REPRESENTATIVE DATASHEET*

Matched-Pair Antibody Set for ELISA of human Kininogen (KN)

Sufficient reagent for **4 x 96 well** plates

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

Store at –10 to -20°C

For Research Use Only
Not for use in diagnostic procedures.

Description of Kininogen (KN)

Kininogens are multi-function proteins that are involved in the processes of coagulation, anticoagulation, fibrinolysis, inflammation and cell adhesion. Kininogens are produced in the liver but have also been found in platelets, granulocytes, renal tubular cells and skin. Two forms of kininogen are identified in plasma, both of which are the result of differential splicing of a single gene. High molecular weight kininogen (HK), previously known as Fitzgerald Factor, is a single chain glycoprotein of 120 kDa with a plasma concentration of 80 µg/mL (660 nM). Low molecular weight kininogen (LK), also known as α-cysteine protease inhibitor, is a single chain glycoprotein of 68 kDa with a plasma concentration of 160 µg/mL (2.35 µM). HK and LK share a common heavy chain and bradykinin domain, but have unique light chains. It is the light chain of HK that is responsible for the coagulant cofactor activity by binding to anionic surfaces and for the ability to bind the zymogens prekallikrein (PK) and factor XI (FXI). HK is cleaved by kallikrein in several sequential steps that result in the release of a potent vasodilator bradykinin and the conversion to a two-chain form of HK with increased cofactor activity. In plasma, most of the PK and FXI circulate in complex with HK. Activation of PK by FXIIa generates kallikrein, which initiates reciprocal activation of PK and FXI. The presence of HK also serves to protect kallikrein and activated FXI from protease inhibitors such as C1-Inhibitor, but regulation of the system may be accomplished through proteolytic inactivation of the HK cofactor activity by these enzymes.[1-2]

Principle of Sandwich-style ELISA

Affinity-purified antibody to KN is coated onto the wells of a microtitre plate. Remaining binding sites on the plate are blocked with an excess of bovine albumin. The plates are washed and plasma or other fluids containing KN are applied. The coated antibody will capture the KN in the sample. After washing the plate to remove unbound material, a peroxidase conjugated second antibody to KN is added to the plate to bind to the captured KN. After washing the plate to remove unbound conjugate, 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 KN present in the sample.

Supplied Materials:

1. Capture Antibody (KN-EIA-C): One yellow-capped vial containing 0.4 ml of polyclonal affinity purified anti-KN antibody for coating plates.

2. Detecting Antibody (KN-EIA-D): One red-capped vial containing 0.4 ml of peroxidase-conjugated polyclonal anti-KN antibody for detection of captured KN.

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:

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. Sample Diluent and 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. Conjugate 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. Other:
Microplates, 96-well Immulon 4-HBX (www.labsystems.fi)
Microplate washer (optional)
Microplate reader.

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 2hrs @ 22°C .

2. Blocking:
Empty contents of plate and add 150 µl of blocking buffer to every well and incubate for 60 minutes @ 22°C.
Wash plate X 3 with wash buffer.

3. Samples:
Reference plasma is diluted 1/1000 (100%) then serial 1/2's down to 1/32000 (3.13%). Sample plasmas are diluted 1/2000, 1/4000 & 1/8000. All dilutions are made in PBS-Tween sample diluent. Apply 100 µl/well and incubate plate @ 22°C for 60 minutes. Wash plate X 3 with wash buffer.

4. Detecting Antibody:
Dilute the detecting antibody 1/100 in HBS-BSA-T20 conjugate diluent and apply 100 µl to each well. Incubate plate @ 22°C for 60 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 **5-10** minutes, and 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 [3,4]. In general, the simplest model that defines the concentration-response relationship should be used [5].
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[5]. 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 [4,5]

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[6].
- 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 1/10, 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. Coleman RW, Schmaier AH; Contact System: A Vascular Biology Modulator With Anticoagulant, Profibrinolytic, Antiadhesive and Proinflammatory Attributes. Blood 90, pp 3819-3843, 1997.

2. DeLa Cadena R, Watchtfogel YT, Colman RW, in Hemostasis and Thrombosis, 3rd Edition, eds. RW Colman, J Hirsh, VJ Marder and EW Salzman, pp. 219-240, J.B. Lippincott Co., Philadelphia, 1994.

3. Nix,B, Wild D, in Immunoassays, A Practical Approach, editor J.P. Gosling, pp. 239-261, Oxford University Press, 2000.

4. 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

5. FDA Guidance for Industry. Bioanalytical Method Validation; May 2001, available on the internet: www.fda.gov/cder/guidance/index.htm