Affinity Biologicals™ INC.

REPRESENTATIVE DATASHEET

Matched-Pair Antibody Set for ELISA of human Activated Protein C- α_1Antitrypsin complex (APCAT)

Sufficient reagent for **5 x 96 well** plates

Product #: APCAT-EIA
Lot #: XXXX
Expiry Date: XXXX

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Store at -10 to -20°C

For Research Use Only
Not for use in diagnostic procedures.

Description of APC- $\alpha_1$antitrypsin Complex (APCAT)

Activation of coagulation leads to the generation of thrombin which, in the presence of thrombomodulin, will activate Protein C to the enzyme activated Protein C (APC). Unless regulated, APC will exert its anticoagulant function through proteolytic inactivation of factor Va and factor VIIIa. In blood, the activity of APC is regulated in part through interaction with protease inhibitors to form inactive enzyme-inhibitor complexes. Based on physiological concentrations and the kinetics of inhibition, the primary inhibitor of APC in blood is Protein C Inhibitor (PCI, also known as plasminogen activator inhibitor-3), followed by $\alpha_1$antitrypsin (α_1AT, also known as $\alpha_1$proteinase inhibitor) and $\alpha_2$macroglobulin.
The APC-α_1AT complex (APCAT) results when APC cleaves a scissile bond near the C-terminus of α_1AT, forming a covalent, 1:1 acyl enzyme intermediate with α_1AT with an apparent mass of 110 kDa. Calcium is not required for this interaction and there is no significant rate enhancement in the presence of heparin. The clearance rate for APCAT clearance from the circulation (half-life of 72 minutes) is slow relative to clearance rates reported for APC-PCI as well as thrombin-antithrombin and thrombin-heparin cofactor II complexes (half-lives of 10-19 minutes). The prolonged survival of APCAT may be an asset in the use of APCAT as a marker of Protein C activation.[1-4].

Principle of Sandwich-style ELISA

Affinity-purified antibody to human Protein C is coated onto the wells of a microtitre plate. Any remaining binding sites on the plastic wells are blocked with bovine serum albumin. The plates are washed and plasma or other fluids are applied. The coated antibody will capture the APC and APC-inhibitor complexes in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to α_1AT is added to the plate to bind to the captured APCAT complexes. 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_2SO_4 and the colour produced is quantified using a microplate reader. The colour generated is proportional to the concentration of APCAT complex present in the sample.

Supplied Materials:

1. Capture Antibody (APCAT-EIA-C): One yellow-capped vial containing 0.5 ml of polyclonal affinity purified anti-Protein C antibody for coating plates.

2. Detecting Antibody (APCAT-EIA-D): One red-capped tube containing 0.5 ml of peroxidase conjugated polyclonal anti-α_1AT antibody for detection of captured APCAT complex.

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_2CO_3 and 2.93g of $NaHCO_3$ 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_2HPO_4, 0.2g KH_2PO_4 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 and Detecting Antibody 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_2O. 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_2O.
Aliquot and store frozen at -20°C.

6. Substrate Buffer: Citrate-Phosphate buffer pH 5.0
2.6g Citric acid and 6.9g Na_2HPO_4 up to a final volume of 500 ml with purified H_2O. 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_2O_2. Do not store.

8. Stopping Solution: 2.5 M H_2SO_4
Caution: VERY CORROSIVE! GENERATES HEAT ON DILUTION! Where stock sulphuric acid is 18 Molar, add 13.9 ml to 86 ml H_2O.
Store at room temperature.

9. Materials for making reference standards:
- Protein C deficient plasma, 1 mL vials (Affinity, cat# PC-LDP).
- Human Activated Protein C, Cat# APC is available from Enzyme Research Labs, South Bend, IN (tel: 574-288-2268).
- Human α_1Antitrypsin is available from Sigma-Aldrich, Inc. (www.sigmaaldrich.com), Cat# A9024.
- PPACK (Phe-Pro-Arg-CMK) is available from Calbiochem (www.emdbiosciences.com), Cat # 520222.

10. Other:
Microplates, 96-well Immulon 4-HBX (http://www.labsystems.fi)
Microplate washer (optional)
Microplate reader.
Optional: VisuLize™ Buffer Pak

Assay Procedure:

1. Preparation of APCAT reference standard:
Purified APC (2 µg/mL = 33 nM) in 50 mM HEPES, 0.15 M NaCl, pH 7.4, is incubated with an excess of purified α_1AT (1.5 mg/mL = 27.3 µM) at 37°C. Complex formation is monitored by measuring residual APC activity using anticoagulant or chromogenic assay. When approximately half of the APC has been inhibited the reaction is quenched by the addition of PPACK to a final concentration of 50 µM to prevent further complex formation. The concentration of complex is calculated from the amount of APC activity inhibited by α_1AT before the addition of PPACK. A series of standards can be made by diluting this stock APCAT complex into PC deficient plasma. The highest APCAT concentration should be 2.5 nM, serially diluted into PC-DP to obtain APCAT concentrations of 1.25, 0.625, 0.313, 0.156 and 0.078 nM respectively.

2. 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 2 hours at room temperature or overnight at 2-8°C.

3. 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.

4. Standards and Test Samples:
Test samples and the reference standards prepared above are each diluted 1/100 in **HBS-BSA-T20** diluent. Apply 100 µl/well and incubate plate @ 22°C for 120 minutes. Wash plate X 3 with wash buffer.

5. Detecting Antibody:
Dilute the detecting antibody 1/100 in HBS-BSA-T20 detecting antibody diluent and apply 100 µl to each well. Incubate plate @ 22°C for 60 minutes. Wash plate X 3 with wash buffer.

6. 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_2SO_4. The plate can be read at 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 [5,6]. In general, the simplest model that defines the concentration-response relationship should be used [7].
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[7]. 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[6,7].

In the quality control of this product we have determined that under the conditions described above, a reference curve that is constructed as described above, 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[6].
- Reference standards may not be stable to freeze-thaw. If standards are aliquoted and stored frozen, the concentration of complex should be retested against freshly prepared standards.
- 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 highest reference point, within 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.
- Stock 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. Bauer KA; Laboratory Markers of Coagulation Activation; Arch Pathol Lab Med 117, pp 71-77, 1993.
2. Broze GJ, Miletich JP; Biochemistry and Physiology of Protein C, Protein S and Thrombomodulin; in Hemostasis and Thrombosis, 3rd Edition, eds. RW Colman, J Hirsh, VJ Marder and EW Salzman, pp 259-276, J.B. Lippincott Co., Philadelphia PA, USA, 1994.
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. Laurell M, Stenflo J, Carlson TH; Turnover of *I-Protein C Inhibitor and *I-α_1Antitrypsin and Their Complexes with Activated Protein C; Blood 76, pp 2290-2295, 1990**5.** Nix,B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.
6. NCCLS. Evaluation of the Linearity of Quantitative Analytical Methods; Proposed Guidline – Second Edition. NCCLS Document EP6-P2 (ISBN 1-56238-446-5, NCCLS, Wayne, Pennsylvania USA, 2001.
7. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available on the internet: www.fda.gov/cder/guidance/index.htm

Related Products:

Cat #: **EIA-PAK-1**	VisuLize™ Buffer Pak: 5 plates, buffers, substrate
Cat #: **GAPC-IG**	Goat anti-human Protein C, whole IgG from antiserum
Cat #: **GAPC-AP**	Goat anti-human Protein C, affinity purified IgG
Cat #: **SAPC-IG**	Sheep anti-human Protein C, whole IgG from antiserum
Cat #: **SAPC-AP**	Sheep anti-human Protein C, affinity purified IgG
Cat #: **SAPC-HRP**	Sheep anti-human Protein C, IgG-peroxidase
Cat #: **MAPC-IG**	Murine anti-human Protein C, IgG from ascites
Cat #: **GA1AT-IG**	Goat anti-human α_1AT, whole IgG from antiserum
Cat #: **GA1AT-AP**	Goat anti-human α_1AT, affinity purified IgG
Cat #: **SA1AT-IG**	Sheep anti-human α_1AT, whole IgG from antiserum
Cat #: **SA1AT-AP**	Sheep anti-human α_1AT, affinity purified IgG
Cat #: **SA1AT-APHRP**	Sheep anti-human α_1AT, APIgG-peroxidase
Cat #: **PC-EIA**	Paired antibody ELISA of Protein C, 5 x 96 wells
Cat #: **APCPCI-EIA**	Paired antibody ELISA of APC-PCI complex, 5 x 96 wells
Cat #: **A1AT-EIA**	Paired antibody ELISA of α_1Antitrypsin, 5 x 96 wells
Cat #: **PC-DP**	Protein C deficient plasma, immune-depleted, frozen
Cat #: **PC-LDP**	Protein C deficient plasma, immune-depleted, lyophilized

Visit our site (www.affinitybiologicals.com) for details.

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