5-ELISA ANNEXIN A1 Antigen

Ref# 5D-55902

ELISA for measurement of ANNEXIN A1

96 tests micro-ELISA plate

Research Use Only. Not for use in diagnostic procedures

Intended use :

The 5-ELISA Annexin A1 Antigen kit is a two-site enzyme immunoassay for measuring Annexin A1 antigen in plasma or in any biological fluid where it must be tested.

Summary and explanation:

This sandwich ELISA is designed with polyclonal antibodies coated onto the plate for capturing ANNEXIN A1 (ANXA1) in the tested sample. Following a washing step, captured ANXA1 is tagged with a peroxidase-labelled polyclonal antibody, which binds onto the free epitopes in a dose-dependent manner. After washing away the excess of immunoconjugate, the substrate, 3,3',5,5' Tetramethylbenzidine (TMB) with hydrogen peroxide (H2O2) is introduced and a blue color develops, which turns yellow when the reaction is stopped with sulfuric acid. This color is measured at 450 nm, and the measured value is directly proportional to the amount of ANXA1 present in the tested sample. Results are expressed in ng/ml, by reference to a calibrated internal reference preparation of human recombinant ANXA1.
ANXA1, or Lipocortin I, is a 37 kDa protein from the annexin family with strong anti-inflammatory and pro-resolving activities (Perretti and D'Aquisto, *Nat Rev Immunol* 2009). ANXA1 is highly expressed in neutrophils, monocytes and macrophages and modestly in mast cells, endothelial cells, epithelial cells and fibroblasts.

Reagents:

1. **COAT**: Micro ELISA plate, containing 12x8 well strips, coated with a polyclonal antibody to human ANNEXIN A1, stabilized; the plate is packed in an aluminum pouch sealed in presence of a desiccant.
2. **SD**: 2 vials of 30 mL Sample Diluent containing aggregated rabbit IgGs, ready to use.
3. **WS**: 50 mL vial of 20-fold concentrated Wash Solution.
4. **CAL**: 2 vials of lyophilized Plasma Calibrator, ***already diluted 1:10*** when restored with 2 mL of SD*.
5. **C1 (High)**: 2 vials of lyophilized Control Plasma High, ***already diluted 1:10*** when restored with 1 mL of SD*.
6. **C2 (Low)**: 2 vials of lyophilized Control Plasma Low***, already diluted 1:10*** when restored with 1 mL of SD*.
7. **IC**: 500 µL vial of Anti-(h)-ANNEXIN A1-HRP Conjugate, 50-fold concentrated.
8. **ICD**: 25 mL of Conjugate Diluent, ready to use.
9. **TMB**: 26 mL of Peroxidase Substrate, 3,3',5,5'-Tetramethylbenzidine containing hydrogen peroxide. Ready to use.
10. **SA**: 7.5 mL vial of 0.50M Sulfuric Acid, ready to use.

** The exact ANNEXIN A1 concentrations are indicated for each lot on the flyer included in the kit.*

Warning and cautions:

- Some reagents provided in this kit contain materials of human (control and calibration plasma) 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.
The bovine blood used to prepare BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
However, no test method can offer complete assurance that infectious agents are absent. Therefore, laboratory operators using these reagents must exercise extreme care in full compliance with safety cautions for the manipulation of these biological materials and treat them as if they were infectious.
- Waste should be disposed in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Any incident that has occurred in relation with the device use shall be reported to the manufacturer.
- If the TMB substrate becomes yellow, this indicates the presence of a contaminant.

Reagent preparation:

Bring the kit at room temperature, at least 30 minutes before use, to avoid use of reagents at a too low temperature, which can reduce the assay kinetics.
Store unused reagents at 2-8°C.
Vials are closed under vacuum. Carefully remove the stopper of lyophilized reagents, to avoid any loss of powder when opening the vials.

When appropriately used and stored, according to the recommended protocol and cautions, the kit content can be used over a 1-month period, and strip by strip, if required.

1. **COAT** (Micro ELISA plate): Open the aluminum pouch and take off the required number 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 aluminum pouch, in presence of the desiccant, hermetically closed with the minigrip system and protected from any moisture.
2. **SD** (Sample Diluent): Ready to use.
   This reagent contains 0.05% Proclin-300, and aggregated rabbit IgGs to minimalize the interference of Rheumatoid Factor and heterophilic antibodies. Stability after opening and provided that any contamination or evaporation is avoided, kept in its original vial:
   - 4 weeks at 2-8°C.
3. **WS** (Wash Solution): If necessary, incubate the vial in a water bath at 37°C, until complete dissolution of solids. Shake the vial and dilute the required volume 1:20 with distilled water (the 50 mL contained in the vial allow preparing 1 liter of Wash Solution). Stability after opening and provided that any contamination or evaporation is avoided, kept in its original vial:
   - 4 weeks at 2-8°C.
4. **CAL** (Calibrator): Reconstitute each vial with exactly 2 mL of **SD** sample diluent to get a 10-fold diluted calibrator plasma with a defined ANNEXIN A1 concentration (indicated on the kit flyer). Stability of reconstituted calibrator, in its original vial and provided that any contamination or evaporation is avoided:
   - 8 hours at room temperature (18-25°C).
   - 24 hours at 2-8°C.
   - 2 months at ≤ -20°C.

5,6. **C1, C2** (Control Plasma 1 High and Control Plasma 2 Low): Reconstitute each vial with 1 mL of **SD** sample diluent to get a 10-fold diluted control plasma. Stability of reconstituted control, in its original vial and provided that any contamination or evaporation is avoided:
   - 8 hours at room temperature (18-25°C).
   - 24 hours at 2-8°C.
   - 2 months at ≤ -20°C.

7. **IC** (Anti-(h)-ANNEXIN A1-HRP Conjugate): Stability after opening and provided that any contamination or evaporation is avoided, kept in its original vial:
   - 4 weeks at 2-8°C.

   Just before use, dilute 50-fold the requested volume of the concentrated **IC** (50x) with the **ICD** "Conjugate Diluent" and shake to homogenize.
   Stability of diluted Conjugate:
   - 6 hours at room temperature (18-25°C).
8. **ICD** (Immuno-Conjugate Diluent): Ready to use. This reagent contains 0.05% Proclin-300. Stability after opening, and provided that any contamination or evaporation is avoided, kept in its original vial:
   - 4 weeks at 2-8°C.
9. **TMB**: Ready to use. Stability after opening, and provided that any contamination or evaporation is avoided, kept in its original vial:
   - 4 weeks at 2-8°C.
10. **SA** (Stop Solution): Stop solution containing 0.50M sulfuric acid, ready to use.

Reagents and materials not provided:

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

Specimen collection and preparation:

Blood (9 volumes) should be carefully collected onto 0.109M (3.2%) trisodium citrate anticoagulant (1 volume) by clean venipuncture.
Samples (plasma or serum) should be collected, prepared, and stored in accordance with applicable local guidelines.
For sample storage, please refer to references.

Assay method:

The standard assay protocol used on plasma, includes a **1:10** dilution.
For low Annexin A1 concentration, a plasma dilution of **1:5** can be used.
Measured concentrations with the standard calibration curve must then be divided by **2**.
Measured ANNEXIN A1 concentrations in tested plasmas, using these assay conditions, are deduced from the calibration curve and must be **multiplied by the dilution factor.** If other samples are used, and different dilutions tested, the measured concentration must be multiplied by the dilution factor used for the tested specimen. Tested samples must be diluted at least 1:5 with the **SD** sample diluent.
1. Controls are ready to use (already diluted 1:10).
2. Tested plasma samples should be diluted 1:5 or 1:10 (or more when required) with the **SD** sample diluent.
3. Immunoconjugate IC must be diluted 1:50 with the **ICD** conjugate diluent, just before use.
4. Calibration range: **CAL**, with an ANNEXIN A1 concentration of "c" (of **about 50 ng/ml** for the 10-fold already diluted calibrator, i.e. 500 ng/ml in original plasma pool). The following calibration range must be prepared with **SD**:

	c	**0.75 c**	**0.50 c**	**0.25 c**	**0.10 c**	**0**
CAL µL	50	37.5	25	12.5	5	0
SD µL	0	125	250	375	450	500

5. Procedure: Remove the required number of strips from the aluminium pouch and place them in the frame provided. Introduce the reagents in the microplate wells and perform the assay as indicated on the here below table:

Samples or Reagent	Volume	Protocol
Calibrators, Controls (C1, C2) or tested specimen	200 µL/well	Introduce rapidly into microwells **(a)**
Incubate for 60 minutes at RT (18-25 °C) ***(b) (c)***		
WS	300 µL/well	5 successive washing steps
IC	200 µL/well	Introduce immediately after washing **(d)**
Incubate for 60 minutes at RT (18-25°C) ***(b) (c)***		
WS	300 µL/well	5 successive washing steps
TMB-H_2O_2	200 µL/well	Introduce immediately **(d)**
Incubate for exactly 10 minutes at RT (18-25 °C) ***(b) (c)***		
SA (e)	50 µL/well	Stop after exactly 10 minutes
Homogenize by shaking smoothly and wait for 10 minutes *Read the absorbance at 450 nm within 20 minutes* ***(f)***		

Notes:

a) Distribute controls and specimen as rapidly as possible (within 10 minutes), to obtain homogeneous immunological kinetics. A too long delay between the distribution of the first and the last wells may influence immunological kinetics and generate inaccurate results (last wells distributed underestimated).

(b) Avoid letting the plate in the bright sunlight during incubations and more particularly during color development.

(c) Perform the incubations preferably at 20±1°C.

(d) 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, to prevent the plate from drying, which could damage the immobilized components and reduce reagents' reactivity. 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 to wash the plates gently, and to avoid a too drastic emptying, which could damage coating and lower plate reactivity.

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

(f) For bichromatic readings, a reference wavelength between 620 nm and 690 nm can be used.

Quality control:

Using quality controls allows validating the method compliance, as well as the homogeneity of assays for a same lot of reagents.

Quality control must be included in each series, as per good laboratory practice, to validate test results.

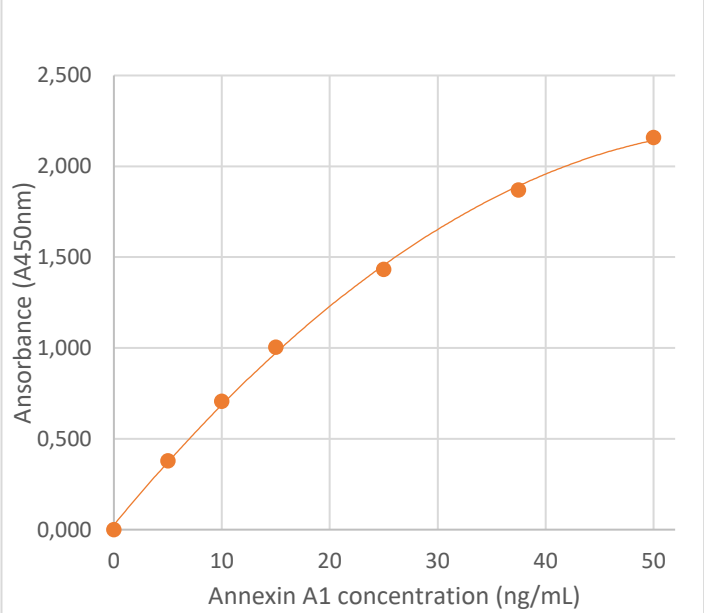
Each laboratory should establish acceptance ranges and verify expected performances in its analytical system, in case of assay automatization, the user agrees to conduct a complete study to validate the analytical performance according to the standards in force.

Results:

Results are calculated from the calibration curve, using the obtained A450 for the tested sample.

The measured concentration must be **multiplied by the sample dilution factor**, to get the actual concentration in the tested specimen.

Example of calibration curve (must not be used for A1 measurements; only the calibration curve generated for the ongoing tested series must be used):



Limitations:

- To ensure optimum test performance and to meet the specifications, the technical instructions should be followed carefully.
- Any reagent presenting no limpid appearance or showing signs of contamination must be rejected.
- Any suspicious samples must be rejected.
- If the washing step is not correctly performed, this can produce a high absorbance value. To avoid non-specific color development, check that the washing step is performed efficiently.
- It is the responsibility of the user to validate any modification to these instructions of use of the reagents.
- Erroneous results can occur from bacterial contamination of reagents, inadequate incubation periods, inadequate washing of test wells, exposure of substrate to bright light/sunlight, omission of test reagents, exposure to temperatures higher or lower than prescribed requirements or omission of steps.
- Aggregated rabbit IgGs are added to the sample diluent **(SD)** to minimalize any possible interference of Rheumatoid Factor or of heterophilic antibodies.

Performances:

Dynamic range: 5.0 to about 500 ng/mL ANNEXIN A1 in plasma (i.e. 0.5 to 50 ng/mL in the tested 10-fold dilution)
Intra-assay CV: C1 (38.72 ng/mL): 4.60%; C2 (14.72 ng/mL): 4.22%
Inter-assay CV: C1 (38.72 ng/mL): 3.65%; C2 (14.72 ng/mL): 3.55%
Recovery: 90-110 % for purified ANNEXIN A1 spiked in citrated plasma.

Normal concentration in plasma:

ANXA1 can be present in plasma as result of neutrophil activation, neutrophil extracellular trap formation, release from monocytes and macrophages, and apoptosis. It serves then as biomarker for ongoing inflammation and pathological processes.

Additional information:

ANXA1 is a member of the annexin protein family. Like most members it binds negatively charged phospholipids in Ca^{2+}-dependent manner. In addition, it binds the FPR2/ALX receptor and elicits anti-inflammatory responses upon FPR2/ALX ligation. ANXA1 is regarded as an important mediator of resolution of acute inflammation. Blood plasma ANXA1 originates mostly from activated and dying cells of the innate and adaptive immune system, including neutrophils, monocytes, macrophages, and dendritic cells. Plasma ANXA1 reflects ongoing inflammation and an activated immune system, especially in cancer. Plasma ANXA1 can be proteolytically cleaved into an N-terminal domain of around 3 kDa and a C-terminal domain of circa 34 kDa. Both the full-length ANXA1 and the 34 kDa C-terminal domain can be measured in citrated plasma using the sandwich ELISA. Plasma ANXA1 is a useful biomarker for immune-inflammatory states associated with infectious diseases, malignancy and autoimmunity.

References:

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