para utilização em diagnóstico *in vitro*
Medicinsk udstyr til in vitro-diagnostik
In vitro diagnostisk medicinsk produkt
Προϊόν για διαγνωστική χρήση *In vitro*



Use by
Verwendbar bis
Fecha de caducidad
Utiliser jusque
Utilizzare entro
Data limite de utilização
Anvendelsesdato
Användning
Χρήση έως

LOT

Batch code
Chargenbezeichnung
Codigo de lote
Code du lotto
Codice del lotto
Número de lote
Batchkoden
Tillverkningskod
Αρ. Παρτίδας



Temperature limitation
Zulässiger Temperaturebereich
Limite de temperatura
Limites de température
Limite di temperatura
Limite de temperatura
Temperaturbegrensning
Temperatur gräns
Θερμοκρασίας Περιορισμοί



Biological risks
Biologisches Risiko
Riesgo biológico
Risque biologique
Rischio biologico
Risco biológico
Biologisk fare
Biologiska risker
Βιολογικοί κίνδυνοι



Consult instructions for use
Gebrauchsanweisung beachten
Consulte las instrucciones de uso
Consultez les instructions d'utilisation
Consultare le istruzioni per l'uso
Consultar as instruções de utilização
Se instruktion for brug
Ta del av instruktionen före användning
Συμβουλευτείτε τις οδηγίες χρήσης



Manufacturer
Hersteller
Fabricante
Fabricant
Fabricante
Fabricado por
Producent
Tillverkare
Κατασκευαστής

EC REP

Authorised Representative in the European Community
Bevollmächtigter in der Europäischen Gemeinschaft
Representante autorizado en la Comunidad Europea
Mandatataire dans la Communauté européenne
Mandatario nella Comunità Europea
Representante autorizado
Repræsentant i det Europæiske Fællesskab
Auktoriserad representant
Εξουσιοδοτημένος αντιπρόσωπος