Cat. No. 5291

Protein C ELISA Kit

INTENDED USE

The Protein C Kit is an enzyme-linked immunosorbent assay (ELISA) for the quantitative determination of Protein C Antigen in citrated human plasma.

SUMMARY

Protein C is a vitamin K-dependent protein synthesized primarily by hepatocytes in the liver and plays an important physiologic role in the Protein C Anticoagulant System.[1, 2] Protein C through complex interactions with thrombin from blood clots, endothelial cells, and other factors of the coagulation cascade, contribute to the maintenance of normal hemostatic mechanisms by down-regulating clot formation and by promoting fibrinolysis. The Protein C Anticoagulant System is activated by the binding of thrombin to thrombomodulin, a transmembrane protein receptor on endothelial cells.[3] The thrombin-thrombomodulin binding on endothelial cell membranes activates circulating Protein C. Activated Protein C binds to Protein S on the membrane of endothelial cells or platelets. In this Protein C- Protein S complex, activated Protein C is now capable of inactivating the coagulation cascade factors Va and VIIIA, down-regulating clot formation. Activated Protein C also inhibits the function of tissue plasminogen activator (TPA) by dissociating this molecule from its inhibitor, plasminogen activator inhibitor-1 (PAI-1), thereby facilitating clot dissolution or fibrinolysis.[1-3] Protein C deficiency, either congenital or acquired, may lead to serious thrombotic events such as thrombophlebitis, deep vein thrombosis, or pulmonary embolism.[4] Patients with a congenital heterozygous deficiency may present with venous thrombosis (purpura fulminans) during the neonatal period.[5] The prevalence of Protein C deficiency in the general population has been estimated at 1 in 300. In younger patients (<40-45 years) with recurrent venous thrombosis, the frequency of Protein C deficiencies may be as high as 10-15%.[6-7] Acquired Protein C deficiency may be seen in liver diseases, extensive thrombotic episodes, surgery, oral anticoagulation, antiphospholipid syndrome, etc. A decreased Protein C activity in plasma may be the result of low concentrations and function (type I) or only low function (type II).[6]

The laboratory diagnosis of Protein C deficiency may require both quantitative and qualitative (functional) determinations. Quantitative determinations of Protein C Antigen are based on immunologic procedures such as radial immunodiffusion in gel, Laurell Rocket immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA).[6, 8] 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.

PRINCIPLE

The Protein C Antigen assay is a sandwich ELISA. The capture antibody specific for human Protein C is coated to 96-microwell polystyrene plates. Diluted patient plasma is incubated in the wells, allowing any available Protein C to bind to the anti-human Protein C antibody on the microwell surface. The plates are washed to remove any unbound proteins or other plasma molecules. Bound Protein C is quantitated using a horseradish peroxidase (HRP) conjugated anti-human Protein C detection antibody. Any unbound conjugated anti-human Protein C is washed away after an incubation period. 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 (O.D.) units. Protein C Antigen relative percent concentration in patient plasma is determined against a curve prepared from a reference plasma.

REAGENTS

WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY.

1. **Protein C Antigen Microwells**
   Ingredients: 96 stabilized antibody coated microwells (12 strips of breakaway wells), with frame holder. Wells are coated with anti-human Protein C 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 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 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 C Conjugate Solution**
   Ingredients: The blue solution contains antibodies, specific for Protein C 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: Discard if product shows signs of microbial growth.

5. **Substrate**
Ingredients: 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: Substrate should be clear and almost colorless.
6. **Stopping Solution**
Ingredients: The solution is 0.36 N Sulfuric Acid.
WARNING: DO NOT INGEST, IRRITANT. 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 to 1 liter with deionized water. The pH of the final solution should be 7.4 ± 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. 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 C:Ag Microwells (96)
ELISA Reference Plasma (3 x 0.5 mL)
Protein C 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 1 (S.A.C. 1) - 5301
Specialty Assayed Control 2 (S.A.C. 2) - 5302
Deionized water
Graduated cylinders
Pipettors (5 and 1000 µL)
Plastic squeeze bottle
Plate reading spectrophotometer capable of reading absorbance at 450 nm
Multichannel pipettors capable of delivering to 8 wells

Procedural Notes

1. Bring plasma samples and kit reagents to room temperature (15-30°C) and mix well before using, avoid foaming. Return all unused samples and reagents to refrigerated 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 deionized 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 calibration 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 (15 to 30°C) may contribute to inaccurate results.
11. Avoid microbial and cross-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 plastic pouch.
2. Assay each reference plasma dilution in duplicate. 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 a 1:26 dilution of patient sample and control plasma in Sample Diluent (blue-green solution); e.g. 20 µL sample added to 500 µL Sample Diluent = 1:26 dilution. Mix thoroughly.
5. Add 100 µL of the dilutions (reference plasmas x 6, patient samples and controls) to the appropriate microwells.
6. Add 100 µL of Sample Diluent to the reagent blank well. Place nothing in the well intended for the water blank.
7. Incubate 40 minutes at room temperature. After the incubation is complete, carefully invert the microwells and decant the sample fluid. Take care to prevent sample from one microwell to flow into another.
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 C Conjugate Solution (blue) to each well (except for the water blank well).
10. Incubate for 10 minutes at room temperature. After the incubation is complete, carefully invert the microwells and decant the Conjugate Solution.
11. Wash 4 times with working PBS solution as in step 8. PBS solution in the water blank well does not interfere with the procedure. Use a snapping motion to drain the liquid, and blot on absorbent towels 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. Substrate in wells incubated with positive samples will turn blue.
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 stopping solution 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. For best results, the O.D. values should be measured immediately after the addition of stopping solution.

Quality Control

1. 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.
2. O.D.s for the duplicates of the reference plasma dilutions, plasma controls or patient samples should be within 20% of the mean O.D. for samples with absorbance readings greater than 0.200.
3. The Protein C Antigen values obtained for the controls should be within manufacturer's assayed ELISA ranges.

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 against the corresponding value of the reference level. The curve may be plotted on a semi-log (if semi-log, plot O.D. on linear axis) or log-log graph. Draw a line to connect the points.
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. To calculate Protein C Antigen levels in % of normal, multiply control and patient relative values obtained from the reference curve by the 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 Protein C Antigen value (as % of normal): 40 x 1.05 = 42%
4. Ensure that all quality control parameters have been met (see Quality Control) before reporting test results.

REFERENCE RANGE

Protein C Antigen values are generally expressed in relative percent (%) as compared to pooled normal plasma. The reference range when normal plasma samples were tested by the Helena Protein C Antigen assay was 72-160% (mean 110%, SD 24%). This is consistent with normal ranges published in the literature [6, 8] and reported by other commercially available assays (65-150%). Samples with values above the range of the reference curve may need to be diluted and retested for accurate results.
Each laboratory should periodically determine their own reference range for this assay.

PERFORMANCE CHARACTERISTICS

Detection range:
The detection range for Protein C Antigen assay has been determined as 5-200%. 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 Protein C 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 CV of 7.0% across three lots. In addition, ninety-nine (99) patient samples with

Protein C 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 CV for duplicates was 6.0%.

Inter-assay precision:

Six (6) commercially prepared, assayed plasma samples with Protein C values ranging from 39-112% were tested in duplicate on three lots to determine assay precision between lots. The mean inter-assay CV was 7.5%, as seen in the table.

Intra-assay precision (variability within a plate)	Protein C range (% of normal)	CV range (3 pilot lots)	Overall mean CV:
Replicates (x16):	82-89%	4.6-12.6%	
	44-116%	2.9-13.0%	
	37-41%	2.9-21.7%	7.0%
Duplicates:	Entire range		6.0%
Inter-assay inter-lot precision (variability between lots)			
Duplicates:	39-112%	1.4-10.0%	7.5%

Linearity

Serial two-fold dilutions of Protein C reference plasma samples tested on three (3) lots of Helena Protein C Antigen assay demonstrated curves with a mean coefficient of determination (r-squared) of 0.992 and individual point recovery ranged from -15.2% to + 21.3%.

Accuracy

Accuracy was determined by testing mixtures of Protein C reference plasma with predetermined values on Helena Protein C Antigen assay to assess the recovery of their theoretical values. The overall mean percent recovery across 3 lots was 99.4% with an average variation CV of 6.1%.

LIMITATIONS OF THE TEST

The Protein C Antigen 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 C may have undetectable levels of Protein C, while those with heterozygous deficiency typically have levels between 30% to 60%. Acquired Protein C deficiency may be seen with numerous clinical conditions: neonates show 20 to 50% lower levels than adults, severe liver diseases, oral anticoagulants, post-operative period, disseminated intravascular coagulation (DIC), antiphospholipid syndrome, etc.[6-8] Increased levels of Protein C may be seen in patients with renal disease. Plasma samples could be inadvertently depleted or degraded of Protein C by improper collection or laboratory processing.

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Distributed By:

HELENA LABORATORIES

Beaumont, Texas USA 77704

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