ZYMUTEST PROTEIN C

 # RK027A-RUO

Complete ELISA kit for the assay of human Protein C

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

HYPHEN BioMed
www.hyphen-biomed.com
155, rue d'Eragny
95000 NEUVILLE SUR OISE
FRANCE
Tel. : +33 (0)1 34 40 65 10
Fax : +33 (0)1 34 48 72 36
info@hyphen-biomed.com

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INTENDED USE:

The ZYMUTEST Protein C kit is a highly sensitive two-site immuno-assay for measuring human Protein C in citrated plasma, or in any biological fluid where Protein C can be present. **This assay is for research use only and should not be used for patient diagnosis or treatment.**

ASSAY PRINCIPLE:

In a first step, the diluted test sample is introduced into a microwell coated with a polyclonal antibody specific for human Protein C. When present, this protein is captured onto the solid phase. Following a washing step, the immunoconjugate, a rabbit polyclonal antibody, specific for human Protein C, and coupled to horse-radish-peroxidase (HRP), is introduced, and binds to free epitopes of immobilized PC. Following a new washing step, the peroxidase substrate, 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. The amount of colour developed is directly proportional to the concentration of Protein C in the tested sample.

TEST SAMPLE:

- Trisodium Citrate anticoagulated human plasma.
- Any biological fluid where Protein C must be assayed.

REAGENTS:

1. **COAT: Micro ELISA plate** containing 12 strips of 8 wells, coated with a rabbit polyclonal antibody specific for human Protein C, then stabilised; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
2. **SD:** 2 vials containing 50 ml of **Sample Diluent**, ready to use.
3. **CAL:** 3 vials of **Plasma PC Calibrator**, lyophilised. When restored with **2 ml** of Sample Diluent, a plasma containing a concentration "**C"** (expressed in %) of human PC is obtained. This concentration (in the range 110-150% according to the lot), established by reference to the NIBSC international standard, is accurately determined for each lot.
4. **CI**: 1 vial containing **0.5 ml** of lyophilised **PC Control I** (human plasma, high).
5. **CII:** 1 vial containing **0.5 ml** of lyophilised **PC Control II** (human plasma, low).

> ***Note:*** The Protein C concentrations and acceptancy ranges for control plasma I and II, and calibrator, can vary from lot to lot, but are precisely indicated for each lot on the flyer provided in the kit.

6. **IC:** 3 vials of **Anti-(H)-PC-HRP-immunoconjugate**, a rabbit polyclonal antibody specific for human Protein C coupled to HRP, lyophilised.
7. **CD:** 1 vial of 25 ml of **Conjugate Diluent**, ready to use.
8. **WS:** 1 vial of 50 ml of 20 fold concentrated **Wash Solution**.
9. **TMB:** 1 vial of 25 ml peroxidase substrate: **3,3',5,5' – Tetramethylbenzidine** containing hydrogen peroxide; ready to use
10. **SA:** 1 vial of 6 ml of **0.45 M Sulfuric Acid** (Stop Solution); ready to use.

> ***Note:*** *Use only components from a same kit lot. Do not mix components from different lots of kits, when running the assay.*

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **8-channel** or repeating **pipette** allowing dispensing 50-300 µl.
- **1-channel 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.
- Distilled water.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

1. **Micro ELISA plate:** open the plastic pouch and take off the required amount of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at **2-8°C** for **4 weeks** in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
2. **Sample Diluent:** It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
3. **Plasma PC Calibrator:** restore each vial with **2 ml** of Sample Diluent in order to obtain a plasma containing a PC concentration **"C",** already diluted 1:50 (fifty fold). This solution is stable for at least **24 hours at room temperature** or **72 hours at 2-8°C.**
4. **PC Control I** (human plasma, high): restore with **0.5 ml** distilled water.
5. **PC Control II** (human plasma, low): restore with **0.5 ml** distilled water.

> ***Note***: Following reconstitution, control plasmas I and II are stable **24 hours** at Room Temperature (18-25°C), or **72 hours at 2-8°C.** They can be kept frozen at **–20°C** or colder for up to **2 months**.
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> ***Warning:*** Plasma PC calibrator (3) and controls (4&5) are prepared with normal human plasma. This latter was tested with registered methods 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.

6. **Anti-(H)-PC-HRP immunoconjugate**: each vial must be restored with **7.5 ml** of Conjugate Diluent. Let the pellet to be completely dissolved before use, and shake the vial gently in order to homogenize the content. The restored conjugate is stable for at least **24 hours** at room temperature or for at least **4 weeks at 2-8°C**.
7. **Conjugate Diluent**: It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8 °C**, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
8. **Wash Solution**: Incubate the vial for 15-30 minutes in a water bath at **37°C** until complete dissolution of solids, when present. Shake the vial and dilute the amount required 1:20 in distilled water (the 50 ml contained in the vial allow to prepare 1 liter of Wash Solution). The Wash Solution must be stored at **2-8°C** in its original vial and used within **4 weeks** following opening. The diluted Wash Solution must be used within **7 days**, when protected from any contamination, and stored at **2-8°C**. This reagent contains 0.05% Kathon CG.
9. **TMB substrate**: It is ready to use. When open, it can be used for **4 weeks**, stored at **2-8°C**, and provided that any bacterial contamination is avoided during use.
10. **Stop solution:** It is ready to use.

> ***Cautions:***
> Sulfuric acid, although diluted to 0.45M is caustic. As for any similar chemical, handle Sulfuric acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.
>
> ***Note:*** Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.
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> The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

PROCEDURE:

Specimen collection:

Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g; citrated plasma should be tested within **24 hours** or stored frozen at –20°C or colder for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within **8 hours**.
EDTA collected human plasma may also be used. Conditions of storage are the same than those for citrated plasma.

Tested plasma or sample or controls:

The sample must be tested diluted **fifty fold (1:50)** in the Sample Diluent. For expected Protein C concentrations higher than "**C**" (in %), plasma or samples must be tested at a higher dilution, ie **1:100 (D=100), or more.** For low or very low Protein C concentrations lower dilutions can be used.
Controls I and II must be tested diluted **fifty fold (1:50)**, with Sample Diluent

Calibration:
Using the **Plasma PC Calibrator,** with a PC concentration "**C**" (in the range 110-150% according to the lot used), provided in the kit, prepare the following standard solutions.

Protein C concentration (%)	C	C/2	C/4	C/10	C/20	0
Vol. of Plasma PC calibrator at C	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05ml	0 ml
Vol. of Sample diluent	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95ml	1 ml

Mix gently for a complete homogenisation.
The standard solutions are stable for at least **8 hours** at room temperature.

Assay procedure:
Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. In the different wells of the micro ELISA plate, introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Calibrator dilutions or diluted tested sample or sample diluent (blank)	**200µl**	Introduce the Calibrator solutions or the tested sample in the corresponding micro ELISA wells **(a).**
Incubate for 1 hour at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument **(c).**
Conjugate (anti PC polyclonal antibody coupled with peroxidase). Restored with 7.5 ml of conjugate diluent.	**200 µl**	Introduce the Anti-(H)-PC- HRP immunoconjugate in the micro ELISA plate wells.(**c**)
Incubate for 1 hour at room temperature (18-25°C) (b)		
Wash Solution (20 fold diluted in distilled water)	**300 µl**	Proceed to 5 successive washings using the washing instrument (**c**).
TMB/H_2O_2 Substrate	**200 µl**	Immediately after the washing, introduce the substrate into the wells. **Note:** The substrate distribution, row by row, must be accurate and at exact time intervals **(c,d)**.
Incubate for exactly 5 minutes at room temperature (18-25 °C) (b)		
0.45 M Sulfuric Acid	**50 µl**	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing the 0.45 M sulfuric Acid **(d).**
Wait for **10 minutes** in order to allow the colour to stabilize and measure absorbance at **450 nm (A450) (e)**. Subtract the blank values.		

Note:

a) Distribute calibrators, controls and tested specimen as rapidly as possible, in order to obtain an homogeneous immunological kinetics for PC binding. A too long delay (>15 min) between the distribution of the first and the last wells may induce an influence of immunological kinetics and produce wrong results.

b) Avoid letting the plate in the bright sunlight during incubations and more particularly during colour development. A micro-ELISA plate shaker can be used.

c) 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. 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 lower plate reactivity.

d) For addition of the TMB substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction.

e) For bichromatic readings, a reference wavelength at 690 nm or at 620 nm can be used.

RAPID PROCEDURE (ONE STEP METHOD)

The highly sensitive ZYMUTEST Protein C can also be performed with a one step method. All reagents must be restored as for the two-step method, with the exception of anti-PC-HRP immunoconjugate (IC), which must be restored with **2 ml** of Conjugate Diluent (CD).

Tested plasma must be assayed at a **fifty fold (1:50)** dilution or at higher dilutions in Sample Diluent (SD). Controls must be diluted at a **fifty fold (1:50)** dilution as for plasmas, as for the two-step method. The immunoconjugate is introduced (**50µL**) in the microwells of the ELISA plate, followed by the introduction of **200µL** of the calibration solution or the diluted plasma. Following a **1 hour** incubation at room temperature and a washing step, the colour development with TMB (**200µl/well**) is allowed developping for **5 min**, and is then stopped with **50 µl** of 0.45M sulfuric acid (SA). **A450** is then measured. Washing and operating cautions, as well as results interpretation, are the same as recommended for the two step method.

EXPRESSION OF RESULTS:

- On a linear graph paper plot the Protein C concentrations, in %, on abscissae and the corresponding absorbances (**A450**) on ordinates, in order to establish the calibration curve. The curve can also be drawn using a bilogarithmic coordinate.
- Users must construct their own calibration curve, obtained using their calibrator dilutions (See model on the flyer). From the curve obtained, deduce directly the PC concentration for the tested sample. For obtaining the PC concentration in a sample tested at a higher or lower dilution, this value must be multiplied by **D:50** (i.e. x**2 for D=100**, or **x 0.40 for D=20**...).
- For **controls I and II**, the concentrations are directly deduced from the calibration curve.
- Alternatively, an ELISA software (i.e. Dynex, Biolise, etc...) can be used for the calculation of concentrations.
- **The results obtained should be for research purposes only and not used for patient diagnosis or treatment.**

BIOCHEMISTRY:

Plasma protein C is a vitamin-K dependent protein, with a molecular weight of about 60KD. Protein C is present in plasma, at a concentration of 4-5µg/ml, as a proenzyme, which, once activated by thrombin, in the presence of thrombomodulin, calcium and phospholipids, inhibits and regulates coagulation. Protein S acts as a cofactor of activated Protein C, which then cleaves Factors Va and VIIIa, thus neutralizing their coagulant activity. Protein C action is very efficient in micro blood circulation, where the thrombomodulin density is dramatically increased respectively to blood volume.

ASSAY CHARACTERICS:

- The assay is calibrated against the NIBSC international standard for Protein C.
- There is no interference of Rheumatoid Factor.
- No Prozone effect was observed for Protein C concentrations up to 100µg/ml, using the recommended protocol.
- Dynamic range: 0 to about 130% PC.
- Detection threshold ≤ 5%.
- Intra-assay CV: 3-8%.
- Inter-assay CV: 5-10%.
- No significant interference is observed for heparin concentrations < 2IU/mL, bilirubin concentrations < 0.05 mg/ml, and haemoglobin concentrations < 5 mg/ml.