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ZYMUTEST™ Protein C

REF RK027A

96 tests

ELISA assay of human Protein C

English, last revision: 12-2021

INTENDED USE:

The ZYMUTEST™ Protein C kit is a sandwich ELISA method for the *in vitro* quantitative determination of human Protein C (PC) antigen on human plasma.

SUMMARY AND EXPLANATION:

Technical:

Protein C is a glycoprotein, vitamin K dependent, which inhibits coagulation. Its normal concentration in human plasma is about 4 µg/mL. Activated by the thrombomodulin-thrombin complex, the activated Protein C (APC), in presence of his cofactor the Protein S, calcium and PPL, will cleave Factors Va and VIIIa, suppressing their procoagulant cofactor activity[1,2].

Clinical:

Assay of coagulation Protein C in plasma may help in the diagnosis of congenital or acquired Protein C deficiencies[3,4,5,6].

Acquired deficiencies are observed in hepatic diseases, during VKA therapy or in Disseminated Intravascular Coagulation (DIC).

Congenital deficiencies can be quantitative (Type I) or qualitative (Type II) and are associated with recurrent venous thromboses.

Congenital or acquired Protein C deficiency is a risk factor of venous thrombosis[3].

PRINCIPLE:

ZYMUTEST™ Protein C is and ELISA method, based on antigen-antibody reaction: PC antigen of the sample reacts with anti-PC polyclonal antibodies, stabilized.

The tested plasma is introduced into a microwell coated. The PC binds to the immobilized antibody. Following a washing step, the PC fixed on the plate is revealed by immunoconjugate, polyclonal antibody coupled to horse radish peroxidase (HRP), witch binds to free epitopes of immobilized PC. Following a washing step, the peroxidase substrate, 3,3',5,5' – Tetramethylbenzidine (TMB), in presence of hydrogen peroxide (H_2O_2), is introduced and a blue colour develops. When the reaction is stopped with Sulfuric Acid, a yellow colour is obtained that can be read at 450nm. The absorbance at 450nm is directly proportional to the concentration of human PC in the tested sample.

REAGENTS:

1. **COAT ELISA microplate :** 12x8 contaning 12 strips of 8 wells, coated with a rabbit polyclonal antibody, specific for human PC, stabilized and packed in an aluminium pouch in presence of a desiccant. Contains small amounts of sodium azide (0.9 g/L).
2. **SD ELISA Sample Diluent :** 2 vials of 50 mL, ready to use. Contains BSA.
3. **CAL Protein C calibrator :** 3 vials of 2 mL, lyophilized. Each vial should be reconstituted by 2 mL of **SD ELISA** to obtain a standard containing a concentration **"C%"** of human PC (**already diluted at 1:50**), precisely determine for each lot. This concentration "C" Is between 110 and 150% according to the lot. The standard is referenced to the NIBSC international standard. Contains BSA.
4. **CI Protein C Control high :** 1 vial of 0.5 mL, lyophilized.
5. **CII Protein C Control low :** 1 vial of 0.5 mL, lyophilized.
6. **IC Anti-(h)-PC-HRP immunoconjugate :** 3 vials of 7.5 mL, a polyclonal antibody, specific for PC and coupled to Horse-Radish-Peroxidase (HRP), lyophilized. Contains BSA.
7. **CD ELISA Conjugate diluent :** 1 vial of 25 mL, ready to use. Contains BSA.
8. **WS ELISA Wash solution :** 1 vial of 50 mL, 20x 20 fold concentrated.
9. **TMB 3,3',5,5'-Tetramethylbenzidine:** 1 vial of 25 mL of substrate, ready to use. Contains hydrogen peroxide.
10. **Stop 0.45M Sulfuric acid:** 1 vial of 6 mL, ready to use.

The control and calibrator concentrations may vary slightly from one batch to another. For the assay, see the exact values indicated on the flyer provided with the kit used.

WARNINGS AND PRECAUTIONS:

- Some reagents provided in these kits contain materials of human and animal origin. Whenever human plasma is required for the preparation of these reagents, approved methods are used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV, and for hepatitis B surface antigen, and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- This device of *in vitro* diagnostic use is intended for professional use in the laboratory.

REAGENT PREPARATION:

Allow the strips and reagents to stabilize for at least 30 min at room temperature before use. Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

COAT Open the aluminum pouch and take off the required amounts of strips for the test series. The strips must be used within 30 minutes.

Reconstitute the contents of each vial with exactly:

CI **CII** ➔ **0.5 mL of distilled water**. Shake vigorously until complete dissolution.

CAL ➔ **2 mL of SD ELISA in order to obtain a solution containing** "C" **% of PC (already diluted at 1:50)**. Shake vigorously until complete dissolution.

IC ➔ **7,5 mL of CD ELISA** at least 15 minutes before use. Shake gently until complete dissolution.

SD ELISA **TMB** **Stop** **CD ELISA**
Reagent ready to use.

WS ELISA Shake the vial and dilute the wash solution **1:20 in distilled water** (the 50 mL of concentrated solution allow to prepare 1 litter of wash solution after dilution).

Incubate, if necessary, the vial in a water bath at **37°C**, until complete dissolution of solids.

STORAGE AND STABILITY:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

COAT Unused strips can be stored at 2-8°C **for 4 weeks** in their original aluminum pouch (hermetically closed, in presence of the desiccant), stored in the provided plastic microplate storage bag (minigrip), protected from any moisture.

Reagent stability after reconstitution, free from any contamination or evaporation, and stored closed, is of:

CAL
➔ **72 hours at** 2-8°C.
24 hours at room temperature (18-25°C).

CI **CII**
➔ **72 hours at** 2-8°C.
24 hours at room temperature (18-25°C).
2 months frozen at -20°C or less*

IC
➔ **4 weeks** at 2-8°C.
24 hours at room temperature (18-25°C).

*Thaw only once, as rapidly as possible at 37°C and use immediately.

Reagent stability after opening, free from any contamination or evaporation, and stored closed, is of:

SD ELISA **CD ELISA** **TMB**
➔ **4 weeks** at 2-8°C.

WS ELISA ➔ **4 weeks** at 2-8°C.
7 days at 2-8°C for the diluted solution.

Stop ➔ **8 weeks** at 2-8°C.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.

Materials:

- 8-channel or repeating pipette allowing dispensing volumes of 50-300 µL.
- Pipettes at variable volumes from 0 to 20 µL, 20 to 200 µL and 200 to 1000 µL.
- Micro ELISA plate washing equipment and shaker.
- Micro ELISA plate reader with a wavelength set up at 450 nm.

SPECIMEN COLLECTION AND PREPARATION:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M, 3.2%) by clean venipuncture. Discard the first tube.
Specimens should be prepared and stored in accordance with applicable local guidelines (for the United States, see the CLSI GP44-A4[7] (and CLSI H21-A5[8]) guideline for further information concerning specimen collection, handling and storage).
For plasma storage, please refer to references.

PROCEDURE:

Assay method:

1. Specimens and controls should be diluted using **SD ELISA** as described in the table below:

Specimens	Dilution
CI and **CII**	**1:50**
Specimens	**1:50**

For expected PC concentrations above "C"%, dilute specimens at **1:100** (D=100), or more.

2. Using the Calibrator **CAL** with a concentration "C" in %, prepare the calibration range as described in the table below:

Concentration of PC (%)	C	C:2	C:4	C:10	C:20	0
Vol. of **CAL**	1 mL	0.5 mL	0.25 mL	0.1 mL	0.05 mL	0 mL
Vol. of **SD ELISA**	0 mL	0.5 mL	0.75 mL	0.9 mL	0.95 mL	1 mL

Mix for homogenization.
The dilutions are stable for **8 hours** at room temperature (18-25°C).

3. Put strips in the frame provided. Introduce the reagents in the micro ELISA plate wells and perform the assay as indicated on the following table:

Reagent	Volume	Procedure
CAL or **CI** or **CII** or **Specimens to test diluted** or **SD ELISA** **(blank)**	**200µL**	Introduce the standard solutions or the tested specimen in the corresponding micro ELISA plate well
Incubate for 1 hour at room temperature (18-25°C) (a)		
WS ELISA	**300µL**	Proceed to 5 successive washings **(b)**
IC	**200µL**	Introduce the **IC** in the micro ELISA plate wells
Incubate for 1 hour at room temperature (18-25°C) (a)		
WS ELISA	**300µL**	Proceed to 5 successive washings **(b)**
TMB	**200µL**	Immediately after the washing, introduce the substrate into the wells **(b,c)**. **Nota:** The substrate distribution, raw by raw, must be accurate and at exact time intervals.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (a)		
Stop	**50µL**	Following exactly the same time intervals, raw to raw, than for the addition of substrate, stop the colour development by introducing the 0.45M sulfuric acid **(c)**.
Wait for 10 minutes in order to allow the colour to stabilize then measure absorbance at 450 nm. Subtract the blank values (d).		

Distribute calibrator dilutions, controls and specimens as rapidly as possible, in order to obtain homogeneous kinetics of the dosage. A too long delay (>10 min) between the first and the last distribution wells may have incidence on immunological kinetics and produce inaccurate results (underestimated value for the last wells).

(a) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro ELISA plate shaker can be used.
(b) Never let the plates empty between the addition of the reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components and reduce the reactivity plate. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could damage coating and lower plate reactivity.
(c) For addition of the substrate, the time interval between each row must be accurate and exactly determined.
(d) For bichromatic readings, a reference wavelength at 620 nm or at 690 nm can be used.

One step method:

The PC assay can also be performed with a one step method. In this case, the calibration curve is considered from 0 to C%.
All reagents are reconstituted as for the two-step method, except for **IC** which must be restored with 2 mL of **CD ELISA**.
Tested plasma is analyzed at 1:50 dilution in **SD ELISA**, or at higher dilutions if necessary. Controls and specimens must be diluted at 1:50, as for the two-step method.
In the microwells of the ELISA plate, introduce 50 µL of **IC**, followed by the introduction of 200 µL of the calibration solution or the diluted plasma. Following a 1 hour incubation at room temperature and a washing step, the **TMB** (200µL/well) is added and the reaction is stopped after 5 min. by 50 µL of **Stop**. A450 is then measured. Washing, operating cautions and results interpretation, are the same as recommended for the two step method.

QUALITY CONTROL:

Using quality controls allows validating the method compliance, as well as the homogeneous of assays for a same lot of reagents.
Quality control plasmas must be included in each series, as per good laboratory practice, in order to validate test results. A new calibration curve must be carried out for each test series.
Each laboratory can establish acceptance ranges and verify expected performances in its analytical system.

RESULTS:

- Obtained OD450 can vary according to the effective temperature during the assay run.
- Plot the calibration curve with the OD 450 nm along the Y-axis and the PC concentration in %, along the X-axis by choosing the "best fit" interpolation mode (refer to the flyer in the kit).
- Results are expressed with the obtained OD450 for specimens and controls using the calibration curve.
- The concentration of PC (%) in the test specimen is directly inferred from the calibration curve, when the standard dilution 1:50 is used.
- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor used.
- Alternatively, a specific software (i.e., Dynex, Biolise, etc...) can be used for the calculation of concentrations.
- For **CI** and **CII**, the concentrations are directly deduced from the calibration curve.
- The results should be interpreted according to the patient's clinical and biological condition.

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- If the washing step is not correctly performed, the negative control can produce a high absorbance value. In order to avoid non-specific colour development, check that the washing step is performed efficiently.

EXPECTED VALUES:

By definition, the 100 % PC 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 or disease. The PC concentration in adults is usually between 70 and 140%.
The PC concentration is decreased in neonates, by hepatic immunity. It is then independent from age or gender.

PERFORMANCES:

- The assay is calibrated against the NIBSC international standard for PC.
- There is no interference of Rheumatoid Factor.
- No Prozone effect was observed for PC concentrations up to 100 µg/mL, using the recommended protocol.
- Dynamic range: 0 to 130%.
- Detection threshold ≤ 5%.
- Intra-assay CV: 3-8 %.
- Inter-assay CV: 5-10 %.
- Interferences: No interference was observed with the molecules and up to following concentrations:

Heparin	Bilirubin	Hemoglobin
2 IU/mL	0.05 mg/mL	5 mg/mL

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6. Pabinger I. Clinical relevance of Protein C. Blut. 1986.
7. CLSI Document GP44-A4: "Procedures for the handling and processing of blood specimens for common laboratory tests".
8. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". 2008.

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

SD ELISA **CD ELISA** **WS ELISA** H317 : May cause an allergic skin reaction.

Changes compared to the previous version.

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