HEMOCLOT™ Protein C

Ref CK031K, R1, R2: 3 x 1 mL
Ref CK032K, R1, R2: 3 x 2 mL

Measurement of Protein C activity with a clotting method

For *in vitro* diagnostic use only

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Last revision: 12-2021

INTENDED USE:

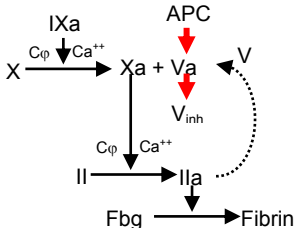
The HEMOCLOT™ Protein C kit is an in vitro method for the quantitative determination of Protein C in human citrated plasma by clotting assay, using a manual or automated method.

SUMMARY:

Protein C (PC) is a vitamin K dependent human Protein, which inhibits and regulates coagulation through specific cleavages of Factors Va and VIIIa, suppressing their procoagulant cofactor activity.
Congenital or acquired Protein C deficiency is a risk factor for venous thrombosis.

ASSAY PRINCIPLE:

The HEMOCLOT™ Protein C method is an APTT like clotting assay, performed in presence of Protein C Activator (Protac®, extracted from snake venom Agkistrodon Contortrix), phospholipids, contact phase activator, and calcium.
In the first step, the diluted assayed plasma is mixed with Protein C deficient plasma (R1). Then, the Activator Reagent (R2), in a constant and optimised concentration, is added. Clotting is initiated by the addition of Calcium (Ca2+). Clotting time is then recorded. Protein C being the limiting factor, there is a direct linear relationship, on a bilogarithmic graph paper, between the Protein C concentration and the corresponding clotting time.



REAGENTS:

R1: *Reagent 1:* Protein C Deficient Plasma.
Protein C Deficient plasma, optimized for the test, lyophilized in the presence of an heparin neutralizing agent.

R2: *Reagent 2:* Activator reagent
Protac® (highly purified enzyme, extracted from the Agkistrodon C Contortrix snake venom, able to specifically activate Protein C), containing phospholipids, in an optimized concentration.

Ref CK031K → R1, R2 : 3 vials of 1 mL.
Ref CK032K → R1, R2 : 3 vials of 2 mL.

Precaution and warnings:

- The Human plasma used for the preparation of the reagents was tested and found negative for HIV antibodies, HBs Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.
- All the required cautions must be respected in order to avoid any risk of ingestion or accidental introduction of reagents in body. In case of skin contact, wash extensively with water. In case of contact with a wound, address to the appropriate medical service, and indicate the biological origin and the nature of the product.
- Avoid contact with skin and eyes. Do not empty into drains. Wear suitable protective clothing.
- For in vitro use only.
- The Protac® concentration may present variations from lot to lot, but it is exactly adjusted for each new lot of reagent.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, preferentially sterile.
- Calcium chloride 0.025M (ex: Ref AR001B/AR001K/AR001L).
- Imidazole buffer (ex: Ref AR021B/AR021K/AR021L/AR021M/AR021N)
- Plasma Calibrator (ex: BIOPHEN™ Plasma Calibrator Ref 222101).
- Normal and Abnormal Quality Control Plasmas (ex: BIOPHEN™ Normal Control Plasma Ref 223201, and BIOPHEN™ Abnormal Control Plasma Ref 223301).

Material:

- Electromagnetic water bath or semi automatic or automatic clotting instruments.
- Chronometer.
- Calibrated pipettes.

STORAGE CONDITIONS:

HEMOCLOT™ Protein C kits must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

Note: The stability studies at 30°C show that all the reagents can be shipped at room temperature without damage.

PREPARATION AND STABILITY OF REAGENTS:

Preparation:

R1: Reagent 1: PC Deficient Plasma

Reconstitute each vial with exactly:
Ref CK031K → 1 mL of distilled water
Ref CK032K → 2 mL of distilled water

Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 15 min, while shaking the vial from time to time. Homogenize the content before each use.

R2: Reagent 2: Activator reagent

Reconstitute each vial with exactly:
Ref CK031K → 1 mL of distilled water
Ref CK032K → 2 mL of distilled water

Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 15 min, while shaking the vial from time to time. Homogenize the content before each use.

Stability:

Stability of the reconstituted R1 and R2 reagents, stored in their original vials, is of:

24 hours	8 hours	1 month
2 – 8 °C	Room Temperature (18-25°C)	Deep frozen at -20°C or less

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use.
- Reagents must be handled with care, in order to avoid any contamination during use.

Note:

- R1 and R2 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between R1 and R2 must be strictly respected.
- Use only reagents from kits with a same lot number. Do not mix reagents from kits with different lots when running the assay. Reagents R1 and R2 are optimized for each lot of kits.

PREPARATION OF PLASMA (SPECIMEN COLLECTION):

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), with great care, in a silicon glass or a plastic tube. Sampling must be performed through a net venipuncture, avoiding any blood activation.

- Within 2 hours, blood must be centrifuged at 2,500 g for 15 min, and plasma decanted into a plastic tube, using a plastic pipette.
- Storage of plasma:
  - Up to 4 hours at Room Temperature (18-25°C).
  - Up to 1 month frozen at –20°C or below.

Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage.

TEST PROCEDURE:

The HEMOCLOT™ Protein C kit is a clotting method, manual or automated. Adaptations on automates are available upon request. The assay is performed at 37°C, and the clotting time, triggered by calcium addition, is measured.

CALIBRATION:

Prepare 2 ml of normal citrated human pooled plasma diluted **1:5** with imidazole buffer (ex: ref AR021). By definition, the ten fold dilution of the normal citrated human plasma pool corresponds to a concentration of 100% of Protein C, and the 1:5 dilution to 200% Protein C.
Using the 1:5 preparation, the calibration curve is obtained as follows:

PC	25%	50%	100%	200%
Dilution	**1:40**	**1:20**	**1:10**	**1:5**
Plasma Pool 1:5	0.125 mL	0.250 mL	0.500 mL	1 mL
Imidazole buffer	0.875 mL	0.750 mL	0.500 mL	0 mL

HEMOCLOT™ Protein C kit can also be calibrated with the **BIOPHEN™ Plasma Calibrator (ref 222101),** which has a well defined Protein C concentration, "**C**". The ten fold dilution of the calibrator then corresponds to a concentration of "C%" of Protein C, indicated for each lot. Use the 1:5 dilution of the calibrator to prepare the calibration curve as here above, from "**2xC**%".

TEST PLASMA:
Dilute the tested samples, and the controls **1:10** with the imidazole buffer.

Caution: to ensure optimal performances of the assay, perform all testings (calibration, samples, controls) extemporaneously and successively without interruption.

ASSAY PROTOCOL:

Assay	Manual Method
Calibrators; or 1:10 diluted plasma or control	100µl
R1 reagent	100µl
Mix and incubate for 2 minutes at 37°C	
R2 reagent	100µl
Mix and incubate for 5 minutes at 37°C	
CaCl2 0.025M (preincubated at 37°C, and stirred)	100µl
Record Clotting Times	**CT**

Automated methods:
Detailed instrument settings including instructions for the preparation of the reagents for a variety of automated instruments are available upon request.

Note: Isotonic Imidazole buffer (ref AR021) is recommended for diluting calibrators, controls and assayed plasmas for the measurement of Protein C with HEMOCLOT™ Protein C, as it gives the best reproducibility. The same type of buffer must be used throughout the assay, for diluting calibrators, controls and plasmas.

QUALITY CONTROL:
Use of quality control plasmas allows validating the calibration curve, as well as the homogeneous reactivity of the HEMOCLOT™ Protein C assay from run to run, and from series to series, when using a same lot of reagents. Various control plasmas are available:
BIOPHEN™ Normal Control Plasma: (ref 223201).
BIOPHEN™ Abnormal Control Plasma: (ref 223301).
Each laboratory should verify and validate its own target value and acceptance range, for each new lot of quality controls used, according to the instrument used and in the laboratory working conditions.
The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range.

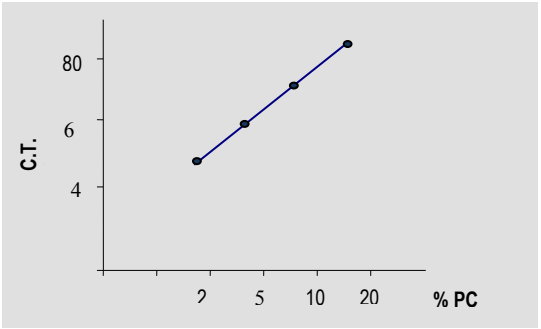
Note: **Include at least one quality control (at different levels) in each series, in order to validate it. A new calibration curve must be carried out for each new batch of reagents, after an important maintenance of the instrument, or if measured values are not in compliance with the one expected.** Each laboratory can define its own acceptance range, according to the protocols and instruments used.

LIMITATIONS OF THE PROCEDURE:
- The assay can be performed in patients with heparin therapy (up to 1 IU/ml), or dicoumarol treated (PC activity is then decreased). In presence of an abnormally prolonged CT, confirming the diagnosis with another method is recommended.
- Special caution should be taken for patients with known high levels of FVIII:C or with LA, and result confirmed with another method.
- Aprotinin inhibits Activated Protein C. The "apparent" Protein C activity could be decreased in patients treated with aprotinin [7]. Using a different method for testing protein C (i.e. immunoassay) is then recommended.
- For a better accuracy, samples measured ≤10% can be tested at the 1:5 dilution, and obtained results divided by 2; for samples measured >140%, the 1:20 dilution can be used and obtained results multiplied by 2. If a dilution different from 1:10 is used, the concentration must be corrected by the complementary dilution factor used, i.e. x2 for 1:20, or x0.5 for 1:5).
- Presence of activated clotting factors may shorten Clotting Times.
- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.
- To ensure optimal performances of the assay, perform all assays (calibration, samples, controls) extemporaneously and successively without interruption
- For a same reagent lot and a same tested specimen, the Clotting Time may present variations according to the instrument used, and to the clot detection sensitivity adjustment.
- The assay performances can slightly vary according to the reagents used, and the instrument used in the laboratory. Performances, as well as the normal range, and target values and acceptance ranges for each new lot of quality controls used, must then be confirmed (and adjusted if necessary) in the laboratory working conditions.

RESULTS:
The Protein C concentration in the tested sample is directly obtained from the calibration curve.
Results are expressed as %.
Using a bilogarithmic graph paper, the assay is linear in the range 25- 200% PC.
- For the manual method, on a bilogarithmic graph paper, plot on abscissa the PC concentrations (%) and on ordinates the corresponding clotting times (sec). On the calibration curve obtained, interpolate directly the corresponding PC concentration for the tested plasma.

The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range.
- Using automated methods, the Protein C concentrations are directly calculated by the analyser, respectively to the calibration curve.

EXAMPLE OF CALIBRATION CURVE:
The calibration curve below, obtained using the KC-10 instrument, is indicated as an example only. Only the calibration curve generated for the series of measures performed must be used.



VALIDATION OF THE CALIBRATION CURVE:
The calibration curve is acceptable when the concentrations measured for the Control Plasmas are within the acceptance range.

PERFORMANCES AND CHARACTERISTICS:
- The detection threshold is calculated by measuring the "apparent" Protein C concentration, which corresponds to the mean clotting time obtained for a Protein C deficient sample plus 3 standard deviations (SD). This detection threshold is of about 10%.
- Dynamic range: 25-200%
- Example of Intra-Assay and Inter-Assay reproducibilities obtained for samples with variable Protein C concentrations, using the KC10 instrument:

Samples	PC concentrations %	Intra-Assay CV%	N	Inter-Assay CV%	N
Sample 1	87%	6.7	10	8.4	9
Sample 2	63%	7.3	10	5.7	9

Intra and inter assay CV on obtained clotting times are <5%.

EXPECTED VALUES:
By definition, the 100 % Protein C concentration corresponds to the concentration in a normal human citrated plasma pool, obtained by pooling plasmas from healthy males or females aged from 18 to 55 years, and out of any medication. The Protein C concentration in adults is usually between 70 and 140%[3].
The Protein C concentration is decreased in neonates.

CLINICAL VARIATIONS:
- A Protein C concentration ≤ 60 % indicates the presence of a deficiency, which must be confirmed by another measurement, or another sample collected from the patient [6].
- Protein C activity is reduced during dicoumarol therapy, in hepatic diseases, in DIC, or in presence of a congenital or acquired deficiency.

BIOCHEMISTRY:
Protein C is a vitamin K dependent human protein of the coagulation system, synthesized in the liver, of about 62 KDa. PC is usually present in plasma as a proenzyme, at a concentration of about 4-5 µg/ml. When activated by thrombin (and thrombomodulin), in presence of calcium and phospholipids, activated Protein C, in presence of Protein S, inhibits and regulates coagulation through specific cleavages of Factors Va and VIIIa, suppressing their procoagulant cofactor activity.

CLINICAL INFORMATIONS:
Protein C deficiencies can be:
- Acquired: they are observed in hepatic diseases, during dicoumarol therapy or in DIC.
- Congenital: they are then associated with recurrent venous thromboses.

Protein C deficiencies can be quantitative (type I) or qualitative (Type II).

REFERENCES:

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6. Pabinger I. : Clinical relevance of Protein C. Blut 53, 63-75 (1986).
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