



## Enzyme Research Laboratories

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### Matched-Pair Antibody Set for ELISA of human Protein C antigen (PC)

Sufficient reagent for 5 x 96 well plates

**Product #:** PC-EIA  
**Lot #:** SAMPLE  
**Expiry Date:** SAMPLE

Store at -10 to -20°C

For Research Use Only  
Not for use in diagnostic procedures.

#### Description of Protein C (PC)

Protein C (PC) is a vitamin K-dependent glycoprotein produced in the liver. The concentration of PC in plasma is ~4 µg/ml (~60 nM). A deficiency of Protein C (quantitative or qualitative) is a risk factor for vascular thrombosis. Protein C is expressed as a two-chain molecule with a molecular weight of 62 kDa. The light chain (21 kDa) of PC consists of two EGF-like domains and an amino-terminal domain containing one hydroxyaspartic acid and 11 γ-carboxyglutamic acid (gla) residues. These residues allow PC to bind to membranes that contain acidic phospholipids in a calcium dependent manner. The heavy chain of PC (41 kDa) consists of the catalytic domain and an activation peptide. Activation of Protein C results from cleavage at residue Arg<sup>12</sup> in the heavy chain by a complex of thrombin and a cell surface cofactor thrombomodulin. The activation of PC is associated with the release of a small activation peptide (2-3 kDa, called Protein C peptide, or PCP) from the N-terminal of the heavy chain. Activated Protein C (APC) is a serine protease with anticoagulant activity. APC, in complex with a phospholipid membrane, calcium and the Protein S cofactor, exhibits anticoagulant activity through the proteolytic inactivation of coagulation cofactors Va and VIIIa. The primary inhibitor of APC activity in plasma is Protein C Inhibitor (PCI, also called Plasminogen Activator Inhibitor-3, PAI-3) and to a lesser extent by α<sub>1</sub>antitrypsin and α<sub>2</sub>macroglobulin. The inhibitory activity of PCI is stimulated approximately 10 fold by heparin<sup>1-3</sup>.

#### Principle of Sandwich-style ELISA

Purified monoclonal antibody to PC is coated onto the wells of a microtitre plate. Any remaining binding sites on the plastic wells are blocked with an excess of bovine serum albumin. The plates are washed and plasma or other fluids containing PC are applied. The coated antibody will capture the PC in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to PC is added to the plate to bind to the captured PC. After washing the plate to remove unbound conjugated antibody, the peroxidase activity is expressed by incubation with o-phenylenediamine (OPD). After a fixed development time the reaction is quenched with the addition of H<sub>2</sub>SO<sub>4</sub> and the colour produced is quantified using a microplate reader. The colour generated is proportional to the concentration of PC in the sample.

#### Supplied Materials:

- 1. Capture Antibody (PC-EIA-C):** One yellow-capped vial containing 0.5 ml of purified monoclonal anti-PC antibody for coating plates.
- 2. Detecting Antibody (PC-EIA-D):** One red-capped vial containing 0.5 ml of peroxidase conjugated polyclonal anti-PC antibody for detection of captured PC.

**Note:** Antibodies are supplied in a 50% (v/v) glycerol solution for storage at -10 to -20°C. Keep vials tightly capped. Do not store in frost-free freezers.

#### Materials Required but not Provided:

This paired antibody set has been optimized for performance using the buffers and conditions described below. Most reagents are available as part of the VisuLize™ Buffer Pak (see Related Products section) or may be prepared as described below.

- 1. Coating Buffer:** 50 mM Carbonate  
1.59g of Na<sub>2</sub>CO<sub>3</sub> and 2.93g of NaHCO<sub>3</sub> up to 1 litre. Adjust pH to 9.6. Store at 2-8°C up to 1 month.
- 2. PBS:** (base for wash buffer and blocking buffer)  
8.0g NaCl, 1.15g Na<sub>2</sub>HPO<sub>4</sub>, 0.2g KH<sub>2</sub>PO<sub>4</sub> and 0.2g KCl, up to 1 litre. Adjust pH to 7.4, if necessary. Store up to 1 month at 2-8°C, discard if there is evidence of microbial growth.
- 3. Wash Buffer:** PBS-Tween (0.1%,v/v)  
To 1 litre of PBS add 1.0 ml of Tween-20.  
Check that the pH is 7.4. Store at 2-8°C up to 1 week.
- 4. Blocking Buffer:** PBS-BSA (1%, w/v)  
Dissolve 2.5 g of Bovine Serum Albumin (Sigma-RIA grade) in 200 ml of PBS. Adjust pH to 7.4, if required, then make up to 250 ml with PBS. Aliquot and store frozen at -20°C.
- 5. Sample Diluent:** HBS-BSA-T20  
5.95g HEPES (free acid), 1.46 g NaCl, 2.5 g Bovine Serum Albumin (Sigma, RIA grade) dissolved in 200 ml H<sub>2</sub>O. Add 0.25 ml of Tween-20, check and adjust pH to 7.2 with NaOH, then make up to a final volume of 250 ml with H<sub>2</sub>O.  
Aliquot and store frozen at -20°C.
- 6. Substrate Buffer:** Citrate-Phosphate buffer pH 5.0  
2.6g Citric acid and 6.9g Na<sub>2</sub>HPO<sub>4</sub> up to a final volume of 500 ml with purified H<sub>2</sub>O. Store at 2-8°C up to 1 month.
- 7. OPD Substrate:** (o-Phenylenediamine.2HCl) **Toxic!**  
(5mg tablets: Sigma # P-6912). Make up immediately before use. Dissolve 5mg OPD in 12 ml substrate buffer then add 12 µl 30% H<sub>2</sub>O<sub>2</sub>. Do not store.

- 8. Stopping Solution:** 2.5 M H<sub>2</sub>SO<sub>4</sub>  
**Caution: VERY CORROSIVE! GENERATES HEAT ON DILUTION!** Where stock sulphuric acid is 18 Molar, add 13.9 ml to 86 ml H<sub>2</sub>O.  
Store at room temperature.

- 9. Other:**  
Microplates, 96-well Immulon 4-HBX (<http://www.labsystems.fi>)  
Microplate washer (optional)  
Microplate reader.  
Optional: VisuLize™ Buffer Pak  
Optional: VisuCal™ Antigen Calibrator Plasma

## Assay Procedure:

### 1. Coating of plates:

Dilute the capture antibody 1/100 in coating buffer (preferably in a polypropylene tube) and immediately add 100 µl to every well in the plate. Incubate overnight at 2-8°C or for 2 hours at 22 °C.

### 2. Blocking:

Empty contents of plate and add 150 µl of blocking buffer to every well and incubate for 90 minutes @ 22°C. Wash plate X 3 with wash buffer.

### 3. Samples:

Reference plasma is diluted 1/100 (100%) then serial 1/2's down to 1/3200 (3.13%). Sample plasmas are diluted 1/200, 1/400 & 1/800. All dilutions are made in HBS-BSA-T20 sample diluent. Apply 100 µl/well and incubate plate @ 22°C for 90 minutes. Wash plate X 3 with wash buffer.

### 4. Detecting Antibody:

Dilute the detecting antibody 1/100 in HBS-BSA-T20 sample diluent and apply 100 µl to each well. Incubate plate @ 22°C for 90 minutes. Wash plate X 3 with wash buffer.

### 5. OPD Substrate:

Apply 100 µl of freshly prepared OPD substrate to every well. Allow colour to develop for 10-15 minutes then stop colour reaction with the addition of 50 µl/well of 2.5 M H<sub>2</sub>SO<sub>4</sub>. The plate can be read at a wavelength of 490 nm.

## Calculation of Results:

The construction of a proper reference curve is of no less importance than any other aspect of the assay. A reference curve should be constructed by plotting the known concentration of standards versus absorbance. This can be done manually using graph paper, or by using curve-fitting computer software. In our experience, the dose response curves of most immunoassays tend to be sigmoid in shape. Although linear regions can be identified within the curve, the best overall fit is often obtained using an algorithm that provides a weighted theoretical model of fit throughout the entire curve, such as a 4-parameter or 5-parameter logistic curve fit<sup>4,5</sup>. In general, the simplest model that defines the concentration-response relationship should be used<sup>6</sup>.

The "back-fit" test is a simple and reliable method to determine if a curve-fitting method is appropriate. In this test, the apparent concentrations for the absorbance values of each standard point are read from the reference curve. The derived values are compared to the assigned values. An appropriate curve fitting method will produce derived values that closely match assigned values throughout the range of the curve, within user-defined limits<sup>6</sup>. The coefficient of determination ( $R^2$ ) is a valuable indicator of the overall fit, but should not be used by itself in the selection of a curve fitting method, as a poor fit in a particular region of the curve may not be evident from this value alone<sup>5,6</sup>.

**In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed using serial dilutions of normal pooled plasma, will produce a correlation coefficient ( $R^2$ ) of at least 0.980 using a log-log fit, and an  $R^2$  of at least 0.990 using a 4-parameter logistic curve fit algorithm.** However, the performance characteristics of in-house assays developed using this product in other laboratories may vary slightly from ours. Different curve fitting methods may be employed but we recommend that the back-fit test be applied as evidence that the fitting method is appropriate.

## Technical Notes:

- This paired antibody product is intended to facilitate the end user in establishing an in-house immunoassay for research purposes only. It must not be used for diagnostic applications. Assay validation is the responsibility of the end user and should be done according to user-defined protocols<sup>6</sup>.
- Reference calibrators should be of the same matrix and anticoagulant as the samples to be tested (example serum or plasma, citrate or EDTA).
- Do not use samples diluted less than 1/20, as falsely high readings may result.
- The optimal colour development time should be determined empirically as the time required to obtain an absorbance of at least 1.000 at 490 nm for the 100% reference point, not to exceed 20 minutes.
- Rheumatoid factor in samples may interfere in ELISA by binding to the capture and/or detecting antibodies.
- The wells should not be allowed to become dry. Keep plate covered or in a humid chamber during incubations.
- Antibodies are supplied in a 50% glycerol solution and can be centrifuged briefly in a micro-centrifuge to gather residual reagent from the cap and walls of the tube.

## References:

1. Broze GJ, Miletich JP; Biochemistry and Physiology of Protein C, Protein S and Thrombomodulin; in Hemostasis and Thrombosis, 3<sup>rd</sup> Edition, eds. RW Colman, J Hirsh, VJ Marder and EW Salzman, pp 259-276, J.B. Lippincott Co., Philadelphia PA, USA, 1994.
2. Esmon CT, Esmon NL, Le Bonniec B, Johnson AE; Protein C Activation; Methods in Enzymology 222, pp 359-385, 1993.
3. Heeb MJ, Mosher D, Griffin JH; Activation and Complexation of Protein C and Cleavage and Decrease of Protein S in Plasma of Patients With Intravascular Coagulation; Blood 73, pp 455-461, 1989.
4. Nix, B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.
5. NCCLS. Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guideline - Second Edition. NCCLS Document EP6-P2 (ISBN 1-56238-446-5, NCCLS, Wayne, Pennsylvania USA, 2001.
6. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available on the internet: [www.fda.gov/cder/guidance/index.htm](http://www.fda.gov/cder/guidance/index.htm)

## Related Products:

Cat #:	<b>EIA-PAK-1</b>	VisuLize™ Buffer Pak: 5 plates, buffers, substrate
Cat #:	<b>EIA-CSA-1</b>	VisuCal™ Antigen Calibrator, 1 x 1ml
Cat #:	<b>EIA-CSA-5</b>	VisuCal™ Antigen Calibrator, 5 x 1ml
Cat #:	<b>GAPC-IG</b>	Goat anti-human Protein C, whole IgG from antiserum
Cat #:	<b>GAPC-AP</b>	Goat anti-human Protein C, affinity purified IgG
Cat #:	<b>SAPC-IG</b>	Sheep anti-human Protein C, whole IgG from antiserum
Cat #:	<b>SAPC-AP</b>	Sheep anti-human Protein C, affinity purified IgG
Cat #:	<b>SAPC-HRP</b>	Sheep anti-human Protein C, IgG-peroxidase
Cat #:	<b>MAPC-IG</b>	Murine anti-human Protein C, IgG from ascites
Cat #:	<b>APCAT-EIA</b>	Paired antibody ELISA of APC-α <sub>2</sub> AT complex, 5 x 96 wells
Cat #:	<b>APCPCI-EIA</b>	Paired antibody ELISA of APC-PCI complex, 5 x 96 wells
Cat #:	<b>PC-DP</b>	Human plasma deficient in PC, immune depleted
Cat #:	<b>PC-LDP</b>	Human plasma deficient in PC, lyophilized