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. Protein C through complex interactions with thrombin forms a complex that inactivates the coagulation cascade, contributes to the maintenance of blood clots, endothelial cells, and other factors of the coagulation cascade, contribute to the maintenance of normal physiological mechanisms. Protein C deficiency is a rare condition with implications for the diagnosis and management of thrombosis. In this Protein C Anticoagulant System is activated by the binding of thrombin to thrombomodulin, a transmembrane protein receptor on endothelial cells. The thrombin-thrombomodulin/endothelial cell membrane activates circulating Protein C. Activated Protein C binds to Protein S on the membrane or 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 Vila, 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. Protein C deficiency, either congenital or acquired, may lead to serious thrombotic events such as thrombophlebitis, deep vein thrombosis, or pulmonary embolism. Patients with a congenital heterozygous deficiency may present with very low Protein C•Factor Va•Protein C levels during the neonatal period. 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%. Acquired Protein C deficiency may be seen in liver disease (cirrhosis, acute liver failure, chronic liver disease, and hepatitis), cancer, surgery, oral anticoagulation, antiphospholipid syndrome, etc. A serum clinical deficiency of Protein C activity in plasma may be the result of low concentrations and function (type I) or only low function (type II). 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). 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-well microwell 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 (H₂O₂) 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 percentage recovery in patient plasma is determined against a curve prepared from a reference plasma.

**REFERENCES**

1. 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). 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.

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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 it 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.

Substrate should be clear and colorless. Discard if product shows signs of microbial growth.

6. Stopping Solution

Ingredients: The solution is 0.36 N Sulfuric Acid.

WARNING: DO NOT INGEST.

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 25 °C) to make in accurate results.

11. Avoid microbial and cross-contamination of reagents and substrates by opening and removing aliquots from the primary pack.

12. Do not use kit components beyond expiration date.

13. Do not use kit components from different lot numbers.

5. Deionized water

Materials Required but not Supplied:

- Specially Assayed Control 1 (S.A.C. 1) - 5301
- Specially Assayed Control 2 (S.A.C. 2) - 5002
- Deionized water
- Graduated cylinders
- Pipettors (5 and 1000 µL)
- Plastic squeeze bottle
- Plate reading spectrophotometer capable of reading absorbance at 450 nm

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 their refrigerated state 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 at the beginning of 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” this water blank 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 microliter 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.

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12. Do not use kit components beyond expiration date.

13. Do not use kit components from different lot numbers.
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%-tetramethyl-1,2-phenylenediamine hydrochloride.

WARNING: IRRITANT, DO NOT PIPEET 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 colorless.

6. Stopping Solution

Ingredients: The solution is 0.36 N Sulfuric Acid.

WARNING: DO NOT INGEST. IRRITANT. DO NOT PIPEET BY MOUTH. AVOID CONTACT WITH EYES OR SKIN. - The solution is an irritant. Use gloves and protective 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.

Materials Required but not Supplied:

Specially Assayed Control 1 (S.A.C.1) - 5301
Specially Assayed Control 2 (S.A.C.2) - 5002
Deionized water
Graduated cylinders
Pipettors (1 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.

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 by adding 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” this water blank 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 25 °C) to be inadequate results.

11. Avoid microbial and cross-contamination of reagents during opening and removing aliquots from the primary trays.

12. Do not use kit components beyond expiration date.

13. Do not use kit components from different kit lot numbers.

SAMPLE-STEP METHOD

1. Remove any microwell strips that will not be used from the frame holder and store them in the plastic pouch. 

2. Add 15 µL + 500 µL = 75 µL of plasma to the well intended for the water blank. Add 200 µL of deionized water 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. It is advisable that duplicate determinations be made for all samples. One well should be run as a reagent blank; sample 0 µL + 1.5 µL of deionized water 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. 

3. Using the Reference Plasma provided with the kit, prepare six reference dilutions as described below:

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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.

7. Place nothing in the well intended for the water blank.

8. Wash times with working PBS solution. Each well should be filled with PBS solution per wash. PBS solution to the wells in the same order and at the same rate. Substrate in wells incubated with positive samples will turn blue.

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 sample fluid. Take care to prevent sample from one microwell to flow into another.

11. Wash 4 times with working PBS solution.

12. Add 100 µL Substrate to each well (except for the water blank well) to stop the reaction. Substrate in wells incubated with positive samples will remain colorless. Do not add Stopping Solution to the water blank well.

13. Add 100 µL of the Stopping Solution (0.36 N sulfuric acid) to each well (except for the water blank well).

14. Incubate for 10 minutes at room temperature. After the incubation is complete, carefully invert the microwells and decant the substrate solution.

15. Place the plate reading spectrophotometer against the water blank well. Read the O.D. values 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 preparation of the substrate.

2. O.D.s for the duplicates of the reference plasma dilutions, plasmas and controls should be within 20% of the mean O.D. for samples with absorbance readings greater than 0.001.

3. The mean 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.

3. Using the mean O.D., determine the control and patient relative values from the graph, or, alternatively, calculate the relative values from the graph. To calculate the 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%

Actual patient Protein C Antigen value (as % of normal): 40 x 1.05 = 42%

4. Ensure that all results have been met (see Quality Control) before reporting test results.

REFERENCE RANGE

Protein C Antigen values are generally expressed in relative percent (% of normal) 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 113%, SD 24%). This is consistent with normal plasma ranges published in the literature 6,7 and reported by other commercially available assays (65-150%). Samples with values outside the reference range are to be diluted and retested for accurate results. Each lab 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 to be 0.5-200%. It is recommended that the effective range for each run will depend on the assigned 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

Inter-Assay
To determine variability within a plate, three plasma samples with known Protein C levels (one each high, medium, 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 all lots. In addition, ninety-nine (99) patient samples with...
REFERENCES

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. 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 thrombotic mechanisms. The Protein C Anticoagulant System is activated by the binding of thrombin to thrombomodulin, a transmembrane protein receptor on endothelial cells. The thrombin-thrombomodulin complex cell membrane 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 Vθ, 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 (P-AI), thereby facilitating clot dissolution or fibrinolysis. Protein C deficiency, either congenital or acquired, may lead to serious thrombotic events such as thrombophlebitis, deep vein thrombosis, or pulmonary embolism.

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. Serum samples depleted or degraded of Protein C by improper collection or storage and Stability:

Protein C Antigen Microwells

Ingredients: 96 stabilized antibody coated microwells

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.

Shaded areas indicate that text has been modified, added or deleted.